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LKB1 signaling in cephalic neural crest cells is essential for vertebrate head development

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ABSTRACT

Head development in vertebrates proceeds through a series of elaborate patterning mechanisms and cell-cell interactions involving cephalic neural crest cells (CNCC). These cells undergo extensive migration along stereotypical paths after their separation from the dorsal margins of the neural tube and they give rise to most of the craniofacial skeleton. Here, we report that the silencing of the *LKB1* tumor suppressor affects the delamination of pre-migratory CNCC from the neural primordium as well as their polarization and survival, thus resulting in severe facial and brain defects. We further show that LKB1-mediated effects on the development of CNCC involve the sequential activation of the AMP-activated protein kinase (AMPK), the Rho-dependent kinase (ROCK) and the actin-based motor protein myosin II. Collectively, these results establish that the complex morphogenetic processes governing head formation critically depends on the activation of the LKB1 signaling network in CNCC.

1. Introduction

The neural crest is a transient embryonic structure that arises at the dorsal lips of the folding neural tube (Le Douarin and Kalcheim, 1999). Cephalic neural crest cells (CNCC) constitute a population of invasive multipotent cells that originate from mid- and hindbrain levels and give rise to a large part of the head skeleton as well as to musculo-connective derivatives (Minoux and Rijli, 2010). These embryological observations coupled with paleontological arguments suggested the emergence of the neural crest as an evolutionarily novelty – one that was key to the development of the "new head" that epitomizes vertebrates (Gans and Northcutt, 1983; Manzanares and Nieto, 2003). Once specified, CNCC undergo an epithelium to mesenchymal transition (EMT), delaminate from the neuroepithelium and migrate into three main streams that colonize the nasofrontal bud, the branchial arches and the heart (Théveneau and Mayor, 2012). Although the persistent directionality and polarized morphology of migrating CNCC have been recognized for more than four decades, it is only recently that a series of studies have conclusively shown that CNCC migrate collectively rather than individually (Théveneau and Mayor, 2012). Furthermore, homotypic contact inhibition of locomotion (CIL), a phenomenon by which cells change their direction of migration after contact with another cell of the same type confines the extension of cell protrusions to the free edge, thereby reinforcing their ability to respond to chemoattractants (Théveneau et al., 2010). Yet, our knowledge of the signaling networks that govern

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the migratory properties of neural crest cells (NCC) is still limited.

Inactivating germ line mutations of the human *LKB1* tumor suppressor gene (also named STK11) are responsible of the Peutz-Jeghers syndrome, an autosomal-dominant disorder characterized by pigmented macules of the lips, multiple gastrointestinal polyps and an increased risk of various cancers (Hemminki et al., 1998). LKB1 is a serine/threonine kinase that regulates cell polarization and acts as a metabolic sensor by phosphorylating and activating the AMP-activated protein kinase (AMPK), a heterotrimer composed of a catalytic (AMPKα) subunit and two regulatory (AMPKβ and AMPKγ) subunits (Alessi et al., 2006; Shackelford and Shaw, 2009; Hezel and Bardeesy, 2008). In addition, LKB1 phosphorylates 12 additional AMPK-related kinases that are involved in distinct biological processes including the regulation of hepatic gluconeogenesis as well as the polarization and axon branching of cortical neurons (Alessi et al., 2006; Shackelford and Shaw, 2009; Hezel and Bardeesy, 2008). LKB1 associates with the pseudokinase STRAD (STRADα or STRADβ) and the scaffolding molecule MO25 to form the LKB1 holoenzyme complex (Alessi et al., 2006; Shackelford and Shaw, 2009; Hezel and Bardeesy, 2008). Homozygous disruption of *Lkb1* in mice causes a defect in neural tube closure and an absence of the first branchial arch (Ylikorkala et al., 2001; Bardeesy et al., 2002), a phenotype compatible with, at least in part, a dysgenesis of CNCC. These data suggest that the LKB1 pathway may be involved in the ontogenesis of CNCC.

To gain insight into this question, we used the tractable system provided by the avian embryo. With this model, we found that the Lkb1 signaling pathway controls both the polarized migration and the survival of CNCC. As a consequence of *Lkb1* inactivation, expression of morphogens that pattern the prosencephalic region was abrogated in the anterior neuroepithelium and inhibited forebrain development. Consistent with these observations, we found that genetic ablation of *Lkb1* in mouse neural crest cells at the time of their emigration from the neural primordium also led to severe craniofacial defects. Finally, delineation of the Lkb1 pathway active in CNCC revealed that the signal converges on myosin II *via* AMPK and ROCK kinases. Collectively, our results establish that the Lkb1 network orchestrates several aspects of CNCC development that are crucially required during cephalogenesis.

2. Results

2.1. Chick Lkb1 gene is expressed in delaminating and migrating neural crest cells

For the purpose of this study, we cloned and sequenced the chicken homolog of *Lkb1* cDNA. Sequence analysis revealed that the human and chicken LKB1 proteins are 90% identical at the amino acid level, and all of the phosphorylation sites and post-translational motifs in human LKB1 are conserved in the chick *Lkb1* homolog. To determine *Lkb1* gene expression patterns at neurula stages, we performed a series of *in situ* hybridization analyses. The *Lkb1* transcript was detected at 6 somites stage (ss) when the neural folds elevate before the neural tube closure. The accumulation of *Lkb1* transcripts intensified at later stages and was detected throughout the neural primordium before the egress of CNCC from the neural tube (Fig. 1A-C). From this stage on, *Lkb1* expression was detected in the migrating CNCC, which progressed along the cephalic vesicles and populated the naso-frontal and maxillo-mandibular regions, as well as the more caudal branchial arches (Fig. 1D-G).

2.2. Lkb1 expression in CNCC is crucial for head development

To inhibit *Lkb1* expression in CNCC, we used the RNA interference (RNAi) approach combined with a triple electrode system enabling a bilateral electroporation of the cephalic neural folds with double-stranded RNA (*dsRNA)* molecules before the emigration of CNCC out of the neural tube (at 4–5 ss ie HH8) (Creuzet, 2009; Garcez et al., 2014; Aguiar et al., 2014). Several studies based on electroporation of long dsRNA to silence gene expression in chicken embryos have successfully been performed and validated (Pekarik et al., 2003; Creuzet, 2009). Using rhodamine-dextran to label transfected cells, we verified that this electroporation procedure selectively transfected the rostral dorsal region of the neural folds and consequently targeted the delaminating NCC (Fig. 2A and B), although non-NCC can also be electroporated. To assess to what extent *Lkb1* gene expression is affected after CNCC electroporation of dsRNA specifically targeting chick *Lkb1*, we performed quantitative PCR on dissected faces of electroporated embryos. After dsRNA electroporation, *Lkb1* gene expression in the head of HH14 (20–21 ss) embryos was significantly reduced (Fig. 2C). Upon *Lkb1* knockdown, we observed that the craniofacial region was clearly underdeveloped 90 h (E5 stage ie HH27) post-electroporation (Fig. 2D and E). Fifty-six *dsRNA* electroporated embryos were analyzed in 12 independent experiments and the craniofacial defects observed were highly reproducible. Micrographs of scanning electron microscopy of embryos at E5 revealed that the silencing of *Lkb1* in CNCC compromised the development of nasofrontal and maxillo-mandibular processes, which appeared distorted and eventually failed to fuse together (Fig. 2F and G). To visualize the effects of the loss of *Lkb1* on the formation and the migration of CNCC, we labeled embryos with the monoclonal antibody HNK1 that recognizes a glycolipid epitope expressed on chicken pre-migratory and migratory NCC (Vincent et al., 1983). As shown in Fig. 2I, a marked reduction of the CNCC population that colonizes the nasofrontal region was observed at 25–30ss following *Lkb1* knockdown (Fig. 2H and I). To address further the role of Lkb1 in head skeletogenesis, we used Alcian blue and Alizarin red to stain cartilage and ossified bone, respectively. At E11 (HH37), the chondrogenic differentiation of the nasal septum and capsule and Meckel's cartilages of *Lkb1*-deprived embryos were reduced and calcification as seen by alizarin red staining, a proxy for osteogenic differentiation, was absent (Fig. 2J and K). Finally, to ascertain that the phenotypic effects observed were specific to the silencing of *Lkb1*, we co-electroporated *dsRNA-Lkb1* together with a vector expressing the human *Lkb1* insensitive to the *dsRNA* targeting its chicken homolog. Using this strategy, we observed that the craniofacial development was rescued with the human LKB1 wild type, whereas the kinase-dead LKB1 failed to restore cephalic development (Fig. 2L and M; Supp Fig. 1A-C). Cumulatively, these results indicate that Lkb1 activity is required for the development of CNCC.

Brain and craniofacial development is coordinated (Aoto and Trainor, 2015) and neural tube closure defects were indeed observed either after the ablation of CNCC in birds (Creuzet et al., 2004; Creuzet, 2009) or *Lkb1* null-mutation in mice (Ylikorkala et al., 2001). Yet the phenotypes resulting from *Lkb1* dsRNA electroporation at 4–5 ss to target NCC did not perturb neural tube closure. We reasoned that *Lkb1* silencing in NCC at 4–5ss may occur too late to affect this process. To investigate if we could reproduce the anencephalic phenotype by earlier silencing of *Lkb1* during development by electroporating a broader area in the neural plate, we bilaterally transfected *Lkb1-dsRNA* at 1ss at the neural plate border (Supp Fig. 1D and E). When performed in early neurula, the inhibition of *Lkb1* generated consistent neural tube defects and resulted in anencephalic embryos. These observations indicated that the spatio-temporal silencing of *Lkb1* expression