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Deletion of the T-box transcription factor gene, *Tbx1,* in mice induces differential expression of genes associated with cleft palate in humans

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ABSTRACT

Objective: We examined the function of the T-box transcription factor 1 (TBX1) in palatogenesis.
Design: *Tbx1*-knockout mice were histologically examined by hematoxylin and eosin staining. Next, secondary palatal shelves dissected from wild type or *Tbx1*-knockout mice embryos at embryonic day 13.5 were investigated with microarray analysis, gene ontology analysis, and real-time quantitative polymerase chain reaction. We performed gene profiling of developing palatal shelves from wild type and *Tbx1*-knockout embryos. We also analyzed the association of mouse genes linked to cleft palate with biological processes and compared the results with those of our ontology analysis of dysregulated genes in *Tbx1*-knockout palatal shelves.
Results: Histological analysis of *Tbx1*-knockout palate with complete cleft palate at postnatal day 1 showed aplasia of secondary palates associated with a small mandible and a small tongue compared to wild type littermates. Gene ontology analysis indicated that genes associated with development of the nervous system, muscle, and biomineral tissue were dysregulated in *Tbx1*-knockout palatal shelves. Furthermore, in *Tbx1*-knockout palatal shelves, genes associated with human cleft palate, specifically, myosin heavy chain 3 (*Myh3*) and nebulin (*Neb*), were downregulated and gamma-aminobutyric acid type A receptor beta 3 subunit (*Gabrb3*) was upregulated.
Conclusions: Our findings demonstrate that TBX1 maintains normal growth and development of palatal shelves, mediated through the regulation of genes involved in muscle cell differentiation, nervous system development, and biomineral tissue development. Multiple factors in *Tbx1*-knockout mice may lead to various subtypes of cleft palate.

1. Introduction

Cleft lip and/or palate are the most frequent craniofacial birth-defects in humans occurring in 1 in every 700 births worldwide (Tolarova & Cervenka, 1998). Cleft palate phenotypes observed in humans include various subtypes of cleft palate termed complete cleft palate, incomplete cleft palate, and submucosal cleft palate. Genes associated with cleft lip and/or palate are genetically different from complete cleft palate, incomplete cleft palate, and submucosal cleft palate (Funato & Nakamura, 2017). Since the secondary palate consists of both a bone-lined hard palate and a bone-free soft palate, incomplete cleft palate includes the mildest forms of cleft palate, which are soft palate cleft and bifid uvula. Among cleft palate phenotypes, approximately 46% of patients have soft palate cleft (Andersson, Sandvik, Abyholm, & Semb, 2010). The soft palate mainly consists of muscle, and is crucial for proper speech, swallowing, and feeding (Kogo, Hamaguchi, & Matsuya, 1996; Marrinan, LaBrie, & Mulliken, 1998). Because most genetically mutant mice exhibit complete cleft palate, studies of palatal development in mice have inherently focused on complete cleft palate, whereas the etiology of soft palate cleft and incomplete cleft palate is not well understood.

Chromosome 22q11.2 deletion syndrome, which manifests as DiGeorge syndrome (Online Mendelian Inheritance in Man; OMIM #188400), velocardiofacial syndrome (OMIM #192430), or conotruncal anomaly face syndrome (OMIM #217095), affects 1 in every 4000 human births. It is characterized by a set of phenotypic craniofacial anomalies that include different subtypes of cleft palate, velopharyngeal insufficiency, and facial muscle hypoplasia. Mutations in *TBX1,* which is a member of the T-box transcription factor gene family, have been reported in patients with characteristic phenotypes of

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22q11.2 deletion syndrome, conotruncal anomaly face syndrome, and nonsyndromic cleft palate (Jaouadi et al., 2018; Osoegawa et al., 2008; Yagi et al., 2003). These findings indicate that *TBX1* is a disease-causing gene responsible for phenotypically variable forms of nonsyndromic cleft palate and 22q11.2 deletion syndrome. *Tbx1*-knockout mice largely exhibit features of 22q11.2 deletion syndrome found in humans (Funato, Nakamura, Richardson, Srivastava, & Yanagisawa, 2015; Hu et al., 2004; Jerome & Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001) and present various types of the cleft palate phenotype (Funato, Nakamura, Richardson, Srivastava, & Yanagisawa, 2012). The secondary palatal shelves are composed of ectoderm-derived epithelium and neural crest-derived mesenchyme. *Tbx1* is expressed in the epithelium of palatal shelves in mice and is implicated in the proliferation, differentiation, and cell orientation of the oral epithelium and palatal mesenchyme (Brock, Economou, Cobourne, & Green, 2016; Funato et al., 2012; Goudy, Law, Sanchez, Baldwin, & Brown, 2010). The phenotypic variation found in cleft palate in *Tbx1*-knockout mice strongly suggests that multiple stochastic factors affect the palatal phenotype of *Tbx1*-knockout mice.

In this study, we demonstrate that TBX1 is required for gene expression in muscle development in secondary palatal shelves. TBX1 affects the expression of homologous genes associated with human cleft palate, specifically, myosin heavy chain 3 (*Myh3*), nebulin (*Neb*), and gamma-aminobutyric acid type A receptor beta 3 subunit (*Gabrb3*) in developing palatal shelves in mice. These findings highlight a previously unknown role of TBX1 in palatal development and identify novel connections between TBX1 and disease-causing genes of cleft palate. The contributions of multiple factors may result in various cleft palate phenotypes, including submucosal cleft palate and soft palate cleft present in 22q11.2 deletion syndrome.

2. Materials and methods

2.1. Tbx1-knockout mice

Tbx1^{neo} mice (*Tbx1*^{tm1Dsr}; Mouse Genome Informatics MGI ID: 3510038) were used in this study. *Tbx1*$^{neo/+}$ mice were mated with *More-Cre* mice to ubiquitously delete exons 4–8 resulting in the heterozygous *Tbx1* null allele (*Tbx1*$^{+/-}$), as previously described (Funato et al., 2012; Hu et al., 2004). *Tbx1*-knockout (*Tbx1*$^{-/-}$) mice have been described previously (Funato, Nakamura, Richardson et al., 2015; Hu et al., 2004). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permit Number: 0160215A). All experiments were performed in strict accordance with approved guidelines and all efforts were taken to minimize pain experienced by the mice.

2.2. Histology

Embryos were fixed in 4% paraformaldehyde/phosphate buffered saline and embedded in paraffin. Sections were stained with hematoxylin & eosin for routine histology.

2.3. Microarray analysis

Secondary palatal shelves dissected from eight wild type or *Tbx1*-knockout embryos at embryonic day 13.5 (E13.5) were processed for total RNA extraction using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany), and were pooled prior to analysis. Concentration and purity were measured with a NanoDrop ND-1000 spectrometer. Quality was checked using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and all RNA integrity number scores were 9.9 and 10. Microarray analysis was performed by the KURABO Bio-Medical Department using the GeneChip Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA). Data were analyzed with Affymetrix Expression Console Software ver.1.3.0 using Affymetrix default analysis settings. The Gene Expression Omnibus (GEO) database accession number for all data sets reported in this paper is GSE79514.

2.4. Gene ontology analysis

Genetically mutant mice with oral cleft phenotypes were screened using the Mouse Genome Informatics (MGI) database from the Jackson Laboratory (http://www.informatics.jax.org). The database was searched using the term “cleft palate” and the search was completed on July 20, 2018 (Table S2). The proteins corresponding to each gene were identified using the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene). We also investigated syndromes/diseases using the Online Mendelian Inheritance in Man (OMIM; http://omim.org). Genes were analyzed using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) database (http://pantherdb.org) (Mi, Muruganujan, Casagrande, & Thomas, 2013), and the Reactome pathway database (http://www.reactome.org). Statistically significant enrichment of the data set was determined using binomial testing with Bonferroni corrections for multiple testing (Thomas et al., 2003). The Bonferroni-corrected P value < 0.05 was considered statistically significant. Because the results contained many classifications with similar gene constituents, the gene counts of those classifications that were a complete subset of another classification were excluded. Gene interaction networks were constructed using ToppGene (https://toppgene.cchmc.org).

2.5. Real-time quantitative polymerase chain reaction (PCR)

Real-time quantitative polymerase chain reaction (qPCR) was performed in triplicate as described previously (Funato, Nakamura, Richardson et al., 2015). Primer sequences are shown in Table S1. All data were normalized to *ActB* (β-actin) expression. Data were analyzed using an unpaired Student’s t-test and expressed as the mean ± standard error of the mean (s.e.m.). P values less than 0.05 were considered statistically significant for all experiments.

3. Results

3.1. Gene ontology analysis of dysregulated genes in Tbx1-knockout palatal shelves

Secondary palates consist of the hard palate, which provides a bony barrier between the nasal and oral cavities, and the soft palate, which forms a barrier between the nasopharynx and the oropharynx. Cleft secondary palate in *Tbx1*-knockout mice is 100% penetrant (n = 17). All *Tbx1*-knockout mice (n = 17) in our study exhibited cleft secondary palate (Funato et al., 2012) and small mandible (Funato, Nakamura, Richardson et al., 2015), including complete cleft palate (41%), incomplete cleft palate (47%), and soft palate cleft (12%), in combination with submucosal cleft palate (59%) (Funato et al., 2012). Histological analysis of *Tbx1*-knockout palate with complete cleft palate at postnatal day 1 showed aplasia of secondary palates associated with a small mandible and a small tongue compared to wild type littermates (Fig. 1A).

We have previously shown that the impaired adhesion separation of the oral epithelium is an underlying cause for various forms of cleft palate phenotypes in *Tbx1*-knockout mice (Funato et al., 2012). Although *Tbx1* is localized specifically to the oral epithelium (Funato et al., 2012; Zoupa, Seppala, Mitsiadis, & Cobourne, 2006), decreased proliferation of palatal mesenchymal cells suggests that epithelial TBX1 indirectly controls palatal mesenchyme development (Funato et al., 2012; Goudy et al., 2010). To understand better the genetic basis