Tbx1 regulates oral epithelial adhesion and palatal development

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Cleft palate, the most frequent congenital craniofacial birth defect, is a multifactorial condition induced by the interaction of genetic and environmental factors. In addition to complete cleft palate, a large number of human cases involve soft palate cleft and submucosal cleft palate. However, the etiology of these forms of cleft palate has not been well understood. T-box transcriptional factor (Tbx) family of transcriptional factors has distinct roles in a wide range of embryonic differentiation or response pathways. Here, we show that genetic disruption of Tbx1, a major candidate gene for the human congenital disorder 22q11.2 deletion syndrome (Velo-cardio-facial/DiGeorge syndrome), led to abnormal epithelial adhesion between the palate and mandible in mouse, resulting in various forms of cleft palate similar to human conditions. We found that hyperproliferative epithelium failed to undergo complete differentiation in Tbx1-null mice (Tbx1−/−). Inactivation of Tbx1 specifically in the keratinocyte lineage (Tbx1KCKO) resulted in an incomplete cleft palate confined to the anterior region of the palate. Interestingly, Tbx1 overexpression resulted in decreased cell growth and promoted cell-cycle arrest in MCF7 epithelial cells. These findings suggest that Tbx1 regulates the balance between proliferation and differentiation of keratinocytes and is essential for palatal fusion and oral mucosal differentiation. The impaired adhesion separation of the oral epithelium together with compromised palatal mesenchymal growth is an underlying cause for various forms of cleft palate phenotypes in Tbx1−/− mice. Our present study reveals new pathogenesis of incomplete and submucous cleft palate during mammalian palatogenesis.

INTRODUCTION

Tbx1, a member of the T-box family of transcription factors, has been identified as a major candidate gene for 22q11.2 deletion syndrome (Velo-cardio-facial/DiGeorge syndrome). 22q11.2 deletion syndrome is a common congenital disorder affecting approximately 1 in 4000 live births, characterized by craniofacial defects, thymic hypoplasia, cardiovascular anomalies, velopharyngeal insufficiency and skeletal muscle hypotonia (1–4). The craniofacial malformations occur in ~60% of 22q11.2 deletion syndrome patients (5). The most frequent features include microglossia, ear abnormalities, blunted nose, hypertelorism, complete cleft palate, submucosal and soft palate cleft (5) and single central incisor (6). In humans, Tbx1 mutation is responsible for the major phenotypes of 22q11.2 deletion syndrome as well as non-syndromic submucous cleft palate (7,8), suggesting that Tbx1 is a regulator of palatogenesis. It is reported that homozygous-null mice of Tbx1 have most features of 22q11.2 deletion syndrome, including cleft palate, microglossia and ear abnormalities (1,9); however, the etiology of cleft palate in Tbx1−/− mice and biological roles of Tbx1 in palatal epithelium have not been determined.

Cleft palate is the most frequent congenital craniofacial birth defect in the human population, occurring in 1 in 500 to 1 in 1000 births worldwide (10). Higher incidences of cleft palate are reported in offspring of individuals with cleft palate than in those without a family history (11), suggesting
a multifactorial etiology, involving both genetic and environmental factors. Cleft palate as part of a syndrome accounts for 55% of reported cases (12) and several cleft-causing mutations have been identified in the IRF6, TP63, MSX1, PVRL1, and Tbx22 genes (13–19). In humans, cleft palate manifests as cleft lip and/or palate. In addition to isolated cleft palate, a large number of cleft palate cases include incomplete cleft palate, soft palate cleft and submucosal cleft palate, and the underlying mechanism for each cleft appears to be distinct. As initial palatal closure occurs in the anterior third of the palatal shelves, the mildest form of cleft palate in humans is bifid uvula or soft palate cleft (20). Cleft palate occurs in a total of 17 cases (27). Eight (47%) had anterior cleft, which was present at the junction between the primary and secondary palates, and an incomplete cleft palate (Fig. 1Ac). Two (12%) showed anterior cleft and soft palate cleft (Fig. 1Ad). In wild-type and Tbx1+− mice, none of the newborns had any type of cleft palate (Fig. 1Aa and data not shown). Alcian blue and alizarin red staining demonstrated that despite the membranous fusion of the palatal shelves, 59% (10 of 17) of Tbx1−− mice had submucosal cleft palate (Fig. 1Ag and Ah). These observations are in accordance with 22q11.2 deletion syndrome phenotype in humans, which includes soft palate cleft and submucosal cleft palate. 22q11.2 deletion syndrome phenotype also includes single central incisor (6). Interestingly, the upper incisors were absent in 30% of Tbx1−− mice (Fig. 1Af and Ah and data not shown).

RESULTS

Tbx1 expression is confined to the epithelium of the developing palatal shelves

The abnormal epithelium observed in Tbx1−− mice suggested that cleft palate may arise from a primary defect in the palatal epithelium. To determine whether Tbx1 was expressed in a pattern compatible with such a phenotype, whole-mount in situ hybridization was performed using wild-type embryos between E12.5 and E15.5. At E12.5, Tbx1 expression appeared in the posterior edge of the palatal tissue folds (Fig. 2Aa). At E13.5, Tbx1 was highly expressed in both the anterior and posterior edges of the paired palatal shelves (Fig. 2Ab). High Tbx1 expression was also seen in the hair follicles and tooth germs (Fig. 2Ab). At E14.5, Tbx1 expression was somewhat downregulated in the fused palate (Fig. 2Ac) and subsequently disappeared by E15.5 (Fig. 2Ad). We also analyzed Tbx1 expression in E13.5 palatal shelves by section in situ hybridization. Tbx1 was detected in the oral epithelium, particularly in the palatal shelves, floor of the mouth and the tongue, but not in the mesenchyme of the palate (Fig. 2Ba). Tbx1 was also expressed in the epithelium of early facial processes (data not shown), including developing tooth germs as previously reported (28,29).
Figure 1. Phenotypic analysis of Tbx1<sup>1−/−</sup> mutant mice. (A) View of the palate from wild-type and Tbx1<sup>1−/−</sup> mice. (a–d) Ventral view of the maxilla of newborn wild-type (a) and Tbx1<sup>1−/−</sup> mutants (b–d). Wild-type palate was completely closed with clear symmetrical rugae (a). Tbx1<sup>1−/−</sup> mice showing various palatal fusion defects (b–d), which include complete cleft palate (b), incomplete cleft palate with anterior cleft (c) and soft palate cleft associated with anterior cleft (d). An anterior cleft (arrowhead in c and d) is present at the junction between the primary and secondary palates. Arrows point to posterior cleft. pp, primary palate; sp, secondary palate; pt, pterygoid bone; ns, nasal septum. (e–h) Ventral view of the skull of newborn wild-type (e) and Tbx1<sup>1−/−</sup> mutants (f–h) stained with alcian blue and alizarin red. Fusion of the bilateral palatine processes (pa) observed in the wild-type (e, dashed line) is absent in the Tbx1<sup>1−/−</sup> mutants (f–h, dashed lines). Note the visible presphenoid bone (ps). The palatal shelves of maxilla (mx) in the Tbx1<sup>1−/−</sup> mutants with complete cleft palate (indicated oval dashed line in f) failed to grow toward the midline. Basisphenoid (bs) and basioccipital bones (bo) are deformed in the mutants (f–h). ic, upper incisor. Table: diagnosis and frequency of each palatal fusion phenotype. (B) H&E staining of sagittal sections of the head from P1 wild-type (a, b) and Tbx1<sup>1−/−</sup> mutants with complete cleft palate (d, e). Asterisk indicates palatal defect. Tags of epithelial tissue adhere to the surface of palatal epithelium (arrow in e). Thickened epithelium on the surface of the palate was also observed (arrowhead in e). (c, f) Higher magnification of the palatal epithelium. Note that the thickened Tbx1<sup>1−/−</sup> epithelium lacks distinct layers (f) seen in the wild-type epithelium (c). Epithelial nests are observed in the mesenchymal stroma (arrows in f). pp, primary palate; sp, secondary palate; ns, nasal septum; t, tongue; str, stroma; epi, epithelium; BL, basal layer; SP, spinous layers; GR, granular layers. Scale bars: a, b, d, e, 400 μm; c, f, 10 μm.
Conditional analysis of Tbx1 in the epithelium

To further study the role of Tbx1 in the keratinocyte lineage, we crossed embroy (Fig. 4Ae and Ag), the palate of palatal epithelium disappeared in the E15.5 wild-type binase. Although the palatal shelves had fused and the marker for keratinocytes upon recombination by Cre recom-

resulting embryos, 

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nations. For this purpose, we used distribution of keratinocytes, as they move toward their desti-

Figure 2. Tbx1 expression is confined to the palatal epithelium. (A) Whole-mount in situ hybridization analysis of Tbx1 expression in E12.5 (a), E13.5 (b), E14.5 (c) and E15.5 (d) palates. At E12.5, Tbx1 expression appears in the most posterior domain of the palatal tissue folds (arrowheads in a). At E13.5, high Tbx1 expression is observed in the palatal shelves (ps), upper incisor tooth germs (ic) and hair follicles. In the palate, strong expression is observed in the medial and posterior edge of the secondary palate before and during fusion (arrowheads in b and c). (B) Section in situ hybridization analysis of Tbx1 expression at E13.5 (a). Tbx1 expression is detected in the oral epithelium of the palatal shelves (p), floor of the mouth and the tongue (t). Adjacent coronal sections are stained with hematoxylin (b). Scale bars, 200 μm.

Tbx1−/− mutants exhibit impaired palatal shelf growth and intraoral epithelial fusion

To pinpoint which palatal developmental processes require Tbx1 gene function, we examined the palatal shelves of wild-type and null embryos from E13.5 to E16.5. As shown in Fig. 4A, at E13.5, palatal outgrowth and initial downward growth were observed in the wild-type (Fig. 4Aa–c). In con-

contrast, retardation of the palatal shelves and anomalous intraoral epithelial adhesion were observed in the posterior domain of the Tbx1−/− palate (Fig. 4Ah–k). At E14.5, the wild-type palatal shelves were elevated above the dorsum of the tongue and coalesced at the midline along the anteroposterior axis (Fig. 4Ba–d). The anterior palatal shelves of Tbx1−/− were elevated over the tongue, but failed to appose at the midline (Fig. 4Be and Bf). In Tbx1−/− posterior palatal shelves, abnormal adhesions between the palatal epithelium and the oral surface of the palate were noted (Fig. 4Bg). More evidence of a disordered epithelium in Tbx1−/− mutants was exemplified by the occurrence of epithelial outgrowths on the palatal shelves and mandible (Fig. 4Aj, Ak and Bh), as well as patches of thickened epithelium on the oral surface of the palatal shelves (arrows in Fig. 4Bf). The fused epithelium was disorganized and unevenly formed (Fig. 4Bh). By E16.5, wild-type palatal shelves were completely fused (Fig. 4Ca). In the Tbx1−/− mutants, most abnormal adhesions broke after the palatal shelves elevated above the tongue (Fig. 4Cc). In a subset of embryos, persistent intraoral epithelial adhesions with thickened epithelium were observed (Fig. 4Cc and d). Restricted expression of Tbx1 to the oral epithelium coupled with oral adhesion seen in the Tbx1−/− mutants indicates that dysregulation of epithelial adhesion-separation behavior may be a cause of cleft palate in Tbx1−/− mice.

Absence of Tbx1 results in hyperplasia of the epithelium at the early stage of epithelial differentiation

Next, we focused on elucidating the underlying mechanism of epithelial defects, which were dramatic and largely unexpect-

ed. Development and subsequent maintenance of the epithelium depends on the intricate balance between proliferation and differentiation of a resident stem-cell population (34,35).
To determine whether the expanded suprabasal layer resulted from increased cell proliferation and/or decreased apoptosis, we examined phosphorylated-Histone H3 (pH3) levels and performed TdT-mediated dUTP nick end labeling (TUNEL) analysis, respectively. At E14.5, the adhered epithelium was thickened, with pH3-positive cells extending into the suprabasal layers in the Tbx1<sup>−/−</sup> embryo, whereas proliferating cells in the wild-type epithelium were confined to the basal layer (Fig. 5Aa and Ab, and Supplementary Material, Fig. S2). These results suggest that abundant suprabasal cells had retained their proliferative potential in Tbx1<sup>−/−</sup> oral epithelium. An average mitotic index in the Tbx1<sup>−/−</sup> embryo was significantly higher than that of the wild-type at E14.5 (Fig. 5C, *P*, 0.05), suggesting that the adhered Tbx1<sup>−/−</sup> epithelium was hyperproliferative. TUNEL-positive cells were hardly detected in the developing palatal epithelium of wild-type or Tbx1<sup>−/−</sup> embryos at E14.5 (Fig. 5D). We further characterized the oral epithelium by immunostaining with antibodies to keratin 14 (K14), a marker for the basal layer (Fig. 5Ac). Although K14 was confined to the basal layer in the palatal epithelium of wild-type mice (Fig. 5Ac), it was extended to the surface of the Tbx1<sup>−/−</sup> epithelium (Fig. 5Ad). Although Tbx1<sup>−/−</sup> epithelium was thickened and showed signs of early differentiation (Fig. 5Ad), late differentiation did not occur (data not shown). These results suggest that increased proliferation of basal cells contributed to an increase in the thickness of the Tbx1<sup>−/−</sup> epithelium.

As we observed the retarded palatal growth in Tbx1<sup>−/−</sup> embryos at E13.5 (Fig. 4A), mesenchymal cell proliferation and apoptosis were also examined. Although Tbx1 is localized only in the epithelium of the palatal shelf (Fig. 2B), a significant decrease in cell proliferation, assayed by pH3 staining, was detected in the mesenchyme of the Tbx1<sup>−/−</sup> palate (Supplementary Material, Fig. S3, *P* < 0.05), consistent with the previous report (36). No increase in apoptosis was found in the palatal shelves of Tbx1<sup>−/−</sup> embryos at E13.5 (data not shown). Retarded palatal shelf growth due to decreased cell proliferation in the palatal mesenchyme suggests that epithelial Tbx1 directly or indirectly induces a secreted factor that mediates cell proliferation in the palatal mesenchyme.

**Absence of Tbx1 results in an incomplete terminally differentiation of keratinocytes**

To examine the late differentiation of palatal epithelium, we examined pH3 levels and performed TUNEL analysis at...
E18.5. pH3 immunostaining and TUNEL staining showed no significant differences between the wild-type and Tbx1−/− palatal epithelium at E18.5 (Fig. 5Ba, d, C and D). Consequently, we analyzed E18.5 embryos using a panel of antibodies raised against proteins expressed at defined stages of epithelial differentiation. K14, which is confined to the basal and suprabasal layer in wild-type mice (Fig. 5Be), was expressed ectopically in the granular layer of the epithelium from Tbx1−/− mice (Fig. 5Bf), suggesting that a loss of Tbx1 failed to silence K14 in the granular layer. In contrast, loricrin and filaggrin, which are present in the keratohyalin granules of the granular cells, were expressed in the granular layer of the wild-type oral epithelium (Fig. 5Bg and Bi), whereas expression of filaggrin-positive cells were seen in the patches in the Tbx1−/− epithelium (Fig. 5Bh). Especially, expression of loricrin in the Tbx1-deficient epithelium was reduced considerably (Fig. 5Bj). In the head skin, immunostaining of epithelial differentiation markers showed no
differences between wild-type and Tbx1<sup>2/2</sup> embryos at P1 (data not shown). These data suggest that hyperproliferation and incomplete terminal differentiation of keratinocytes contributes to the abnormal oral epithelial morphogenesis in Tbx1<sup>2/2</sup> mice.

Expression of genes implicated in palatogenesis in Tbx1<sup>2/2</sup> mutants

Fusion between unelevated palatal shelves and the tongue was previously observed in mice deficient for Jag2, which encodes a cell surface ligand for the Notch family receptors (37). Jag2 is downregulated in the palatal shelves of Fgf10<sup>2/2</sup> mice, which develop complete cleft palate and aberrant adhesion of the palatal shelves (38). Tbx1 regulates Fgf10 expression in the mesodermal core of the mandibular arch and the secondary heart field (34,35). However, expression of Jag2 and Fgf10 were normal in the Tbx1<sup>2/2</sup> palate (Fig. 6A), indicating that these genes are not downstream effectors of Tbx1 in the palatogenesis. The Eph family of receptor tyrosine kinases and their membrane-anchored ephrin ligands are a large group of highly conserved molecules that function in diverse cell-cell adhesion and fusion events, including axon pathfinding and formation of the cardiovascular network (39). As EphB2/EphB3 double mutants and ephrinB1-null mice have a cleft palate phenotype (39–43), we hypothesized that Tbx1 might regulate these genes. However, expression of ephrinB1, ephrinB2, EphB2 and EphB3 appeared normal in the Tbx1<sup>−/−</sup> palate at E13.5 (Fig. 6B), indicating that these genes are not downstream effectors of Tbx1 in palatal development.

The defects in palate development (Fig. 4A) and the reduction in cell proliferation in the palatal mesenchyme in
Tbx1\(^{-/-}\) mutants (Supplementary Material, Fig. S3) indicated that dynamic molecular changes occurred during palate development. To establish a genetic hierarchy between Tbx1 and genes implicated in palatal development, we also examined the expression of a number of candidate genes at E13.5. Bmp4 signaling is required for normal cell proliferation and survival in the developing secondary palate, and application of exogenous Bmp4 can stimulate cell proliferation in the palatal mesenchyme (33, 44). Tbx1 interacts with Smad1 and suppresses the Bmp4/Smad1 signaling (45). In the Tbx1\(^{-/-}\) palatal shelves, Bmp4 was upregulated in the posterior regions (100%, \(n = 3\)) (Fig. 6C). Bmp-mediated signaling has been shown to repress expression of Pax9 and Barx1 in the palate and mandible, and mutations in Pax9 lead to cleft palate and adontogenesis (46–48). Pax9 expression is previously shown to be downregulated in the pharyngeal region of Tbx1\(^{-/-}\) embryos (49). Whole-mount in situ hybridization showed that Pax9 expression domain was moderately reduced in the posterior palatal shelves of Tbx1\(^{-/-}\) mutants (100%, \(n = 3\)), whereas expression of Barx1 was unaltered (Fig. 6C). We cannot exclude the possibility that the increase in Bmp4 signals is due to an increase in the number of cells expressing Bmp4, as the Tbx1 expression domain does not completely overlap with that of Bmp4 or Pax9 (Fig. 2Ab and Fig. 6Ci). However, Tbx1\(^{-/-}\) embryos exhibit decreased level of mesenchymal proliferation in middle and posterior regions of palatal shelves (Supplementary Material, Fig. S3). Together with the notion that Tbx1 may suppress Bmp4 signaling directly (45) or indirectly (50), it is likely that a loss of Tbx1 indirectly restricts expression of Bmp4 and Pax9 in the palatal shelves.

The Msx1 homeobox gene, in which mutation leads to a complete cleft palate, is exclusively expressed in the anterior mesenchymal cells of the developing palate (33). As expected, expression of Msx1 was not observed in the posterior palatal shelves in either wild-type or Tbx1\(^{-/-}\) mutants (Fig. 6C). Tbx1 regulates expression of Pitx2, a paired-bisoid homeobox gene, and Fgf8 in the secondary heart field (9, 51) and Pitx2 positively regulates Fgf8 and restricts Bmp4 in oral ectoderm (52). However, our results demonstrated normal levels of

**Figure 6.** Expression of genes implicated in palatogenesis in Tbx1\(^{-/-}\) mutants. (A) Analysis of Jag2 and Fgf10 in Tbx1\(^{-/-}\) embryos. Expression of Jag2 (a, c) and Fgf10 (b, d) was detected by whole-mount in situ hybridization of palates from wild-type (a, b) and Tbx1\(^{-/-}\) (c, d) littermates at E13.5. Jag2 and Fgf10 are similarly expressed in the palatal shelves of both wild-type and Tbx1\(^{-/-}\) mutant embryos. ps, palatal shelf; ic, upper incisor bud. (B) Transcripts for indicated markers of palatogenesis were detected by whole-mount in situ hybridization of wild-type (top row) and Tbx1\(^{-/-}\) mutants (bottom row) at E13.5. Note that the palatal shelves are shortened in the Tbx1\(^{-/-}\) mutants. Expression of ephrinB1 (a, c, e), ephrinB2 (b, d, f), EphB2 (g, h) and EphB3 (i, j) is not altered in the Tbx1\(^{-/-}\) embryos. ps, palatal shelf; ic, upper incisor bud. (C) Transcripts for indicated markers of palatogenesis were detected by whole-mount in situ hybridization of wild-type (top row) and Tbx1\(^{-/-}\) mutants (bottom row) at E13.5. Bmp4 expression is upregulated in Tbx1\(^{-/-}\) posterior palatal shelves (arrowheads in h). Pax9 expression domain is reduced in the Tbx1\(^{-/-}\) posterior palatal shelves (brackets in i). Note that the palatal shelves are shortened in the Tbx1\(^{-/-}\) mutants. Expression of Msx1, Barx1, Pch1, Pitx2 and Fgf8 is not altered in the Tbx1\(^{-/-}\) embryos. ps, palatal shelf; ic, upper incisor bud.
expression of Pitx2 and Fgf8 in the Tbx1−/− palatal shelves (Fig. 6C). Although Pch1 is implicated in craniofacial development (53), comparable levels of Pch1 expression was found in wild-type and Tbx1−/− embryos (Fig. 6C).

Overexpression of Tbx1 resulted in a decreased cell proliferation and promoted cell-cycle arrest

Absence of Tbx1 results in hyperplasia of the oral epithelium. To postulate that Tbx1 is an important component of cell-cycle regulation, we examined the effects of ectopic expression of Tbx1 on epithelial cell proliferation, using human epithelial cell line MCF7 cells. MCF7 cells do not express readily detectable amount of endogenous Tbx1 (Fig. 7A). The adenovirus-mediated Tbx1 expression was verified in MCF7 by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR; Fig. 7A). The nuclear localization of Tbx1 was confirmed using immunofluorescent staining (data not shown). Proliferation assays comparing Tbx1-transfected MCF7 cells with control enhanced green fluorescent protein (EGFP)-transfected cells revealed a reduction in total cell numbers following adenovirus infection (Fig. 7B). Because of the decreased growth rate found in the Tbx1-expressing cell lines, we examined the effect of Tbx1 expression on the cell-cycle kinetics, measuring DNA content by flow cytometer. There was an increased accumulation of cells in G1 phase in Tbx1-expressing cells (46.3%) at 96 h, compared with the EGFP-expressing control cells (37.7%), representing a trend toward a more quiescent cell-cycle profile (Fig. 7C, Supplementary Material, Fig. S4).

DISCUSSION

The process of palatogenesis involves the delicate integration of several stages, each under the influence of genetic and environmental factors. We have now shown that Tbx1−/− mice exhibit various forms of cleft palate phenotypes, including submucosal cleft palate and soft palate cleft. The cleft
palate phenotypes in the Tbx1<sup>−/−</sup> mice are clearly different from those previously reported in genetically mutated mice. Tbx1<sup>−/−</sup> mice also represent a unique model for studying the etiology of submucosal cleft palate. The phenotypic variation in the Tbx1<sup>−/−</sup> mutants strongly suggests the effects of stochastic factors and/or modifier genes in Tbx1 function. Cleft palate observed in Tbx1<sup>−/−</sup> mice is likely to result from abnormalities in the adhesion-separation behavior of the oral epithelium and palatal mesenchyme proliferation. Although it is unclear whether hyperproliferation and inhibition of complete terminal differentiation of the palatal epithelium is a primary or secondary defect, ablation of Tbx1 results in the self-adhesiveness of the oral epithelia, which induces cleft palate by inhibiting or delaying palatal elevation. These findings provide insights into the pathogenesis underlying the variety of cleft palate phenotypes observed in human patients.

**Oral adhesions in Tbx1<sup>−/−</sup> embryos**

A remarkable finding of the present study is that absence of Tbx1 profoundly changes the dynamics of the oral epithelium. Defects in cell behavior led to uncontrolled growth of keratinocytes and appearance of large epithelial masses in the oral cavity. As adhered Tbx1<sup>−/−</sup> epithelium was disorganized, abnormal oral adhesion may be induced by distorted cell polarity with cell division. Tbx1<sup>−/−</sup> mutants showed early differentiation as well as signs of impaired terminal differentiation of the epithelium. As the elevation of palatal shelves are inhibited by oral adhesion, prolonged adhesion-activated signals may alter the late differentiation of the epithelium from Tbx1<sup>−/−</sup> mice. It is also possible that signaling from the mesenchyme, which is directly or indirectly modulated by epithelial Tbx1, may influence the late stage of epithelial differentiation.

In the Tbx1<sup>−/−</sup> palatal epithelium, pH3 and K14 staining delineated the expansion of overlying epithelium. These findings suggest that dysregulation of basal keratinocytes in early palatal development may be an underlying cause for abnormal fusion of the oral epithelium. Interestingly, mice homozygous for a null allele of Irf6 also have a hyperproliferative epithelium, resulting in soft tissue fusions (26,54). Tbx1<sup>−/−</sup> mice failed to undergo complete differentiation similar to Irf6 mutant mice, although the phenotype of Tbx1<sup>−/−</sup> mice was mild. Several mutant mice, including Jag2<sup>−/−</sup> (37) and Fgf10<sup>−/−</sup> mice (55), are also reported to develop abnormal oral epithelial fusion causing mechanical hindrance to palatal shelf elevation. However, the fusion phenotype in these mutants is different from that of Tbx1<sup>−/−</sup> mice in several ways. In Fgf10<sup>−/−</sup> mice, the adhesions are unilateral and involve only the oral epithelium. Also, increased apoptosis is observed in the oral epithelium. Jag2<sup>−/−</sup> mice exhibit fusion of the palatal epithelium predominantly with tongue but the organization of palatal epithelium itself remains intact (37,56). Neither mouse shows over-proliferation of the epithelium. The expression domains of Jag2 and Fgf10 are also different from Tbx1; Jag2 is expressed in the epithelium of anterior palatal shelves, and Fgf10 in the palatal mesenchyme (37,55). These observations suggest that a different mechanism underlies palatal epithelial fusion in Tbx1<sup>−/−</sup> mice.

**Tbx1<sup>KCKO</sup> mutant mice exhibit an anterior cleft palate**

Consistent with the Tbx1 expression in anterior palatal shelves, Tbx1<sup>KCKO</sup> mutant mice show an anterior cleft palate. Although the anterior cleft palate was rare both in genetically engineered and naturally occurring mutant mice reported previously, the phenotype was observed in 12% of Tbx1<sup>−/−</sup> mice and 100% of Tbx1<sup>KCKO</sup> mutant mice. The phenotype of Tbx1 mutant mice suggests Tbx1 a potential candidate gene for this rare type of cleft.

In all experiments, a Tbx1<sup>KO</sup> allele was introduced to increase the efficiency of Cre-mediated recombination; however, the phenotype of Tbx1<sup>KCKO</sup> mutant mice was not as severe as that of Tbx1<sup>−/−</sup> mice. β-Galactosidase activity patterns in K14-Cre;R26R mice and the K14 expression in the oral epithelium indicated that the palatal epithelium was a descendant of K14-positive cell lineage. As inactivation of Tbx1 in the pharyngeal endoderm results in complete cleft palate (57), mild palatal phenotype in Tbx1<sup>KCKO</sup> mutant mice may be due to the relatively late activation of Cre-induced recombination of the Tbx1-foxed allele in keratinocytes. In addition, we cannot rule out the possibility that K14-Cre might be expressed at reduced levels in transgenic animals. It is also possible that Tbx1 expression in other tissues may involve the palatal phenotype of Tbx1<sup>−/−</sup> mice. Tbx1 is expressed in the digastric muscle and masseter muscle (Supplementary Material, Fig. S5), and branchiomeric muscle development is severely perturbed in Tbx1 mutant mice (58). As Tbx1 regulates the onset of branchiomeric myogenesis, Tbx1 may also be involved in the elevation of palatal shelf indirectly.

**Tbx1-expressing cells negatively modulate proliferation and cell-cycle progression**

Coordination between proliferation and differentiation is critical for proper palatal development and epithelial differentiation. Tbx1<sup>−/−</sup> mice have abnormal epithelial adhesions and hyperproliferative epithelium between the palate and mandible at the early stage of palatogenesis. These findings support a putative role for Tbx1, in regulating cell growth and promoting the early phase of differentiation of oral epithelial cells. The hypothesis is consistent with the findings that overexpression of Tbx1 in MCF7 cells results in decreased proliferation. The result is also in line with a recent report, which demonstrates that ectopic Tbx1 expression resulted in decreased cell growth and reduced development into multilayered colonies of mouse spindle carcinoma cells (59). The ability of overexpressed Tbx1 to reduce cell proliferation in MCF7 cells suggests that Tbx1 negatively modulates cell-cycle progression by promoting entry into the G1 phase, thereby promoting cellular differentiation of epithelial cells.

A question remains regarding the link between Tbx1 and cell-cycle. We did not obtain any evidence to support that Tbx1 regulates cyclin-dependent kinase inhibitor, Cdkn1a/p21, Cdkn1c/p57 or E2F promoters (N.F., unpublished data). It is therefore possible that cell-cell adhesion in the absence of Tbx1 may induce adhesion-activated signals, causing
hyperproliferation of keratinocytes and leading to an abnormal differentiation of the epithelium.

**Tbx1 is an intrinsic regulator of secondary palate development**

In the present study, we have shown that Tbx1 expression is specifically activated in the palatal shelf epithelium at the onset of palatal outgrowth prior to elevation and persists until fusion is complete in both anterior and posterior directions. The Tbx1 expression in the palatal shelves and Tbx1\(^{-/-}\) palatal phenotype make Tbx1 a potential candidate gene for submucosal cleft palate and incomplete cleft palate cases in humans. For a gene to be a strong non-syndromic cleft palate candidate, it must show a relevant spatiotemporal gene expression pattern during palatal development and cause a strong phenotype when mutated (11).

Recent studies have provided evidence that several genes responsible for Mendelian syndromic forms of cleft palate may also play an important part in the etiology of non-syndromic cleft palate (60). Mutations in the TP63 gene found in five human malformation syndromes can also be found in patients with only isolated cleft palate (14). Although cleft lip/palate-ectodermal dysplasia 1 (CLPED1), an autosomal recessive syndrome, was shown to have mutations in the poliovirus receptor related-1 (PVRL1) gene (15), heterozygous PVRL1 mutation was also identified in non-syndromic cleft palate (17). This is similarly true for cleft palate in Tbx22 mutations with X-linked inheritance (CPX) where the ankyloglossia may be mild or overlooked (16,61), or for IRF6 mutations in Van der Woude syndrome without penetrance of the lip pit phenotype (18). Indeed, Tbx1 gene mutation or 22q11.21 deletion was reported in patients with incomplete cleft palate without clinical diagnosis of 22q11.21 deletion syndrome (7,8). Thus, Tbx1 may be one of candidate genes for non-syndromic cleft palate, especially incomplete cleft palate and submucosal cleft palate.

In summary, our study shows a new role for Tbx1 in mammalian secondary palate development, controlling the adhesion-separation behavior of oral epithelial cells. Our data reveal that loss of Tbx1 not only affects proliferation and differentiation of oral epithelial cells, but also results in abnormal adhesion of the oral epithelium. Tbx1\(^{-/-}\) mice represent a unique model for studying the etiology of cleft palates, as the oral adhesion could be one of the environmental/stochastic causes which contribute to the variety of cleft palate phenotypes. Understanding the palatal epithelial functions during palatal development may lead to invention of novel therapeutic and/or diagnostic methods and tools for cleft palates.

**MATERIALS AND METHODS**

**Mouse strains**

Tbx1\(^{loxP/loxP}\), Wnt1-Cre (The Jackson Laboratory, Bar Harbor, Maine), K14-Cre (The Jackson Laboratory), Twist2-Cre (The Jackson Laboratory), R26R (The Jackson Laboratory), Tbx1-Cre and More-Cre (a kind gift from Dr M. Tallquist) mice have been described (9,62,63). Wild-type littermates were used as controls. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center and Tokyo Medical and Dental University.

**Cartilage and bone staining**

Embryonic or newborn mice were skinned, eviscerated and fixed in 95% ethanol. Skeletal preparations were stained using alcian blue for cartilages and alizarin red for bones, as described previously (64).

**Histology and TUNEL staining**

Embryos were harvested and fixed in 4% paraformaldehyde at 4°C overnight. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E) for routine histology. TUNEL staining was performed according to the manufacturer’s instructions (Roche). TUNEL-positive cells were quantified (average number per 400 μm distance) in epithelium of wild-type and Tbx1\(^{-/-}\) posterior palatal shelves at E14.5 and E18.5. More than five sections were analyzed per palate (n = 3 per genotype).

**Section in situ hybridization**

E13.5 embryos were fixed in 4% paraformaldehyde at 4°C overnight. Riboprobes for Tbx1 were labeled with \(^{35}\)S-UTP (Amersham, GE Healthcare, Piscataway, NJ) using the MAXIscript in vitro Transcription Kit (Ambion, Life Technologies, Grand Island, NY). In situ hybridization of sectioned tissues was performed as previously described (65).

**β-Galactosidase staining**

For whole-mount staining, E15.5 embryos were fixed in 4% paraformaldehyde/lacZ rinse buffer (0.2 mM sodium phosphate, pH 7.3, 2 mM MgCl\(_2\), 0.02% NP-40, 0.01% sodium deoxycholate) at 4°C for 1 h, washed in lacZ rinse buffer and stained in lacZ rinse buffer containing 5 mM K\(_4\)Fe(CN)\(_6\), 5 mM K\(_3\)Fe(CN)\(_6\) and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at room temperature for overnight. Embryos were postfixed in 4% paraformaldehyde/phosphate-buffered saline at 4°C overnight. For histology, stained embryos were dehydrated in alcohol, and cleared in xylene. Then they were embedded in paraffin and sectioned into 5 μm thickness and counterstained with eosin.

**Immunohistochemistry**

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Primary antibodies were incubated at 4°C overnight at the following dilutions: phospho-histone 3 (pH3) (rabbit, 1:100; Upstate, Millipore, Billerica, MA), keratin 14 (rabbit, 1:1000; Covance, Princeton, NJ), filaggrin (rabbit, 1:1000; Covance) and loricrin (rabbit, 1:1000; Covance). Secondary antibodies coupled to fluorescein isothiocyanate (Vector, Burlingame, CA) were diluted 1:200 and incubated
1 h at room temperature. Immunocytochemistry was performed, using rabbit anti-Tbx1 (rabbit, 1:100; Zymed, South San Francisco, CA). pH3 antibody was used according to the manufacturer’s instructions to evaluate cell proliferation. Epithelial proliferation of the wild-type and Tbx1<sup>+/−−</sup> epithelium was calculated on E14.5 and E18.5 palatal sections as the ratio of the number of cells that stained positive for pH3 in the basal layer over the total number of cells as determined by 4′,6-diamidino-2-phenylindole (DAPI) staining. More than five sections were analyzed per palate (n = 3 per genotype). Images were captured using fluorescence microscopy BX41 and DP controller (Olympus), and processed with Photoshop CS5 (Adobe).

Whole-mount <em>in situ</em> hybridization

Embryos were harvested at E12.5, E13.5, E14.5 or E15.5 and fixed in 4% paraformaldehyde at 4°C overnight. Whole-mount <em>in situ</em> hybridization was performed using digoxigenin-labeled riboprobes for Bmp4, Fgf10, Pitx2, Jag2 (37), Barxl (48), Pax9 (66), Fgf8 (67), Pchtl (68), Msxl (69), ephrinB1, ephrinB2, EphB2 and EphB3 (kind gifts of Dr M. Henkemeyer).

Adenovirus transfection and cell proliferation assay

Recombinant adenoviruses for Tbx1 and EGFP (Clontech, Mountain View, CA) were newly generated using the ViraPower™ adenoviral expression system (Invitrogen, Life Technologies, Grand Island, NY). The human epithelial cell line MCF7 was seeded into six-well culture plates at a density of 1 × 10<sup>4</sup> cells per well. Tbx1 and control EGFP transfection were performed by using the pAd-CMV adenovirus construct at 100 multiplicity of infection. For viral infection, cells were treated with virus in antibiotic-free Roswell Park Memorial Institute (RPMI) medium containing 1% fetal bovine serum (FBS) for 2 h, then complete RPMI medium, supplemented with 5% FBS and penicillin streptomycin, was added to the cells. Proliferation was determined by cell counting using 0.4% Trypan Blue (Invitrogen). The experiments were performed and analyzed in triplicate.

RT–PCR

Total RNA was isolated using Trizol (Invitrogen) without pooling the samples, then cDNA synthesis by Transcriptor First Strand cDNA Synthesis Kit (Roche). Sequences of PCR primers are available upon request.

Cell-cycle analysis

MCF7 cells were seeded at a density of 5 × 10<sup>4</sup> cells per 6 cm dish. Cells were transfected with either Tbx1 or EGFP, and cultured in the media as described above. Cells were harvested and analyzed, as described previously (70).

Statistical analysis

The experimental data were analyzed by two-tailed Student’s t-test and expressed as mean ± SEM. A P-value <0.05 was considered to be significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at <HMG> online.

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Conflict of Interest statement. None declared.

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