Mesenchymal cell re-modeling during mouse secondary palate re-orientation

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Abstract

The formation of mammalian secondary palate requires a series of developmental events such as growth, elevation and fusion. Despite recent advances in the field of palate development, the process of palate elevation remains poorly understood. The current consensus on palate elevation is that the distal end of the vertical palatal shelf corresponds to the medial edge of the elevated horizontal palatal shelf. We provide evidence suggesting that the prospective medial edge of the vertical palate is located toward the interior side (the side adjacent to the tongue), instead of the distal end, of the vertical palatal shelf and that the horizontal palatal axis is generated through palatal outgrowth from the side of the vertical palatal shelf rather than rotating the pre-existing vertical axis orthogonally. Since palate elevation represents a classical example of embryonic tissue re-orientation, our findings here may also shed light on the process of tissue re-orientation in general.

Keywords

palate development; palate elevation; tissue re-orientation

Introduction

The formation of mammalian secondary palate involves multiple developmental events, including growth, elevation and fusion (Ferguson, 1988; Hilliard et al., 2005; Chai and Maxson, 2006; Gritli-Linde, 2007). During mouse development, the two developing palatal shelves first grow vertically along the lateral sides of the tongue on embryonic days 12.5 and 13.5 (E12.5 and E13.5) (Ferguson, 1988; Murray and Schutte, 2004; Gritli-Linde, 2007). On E14.5, however, the two vertical palatal shelves re-orientate to a horizontal position above the dorsal side of the tongue, a process termed palate elevation (Ferguson, 1988; Gritli-Linde, 2007). The two elevated palatal shelves then grow horizontally towards each other to meet along the facial midline and form the medial edge epithelial (MEE) seam. The MEE

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seam then undergoes degeneration giving rise to a fused palate with mesenchymal confluence, a process referred to as palate fusion (Ferguson, 1988; Hilliard et al., 2005).

Significant efforts have been made to understand the cellular mechanisms of palate fusion, especially the degeneration of the MEE seam. A number of lineage tracing studies have suggested that MEE seam degeneration is mediated by epithelial-mesemchymal transition (EMT)(Griffith and Hay, 1992), cell migration (Carette and Ferguson, 1992), apoptotic cell death (Cuervo and Covarrubias, 2004; Vaziri Sani et al., 2005), or a combination of all three (Jin and Ding, 2006a). At the molecular level, Smad2 mediated TGF-β3 signaling has been demonstrated to be essential for palate fusion (Kaartinen et al., 1995; Proetzel et al., 1995; Cui et al., 2005). Consistently, epithelial- specific deletion of the TGF-β type II receptor gene also causes cleft palate in mouse embryos (Xu et al., 2006).Conversely, *Jagged2* (*Jag2*) expression may prevent epithelial fusion because *Jag2* mutant embryos display premature fusion of the palatal shelves with the tongue, probably due to precocious differentiation of periderm cells (Jiang et al., 1998; Casey et al., 2006). It has been reported that Msx1 and BMP4 are responsible for anterior palate growth (Zhang et al., 2002). Other regulatory factors involved in mouse palatal growth include the BMP receptor 1A (Alk3) (Liu et al., 2005), the growth factor PDGF-C (Ding et al., 2004) and the transcription factor Osr2 (Lan et al., 2004).

In contrast to palatal growth and fusion, the mechanism of palate elevation is poorly understood and little progress has been made in the past decades (Gritli-Linde, 2007). The current consensus on palate elevation is that the distal end of the vertical palatal shelf corresponds to the medial edge region in the horizontal palatal shelf (Gritli-Linde, 2007). In this model, the palatal shelves must overcome the physical obstruction imposed by the tongue during elevation. Previous studies have suggested that the forward displacement of the mandible and the tongue lowers the level of the tongue during palate elevation (Seegmiller and Fraser, 1977; Lavrin et al., 2001). However, this forward displacement of the tongue may not be sufficient to position the palatal shelf above the tongue. In fact, palate re-orientation does not require the dorsal level of the tongue to be below the distal end of the palatal shelf. It has been observed that the two palatal shelves do not elevate in unison, but sequentially in a very short time frame (Greene and Kochhar, 1973; Luke, 1984). Often one shelf is completely horizontal, whilst the other remains vertical with its distal end far below the dorsal level of the tongue (Greene and Kochhar, 1973; Luke, 1984), indicating that the palatal shelves can re-orient without moving above the dorsal level of the tongue. This observation also suggests that palate elevation involve complicated movements beyond a simple rotation. Consistent with this notion, previous histological studies observed that the palate shelves bulge out medially and squeeze into the space above the tongue, and it has been speculated that that palate shelves may "flow" over the tongue during elevation (Greene and Kochhar, 1973; Diewert and Tait, 1979). In addition, a recent in vitro study using carbon labeling suggested tissue remodeling in the anterior and posterior palate development (Chou et al., 2004). The mechanism driving the palate elevation is still poorly understood. However, a correlation between hyaluronic acid synthesis and palate elevation has been observed although a causative relationship has not been established (Ferguson, 1988). In addition, it has been recently reported that loss-of-function mutations in *Snai1,2* and *Wnt5A* resulted in vertical palate shelves (Murray et al., 2007; He et al., 2008), indicating that the functions of these genes are involved in palate elevation.

We previously reported that *Zfhx1a* mutant mice displayed delayed palate elevation by 24 to 48 hours (Jin et al., 2008). In this study, we used *Zfhx1a* and *Jag2* mutant mice to investigate the process of palate elevation and found that the prospective medial edge region is not located in the distal end of the vertical palatal shelf and the horizontal palatal axis is

generated by palatal outgrowth from the side rather than rotating the pre-existing vertical axis orthogonally.

Results and Discussion

As mentioned above, the current model for palate elevation assumes that the distal end of the vertical palatal shelf corresponds to the medial edge region of the horizontal palatal shelf (see supplementary Fig. 1A and B). However, several observations prompted us to suspect that this assumption may not be correct. For example, in the posterior region of the vertical E13.5 palate, the prospective nasal surface is longer than the prospective oral surface. After palate re-orientation to a horizontal position on E14.5, however, the definitive nasal surface in the posterior region is shorter than the oral surface (Supplementary Fig.1C and D). This phenomenon is difficult to explain if the distal end of the vertical palate corresponds to the medial edge area of the horizontal palate. Similarly, it was reported before that the TGF-β3 expression domain on E14.5 is mainly located in the medial edge epithelium with equal extension to the oral and nasal surfaces, whereas on E13.5, when the palatal shelves are vertical, the TGF-β3 expression domain is mainly located within the prospective nasal epithelium, the surface facing the tongue (Fitzpatrick et al., 1990; Pelton et al., 1990). We therefore hypothesized that the prospective medial edge region of the vertical palate is located on the interior side, the side facing the tongue, rather than on the distal end, and that palate re-orientation is through horizontal outgrowth from the interior side of the vertical shelf towards the midline. To test this hypothesis, we used *Zfhx-1a* (also known as *ZEB-1* and *δEF1*) mutant mice (Takagi et al., 1998; Postigo, 2003). Palate re-orientation occurs very rapidly within a short time window in wild type mice (Gritli-Linde, 2007), making it difficult to study the sequential events during re-orientation in detail. In our previous study, we reported that *Zfhx-1a* mutant embryos were delayed in palate elevation by 24–48 hours (Jin et al., 2008). Briefly, on E14.5, 100% of palatal shelves from *Zfhx-1a* mutants remain vertical along the entire length from anterior to posterior. On E15.5, 75% of the *Zfhx-1a* mutant palate shelves are vertical in the anterior and middle regions, but the extreme posterior region was proceeding to a horizontal position, while the other 25% were fully horizontal from anterior to posterior (Jin et al., 2008). Interestingly, regional specification of the medial edge epithelium occurred normally, as determined by both marker analysis and an in vitro fusion assay (Jin et al., 2008). Thus, the *Zfhx-1a* mutant line provides a model to examine medial edge epithelium (MEE) markers in an E14.5 palatal shelf that is still vertical. If our hypothesis is correct, the MEE specific markers of horizontal palates should be located on the interior side, rather than on the distal end, of E14.5 *Zfhx-1a* mutant vertical palates. In wild type embryos, *MMP13* is not expressed in the vertical palate prior to E13.75 (data not shown), however, once the palatal shelves become horizontal on E14.5, *MMP13* is expressed exclusively in the MEE and this MEE specific expression continues until early E15.5 before the MEE degenerates (Fig.1A and B), consistent with previous reports (Blavier et al., 2001; Jin et al., 2008). We therefore examined *MMP13* expression in the vertical *Zfhx-1a* mutant palate on E14.5 and E15.5 to determine whether the expression is on the interior side or distal end of the mutant vertical palate. As shown in figure 1, the expression of *MMP13* is exclusively located on the interior side of the E14.5 *Zfhx-1a* mutant vertical palatal shelves in the middle and posterior regions (Fig. 1D and E). In the most anterior region, the expression resides primarily within the interior side and covers only a portion of the distal tip (Fig. 1C). As mentioned previously, by E15.5, 25% of *Zfhx-1a* mutant palatal shelves were fully horizontal (Jin et al., 2008). In these embryos, *MMP13* was exclusively expressed in the MEE region from anterior to posterior (Fig.1I). The other 75% remained vertical in the anterior and middle regions. In these mutants, *MMP13* expression was exclusively located on the interior side in the middle vertical region (Fig. 1G), and in the most anterior region, the expression was primarily located on the interior side with slight extension to the distal end (Fig. 1F). The posterior region of these palates was proceeding to

be horizontal and *MMP13* expression was located in a region corresponding to the MEE (Fig. 1H). These results indicate that the prospective medial edge epithelium is located mainly on the interior side of the vertical palate, except for the most anterior region, where a portion of the distal region contributes to the future medial edge region. To further confirm this result, we also examined the expression of *Jag2*. In wild type embryos, the *Jag2* expression domain covers the entire palate epithelium up to E14.5 (Casey et al., 2006; Jin et al., 2008). However, the expression is specifically down-regulated in the MEE around E15.0–E15.25 when palate fusion occurs (Casey et al., 2006; Jin et al., 2008). This down-regulation may be essential for palate fusion because loss of *Jag2* promotes epithelial cell fusion (Jiang et al., 1998). We found that the down-regulation of *Jag2* expression in the MEE region is independent of palate contact, since it occurs in the MEE region of elevated, but not touching, E15.5 *Zfhx-1a* mutant palates (Jin et al., 2008). Importantly, *Jag2* down-regulation also occurs in those unelevated E15.5 *Zfhx-1a* mutant palates. Consistent with the *MMP13* expression in *Zfhx-1a* mutant palates, a *Jag2*–free domain resides exclusively on the interior side in the middle region of the vertical palate (Fig. 1O). In the most anterior region of the vertical palates, *Jag2* down-regulation was located mostly on the interior side with limited extension into the distal region (Fig.1N). The posterior region of these E15.5 mutant palates are proceeding to be horizontal, and the down-regulation of *Jag2* occurs in the MEE region (Fig.1P). Based on these results, *MMP13* expression and *Jag2* down-regulation, we conclude that during palate development, the prospective MEE region is located on the interior side of the vertical palate before palate elevation/re-orientation, with the exception of the most anterior region where a portion of the distal region contributes to the MEE of horizontal palatal shelf. The identification of the prospective medial edge epithelium on the interior side, rather than the distal end, of the vertical palatal shelf raises the possibility that palate re-orientation results from outgrowth from the side of palatal shelf rather than a rotation of the vertical palatal axis. This question is particularly interesting because previous studies suggested that the shelves may "flow" over the tongue (Greene and Kochhar, 1973; Diewert and Tait, 1979). However, due to the lack of MEE markers at that time, these studies were unable to address the relationship between palate re-orientation and the prospective medial edge. We further examined *MMP13* expression in *Zfhx-1a* mutant palates between E15.5 and E16.5 to determine the region of the developing MEE in relation to the outgrowth zone, and found the future MEE, marked by *MMP13* expression, is immediately underneath the outgrowth tip (Supplementary Fig. 2A). We also observed "outgrowth tip/zone" in histological sections of embryos that undergo normal palate re-orientation (Supplementary Fig.2B and C), consistent with the observation from a previous study suggesting that the palate shelves may "flow" over the tongue during elevation (Greene and Kochhar, 1973).

The outgrowth that converts the vertical shelf to the horizontal shelf should, in principle, be driven by the mesenchymal cells. We therefore examined the expression of *goosecoid*, a homeobox gene expressed specifically in the medial edge mesenchyme of the horizontal palatal shelves (Jin and Ding, 2006b). As shown in figure 2, *goosecoid*-expressing cells are organized as a condensed block adjacent to the MEE on E15.5 and E14.5 (Fig. 2A and B). This condensed mesenchyme cell block is visible by HE staining after E16.5 (Fig. 2E). However, the *goosecoid*-expressing cells present on E13.5 are diffuse (Fig. 2D), and become gradually restricted to the interior side of the vertical shelf on E13.75 (Fig. 2C). Thus, it appears that palate re-orientation is accompanied by mesenchymal cell condensation, and this process is delayed in *Zfhx-1a* mutant embryos (Fig. 2F, G and H). Cell movement during mesenchyme cell condensation can, in principle, trigger palatal outgrowth. However, it is also possible that the observed mesenchyme cell condensation is a result of palate re-orientation. To rule out this possibility, we examined mesenchyme cell condensation in *Jag2* mutant embryos. Previous reports demonstrated that loss of *Jag2* leads to abnormal fusion between the palate and tongue, physically preventing palate elevation (Jiang et al., 1998). If

mesenchyme cell condensation is the consequence of palate re-orientation, *goosecoid*-expressing cells should not form a condensed block in the palates of *Jag2* mutant embryos. Conversely, if it is a trigger of the outgrowth, mesenchyme cell condensation in *Jag2* mutants may still occur. As shown in figure 2, the condensed mesenchyme cell block is present in *Jag2* mutant palatal shelves on E15.5 and E16.5 as visualized by both *goosecoid* in situ hybridization and HE staining (Fig. 2I and J). Consistent with our hypothesis, the condensed cell block is located on the interior side of the vertical *Jag2$^{-/-}$* palatal shelves on E15.5 and E16.5 (Fig. 2I and J)., In addition, *Jag2* mutant palatal shelves bulged out toward the tongue on E15.5 and E16.5 (see arrowheads in Fig. 2I and J), indicating that palatal outgrowth indeed occurs. Moreover, in certain areas of *Jag2* mutant embryos on E17.5, the palate shelves were almost horizontal with a medial edge-like region fused with the tongue (Fig. 2L and M), confirming that the interior side, rather than distal end, becomes the medial edge because the distal palate is never in contact with the tongue (Fig. 2K). Also, the formation of a horizontal-like palate in the *Jag2* mutant embryos on E17.5 provides additional support for our hypothesis that the horizontal palate axis is generated by palate outgrowth from the side, since it would be impossible to rotate the palatal shelves in *Jag2* mutants.

Based on the above results, including the MEE marker analysis in two different manners (presence of *MMP13* expression and down-regulation of *Jag2* expression), cell condensation on the interior side of *Jag2* mutant vertical palate shelves and the formation of a horizontal-like palate in *Jag2* mutants with a medial edge fused with the tongue, we propose that the prospective medial edge is located on the interior side of the vertical palatal shelf and the horizontal palate shelf is formed by outgrowth from the side of the vertical palate shelf rather than by rotating the vertical axis (Fig.3). Because *goosecoid* expression (the medial edge mesenchymal cell marker used here) is absent in the extreme anterior region (data not shown), we were not able to visualize the mesenchymal cell condensation in this area. However, we believe that a similar mesenchymal re-modeling also occurs in that region. The prospective MEE marker in the very anterior region is mainly expressed on the interior surface facing the tongue with extension covering only a portion of the distal region, suggesting that the majority of the medial edge in the anterior region is also derived from the interior surface rather than the distal tip. In addition, previous carbon labeling experiments also suggested tissue re-modeling in the anterior palate shelf (Chou et al., 2004).

We then attempted to identify the candidate genes responsible for this outgrowth and *Snai1* came to our attention. *Snai1* has been reported to be expressed in the medial edge mesenchyme cells in the horizontal palatal shelves and its expression is regulated by TGF-β (Martinez-Alvarez et al., 2004; Pungchanchaikul et al., 2005), a signaling pathway that also interacts with *Zfhx-1a* (Nishimura et al., 2006). Recently, a study using Zebrafish embryos demonstrated that *Snai1* function can affect axial mesoendoderm cell migration during gastrulation (Blanco et al., 2007). Palatal outgrowth can be viewed as mesenchyme cell migration or movement. We therefore tested if *Snai1* expression is delayed in the development of *Zfhx-1a* mutant palates. As shown in figure 4, *Snai1* expression in wild type palates begins on E13.5, whereas *Zfhx-1a* mutant palates did not express *Snai1* until E14.5, 24 hours later than wild type embryos (Fig.4). Once *Snai1* expression initiates in *Zfhx-1a* mutant palates on E14.5, the intensity of expression is similar to wild type palates (Fig.4), consistent with a recent study reporting that *Snai1* expression level is not changed in *Zfhx-1a* mutant palate as measured by quantitative RT-PCR (Liu et al., 2008). Importantly, neural crest cell-specific inactivation of the *Snai1* gene on a *Snai2$^{-/-}$* mutant background leads to cleft palate due to failure of palate elevation without abnormal fusion between palate shelf and the tongue or other tissues, demonstrating the function of *Snail* genes in palate re-orientation (Murray et al., 2007). A recent study revealed Wnt5A mediated palate mesenchymal cell migration and loss of Wnt5A leads to the failure of palate re-orientation

in the posterior region, demonstrating the requirement of mesenchymal cell migration palate re-orientation (He et al., 2008).

As mentioned previously, palate re-orientation is the least understood process in palate development (Gritli-Linde, 2007). The assumption that the distal end of the vertical palatal shelf corresponds to the medial edge of the horizontal palatal shelf may have played a negative role in the study of palate re-orientation. In addition, our model explains how palate shelves overcome the physical obstruction of the tongue during re-orientation. Because it is an outgrowth from the side of the vertical shelf, it is not necessary to move the distal end above the dorsal level of the tongue. This is not to say that the forward displacement of the tongue is not important. We think that a combination of our model and the forward displacement of the tongue allow the palatal shelves to overcome the physical obstruction of the tongue during palatal re-orientation.

Experimental Procedures

Zfhx-1a and *Jag2* mutant mouse lines have been described previously (Jiang et al., 1998; Takagi et al., 1998). Mouse embryonic heads were dissected on gestation days 13.5 through 17.5 *post coitum* (the day when vaginal plugs were observed was designated as day 0.5 *post coitum*), placed in cold PBS and processed for HE staining or section in situ hybridization using digoxygenin-labeled antisense riboprobes as described by Shen (Shen, 2001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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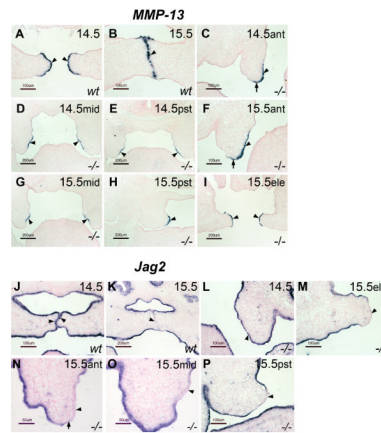
Figure 1. *MMP13* expression (A–I) and *Jag2* down-regulation (J–P) in *Zfhx-1a$^{-/-}$* embryos showing that the prospective MEE is located on the interior side of the vertical palate shelf

(A) *MMP 13* is expressed exclusively in the MEE of E14.5 wild type mouse palatal shelves (arrowheads). (B) *MMP13* expression remains in the MEE of wild type palates on E15.5 (arrowhead). (C) In the anterior region of E14.5 *Zfhx-1a$^{-/-}$* palates, *MMP13* expression is located primarily on the interior side of the vertical palate (arrowhead), with limited extension to a portion of the distal end (arrow). (D and E) In the middle (D) and posterior (E) regions of E14.5 *Zfhx-1a$^{-/-}$* palate, *MMP13* expression is located exclusively on the interior surface (arrowheads). (F) In the anterior region of unelevated E15.5 *Zfhx-1a* mutant palates, *MMP13* expression is located primarily on the interior surface of the vertical palate (arrowhead) with minimal extension to the distal end (arrow). (G) In the middle region of unelevated E15.5 *Zfhx-1a* mutant palates, *MMP13* expression is found exclusively on the interior side of the vertical palate (arrowheads). (H) The posterior region of unelevated E15.5 *Zfhx-1a* mutant palate is proceeding to a horizontal position and *MMP13* expression is located in the region corresponding to the MEE area (arrowhead). (I) *MMP13* expression is located exclusively in the MEE area of elevated E15.5 *Zfhx-1a* mutant palate (arrowheads). (J) *Jag2* is expressed in the epithelium surrounding the entire wild type palate shelf on E14.5 (arrowheads). (K) *Jag2* expression is specifically down-regulated in the MEE region in wild type palate around E15.5 (arrowhead). (L) *Jag2* expression surrounds the entire *Zfhx-1a$^{-/-}$* palate shelf on E14.5 (arrowhead). (M) In the elevated E15.5 *Zfhx-1a$^{-/-}$* palate, *Jag2* is also specifically down-regulated in the MEE area (arrowhead). (N) In the anterior region of the unelevated E15.5 *Zfhx-1a$^{-/-}$* palate, *Jag2* is down-regulated on the interior side (arrowhead) and a portion of the distal tip (arrow). (O) In the middle region of unelevated E15.5 *Zfhx-1a$^{-/-}$* palates, *Jag2* down-regulation occurs exclusively on the interior side of the vertical palatal shelf (arrowhead). (P) The posterior region of unelevated E15.5 *Zfhx-1a$^{-/-}$* palatal shelf is nearing a horizontal position and *Jag2* down-regulation is found in the region corresponding to the MEE area (arrowhead). **Ant:** anterior; **Mid:** middle; **Pst:** posterior; **Ele:** elevated.
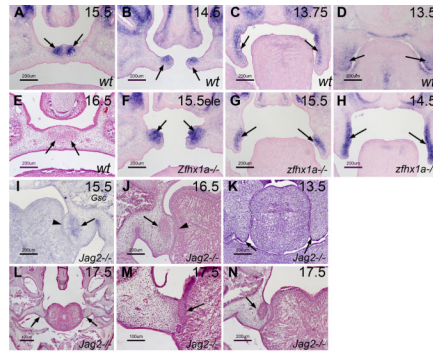
Figure 2. Mesenchymal cell condensation and palatal outgrowth during palate re-orientation
(A and B) *Goosecoid*-expressing cells in the horizontal palates of wild type E15.5 (A) and E14.5 embryos (B) are organized as a condensed block in the medial edge area (arrows). (C) *Goosecoid* expressing cells are formed as a band restricted to the interior side of wild type palate on E13.75 (arrows). (D) *Goosecoid*-expressing cells in E13.5 wild type palates are more widely distributed (arrows). (E) HE staining showing the high density of cells in the medial edge area (arrows). (F) *Goosecoid*-expressing cells are organized as a condensed block in the elevated E15.5 *Zfhx-1a*$^{-/-}$ mutant palates (arrows). (G and H) *Goosecoid*-expressing cells in uneleveted E15.5 (G) and E14.5 (H) *Zfhx-1a* mutant palates are confined in a strip restricted to interior side, similar to E13.75 wild type palates. (I) *Goosecoid* expression showing condensed cells (arrow) formed on the interior side of E15.5 *Jag2*$^{-/-}$ palatal shelves. (J) HE staining showing the formation of a condensed cell block (arrow) on the interior side of E16.5 *Jag2*$^{-/-}$ palates. (K) *Jag2*$^{-/-}$ palates on E13.5 showing that the distal end is not in contact with the tongue (arrow). (L–N) Whole view (L) and high power views (M and N) of HE stained E17.5 *Jag2*$^{-/-}$ palates showing a horizontal-like palate (arrows in L) with the MEE region fused with the tongue (arrow in M) and a condensed cell block located on the interior side (N).
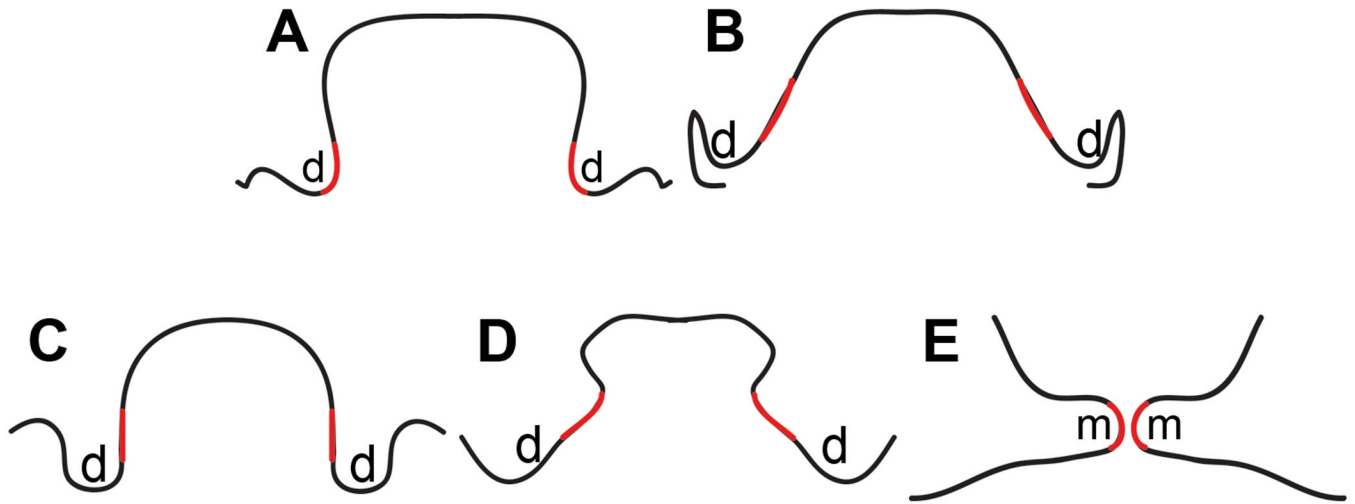
Figure 3. A proposed model for palate re-orientation
The prospective and definitive MEE is shown in red. (A–C) Anterior (A), posterior (B) and middle (C) regions of E13.5 palate. (D) The middle region of palate at an intermediate stage between E13.75 and E14.5. (E) The middle region of E14.5 palate. **d:** distal end; **m:** medial edge area.
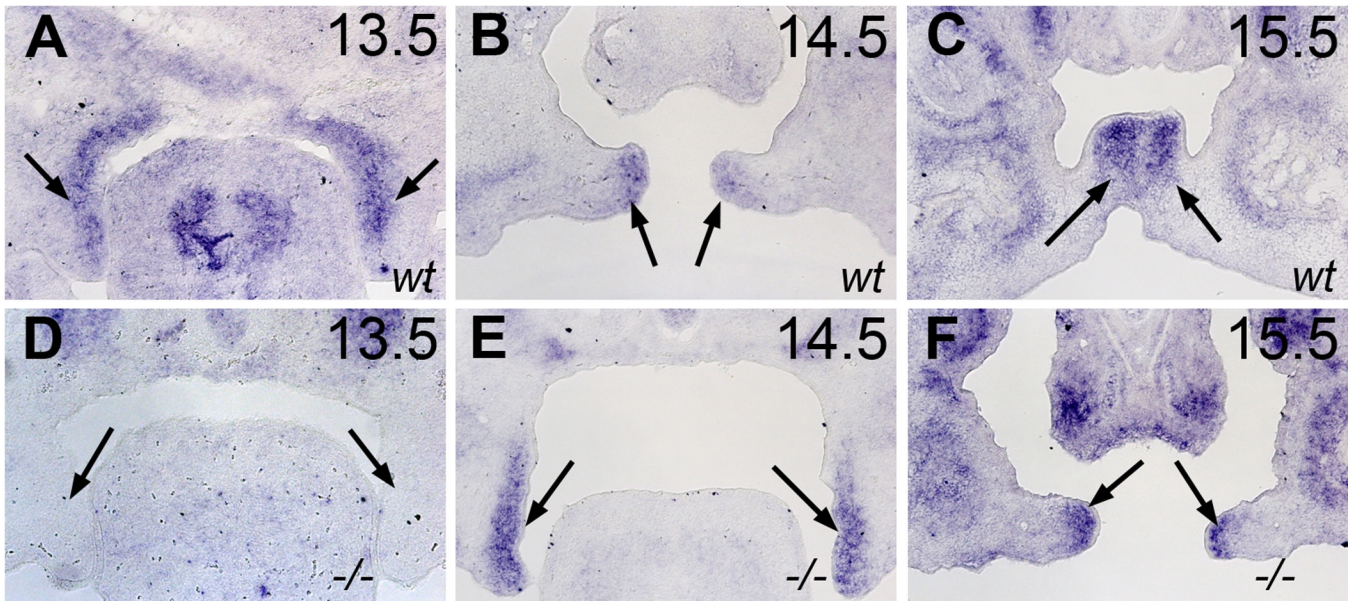
Figure 4. In situ hybridization showing the expression of *Snai1* in *Zfhx-1a*$^{-/-}$ palates is delayed by 24 hours compared wild type palates
Snai1 expression is detected in wild type palatal mesenchymal cells from E13.5 through E15.5 (A–C). *Snai1* expression is not found in E13.5 *Zfhx-1a*$^{-/-}$ palates, but is present in *Zfhx-1a*$^{-/-}$ palates on E14.5 and E15.5.