Tbx22 Expressions During Palatal Development in Fetuses With Glucocorticoid-/Alcohol-Induced C57BL/6N Cleft Palates

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Abstract: T-box transcription factor 22 (Tbx22) belongs to the T-box family of transcription factors and was originally found using an in silico approach to identify new genes in the human Xq12-Xq21 region. Mutations in Tbx22 have been reported in families with X-linked cleft palate and ankyloglossia, but the underlying pathogenetic mechanism remains unknown. The aim of this study was to evaluate the expression of Tbx22 messenger RNA (mRNA) during palatogenesis in glucocorticoid-/alcohol-induced cleft palate in a C57BL/6N mouse model. Palatal development was monitored by histomorphologic and immunohistochemical studies and by in situ hybridization. Thirty pregnant C57BL/6N mice at 8 weeks of age, weighing 20 to 25 g, were used in this study. In the experimental group, 12 mice were exposed to alcohol for 7 days before mating, and 12 mice in the control group were not exposed. Six mice in a sham group were exposed to neither alcohol nor glucocorticoids. A total of 18 fetuses with induced cleft palates each from 102 fetuses in the experimental group, 109 in the control group, and 58 in the sham group were used. In both the experimental and the control groups, glucocorticoids were injected subcutaneously on gestational days (GD) 9.5, 10.5, and 11.5, and each mouse was killed on GD 11.5. Localization in the tongue frenum correlated with the ankyloglossia phenotype in the induced cleft palate animal model.

Key Words: Alcohol, ankyloglossia, cleft palate, C57BL/6N mouse, glucocorticoid, nasal septum, T-box transcription factor, Tbx22

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Orofacial clefts (OFCs), such as a cleft lip with or without a cleft palate (CLP) or a cleft palate only (CP), affect approximately 1 per 1000 live births, with some variation by race and ethnic group. Orofacial clefts are generally considered as a sporadic occurrence with multifactorial inheritance resulting from an interaction between genetic and environmental factors.1,2 Clefts of the secondary palate can be induced in embryonic mice by several physical and chemical exposure techniques. The etiology of cleft palate induction encompasses multiple morphologic processes including disruption of palatal shelf growth, elevation, adhesion of opposing shelves, and events critical to removal of the medial epithelial seam. Despite the high incidence of cleft palate malformations, few genes controlling palate development have been identified or proposed. Mutations in T-box transcription factor 22 (Tbx22) have been found to cause a nonsyndromic form of cleft palate in humans that is associated with ankyloglossia.3 Cleft palate with ankyloglossia (CPX; Online Mendelian Inheritance in Man No. 303400) is inherited as a semidominant X-linked disorder and is characterized by a cleft of the secondary palate and ankyloglossia. The T-box transcription factor Tbx22, which is named after a DNA-binding domain of approximately 200 amino acids,4 was found to be mutated in families with CPX. It is expressed primarily in the palatal shelves and tongue during palatogenesis and plays an important role in both palatal and tongue developments.2,5–6 Missense, nonsense, splice site, and frameshift mutations in Tbx22 gene, whose locus was delimited to a region of Xq21, are predicted to cause a complete loss of Tbx22 function. Because of this, Tbx22 is considered to be an important transcription factor determinant in palatal morphogenesis.7 In addition, we focused also on related proteins such as Pena (proliferation cell nuclear antigen), matrix metalloproteinase 9 (Mmp9), heat shock protein 70 (Hsp70), and zinc finger protein 422 (Krox25) on gestational days (GDs) 10.5 to 15.5.
We developed a C57BL/6N mouse model in which cleft palate can be induced by glucocorticoids with maternal alcohol consumption\(^8,9\) and used this model to examine the messenger RNA (mRNA) expression of Pcna, Mmp9, Hsp70, Krox25, and Tbx22 in the induced cleft defect. Pcna, Mmp9, Hsp70, and Krox25 were not strongly and specifically expressed in the entire facial region during any gestational period, and gaps were identified by immunohistochemical staining on GD 10.5 to 15.5. By whole-mount in situ hybridization (ISH), we also confirmed the localization of mouse Tbx22 in distinct areas of the embryonic head involved in secondary palate and tongue development.

**MATERIALS AND METHODS**

**Animal Model and Mating**

A total of 240 (120 females and 120 males) pathogen-free C57BL/6N mice were obtained from Orient Bio Co (Seoul, Korea) at 6 weeks of age and were held for 2 weeks before mating. They were housed at a temperature of 22°C ± 1°C and a humidity of 50% ± 10% in a 12-hour light/dark cycle. During the study, mice were provided with autoclaved tap water and laboratory chow ad libitum. Individual females were housed overnight with individual males and checked the next morning for the presence of a vaginal plug, denoted as GD 0.5. Plug-positive females were maintained in a vinyl isolator established in a hazard room to prevent environmental exposure. All experimental protocols were approved by the Kangnung National University Animal Care and Use Committee.

**C57BL/6N Mouse Strain Description**

C57BL/6N is a National Institutes of Health subline of C57BL/6, separated from C57BL/6J in 1951. Five single-nucleotide polymorphism differences have been identified that distinguish C57BL/6J from C57BL/6ByJ and C57BL/6NJ. The C57BL/6ByJ and C57BL/6NJ types are as follows: 08-015199792-M is C; 11-004367508-M, A; 13-041017317-M, C; 15-057561875-M, G; and 19-049914266-M, T. C57BL/6J types are as follows: 08-015199792-M is T; 11-004367508-M, G; 13-041017317-M, T; 15-057561875-M, A; and 19-049914266-M, G.

**Glucocorticoid-/Alcohol-Induced C57BL/6N Cleft Palate Model**

A total of 30 pregnant female mice had vaginal plugs. These were divided into 3 groups. Twelve mice were exposed to alcohol, constituting the experimental group, and 12 mice were not exposed, constituting the control group. Six mice in a sham group were given neither alcohol nor glucocorticoid injections. All mice in the experimental group were exposed to alcohol 7 days before the first GD to condition them for a taste preference for alcohol. Two bottles were given for the first 2 days, 1 with water and alcohol at a 4:1 ratio and 1 with alcohol only. For the next 2 days, the 4:1 bottle was exchanged for a 2:1 ratio bottle, and for the final 3 days, bottles contained only alcohol (Fig. 1A).

Subcutaneous injection of a glucocorticoid, dexamethasone, which was selected on the basis of previous reports (Figs. 1B, C), was given to the experimental and control groups as a single dose of 100 mg/kg body weight on GDs 9.5, 10.5, and 11.5 (Fig. 1B).

**Fetus Acquisition**

From GD 10.5 to 15.5, 2 mice in the experimental and control groups and 1 mouse from the sham group were killed, and all fetuses were acquired by cesarean delivery in the Hank buffered salt solution. Live fetuses were grossly examined under an inverted microscope to evaluate the incidence of a cleft palate (Fig. 2) and then fixed in 10% neutral buffered formalin for further histologic examination.

**FIGURE 1.** A, Experimental study design. B, Taste preference cage for alcohol consumption mouse model. C, Subcutaneous injection of glucocorticoid as a single dose of 100 mg/kg body weight. Dexamethasone (5 mg; Daewon Co, Seoul, Korea).

**FIGURE 2.** Fetuses were grossly examined under an inverted microscope to evaluate the incidence of cleft palate. A, Acquired fetuses; B, lateral view of the fetus, GD 14.5; C, frontal view of the fetus, GD 14.5; D, aborted fetus, GD 11.5.
Histologic Procedures With Hematoxylin and Eosin, Masson Trichrome, and Toluidine Blue Staining

The heads of all embryos were fixed in a 10% formaldehyde solution at pH 7.4 and stored for 24 hours at 4°C. Specimens were then decalcified in 9% formic acid/formalin, dehydrated with graded ethanol (50%, 70%, 85%, 95%, and 100%), cleared in xylene, embedded in paraffin cut into slices with an approximately 4-μm thickness in the coronal direction, and stained with hematoxylin and eosin (H&E), Masson trichrome (MT), and toluidine blue.

Immunohistochemical Staining for Pcna, Mmp9, Hsp70, Krox25, and Tbx22

Immunohistochemical analysis was performed on the paraffin-embedded tissue using the streptavidin biotin peroxidase method. The endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes and washed 3 times with phosphate-buffered saline (PBS). Primary antibodies were Pcna (1:100, mouse; Dako Co, Glostrup, Denmark), Mmp9 (1:100, mouse; Santa Cruz Bio, Santa Cruz, CA), Hsp70 (1:100, mouse; Santa Cruz Bio), Krox25 (1:100, mouse; Santa Cruz Bio), and Tbx22 (1:100, mouse; Santa Cruz Bio), diluted in distilled water 1:50, added to the sections overnight at 4°C, and washed 3 times with PBS. A biotinylated secondary antibody solution was placed on the sections for 30 minutes and then washed 3 times with PBS. Then, horseradish peroxidase-conjugated streptavidin-biotin complex (labeled streptavidin-biotin kit K0681; Dako Co) was placed on the sections for 30 minutes, followed by 3 washes with PBS and a peroxidase reaction using 3,3'-diaminobenzidine (Sigma, St Louis, MO) as a chromogen. Finally, the sections were washed 3 times with PBS, dehydrated through graded ethanol, and mounted. Negative control staining was performed for each antibody. Immunostained slides were evaluated for intensity of positive immunostaining and graded as +++, strongly positive (201–300); ++++, extremely strong (more than 401).

### TABLE 1. Counting of Positive Cells and the Degree of the Positive Reactions of Palatal Tissues in the Normal Development and Glucocorticoid-/Alcohol-Induced C57BL/6N Cleft Palate Mouse by Immunohistochemical Stainings (ImageTool Version 2.0, Alpha 3 Computer Analysis Image System)

<table>
<thead>
<tr>
<th>Proteins (Normal)</th>
<th>No. Positive Cells, Mean (SD)</th>
<th>Degree</th>
<th>Proteins (Cleft Palate)</th>
<th>No. Positive Cells, Mean (SD)</th>
<th>Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pcna</td>
<td>192 (6.7)</td>
<td>++</td>
<td>Pcna</td>
<td>205 (17.1)</td>
<td>+++</td>
</tr>
<tr>
<td>Mmp 9</td>
<td>184 (4.5)</td>
<td>++</td>
<td>Mmp 9</td>
<td>217 (11.7)</td>
<td>+++</td>
</tr>
<tr>
<td>Hsp 70</td>
<td>196 (8.6)</td>
<td>++</td>
<td>Hsp 70</td>
<td>270 (25.1)</td>
<td>+++</td>
</tr>
<tr>
<td>Krox 25</td>
<td>286 (21.4)</td>
<td>+++</td>
<td>Krox 25</td>
<td>203 (17.3)</td>
<td>+++</td>
</tr>
<tr>
<td>Tbx 22</td>
<td>84 (7.7)</td>
<td>+</td>
<td>Tbx 22</td>
<td>124 (11.8)</td>
<td>++</td>
</tr>
</tbody>
</table>

* indicates rarely positive (0–50); +, weakly positive (51–100); ++, moderately positive (101–200); ++++, strongly positive (201–300); ++++, extremely strong (more than 401).

**FIGURE 3.** Relative location of the Tbx22 gene in the linkage map of the mouse X chromosome (A) and the Tbx22 gene in the linkage map of the human X chromosome (B), from the NCBI annotation of NCBI Build 36, ChrX: 103870683 – 103891696 bp + strand.
In Silico Identification of Mouse Tbx22

Database searches with a human Tbx22 mRNA resulted in the identification of mouse genomic sequences that were assembled into contigs and used for the in silico prediction of a mouse Tbx22 mRNA sequence.

The Tbx22 gene of the C57BL/6N mouse is located on Xq21.1 (MM_Tbx22, AF515700), between DXGgc2e and Usf1-rs2, spans 8.6 kb from the beginning of the first exon to the first identified polyadenylation signal in genomic sequences of the mouse XD region, and is known to be syntenic with human Xq21. The human Tbx22 gene is also located on Xq31, LOC 302369, according to the linkage map of the X chromosome (Fig. 3). Promoters of human Tbx22 and mouse Tbx22 were compared by analysis of the 5' upstream regions. Sequences of mouse promoters were obtained using an in silico approach as described for the mouse Tbx22 gene.

The nucleotide sequence of the human Tbx22 mRNA (AF251684) was used to identify corresponding mouse genomic reads in the National Center for Biotechnology Information (NCBI) trace database (http://www.ncbi.nlm.nih.gov/Traces/trac.cgi). Tbx22-positive reads were assembled into sequence contigs based on the Mus musculus T-box transcription factor protein 22 mRNA and complete coding sequences from the AF516208 and AF251684 accessions in the NCBI nucleotide database. A 5217 base pair (bp) sequence for C57BL/6N was generated from the ENSEMBL trace archive (http://trace.ensembl.org) and the NCBI trace database (http://www.ncbi.nlm.nih.gov/Traces/trac.cgi). The resulting mouse genomic sequence was subjected to gene prediction using a DNA base-pair polarity conversion program (Lee et al, Gangneung, Korea) for the polymerase chain reaction (PCR) primer sequence selection (Fig. 4).

DNA Purification and Reverse Transcription PCR

Genomic DNAs were purified from the hearts of 35 6-week-old C57BL/6N mice. To confirm the individual genomic DNA, the 5' end of plasmid complementary DNA and the 3' end of the flanking RNA promoter (T7, T3, and SP6) were used as forward and reverse primers. Amplification conditions were 30 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 1 minute, and elongation at 72°C for 1 minute. Polymerase chain reaction products were visualized by 1% agarose gel electrophoresis and ethidium bromide staining with ultraviolet light detection.

Primer Selection for RNA Probes

Digoxigenin–uridine 5'-triphosphate–labeled single strand antisense and sense RNA probes were prepared with T7 RNA polymerase and T3 RNA polymerase, respectively, using an RNA labeling kit (Boehringer Mannheim, Ingelheim, Germany). For the Tbx22 transcript-specific probe, sense and antisense oligonucleotides corresponding to the exon 3a DNA sequence (48 bp) were synthesized using a PCR mate EP 391 DNA Synthesizer (Bioneer, Daejeon, Korea). The oligonucleotides containing T3 and T7 promoter sequences were as follows: (1) sense GAGG TAA TAC GAC TCA CTA TAG GG TGT CAT ATT CTT ACA GGT CAC AGA (1891–1868, GAGG + T7P + 24 bp = 48 bp), (2) antisense GAGG TAA TAC GAC TCA CTA TAG GTC TTA CAG GTC ACA GAT GAC ATT A (1883–1860, GAGG + T7P [−1 bp] + 24 bp = 47 bp). Oligonucleotides were precipitated in ethanol and diluted to 100 pmol/L, and 1 μL of this was used for PCR using Taq DNA polymerase (PerkinElmer, Waltham, MA) with cycling for 30 seconds at 95°C, 30 seconds at 55°C, and 20 seconds at 72°C per cycle. Pure, RNAse-free DNA was acquired using a DNA clean-up kit (Promega, Madison, WI), and optical density was measured at OD260. Purified linearized DNA for in vitro transcription was obtained by cutting at the 5' end for antisense probe and by priming the 3' end of the RNA promoter with RNA polymerase. The double-stranded exon 3a DNA contained T3 and T7 promoter sequences at the 3' and the 5' ends, respectively, and was used to produce RNA probes.

FIGURE 4. Primer selection by using the DNA polarity conversion program; red marked sequences between 721 and 781 numbers were selected.
TABLE 2. Group Designations of the S2 Pregnant C57BL/6N Mice

<table>
<thead>
<tr>
<th>No. pregnant mouse</th>
<th>Experimental</th>
<th>Control</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>No. aborted mouse</td>
<td>8</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>No. selected mouse</td>
<td>12</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>No. renamed mouse</td>
<td>E1–E12</td>
<td>C1–C12</td>
<td>S1–S6</td>
</tr>
</tbody>
</table>

Each of the 20 mice was prepared for the experimental and control groups, and another 12 mice were prepared for the sham group.

Whole-Mount and Histologic Examinations of ISH

Each pregnant C57BL/6N mouse from GD 10.5 to 15.5 was anesthetized with ketamine hydrochloride (Sigma Co) and perfused through the left ventricle with 4% paraformaldehyde in PBS (pH 7.0). Heads, including palatal tissues of all embryos, were fixed in Serra fixative solution (60% ethanol, 30% formalin, and 10% acetic acid) overnight at 4°C and dehydrated with graded alcohol. Tissues were dehydrated in 100% isopropanol and embedded in paraffin, and 5-μm sections were prepared. After deparaffinization, sections were treated with protease K (10 μg/mL) for 15 minutes at room temperature, and endogenous alkaline phosphatases were inactivated using 0.2 N HCl. Hybridizations were performed in 50% formamide, 10 mmol/L of Tris-HCl at pH 7.6, 200 μg/mL of transfer RNA, 1× Denhardt solution, 10% dextran sulfate, 600 mmol/L of NaCl, 0.25% sodium dodecyl sulfate, and 1 mmol/L of EDTA at 50°C for 16 hours in a humidified chamber. The slides were washed with 2× saline sodium citrate (SSC) containing 50% formamide at 55°C for 30 minutes and then rinsed at 37°C for 10 minutes in Tris-NaCl-EDTA buffer solution (10 mmol/L of Tris-HCl at pH 8.0, 500 mmol/L of NaCl, and 1 mmol/L of EDTA). Nonhybridized transcripts were digested with 20 μg/mL of RNase A (Sigma Co) in TNE at 37°C for 30 minutes. The slides were washed in TNE at 37°C for 10 minutes and then once with 2× SSC at 50°C for 20 minutes, and twice with 0.2× SSC at 50°C for 20 minutes.

Detection of ISH was carried out using the Genius Detection system (Boehringer Mannheim Co.). According to manufacturer’s instructions, transcripts were detected specifically with an anti-digoxigenin antibody conjugated to alkaline phosphatase in mixed solution 1 (maleic acid 0.1 M, NaCl 0.15 M, pH 7.5). The slides were washed several times with mixed solution II (100 mmol/L of Tris-HCl at pH 9.5, 100 mmol/L of NaCl, and 50 mmol/L of MgCl2) and immersed in color-developing solution (0.3 mg/mL of nitro blue tetrazolium and 0.15 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 mol/L of NaHCO3; Boehringer Mannheim Co). Color development was stopped by immersing in mixed solution 3 (10 mmol/L of Tris-HCl buffer at pH 8.0 and 1 mmol/L of EDTA). The final specimen was mounted on a silanized RNase-free slide (Muto Pure Chemicals Co, Japan) and kept horizontally in a 55°C oven for 2 hours. All slides were observed under bright- and dark-field illumination using a Zeiss Axiophot microscope (Zeiss Co, Jena, Germany), and image collection was performed with a Nikon 3 charge-coupled device camera (Sony Co, Tokyo, Japan).

RESULTS

Mating and Fetal Mortality

Of a total of 120 female C57BL/6N mice, 45 showed vaginal plugs, of which 5 were false positives. In all, 52 pregnant mice were generated for a mating success rate of 43.3%. From the 52 mice, each experimental and control group received 20, with 12 placed in the sham group. Embryos were aborted from 8 mice in the experimental group on GDs 10.5 to 13.5, from 5 in the control group on GDs 10.5 to 12.5, and from 2 in the sham group on GD 10.5 (Fig. 2D, Table 2). Each aborted embryo was prepared for H&E staining whether or not a morphologic structure had formed.

Cleft Palate Induction and Gross Findings

From a possible 12 mice in the experimental group and 15 mice in the control group, 12 mice from each group (labeled E1 to E12 and C1 to C12) were selected for the final study. Three mice from the control group were excluded because of a small number of fetuses, between 2 and 4. From 10 mice in the sham group, mice with more than 8 fetuses were selected as S1 (GD10.5, 8 fetuses),

TABLE 3. Number Labels of Healthy Fetuses and Fetuses With Induced Cleft Palates From 30 Pregnant C57BL/6N Mice

<table>
<thead>
<tr>
<th>GD</th>
<th>Mouse</th>
<th>Fetuses</th>
<th>Cleft Palate</th>
<th>Mouse</th>
<th>Fetuses</th>
<th>Mouse</th>
<th>Fetuses</th>
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</thead>
<tbody>
<tr>
<td>10.5</td>
<td>E1</td>
<td>9</td>
<td>—</td>
<td>C1</td>
<td>11</td>
<td>S1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>8</td>
<td>—</td>
<td>C2</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>E3</td>
<td>8</td>
<td>—</td>
<td>C3</td>
<td>8</td>
<td>S2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>9</td>
<td>—</td>
<td>C4</td>
<td>11</td>
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<td></td>
</tr>
<tr>
<td>12.5</td>
<td>E5</td>
<td>11</td>
<td>3</td>
<td>C5</td>
<td>12</td>
<td>S3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>E6</td>
<td>8</td>
<td>—</td>
<td>C6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.5</td>
<td>E7</td>
<td>7</td>
<td>3 (PCP 1)</td>
<td>C7</td>
<td>8</td>
<td>S4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>E8</td>
<td>9</td>
<td>1 (PCP 1)</td>
<td>C8</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.5</td>
<td>E9</td>
<td>7</td>
<td>3</td>
<td>C9</td>
<td>9</td>
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<td>8</td>
<td>2</td>
<td>C11</td>
<td>8</td>
<td>S6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>E12</td>
<td>8</td>
<td>2</td>
<td>C12</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>18</td>
<td>109</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCP indicates posterior cleft palate.
S2 (GD 11.5, 9 fetuses), S3 (GD 12.5, 11 fetuses), S4 (GD 13.5, 11 fetuses), S5 (GD 14.5, 10 fetuses), and S6 (GD 15.5, 9 fetuses). Four mice with fewer than 7 fetuses were excluded.

A total of 102 fetuses in the experimental group, E1 and E2 (GD 10.5, 9 and 8 fetuses), E3 and E4 (GD 11.5, 8 and 9 fetuses), E5 and E6 (GD 12.5, 11 and 8 fetuses), E7 and E8 (GD 13.5, 7 and 9 fetuses), E9 and E10 (GD 14.5, 7 and 10 fetuses), E11 and E12 (GD 15.5, 8 and 8 fetuses), were acquired. Cleft palate induction occurred in 18 fetuses: 3 of 11 in E5, 3 of 7 in E7, 1 of 9 in E8, 3 of 7 in E9, 4 of 10 in E10, 2 of 8 in E11, and 2 of 8 in E12. Among these, 1 of 3 in E7 and 1 in E8 were posterior cleft palates.

A total of 109 fetuses in the control group, C1 and C2 (GD 10.5, 11 and 10 fetuses), C3 and C4 (GD 11.5, 8 and 11 fetuses), C5 and C6 (GD 12.5, 12 and 8 fetuses), C7 and C8 (GD 13.5, 8 and 9 fetuses), C9 and C10 (GD 14.5, 9 and 8 fetuses), C11 and C12 (GD 15.5, 8 and 7 fetuses), were acquired. Three fetuses, 1 in C4 and 2 in C7, were mistaken to have cleft palates because of cutting errors during the coronal direction slicing (Table 3).

Cleft palates of these 18 fetuses were observed as having a gap between both palatal shelves, an asymmetric palatal fusion, and a typical morphology, with normal-sized palatal shelves in a vertical position compared with healthy fetuses in the control or sham group.

**FIGURE 5.** Coronal sections through the palatal region of C57BL/6N mouse fetuses from GD 10.5 to 15.5.
Histomorphologic Findings of Healthy Fetuses and Fetuses With Cleft Palates

The H&E and MT stainings of the coronal sections through the palatal region from the control fetuses on GDs 10.5 to 15.5 showed apparently normal skulls with no gross abnormalities in the shape of the skeleton in the vault and base of the cranium. The 2 palatal shelves were fused normally according to their fetal age, and other structures including the eye, nose, tongue, and mandible also appeared to be normal in their development (Fig. 5). On GDs 11.5 to 13.5, the lateral maxillary processes were elevated and oriented toward one another, merging in the midline of the palate region of the maxilla with a smooth furrow between them.

In coronal sections through the palatal region of the E7 fetus on GD 13.5, the 2 palatal shelves failed to meet and fuse, resulting in a wide gap. In this fetus with a unilateral cleft palate, the right palatal process was not elevated and lacked downward movement of the tongue. On the contrary, on the right side, the left palatal process was elevated, with the left side of the tongue descending, as seen in normal palate growth (Fig. 6).

In the E7 fetus with a posterior cleft palate on GD 13.5, downward movement of the tongue was absent, so the left palatal process was not able to approach to the midline and fuse with the medial and lateral nasal processes. The right palatal process was also unable to approach the middle portion of the maxilla because of high tongue posture (Fig. 7).

In another fetus with a posterior cleft palate, E8 on GD 14.5, the left palatal process was absent from the posterior palatal region. Instead, the right lateral nasal process was elongated to fill the space, suggesting a complete unilateral cleft palate (Fig. 8). Under the ×40 and ×100 light magnification, the cleft site was lined with nasal epithelial cells, with prominent, opposing medial epithelial cells connected by squamous epithelial cells from the oral cavity (Figs. 6–8).

Immunohistochemical Findings of Healthy Fetuses and Fetuses With Cleft Palates

Positive cross-reactions were seen for Pcna, Mmp9, Hsp70, and Krox25 in the coronal sections through the palatal region from control fetuses on GDs 10.5 to 15.5, whereas the expression of Tbx22 was moderately positive (Table 1). On GDs 12.5 to 14.5, Tbx22 expression was particularly strong at the peripheral border of the tongue margin and in both lateral maxillary mesenchymal tissues from which the maxillary process originated (Fig. 5). Other proteins, such as Pcna, Mmp9, Hsp70, and Krox25, were not expressed strongly or specifically in the entire facial region for all gestational periods.

In the induced cleft palates of 18 fetuses, counts of positive cells and the degree of palatal tissue positive reaction increased slightly for Pcna, Mmp9, Hsp70, and Tbx22 proteins, whereas cross-reaction decreased for the Krox25 protein (Table 1). In 1 E7 fetus with a posterior cleft palate on GD 13.5, expression of Tbx22 was more intensive than that of Hsp70, and Tbx22 protein was expressed especially strongly in the right maxillary prominence from which the palatal shelf originated and at the peripheral dorsal border of the tongue (Fig. 7). In another E7 fetus with a posterior cleft palate on GD 13.5, the asymmetric palatal processes did not meet at the midline of the maxilla and Tbx22 expression was stronger in the right buccal region of the thick palatal process than in the same region of the left thin palatal process. At the peripheral edges of the downward tongue, the Tbx22 protein was particularly evident compared with Hsp70 or other proteins (Fig. 9). Expressions of Pcna and Hsp70 were evident at the base of the nasal septum and elevated around the site of the palatal shelf fusion during normal development from GD 11.5 to 13.5 and were also evident near the disrupted epithelial seam in fetuses with induced cleft palates from GD 13.5 and 14.5 (Figs. 5 and 7).

Histologic Findings From the ISH of Tbx22 mRNA

During normal development of the palate and the maxilla from GD 11.5, expression of Tbx22 mRNA occurred in distinct regions of the fetal head such as the nasal septum, the posterior palatal shelves before fusion, the tongue, and the mesenchymal tissues surrounding the eye anlage (Fig. 10). In addition, overexpression of Tbx22 mRNA was seen on the lower portions of the anterior tongue during normal face development on GD 10.5,
whereas no Tbx22 expression was seen at posterior regions, where
the broad base of the tongue is attached to the floor of the mouth
(Fig. 11). The strong expression of Tbx22 mRNA before palatal
fusion that was detected in the mesenchymal tissues of both palatal
processes was similar to the strong expression pattern of the Merkel
cartilage in the mandible (Fig. 12).

DISCUSSION

Orofacial clefts are common congenital birth defects in
humans. The embryology of CLP and CP are largely distinct, with
the upper lip and jaw forming between the third and fifth week and
the palate between the fifth and 12th week of human development.
The palate is derived from 2 structures: the primary palate, called the
anterior portion that originates from the intermaxillary segment, and
the secondary palate, called the posterior portion. 10 The latter is
formed by the fusion of 2 lateral palatal processes that grow
downward as the lateral palatal shelves on either side of the tongue
before becoming elevated to a horizontal position above the tongue.
The medial edge epithelia (MEE) of the 2 opposing palatal shelves then
fuse to form the midline seam that subsequently degenerates
through a combination of epithelial-mesenchymal transformation,
cell migration, and apoptosis to create an intact palate separating the
oral and nasal cavities. The palatal shelves also fuse with the nasal
septum dorsally and the primary palate anteriorly. 11 At the same
time, midline fusion proceeds at the free margin of the nasal septum,
which grows down from the fused medial nasal processes, and the
anterior portion fuses with the primary palate.

In our results, the midline seam was fused from GD 13.5 or
14.5 in the C57BL/6N fetus, and palatal shelves were fused with
nasal processes from GD 14.5 or 15.5. Gestational day 10.5 is
comparable to the fifth to sixth human week, so the fusion period of
GD 13.5 to 15.5 represents 8 to 10 weeks after human fertilization.
From the coronal sections, the posterior portions of the head
appeared to fuse earlier than the anterior portions of the palate. In
the mouse, the variety of loss-of-function mutations that result in CLP or
CP is not as clear as in humans. Most mutations occur sporadically
and display a multifactorial mode of inheritance. Thus, the complex
interaction between genes and the environment has hampered efforts
to identify underlying genetic predispositions to OFCs. Numerous
candidate genes have been studied to date, and we focused on the
Tbx22 protein and related proteins such as Pcna, Mmp9, Hsp70,
and Krox25 on GDs 10.5 to 15.5.

In humans, primary palate development occurs mainly in the
fifth and sixth week of development, whereas secondary palatal
formation begins in the sixth week of development. This continues
through to palatal shelf fusion, which occurs in the eighth and ninth
week of development, and then bony ossification and development of
associated structures, such as the uvula, muscles, and glands,
follow at later stages of fetal development. Any disruption during
these complex procedures in the palate development, such as
physical or environmental factors, can result in OFCs.

During palatal development, the inferior nasal septum fuses
with the right and left palatal shelves to form the secondary palate.
We found that Tbx22 was expressed in outgrowths of the nasal
septum that expand in the direction of these shelves. In the GD-10.5
fetus, sloughing of the suprabasal and lateral epithelial cells of both
palatal shelves could be seen near such an outgrowth, a mechanism
that takes place immediately before contact. This is the first step of
mesenchymal bridging of these structures by Tbx22 signaling factor
(Figs. 11D, E).

In a more posterior section where the palatal shelves are more
closely apposed, Tbx22 expression was shown to be confined to the
inferior nasal septum. After fusion of the nasal septum with the
palatal shelves, mesenchymal cells that were thought to have
migrated from the nasal septum and merged with the tissue of the
palatal shelves showed faint Tbx22 expression in the results of our
immunohistochemical (IHC) analysis (Figs. 5G3, G4, H3, and H4).

Only the anterior parts of the palatal shelves fuse with the
nasal septum, whereas no connection is made with the posterior
colateral shelves, leaving an opening, the nasopharynx. A recent study
showed that the expression of Msx1, another gene involved in
secondary palate development, is restricted to the anterior region of
the palatal shelves and that different genetic pathways act in the
anterior and posterior palatal shelf mesenchyme. The Msx1 gene is
now believed to promote growth and inhibit differentiation. Growth
disruption by Msx1 mutation could cause a lack of distal facial bud
outgrowth and consequent primary or secondary palatal clefting in
the mouse model. 12,13

Orofacial clefts are the most frequent congenital orofacial
abnormalities, and most cases are sporadic and thought to be due to
environmental and genetic susceptibility factors. 10,14,15 In the
C57BL/6N embryonic mouse, exposure to glucocorticoids reduces
the palatal shelf size so that opposing shelves cannot come into
contact and fusion does not occur. 8 A coordinated expression of
genes that regulate cell proliferation, differentiation, transformation,
and cell death is required for normal palatogenesis. 16 Many previous
studies have suggested that the relationship between genes and
nonsyndromic CLP or CP might be modified by environmental
factors. Among these, risk estimates for maternal alcohol consump-
tion of more than 4 drinks per month were significantly elevated for

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CLP in a population-based case control study. Maternal alcohol consumption in pregnancy is detrimental to the developing human fetus. Women who abuse alcohol during pregnancy are at significant risk of bearing a child with fetal alcohol syndrome. Affected individuals exhibit prenatal and postnatal growth retardations, craniofacial anomalies, and dysfunction of the central nervous system. Fetal alcohol syndrome represents the mild end of the holoprosencephaly spectrum of anomalies, which can include clefting, and the heavier the consumption, the more likely a CLP/CP phenotype will be a component of the craniofacial defect. In the experimentally aborted 8 fetuses in this study, this kind of craniofacial defect might be attributed to high alcohol consumption.

Synthetic glucocorticoids including dexamethasone, triamcinolone, and hydrocortisone bind to the glucocorticoid receptor and, when administered at pharmacologic doses, are teratogenic, and induce cleft palate. Glucocorticoid receptors are expressed in the developing embryo in both the palatal epithelium and the mesenchyme. These receptors enter the cellular nucleus, bind to specific transcriptional regulatory elements, and alter transcription of target genes. The components of these receptor-mediated pathways are complex and include multiple regulatory proteins, including Hsp90 and Mmp9. In glucocorticoid-related clefts, the primary cause of clefting is thought to be the formation of small palatal shelves. In our induced cleft palate model, glucocorticoids were given subcutaneously from GD 9.5 to GD 11.5 at 100 mg/kg, a dose expected to be minimal based on dose regimens in previous studies that examined the synergistic interaction between 2,3,7,8-tetrachlorodibenzo-p-dioxin and hydrocortisone.

Palatal shelves first appeared approximately on GD 11.5, corresponding to about the sixth to seventh human week. In the mouse, shelves grow rapidly along a vertical plane facing the developing tongue. The important stage in mouse palatogenesis occurs during GDS 12.5 to 13.5, when the palatal shelves undergo a sudden elevation to bring them into horizontal apposition above the flattening tongue. These palatal shelves consist of rapidly proliferating mesenchymal cells, and insufficient mesenchyme is believed to be the most common reason for cleft palate in mice.

Several proteins such as Pcn, Krox25, Mmp9, Hsp70, Msx1, and Lhx8 have been implicated in palatal mesenchymal proliferation; cleft palate might be induced in the mouse if the palatal shelves fail to meet in the horizontal plane. Pcn, Mmp9, Hsp70, and Krox25 were not strongly and specifically expressed in the entire facial region during any gestational period. In the healthy fetuses or fetuses with induced cleft palates, Pcn antibodies reacted moderately in the broad whole facial region during all gestational periods. Pcn expression is representative of rapidly proliferating mesenchymal cells in palatal development, and based on the moderate to strong expression of Pcn in our study, we conclude that Pcn can be considered indicative of the degree of proliferation of palatal mesenchymal cells in the developing palate shelves and tongue.

Mmp9 is a gelatinase B that is broadly expressed in the maxillary prominence region and the tongue during all gestational
These Mmp9 and Hsp70 expressions were that reported strong Tbx22 Expressions in C57BL/6N Mice. The expression of was detected in the mesenchymal cells at the base of the normal development on GDs 11.5 to 13.5. These proteins were also evident near the disrupted epithelial seam in the fetus with an induced cleft palate on GDs 13.5 and 14.5 because of limited mesenchymal cell proliferation.

Krox25, also known as Kox15, Krox26, and Zfp422, plays an important role as a transcription factor in the development of craniofacial bones and dental organs and in the cytodifferentiation and the amelogenesis of enamel epithelium. We found that Krox25 was characteristically expressed in the MEE and mesenchymal tissues of maxillary prominence in healthy fetuses on GDs 11.5 to 13.5. Clearly, epithelial-mesenchymal interactions are crucial in craniofacial development, and specific sites of expression such as the tooth buds in the MEE and mesenchymal tissues of maxillary prominence may function as inductive signaling centers that influence palate morphogenesis later.

In the induced posterior cleft palate of the E7 fetus on GD 13.5, the asymmetric palatal processes did not meet in the midline of the maxilla and Tbx22 protein expression was more intense in the right maxillary prominence region of the thick palatal process region than the same region in the thin left palatal process. Tbx22 protein expression was strong, particularly in the peripheral edges of the downward tongue compared with Hsp70 or other proteins (Fig. 9). The spatiotemporal expression of Tbx22 was reevaluated by ISH, and this transcript was expressed in distinct regions of the fetal head including the nasal septum, posterior palatal shelves before fusion, tongue, and mesenchymal tissues surrounding the eye anlage during the normal development of the palate and the maxilla from GD 11.5 to 13.5. In our ISH results, overexpression of Tbx22 mRNA was seen on the lower portions of the anterior tongue during the normal face development on GD 10.5, whereas no Tbx22 expression was seen in the posterior regions where the broad base of the tongue is attached to the floor of the mouth on GD 13.5. Thus, these findings are consistent with recently published studies that reported strong expression of Tbx22 mRNA in the mesenchymal tissues of both palatal processes and in the region at the base of the tongue, especially on GDs 10.5 to 13.5, before palatal fusion.

The tongue is formed from tissue swellings at the floor of the mouth, and during development, its basal attachment is anteriorly reduced to a thin tissue bridge, the frenum. Ankyloglossia, named tongue-tie, has been reported in most CPX patients. An ankyloglossia, a shortened frenum may restrict movement of the tongue, creating a physical barrier that inhibits palatal shelf elevation. Disturbances of this process can result in an anteriorly extended frenum or a shortened frenum. The expression of Tbx22 was weakly detected in the tissue bridge between the tongue and the floor of the mouth in the IHC and ISH results, and on anterior sections, Tbx22 was detected in the mesenchymal cells at the base of the tongue, in the frenum, and in the adjacent superior layer of the floor of the mouth.

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