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Journal of Cellular Biochemistry 107:818–825 (2009)

Journal of Cellular Biochemistry

A TGF-β-Induced Gene, βig-h3, Is Crucial for the Apoptotic Disappearance of the Medial Edge Epithelium in Palate Fusion

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ABSTRACT

TGF-β3, TβR-I, and TGF-β-activated Smad2 has been suggested to be a series of signaling molecules for secondary palate fusion. In this article, we show that a gene induced by TGF-β, βig-h3, is coincidentally expressed with TGF-β3 in medial edge epithelial (MEE) cells undergoing apoptosis during normal palatal fusion. βig-h3 was also highly expressed in the areas of post-weaning mammary gland cells and developing phalangeal joints in which TGF-β3 or BMP-4-induced apoptosis occurs, respectively. Blocking of βig-h3 expression in E12.5 embryos with antisense oligodeoxynucleotides (ODN) resulted in cleft of the secondary palate in 84% of the treated mice that were born. Moreover, the antisense ODN treatment resulted in a failure of apoptosis in the MEE between palatal shelves in physical contact in organ culture. We conclude that βig-h3 expression in the MEE is stimulated by TGF-β3, causes cell death, and consequently results in complete fusion of the apposed palatal shelves. J. Cell. Biochem. 107: 818–825, 2009.

KEY WORDS: TGF-β3; βig-h3; MEDIAL EDGE EPITHELIUM (MEE); APOPTOSIS; PALATE; CLEFT; ANTISENSE; RGD

Although cleft palate is common in knockout mice and humans, non-syndromic cleft palate involving the secondary palate is uncommon in mice in the absence of any other detectable craniofacial abnormality. At present, at least nine distinct genes causing non-syndromic cleft palate have been identified, three of which are closely related to tongue development. In these phenotypes, the tongue interferes with contact between the palatal shelves. It has been speculated that the cleft palate resulting from the Tbx22 mutation in X-linked cleft palate and ankyloglossia may be due to a primary defect of the tongue [Braybrook et al., 2001], and there is an obvious similarity between the palatal phenotype caused by Foxf2 deficiency and human overt cleft. The Foxf2 gene plays key roles in palatogenesis by reshaping the growing tongue [Wang et al., 2003]. In Jag2-deficient mice, the unelevated palatal shelves fuse on each side with the posterior portion of the tongue; this fusion prevents the proper formation of the secondary palate [Jiang et al., 1988].

In the Lhx8 mutant mouse, cleft palate is not likely to be related to tongue development but shows a very similar phenotype to that found in TGF-β3 [Kaartinen et al., 1995; Proetzel et al., 1995] and TGF-β receptor-II (TbR-II) mutant mice [Ito et al., 2003], in which a failure of the palatal shelves to fuse leads to cleft of the secondary palate. TGF-β3 has been proposed to be the single most important factor for secondary palate fusion. In an organ culture study of the palatal shelves of TGF-β3 knockout mice, pairs of −/− and −/− shelves failed to fuse over 72 h of culture whereas combinations of

Grant sponsor: Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea; Grant numbers: 01-PJ3-PG6-01GN11-0002, 01-PJ5-PG3-20507-0044; Grant sponsor: National Research Laboratory Program; Grant number: M10104000036-01J0000-01610.

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Received 23 March 2009; Accepted 24 March 2009 • DOI 10.1002/jcb.22180 • © 2009 Wiley-Liss, Inc.
Published online 4 May 2009 in Wiley InterScience (www.interscience.wiley.com).

+/+ (wild-type) and +/+, +/− (heterozygous), or −/− (homozygous) shelves fused within the first 48 h. Histological examination of fused +/+ and +/+ shelves showed that the midline epithelial seam had completely disappeared, whereas −/− and +/+ shelf pairs still had some seam remnants [Taya et al., 1999]. These results indicate that there is a problem with the disappearance of medial edge epithelium (MEE) in TGF-β3 knockout animals.

The importance of TGF-β3 is further supported by reports that molecules involved in TGF-β signaling pathways, including TGF-β receptor II [Ito et al., 2003], the TGF-β receptor I gene ALK5 [Dudas et al., 2004], and TGF-β-regulated Smad2 [Cui et al., 2005], are also critically involved in the process of palatal fusion. Previous reports indicated that TGF-β3 plays crucial roles in several steps of palatogenesis: first, TGF-β3 is involved in the adhesion of the palatal shelves through the induction of chondroitin sulfate proteoglycan on the apical surface of MEE cells [Gato et al., 2002]; second, TGF-β3 breaks down basement membrane during palatal fusion by inducing matrix metalloproteinase (MMP)-13 [Blavier et al., 2001] and is important for extracellular matrix (ECM) remodeling; and third, TGF-β3 is very important for the disappearance of MEE cells either by epithelial-mesenchymal transition (EMT) or apoptosis [Martinez-Alvarez et al., 2000]. The molecular mechanism connecting TGF-β3 to the disappearance of MEE cells is still unknown; however, it is likely that a gene downstream of TGF-β3 plays a crucial role.

TGF-β3 has been reported to be an inducer of apoptosis at the calvarial coronal suture [Opperman et al., 2000] and during mammary gland involution [Nguyen and Pollard, 2000], among other sites [Ganan et al., 1996], but none of these studies elucidated the direct cause of the apoptosis. A TGF-β-induced gene, βig-h3, is a fibrillar extracellular matrix protein that has four highly conserved drosophila fasciclin-1-like domains (fas-1) and an RGD domain. Fas-1 domains are involved in cell adhesion through integrin binding [Nam et al., 2000; Kim et al., 2002a]. Proteolytic cleavage of βig-h3 occurs after secretion of the protein and generates a soluble C-terminal peptide containing RGD, which goes into the cell and induces apoptosis [Kim et al., 2003b] probably by activating caspase-3 [Buckley et al., 1999]. In this article, we demonstrate that βig-h3 is a target of TGF-β3 in apoptotic MEE cells. Antisense blocking of βig-h3 expression in the cells causes failure of the MEE cell's programmed death, resulting in cleft of the secondary palate.

RESULTS

Mouse palatogenesis is a complex process that includes budding out of a tissue fold from the lateral wall of the oral cavity at E11.5; vertical downward growth of the tissue beside the tongue at E12.5–13.5 (Fig. 1a,b); the elevation and projection of the palatal shelf primordia over the tongue to abut each other in the midline at early E14 (Fig. 1d); the edge-to-edge adhesion of the shelves at the midline; and the disappearance of the MEE to make the palatal mesenchyme confluent at mid-to-late E14 (Fig. 1c–f). To test whether apoptosis plays a role in the disappearance of the MEE, we performed TUNEL assays to identify apoptotic cells. TUNEL-positive

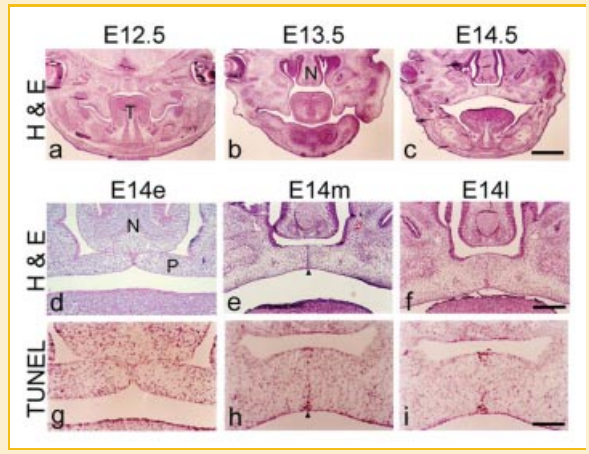


Fig. 1. Normal palatal development in mouse. Coronal sections of developing mouse heads from E12.5 to E14.5 (a–c), and early, mid, and late E14 (E14e, E14m, and E14l, respectively; d–f) were stained with hematoxylin and eosin. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was performed in adjacent sections of early, mid, and late E14 palatal tissue to identify apoptotic cells (g–i). T, tongue; N, nasal septum; P, palatal shelf; arrowheads, medial edge epithelium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

apoptotic cells appeared in the MEE from palatal adhesion at E14.5 until the epithelial seam disappeared (Fig. 1g–i), suggesting that apoptotic cell death could be involved in MEE cell disappearance.

Tgf-β1 and *β3* began to be expressed in the MEE just before palatal adhesion, and their expression ceased when MEE cells disappeared (Fig. 2 a–c and g–i, respectively). In contrast, *Tgf-β2* was mainly expressed in mesenchymal cells in the developing palate (Fig. 2d–f). Interestingly, all three *Tgf-β* isoforms were similarly expressed in the perichondral cells of the nasal and paranasal

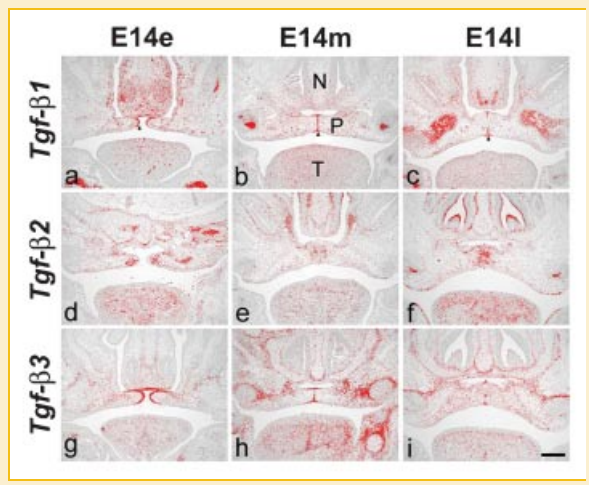


Fig. 2. Expression pattern of *Tgf-β1* (a–c), *-β2* (d–f), and *-β3* (g–i) in developing mouse palatal tissue at early, mid, and late E14. T, tongue; N, nasal septum; P, palatal shelf; arrowheads, medial edge epithelium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cartilages. Although *Tgf-β1* and *-β3* were similarly expressed in MEE cells, we concentrated further studies on Tgf-β3 because the inhibition of Tgf-β3, but not Tgf-β1 or -β2, activity by antisense ODNs or neutralizing antibodies blocked palatal fusion process [Brunet et al., 1995].

The expression pattern of *βig-h3* was very similar to that of *Tgf-β3* and co-localized with TUNEL-positive apoptotic cells at the medial epithelial seam (Fig. 3). However, *βig-h3* expression occurred later in palatogenesis than *Tgf-β3* expression. For example, *Tgf-β3* expression was evident in the apical area of the budding palate at E12.5 when *βig-h3* was not expressed in the same area (Fig. 3, arrowheads). In addition, *Tgf-β3* expression was always caught up by *βig-h3* expression in the next stage, which also suggests *βig-h3* expression is regulated by Tgf-β3. Interestingly, *βig-h3* differed from *Tgf-β3* at E13.5 and E14 in being strongly expressed in the perimuscular fascia area that separates the longitudinal and transverse muscles of the developing tongue.

Because we suspected that Tgf-β3 and βig-h3 induce apoptotic cell death in the MEE, we examined other tissues where apoptotic cell death occurs as part of the normal developmental process. Involuting mammary gland cells are well known to undergo apoptosis in response to TGF-β3 signaling [Nguyen and Pollard, 2000]. βig-h3 expression was strongly increased in involuting mammary gland epithelial cells coincident with their apoptosis. Immunohistochemical staining indicated that βig-h3 was not expressed in mammary epithelial cells at post-weaning day 1 (PW1), when apoptosis has not yet occurred, but is strongly expressed at PW3, when most of the cells are undergoing apoptosis. Digit formation is another model of apoptosis in normal development in which Bmp-4 mediates the apoptotic cell death [Ganan et al., 1996]. Even though the programmed cell death is not initiated by the Tgf-β3 signal in this case, βig-h3 was clearly expressed in the area of cell death in the interdigital tissue and phalangeal joint (Fig. 4 lower two panels; bright and dark field). From our present in vivo evidence and a previous report that βig-h3 is involved in apoptosis [Kim et al., 2003b], we hypothesized that Tgf-β3 induces βig-h3 and βig-h3 subsequently executes programmed cell death in the MEE of the palate.

To test this, we treated HaCaT cells with recombinant human TGF-β3 and examined βig-h3 expression. Recombinant human TGF-β3 strongly stimulated βig-h3 protein expression in HaCaT cells. We then injected an antisense ODN into the amniotic fluid of half of a litter and a scrambled ODN into the amniotic fluid of the other half. The ODNs were injected at E12.5 because βig-h3 expression begins at E13.5. FITC-labeled ODNs were injected in some cases to observe absorption of the ODN into the tissue. After two more days of gestation, we sacrificed the embryos at E14.5 and found that the FITC-labeled ODN was successfully absorbed and remained in the

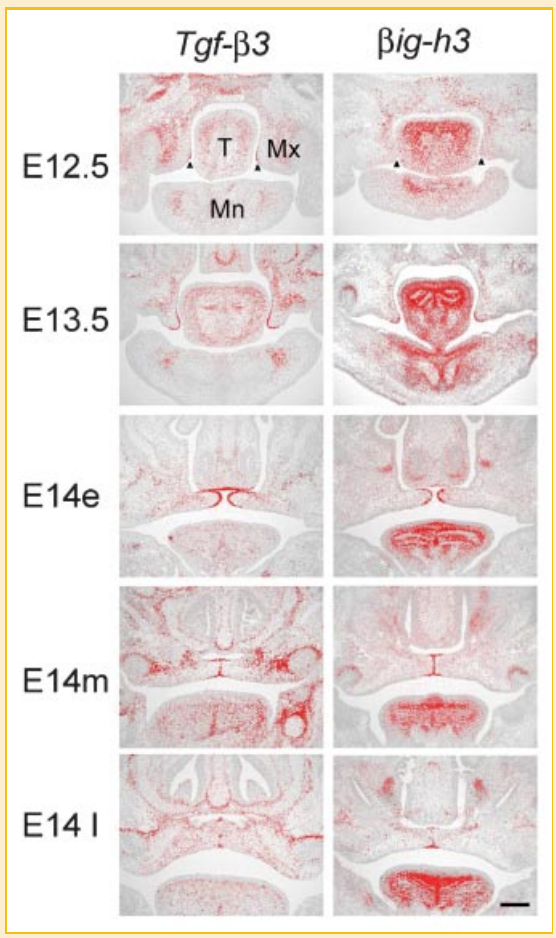


Fig. 3. Expression pattern of *Tgf-β3* and *βig-h3* in developing mouse palatal tissue from E12.5 to late E14. T, tongue; Mn, mandible; Mx, maxilla; arrowheads, budding palatal tissue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

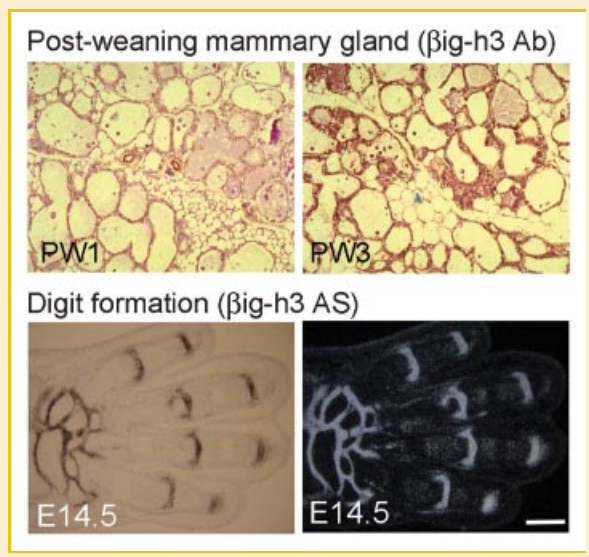


Fig. 4. Expression pattern of βig-h3 protein in mammary gland at post-weaning day 1 (PW1) and day 3 (PW3) (βig-h3-positive cells are dark brown in color). The protein expression was determined by anti-mouse polyclonal rabbit antibody; and expression pattern of *βig-h3* mRNA in developing phalanges at E14.5 was determined by *βig-h3* antisense riboprobe (left, bright field; right, dark field). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

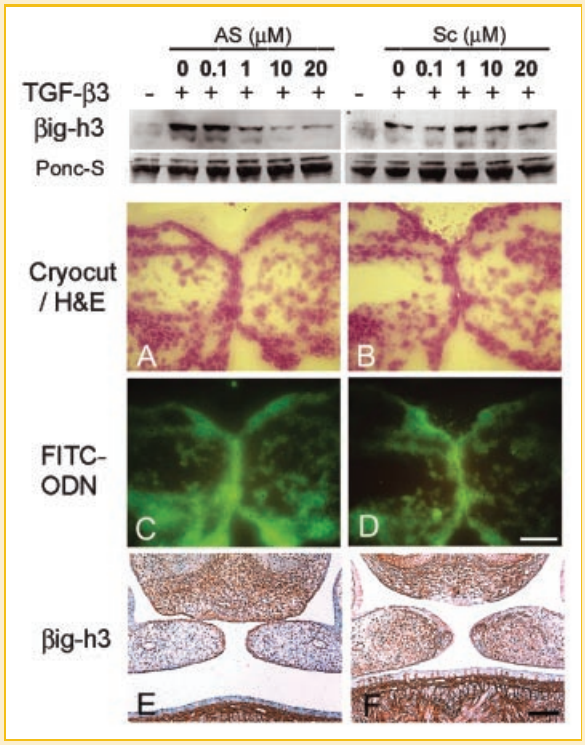
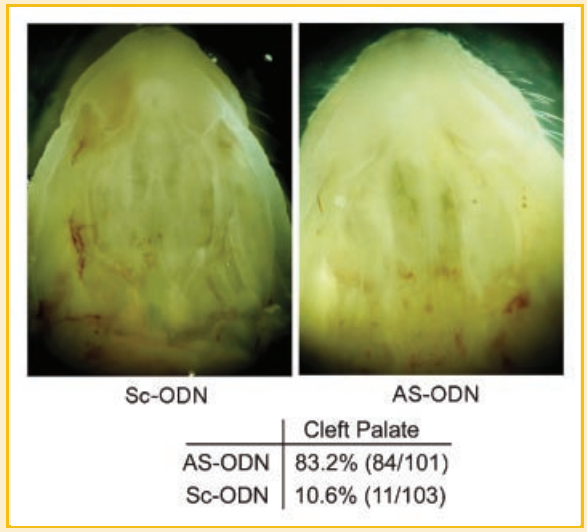


Fig. 5. An antisense ODN strongly suppresses βig-h3 expression. Spontaneously immortalized human keratinocytes, HaCaT cells, were treated with 0.1–20 μM of antisense (AS) or scrambled (Sc) ODN 2 h before treatment with TGF-β3 (2.5 ng/ml). Cells and matrix proteins were harvested 24 h after TGF-β3 treatment, and Western blot analysis was performed with an anti-human βig-h3 antibody. Consistent protein loading was demonstrated by Ponceau S staining (Ponc-S) of the loaded protein. FITC-labeled antisense ODNs (A,C,E) or scrambled ODNs (B,D,F) were injected into the amniotic fluid of E12.5 foetuses, and the pups were sacrificed 48 h after the injection. Frozen sections of palatal tissue were examined after H&E staining (A,B) or under a fluorescent microscope (C,D) to determine the penetration of the ODNs. After regular fixation and paraffin embedding, sections were examined for βig-h3 expression using immunohistochemistry with an anti-mouse βig-h3 antibody (E,F).



	Cleft Palate
AS-ODN	83.2% (84/101)
Sc-ODN	10.6% (11/103)

Fig. 6. Antisense blocking of βig-h3 expression resulted in secondary palate cleft. Antisense (AS-ODN) and scrambled (Sc-ODN) ODNs of *βig-h3* (final concentration 3 μM in amniotic fluid) were each injected into the amniotic fluid of half of a litter at E12.5, respectively. The pups were sacrificed just before or after birth, and the palate was examined under a stereomicroscope. The table summarizes the occurrence of visible secondary palatal cleft in each group.

epithelial cells (Fig. 5A–D). The βig-h3 antisense ODN clearly suppressed the TGF-β3-induced βig-h3 protein expression in a dose-dependent manner, but a scrambled ODN did not (Fig. 5, top). Tissues from embryos treated with scrambled ODN showed strong βig-h3 staining in the palatal mesenchyme, tongue, and nasal septum, whereas embryo tissues treated with antisense ODN showed very weak βig-h3 staining in the palatal shelves, even though strong positive βig-h3 staining remained in the tongue and nasal septum (Fig. 5E,F). These results show that the antisense ODN functioned adequately, at least in superficial tissues like the MEE, and did not interfere with the development of other oral and cranial tissues.

Among the 101 total embryos treated with antisense ODN, 84 showed complete failure of secondary palate fusion. In contrast, only 11 of the 103 littermates treated with scrambled ODN showed secondary palatal cleft (Fig. 6). Only three and two injected pups in the antisense-ODN and scrambled-ODN group, respectively, died during the experimental period, suggesting that the injection procedure itself was not particularly hazardous.

Blocking βig-h3 caused dramatic failure in secondary palatal fusion, but it was not clear whether the palatal clefts were caused by failure of palatal adhesion or of MEE apoptosis. We ruled out the possibility of a failure of the palatal shelves to grow together because the apposed shelves almost made contact at E14.5 (Fig. 5D,E). To address this question, we designed an organ culture of E12.5 palatal shelves. We placed the MEE of two shelves in physical contact and cultured them for 72 h. Treatment with scrambled ODN did not interfere with MEE cell disappearance: the epithelial tissue almost disappeared and the mesenchymal tissue of the two palatal shelves completely mixed together, although there was some remnant of epithelial seam (Fig. 7, Sc). In contrast, we found gaps between the two palatal tissues from mice treated with antisense ODN. Moreover, epithelial cells and basement membrane remained even in the areas of direct cell-to-cell contact (Fig. 7, AS).

DISCUSSION

βig-h3, whose expression is induced by TGF-β, is an ECM protein composed of 683 amino acids containing four homologous internal repeat domains. These domains are homologous to the *Drosophila* protein fasciclin-I and thus are denoted fas-1 domains. Through these domains, βig-h3 interacts with other matrix proteins such as fibronectin, collagen, and laminin [Kim et al., 2002b] and mediates cell adhesion and migration through interactions with integrins [Kim et al., 2000, 2002a]. These domains are also involved in cell growth, differentiation, tumorigenesis, wound healing, and apoptosis. In the introduction, we summarized three proposed roles for TGF-β3 in secondary palate closure: (a) in MEE adhesion, (b) in

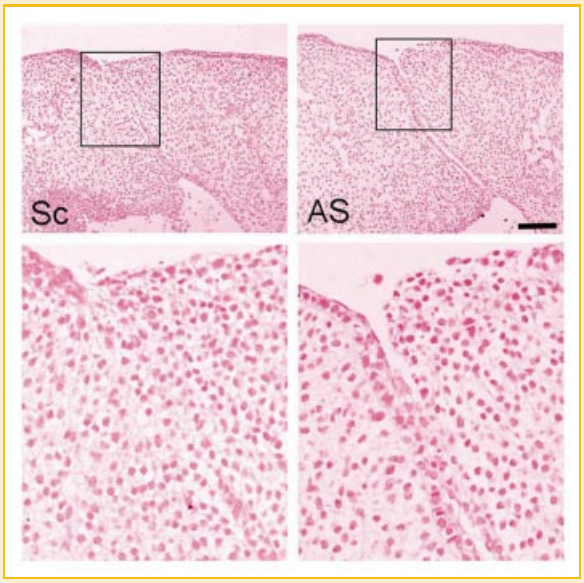


Fig. 7. Antisense blocking of βig-h3 suppressed MEE cell apoptosis. Palatal shelves were dissected from E12.5 mice, and the MEE of each side of the palatal shelf was slightly overlapped to ensure physical contact. The explants were subsequently cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 h in serum-free DMEM containing antisense (AS) or scrambled (Sc) ODNs. The cultured tissues were then fixed, dehydrated, and embedded in paraffin. Thin sections (5 μm) were stained with hematoxylin and eosin and examined histologically. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degradation of underlying basement membrane, and (c) in MEE disappearance. Molecular downstream targets of TGF-β3 that cause MEE adhesion [Gato et al., 2002] and basement membrane degradation [Blavier et al., 2001] have been proposed. However, there has been no reasonable downstream target to account for MEE cell disappearance. We summarized previous reports that account for TGF-β3-related palatal fusion mechanism in Figure 8. We suggest here that βig-h3 could be the critical molecular mediator of MEE cell apoptosis.

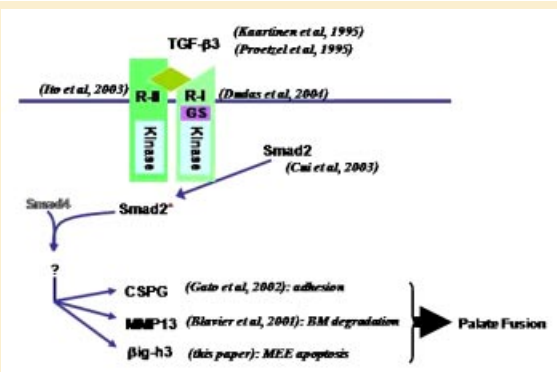


Fig. 8. TGF-β3 signaling pathway and possible candidate target genes were summarized. Their action mechanisms in palatal fusion and related references were depicted. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

βig-h3 IS CRITICAL FOR THE EPITHELIAL SEAM DISAPPEARANCE BY APOPTOSIS

Although we cannot rule out the possibility that an epithelial-mesenchymal transition is responsible for the disappearance of MEE cells, our present results clearly indicate that βig-h3 is involved in MEE cell disappearance by apoptosis. First, βig-h3 expression is co-localized in TGF-β3- and TUNEL-positive MEE cells; second, TGF-β3 and βig-h3 strongly stimulated apoptosis in cultured cells [Kim et al., 2003b] and *Tgf-β3* and *βig-h3* are highly expressed in involuting mammary gland cells undergoing apoptotic cell death; and third, blocking βig-h3 expression with an antisense ODN clearly shut down MEE cell death in palatogenesis in vivo. Moreover, we found patent MEE cells in antisense ODN-treated palates in organ culture, in which MEE cells from the two explants made direct physical contact.

The proteolytic release of an RGD-containing small peptide from βig-h3 plays a role in programmed cell death in MEE. Apoptosis is abolished by mutations of βig-h3 that inhibit proteolytic cleavage of the RGD-containing peptide or modify RGD to RGE [Kim et al., 2003b]. The released C-terminal RGD-containing peptides enter cells and directly promote pro-caspase-3 autoprocessing and activation [Buckley et al., 1999]. Together, these results strongly suggest that a decrease in C-terminal RGD-containing small peptides by any mechanism would be a critical pathogenic mechanism leading to cleft palate in *Tgf-β3*-null mice.

βig-h3 IS PROBABLY ALSO INVOLVED IN PALATAL ADHESION

Before apoptotic cell death occurs, there should be adhesion between MEE cells of the two palatal shelves and formation of a molecular bridge between them. Previous reports have indicated that TGF-β3 induces the formation of chondroitin sulfate proteoglycan filopodia that mediate palatal shelf adhesion on the apical surface of MEE cells [Gato et al., 2002]. βig-h3 has many ECM-molecule and cell-surface-integrin binding domains. Fas-1 domains interact with αVβ3 or α3β1 integrins [Nam et al., 2000], which mediate outside-in signaling [Schlaepfer and Mitra, 2004]. The binding of ECM and integrins activates focal kinases, which may in turn result in changes in cellular architecture such as filopodia formation [Naik and Naik, 2003]. In organ culture, a large portion of the MEE of palates treated with antisense ODN did not firmly contact the co-cultured palate, even after 72 h of culture that began with physical contact between the explants. The evidence strongly suggests that βig-h3 plays a crucial role in providing the molecular bridges between apposed MEE cells. In addition, basement membrane remained in the antisense ODN-treated palatal shelves in organ culture, suggesting that βig-h3 may be involved in basement membrane breakdown as well.

βig-h3 has been reported to be a causative molecule of corneal dystrophy (OMIM *601692). In this congenital anomaly, βig-h3 mutations of Arg124 to Leu, Cys, His, Trp, Ser or Gln; Pro501Thr; Phe540Ser; Ala546Asp; Pro551Gln; and Arg555 to Trp or Gln have been found. Until recently, there was no evidence of linkage between corneal dystrophy and cleft palate. In corneal dystrophy, the mutations result in abnormal accumulation of insoluble βig-h3 protein in the cornea. Thus, it can be speculated that mutations in βig-h3 molecule causing corneal dystrophy have nothing to do with

C-terminal RGD peptide processing or the decrease in βig-h3 levels that are probably associated with palatal cleft.

For this reason, we checked for mutations in the βig-h3 molecule in 25 patients with cleft palate. We collected genomic DNA from the patients' blood and performed direct sequencing. We first focused on a possible mutation in the proteolytic cleavage site at the C-terminal end of the molecule [Kim et al., 2003b], and then sequenced all exons and adjacent intronic sequences. Unfortunately, we did not find any mutation in the genomic DNA of those patients, at least in the C-terminal half of protein coding sequence (data not shown). Further genetic studies should be applied to molecules mediating βig-h3 expression by TGF-β signaling. Future targets of the genetic studies may include a transcription factor that mediates TGF-β3 action to βig-h3 or cis-acting elements in the βig-h3 promoter through which a transcription factor regulates the βig-h3 expression. Furthermore, rescue experiments with βig-h3 protein delivery or a stage- and site-specific *βig-h3* transgenic approach in *Tgf-β3*-null mice should also be performed to definitively test the importance and mechanism of action of βig-h3 as well as Tgf-β3 in palate fusion.

In conclusion, we showed in this article that a TGF-β3-induced gene, βig-h3, is a critical molecule for normal palatal fusion and suggest that βig-h3 is involved in MEE cell apoptosis through the release of its C-terminal RGD-containing peptide. We do not rule out a possibility that βig-h3 may be involved in palatal adhesion through its fas-1 domains, but this also remains to be clarified in further studies.

EXPERIMENTAL PROCEDURES

MATERIALS

Recombinant human TGF-β3 protein was from R&E Systems (Cambridge, MA). Antisense or scrambled ODNs and FITC-labeled ODN were produced from Bionics Co. (Seoul, Korea). Timed-pregnant ICR mice were from Daehan Biolink Co., Ltd (Chungbuk, Korea). [^{35}S]UTP was from Amersham Biosciences (Piscataway, NJ).

TISSUE PREPARATION

The heads of ICR mice were removed on embryonic days 12.5 (E12.5) to E15 [these timed-pregnant mice were purchased from Daehan Experimental Animal, Daejeon, Korea] and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The mouse heads were dehydrated in a series of ethanol steps and embedded in paraffin. Thin (5 μm) coronal sections of the palate were mounted on silanized slides, dried overnight at 37°C, and stored at 4°C. Sections were stained with hematoxylin to assess the histology of the developing tissues. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed as described previously [Rice et al., 1999] to identify programmed cell death in MEE cells. Briefly, dehydrated and deparaffinized tissue sections were treated with 100% ethanol/acetic acid (2:1) for 20 min and then placed in 100% methanol before being treated with 0.5% H_2O_2 in methanol for 30 min. Proteinase K treatment was performed at room temperature, and 5 mM levamisole was used in the washes prior to and also in the color reaction. Following the color reaction, the sections were counterstained with hematoxylin, dehydrated, and washed twice in xylene for 10 min, after which coverslips were mounted.

IN SITU HYBRIDIZATION

To assess the expression patterns of mouse *Tgf-β1*, *Tgf-β2*, *Tgf-β3*, and *βig-h3*, we cloned their complementary DNAs (cDNAs) into pBluscript KS+ plasmids. To minimize possible cross-hybridization among the *Tgf-β* isoforms, the highly homologous mature peptide region was excluded, as described previously [Derynck et al., 1986; Miller et al., 1989; Denhez et al., 1990]. To develop a βig-h3-specific probe, we subcloned the 660-bp 5′-UTR and part of the 5′-coding region from the full-length cDNA [Kim et al., 2003b]. Each cDNA vector was digested with KpnI or SacI, and antisense and sense riboprobes were produced by T3 and T7 RNA polymerase, respectively. For [^{35}S]UTP-labeled riboprobes, the final probe concentration was adjusted to 50,000–60,000 cpm/μl [Park et al., 2001]. After 2 min of denaturation at 80°C, 20–100 μl of probe solution was placed on each slide and covered in Parafilm. After overnight hybridization in a humidified sealed box at 52°C, high stringency washes with 50% formamide and 20 mM dithiothreitol (DTT) were carried out at 65°C. The slides were then prepared for autoradiography. The dehydrated slides were dipped into photographic emulsion (Kodak NTB-2; Eastman Kodak, Rochester, NY), dried, and exposed for 2–3 weeks at 4°C. The slides were then developed (Kodak D-19; Eastman Kodak), fixed (Kodak Unifix; Eastman Kodak), briefly counterstained with hematoxylin, and mounted with DePeX (BDG).

BLOCKING βig-h3 EXPRESSION

In order to block βig-h3 expression, we designed antisense oligodeoxynucleotides (ODNs). For the effective introduction of an antisense ODN, the ODN should be smaller than 18 nucleotides. The βig-h3 mRNA sequence was analyzed with the RNAdraw program Ver1.1 (CABIOS), which can suggest the most probable secondary structures of the mRNA. We found 8 hairpin structures in the RNA and designed antisense ODNs to attack them (Table I). To test the efficiency of antisense ODNs for blocking βig-h3 expression, we cultured spontaneously immortalized human keratinocytes, HaCaT cells, and mouse bone marrow-derived KS483 cells. Cells were treated with several different concentrations of antisense ODNs just prior to treatment with 5 ng/ml TGF-β3 for 24 h. The most

TABLE I. Nucleotide Sequences of Antisense and Scrambled Oligodeoxynucleotides of βig-h3 Used in the Experiments

Name	Sequence	NM_009369[a]
AS1	5′-gatgaaagcaggctcgtg-3′	3–17
AS2	5′-gctggatgttgttggtga-3′	761–775
AS3	5′-gtggttgtttagcaggtc-3′	958–975
AS4	5′-caggaaggtcaactg-3′	1,270–1,284
AS5	5′-accatgtggttcaga-3′	1,350–1,364
AS6	5′-tgtggtacttcaggatg-3′	1,746–1,762
AS7	5′-ctcactacattgttttt-3′	1,849–1,865
AS8	5′-ctcatctcctcgttctt-3′	1,964–1,980
Sc4	5′-tacaagcgtcgagag-3′	

[a]NCBI nucleotide accession number; numbers below indicate nucleotide numbers of each AS matches in the mRNA sequence. AS, antisense; Sc, scrambled. Sc4 has the same nucleotides composition that has AS4 but the nucleotide sequence is mixed up.

effective ODN sequence and its highest effective concentration without cytotoxicity were assessed with a Western blot analysis of the βig-h3 protein level [Kim et al., 2000] and an MTT assay for cell viability [Kim et al., 2004].

To determine the in vivo penetration of the ODNs, we injected FITC-labeled ODNs into the amniotic fluid of E12.5 foetuses, and sacrificed the pups 48 h after the injection. Frozen sections of palatal tissue were examined under a fluorescent microscope.

In order to determine the functional consequence of blocking βig-h3 in palatogenesis in vivo, an antisense ODN was injected into the amniotic fluid (3 μM final concentrations in amniotic fluid; the average volume of amniotic fluid was 120 μl at the stage of injection) of half a litter and a scrambled ODN was injected into the other half at E12.5, when βig-h3 is not expressed in the palatal tissue. The in vivo blocking of βig-h3 protein expression was analyzed by immunohistochemistry 48 h after the antisense injection. To determine the effect of antisense blocking of βig-h3 expression in palatogenesis, mice were sacrificed at E18.5 or P1 and the palatal fusion was checked under the stereomicroscope.

ORGAN CULTURE

We followed previously described organ culture protocols [Kim et al., 2003a]. Briefly, palatal shelves were dissected from E12.5 mice in which the palatal shelves from each side of the oro-nasal cavity did not yet contact each other. The palatal shelves were placed with the apposed MEE made direct physical contact on a Nucleopore filter (0.1-μm pore size) supported by metal grids. The explants were subsequently cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 h in serum-free DMEM containing antisense or scrambled ODN. The medium was supplemented with 100 μg/ml ascorbic acid daily. The cultured tissues were then fixed overnight with 4% PFA and, after a series of dehydration steps, were embedded in paraffin. Thin sections were stained with hematoxylin and examined histologically.

ACKNOWLEDGMENTS

This study was supported by grants from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ3-PG6-01GN11-0002 to H.M.R. and 01-PJ5-PG3-20507-0044 to H.J.K.), and by the National Research Laboratory Program (M10104000036-01J0000-01610 to I.S.K.).

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