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Cdc42 is crucial for facial and palatal formation during craniofacial development

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ABSTRACT

Craniofacial deformities with multifactorial etiologies, such as cleft palate and facial dysmorphism, represent some of the most frequent congenital birth defects seen in humans. Their pathogeneses are often related to cranial neural crest (CNC) cells. During CNC cell migration, changes in cell shape and formation, as well as maintenance of subcellular structures, such as filopodia and lamellipodia, are dependent on the complex functions of Rho family small GTPases, which are regulators of actin cytoskeletal organization. Cdc42, a member of the Rho family of small GTPases, is known to play critical roles in organogenesis of various tissues. To investigate the physiological functions of Cdc42 during craniofacial development, we generated CNC-derived cell-specific inactivated Cdc42 mutant mice (*Cdc42*$^{fl/fl}$;*P0-cre*). Most of the *Cdc42*$^{fl/fl}$;*P0-cre* neonates were viable at birth, though they appeared weaker and no milk was found in their stomachs, and all died within a few days. They had a short face and intracranial bleeding, and abnormal calcification of the cranium. *Cdc42*$^{fl/fl}$;*P0-cre* neonates also demonstrated a cleft palate and there was no fusion of the secondary palate because of failure of palatal shelf elongation for the process of palate closure. Cdc42 is crucial for facial and palatal formation during craniofacial development.

1. Introduction

Cleft lip with or without cleft palate occurs in 1 in 500 to 2500 live births worldwide, and represents the most frequent congenital facial anomaly seen in humans. The condition, which requires complex multidisciplinary treatments and has lifelong implications for affected individuals (Vanderas, 1987; Schutte and Murray, 1999), is caused by various pathogenetic influences, such as genetics and environmental risk factors, as well as others (Wilkie and Morriss-Kay, 2001; Cobourne, 2004). Cleft lip and palate seen in affected humans are caused by abnormal facial development during fusion of the medial nasal process and maxillary process between 5 to 8 weeks after fertilization (Levi et al., 2011). The definitive mammalian palate forms through union of the primary palate and 2 secondary palatal shelves. Palatal development is the process in which the bilateral maxillary processes descend vertically from the maxilla, and occurs between embryonic day (E)12.5 and E15.5. Subsequently, the palatal shelves rotate horizontally, then meet at the midline and fuse by E15.5, followed by disappearance of the midline epithelial seam (Ferguson, 1977; Liu et al., 2007). One of the key features of craniofacial development is formation of neural crest (NC) cells (Le Douarin et al., 2004).

NC cells are embryonic multi-potent stem cells that give rise to various types of cells and tissues (Bronner-Fraser and Fraser, 1988; Shah et al., 1996). Among the various types, cranial neural crest (CNC) cells play important roles in the regulation of craniofacial development (Bronner-Fraser, 1993; Selleck et al., 1993), while it is also known that they form most of the hard tissues of the head, such as the maxilla, mandible, and teeth (Chai and Maxson, 2006). During CNC cell migration, changes in cell shape and formation, as well as maintenance of subcellular structures, such as filopodia and lamellipodia, are dependent on members of the Rho family of small G proteins. Cdc42, a Rho family small G protein, is ubiquitously expressed and functions as a molecular switch, cycling between an active and inactive GDP-bound states (Van Aelst and D'Souza-Schorey, 1997; Etienne-Manneville and Hall, 2002), while it is also known to play critical roles in cellular functions, such

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as actin cytoskeletal reorganization, cell migration, differentiation, and gene expression (Bishop and Hall, 2000; Jaffe and Hall, 2005). Cdc42 conventional knockout mice die before E7.5 (Chen et al., 2000). Using tissue-specific gene knockout technology, Cdc42 has been indicated to play various critical roles in vivo (Hall and Nobes, 2000; Liu et al., 2013).

Recently, Aizawa et al. (2012) demonstrated the functions of Cdc42 during limb development using limb bud mesenchyme-specific inactivated Cdc42 (*Cdc42*$^{fl/fl}$;*Prx1-cre*) mice. Those mice demonstrated a cleft palate because of failure of palatal shelf elongation (Aizawa et al., 2012). Liu et al. (2013) also reported that Cdc42 plays an essential role in NC cell migration, and inactivation of Cdc42 in NC cells impaired craniofacial and cardiovascular development in mice. To investigate the physiological functions of Cdc42 during facial and palatal development, we used a well-characterized transgene in which Cre-recombinase is driven by a promoter of protein 0 (P0), a specific marker of NC cells (Yamauchi et al., 1999). This transgene expresses Cre in tissues derived from NC cells, such as spinal dorsal root ganglia, the sympathetic and enteric nervous systems, and ventral craniofacial mesenchyme during stages later than E9.0.

2. Materials and methods

2.1. Generation of Cdc42 conditional knockout mice

All animal experiments were conducted in accordance with the guidelines of Showa University and the University of Tokyo. The *Cdc42 gene* was knocked out using Cre-loxP recombination by crossing *Cdc42* flox with *P0-cre* transgenic (*P0-cre*) mice (Yamauchi et al., 1999; Aizawa et al., 2012). Timed-mating was set to occur on noon of the day on which a vaginal plug was detected and was considered to be E0.5. Offspring were genotyped by PCR analysis using the following primer pairs: for *P0-Cre*, 5′-GACGATGCAACGAGTGATGA-3′ and 5′-AGCA TTGCTGTCACTTGGTC-3′; and for *Cdc42*, F1 5′-ATCGGTCACTGTTCTACT TTG-3′ and R1 5′-TACTGCTATGACTGAAAACCTC-3′. Both conditional and Δexon2 alleles were identified using F1, R1, and R2 5′-GTTTT GCCTGCATGTATGTCTG-3′ primers (Fig. 1A).

For analyses of expression patterns generated by *P0*-mediated Cre recombination, R26R reporter mice and CAG-CAT-EGFP transgenic mice were used (Soriano, 1999; Kawamoto et al., 2000). R26R reporter mice carry a *loxP-stop-loxP-lacZ* cassette inserted into the ubiquitously expressed ROSA26 locus. Mating of *P0-cre* with *R26R* reporter mice generated double transgenic mice (*R26R*;*P0-cre* mice), then detection of β-galactosidase activity in whole embryos was performed as previously described (Chai et al., 2000). Also, mating of *P0-cre* mice with CAG-CAT-EGFP transgenic mice generated double transgenic mice (*EGFP*;*P0-cre* mice), with detection of EGFP in the palate of E13.5 mice examined using fluorescence stereomicroscopy (MVX100, OLYMPUS). The genetic background of the mice used in this study is a hybrid of the C57BL/6, 129Ola, and ICR strains.

2.2. Quantitative real-time PCR

Total RNA from palates was extracted with TRIzol reagent (Life Technologies), then reverse transcribed using SuperScript III (Life Technologies). Quantitative PCR was performed using a TaqMan real-time PCR system, with the following assay IDs: Cdc42; Mm01194005g1, *CyclinD*$_1$; Mm00432359m1, and *Gapdh*; Mm03302249g1.

2.3. Anatomical and histological analyses

For skeletal staining, mice were skinned and eviscerated, then dehydrated in 95% ethanol overnight. The skeletons were stained

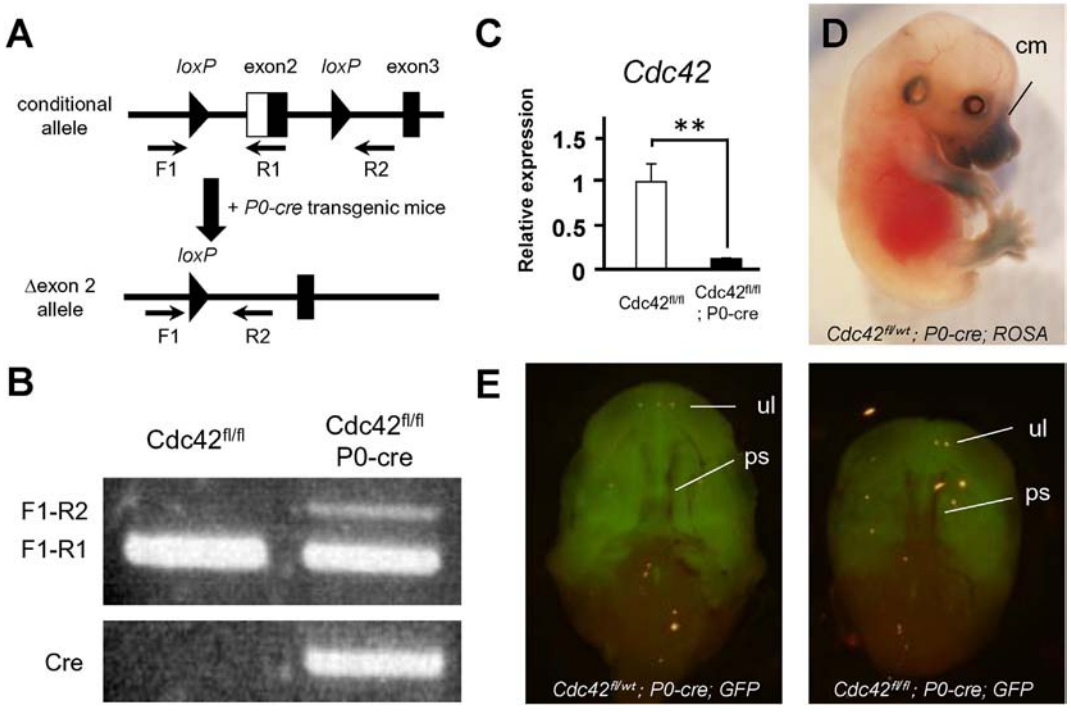

Fig. 1. Generation of *Cdc42* conditional knockout mice. (A) Schematic drawing of targeted strategy for production of *Cdc42* conditional knockout mice. Different primers (F1, R1, R2) were used for PCR assessment of *Cdc42* exon 2 deletion (Δexon2). (B) PCR was performed using *Cdc42*$^{fl/fl}$ and *Cdc42*$^{fl/fl}$;*P0-cre* palate samples obtained on postnatal day 0. Conditional allele specific (F1–R1; 162 bp) and Δexon 2 allele specific (F1–R2; 350 bp) gene expressions were found. (C) The expression level of *Cdc42* was determined using real-time PCR. Amplification signals from the *Cdc42* gene were normalized against those from the *Gapdh* gene. Values are shown as the mean ± SD of 3 samples as compared to the level seen with *Cdc42*$^{fl/fl}$ (**P < 0.01). (D) Detection of β-galactosidase (*lacZ*) activity. Temporal and spatial expressions of *lacZ* in whole-mount X-gal-stained embryos of *Cdc42*$^{fl/wt}$;*P0-cre*;*R26R* mice on E13.5. Lateral views demonstrated that β-galactosidase activity was mostly observed in the area of neural crest migration. cm; cranial mesenchyme. (E) Localization of NC-derived cells in palates of *Cdc42*$^{fl/wt}$;*P0-cre*;*EGFP* and *Cdc42*$^{fl/fl}$;*P0-cre*;*EGFP* mice on E13.5. Stereoscopic fluorescence microscope images of palates. Panels show corresponding fluorescent images. GFP labeled cells (green) were observed in the palates. ul; upper lip, ps; palatal shelf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)