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Inactivation of Cdc42 in neural crest cells causes craniofacial and cardiovascular morphogenesis defects

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ABSTRACT

Neural crest cells (NCCs) are physically responsible for craniofacial skeleton formation, pharyngeal arch artery remodeling and cardiac outflow tract septation during vertebrate development. Cdc42 (cell division cycle 42) is a Rho family small GTP-binding protein that works as a molecular switch to regulate cytoskeleton remodeling and the establishment of cell polarity. To investigate the role of Cdc42 in NCCs during embryonic development, we deleted Cdc42 in NCCs by crossing Cdc42 flox mice with Wnt1-cre mice. We found that the inactivation of Cdc42 in NCCs caused embryonic lethality with craniofacial deformities and cardiovascular developmental defects. Specifically, Cdc42 NCC knockout embryos showed fully penetrant cleft lips and short snouts. Alcian Blue and Alizarin Red staining of the cranium exhibited an unfused nasal capsule and palatine in the mutant embryos. India ink intracardiac injection analysis displayed a spectrum of cardiovascular developmental defects, including persistent truncus arteriosus, hypomorphic pulmonary arteries, interrupted aortic arches, and right-sided aortic arches. To explore the underlying mechanisms of Cdc42 in the formation of the great blood vessels, we generated Wnt1Cre-Cdc42-Rosa26 reporter mice. By beta-galactosidase staining, a subpopulation of Cdc42-null NCCs was observed halting in their migration midway from the pharyngeal arches to the conotruncal cushions. Phalloidin staining revealed dispersed, shorter and disoriented stress fibers in Cdc42-null NCCs. Finally, we demonstrated that the inactivation of Cdc42 in NCCs impaired bone morphogenetic protein 2 (BMP2)-induced NCC cytoskeleton remodeling and migration. In summary, our results demonstrate that Cdc42 plays an essential role in NCC migration, and inactivation of Cdc42 in NCCs impairs craniofacial and cardiovascular development in mice.

Introduction

Craniofacial and cardiovascular malformations comprise more than one-third of human congenital diseases (Hoffman and Kaplan, 2002; Walker and Trainor, 2006). The formation of craniofacial and cardiovascular structures is an orchestrated process and largely depends on the specific interactions between neural crest cells (NCCs) and their surrounding cells, which are derived from the ectoderm, endoderm and splanchnic mesoderm (Hutson and Kirby, 2007; Sauka-Spengler and Bronner-Fraser, 2008; Stoller and Epstein, 2005). NCCs are a multipotent cell population that originate from the border between the neural tube and surface ectoderm. After induction, NCCs undergo epithelial-to-mesenchymal transition, migrate stereotypically to diverse locations and then differentiate into multiple cell types at their destinations (Knecht and Bronner-Fraser, 2002; Trainor, 2005), including craniofacial skeletons, as well as vasculature and smooth muscle cells in the conotruncal structures of the heart (Chai and Maxson, 2006; Hutson and Kirby, 2007). Cardiac NCCs, a subpopulation of cranial NCCs that emigrate from the region between the otic placode and the caudal border of somite three, are necessary for proper septation of the cardiac outflow tract and correct alignment of aortic arch arteries (Hutson and Kirby, 2007; Stoller and Epstein, 2005). During embryogenesis, cardiac NCCs ventrolaterally migrate into pharyngeal arch three, four, and six, and a subpopulation of cells continue migrating to cardiac outflow tract cushions. Experiments on both chicken and mouse embryos have shown that cardiac NCCs are essential for outflow tract septation (Kirby, 1987; Porras and Brown, 2008; Waldo et al., 1998). Interference of NCC functions results in persistent truncus arteriosus (PTA) and a shortened outflow tract (Goddeeris et al., 2007; Snider et al., 2007) that causes malalignment of the outflow tract and cardiac loop defects (Watanabe et al., 2010;

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Yelbuz et al., 2002). The molecular signals that operate in NCCs during craniofacial and cardiovascular formation remain to be addressed.

GTP-binding (G) proteins act as molecular switches that regulate a vast array of cellular activities and biological responses (Bar-Sagi and Hall, 2000; Hall, 1992; Neer, 1995). Cdc42 (cell division cycle 42) together with Rac and RhoA are members of the Rho subfamily in the Ras superfamily of GTPases (Johnson, 1999; Ridley, 1996; Symons, 1996; Van Aelst and D'Souza-Schorey, 1997). It is well documented that Cdc42 plays a critical role in regulating cytoskeleton reorganization, the formation of filopodia and focal adhesion complexes, the establishment of microtubule-dependent cell polarity, gene transcription, intracellular trafficking, and endocytosis (Erickson and Cerione, 2001; Etienne-Manneville, 2004; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Wu et al., 2000). Hence, Cdc42 is a critical regulator of many cellular functions such as cell cycle progression, migration, differentiation, and apoptosis (Cerione, 2004; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). A potential role of Cdc42 in NCCs in craniofacial and cardiovascular development has been suggested by a number of studies. Rho GEF and PH domain-containing protein 1 (FGD1) is a Cdc42 putative guanine nucleotide-exchange factor, and mutations in the human FGD1 gene have been shown to cause faciogenital dysplasia (Pasteris et al., 1994; Zheng et al., 1996). GTP-bound Cdc42 is able to interact with and thereby activate downstream targets, the so-called effector proteins. Over 20 target proteins for Cdc42 have been identified in mammalian cells, including PAK, Cool-1 (cloned-out of library 1), WASP (Wiskott–Aldrich syndrome protein) and IQGAP. PAK1 is one of the first Cdc42 effectors identified, and the overexpression of dominant negative PAK1 inhibits NCC migration (Bisson et al., 2012). In addition, Cdc42 is activated by integrins and focal adhesion kinase (FAK) (Dormond et al., 2001; Etienne-Manneville and Hall, 2001; Feng et al., 2006; Price et al., 1998), and loss of integrin β1 or FAK in NCCs results in craniofacial and cardiovascular developmental defects (Pietri et al., 2004; Vallejo-Illarramendi et al., 2009). Cdc42 is involved in regulating growth factor-initiated signal transduction pathways, including bone morphogenetic proteins (BMPs) (Gamell et al., 2008), fibroblast growth factors (FGFs) (Endo et al., 2009), vascular endothelial growth factors (VEGFs) (Lamalice et al., 2004; Zeng et al., 2002), and the critical functions of these growth factors in NCCs are well accepted (Kulesa et al., 2010). All of this evidence indicates that Cdc42 may be a critical regulator in NCCs during craniofacial and cardiovascular development.

BMPs are transforming growth factor-β (TGF-β) superfamily members and are among the first factors identified in the regulation of NCC development (Correia et al., 2007; Walsh et al., 2010). The evidence generated from studying genetically modified mice has demonstrated that BMP2 is one of the most important growth factors in NCC migration. The deletion of BMP2 caused the absence of migratory NCCs (Correia et al., 2007). Consistently, overexpression of Xnoggin, which is a BMP2 inhibitor, blocked NCC migration (Kanzler et al., 2000). Moreover, NCC-specific deletion of BMP2 receptors, either ActRIA (ALK2) or BMPR1A (ALK3), brought about defective formation of the cardiac outflow tract, including a shortened outflow tract (Dudas et al., 2004; Kaartinen et al., 2004; Stottmann et al., 2004). The importance of BMP2 in cell migration was also documented in cultured C2C12 cells (a type of mouse myoblast). Exposure of C2C12 cells to BMP2 enhanced actin cytoskeleton reorganization and migration, and these effects were abolished when Cdc42 function was inhibited (Gamell et al., 2008).

Consistent with its critical cellular functions, the total deletion of Cdc42 caused embryonic lethality with aberrant actin cytoskeleton organization (Chen et al., 2000). Using tissue-specific gene knockout technology (Cre-LoxP system), Cdc42 has been implicated to play a critical role in neuron apical progenitor cell renewal and cerebral hemispheres separation (Cappello et al., 2006; Chen et al., 2006; Peng et al., 2013). However, relatively little is known about the functions of Cdc42 in craniofacial and cardiovascular development. While a recent study has shown that Cdc42 is important for NCC self-renewal and proliferation, the relatively early embryonic lethality (embryonic day 13.5, E13.5) of these Cdc42 NCC knockout mice made it impossible to conduct a detailed analysis of craniofacial and aortic arch artery defects because the knockout embryos died before these structures were formed (Fuchs et al., 2009). To determine the role and molecular mechanisms of Cdc42 in craniofacial and cardiovascular development, we generated a NCC-specific Cdc42 knockout mouse line by crossing Cdc42 flox mice (Peng et al., 2013) with Wnt1-Cre mice (Danielian et al., 1998). We found that the deletion of Cdc42 in NCCs induced embryonic lethality with craniofacial morphogenetic defects. In addition, we observed that the inactivation of Cdc42 in NCCs caused abnormal great vessel patterns and aortic and pulmonary septation defects. Cellular function analysis showed that Cdc42 is crucial for BMP2-induced cytoskeleton remodeling and migration.

Materials and methods

Generation of NCC Cdc42-specific knockout mice

Cdc42/flox mice (Peng et al., 2013) were crossed with Wnt1-cre mice (Danielian et al., 1998) to inactivate Cdc42 in NCCs. The LacZ enzyme gene was introduced into Cdc42 NCC knockout mice by crossing Cdc42 and Wnt1-cre double heterozygous mice with R26R-STOP-lacZ mice. The genotypes of embryos and pups obtained by crossing Cdc42flox/+;cre+ mice with Cdc42 flox/flox mice were determined by PCR using forward (TGC CTC TAC CTC CTA AGT GC TGG GA) and reverse primers (AGA GGA CCC TTA CAG GCC TCT TCC A). Cycling conditions were as follows: 3 cycles at 94 °C for 3 min, 67 °C for 2 min and 72 °C for 1 min; 30 cycles at 94 °C for 1 min, 67 °C for 1 min and 72 °C for 1 min; 1 cycle at 72 °C for 10 min. The expected sizes of wild-type allele and floxed alleles were 206 bp and 412 bp, respectively. Mice were housed in a pathogen-free facility and handled in accordance with the principles of the *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M Health Science Center.

Skeleton staining

E18.5 embryos were de-skinned, eviscerated and fixed in 95% ethanol for 2 days. The skeleton was then stained in Alcian blue staining solution (0.03% Alcian Blue, 80% ethanol, 20% acetic acid) for 3 days. After washing with 95% ethanol for 1 day, and 95% ethanol with 2% KOH for 2 days at room temperature, Alizarin Red solution (0.03% Alizarin Red, 1% KOH in water) was added and the embryo stained for another 2 days. Finally the embryos were cleared in 1% KOH/20% glycerol solution and stored in 1:1 glycerol/95% ethanol for imaging.

Histological analysis and immunofluorescence staining

Embryos were harvested at stages from E9.5 to E18.5 and fixed in 4% paraformaldehyde at 4 °C for overnight. Paraffin embedded embryos were then sectioned and stained with H&E or eosin. Immunofluorescence staining was performed on cryosections, as described previously (Peng et al., 2008), and information on the antibodies follows: Phalloidin (p1951; Sigma; 1:100), Phosphohistone H3 (06-570; Upstate Biotechnology; 1:100), Smooth Muscle Actin (M0851; DAKO; 1:5000), SM22alpha (ab14106; Abcam; 1:100), Troponin T Ab-1 (MS295PO; Thermo Scientific; 1:100),