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Title: Cell cycle-related kinase is a crucial regulator for ciliogenesis and Hedgehog signaling in embryonic mouse lung development

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MATERIALS AND METHODS

Generation of mice

All mice were housed and bred in a C3Heb/FeJ background in the animal facilities of the Dongguk and Yonsei University. Experiments, housing, and breeding protocols were approved by the Institutional Animal Care and Use Committee of the Dongguk and Yonsei University. Genotyping was performed using DNA isolated from yolk-sac or tail tissue. When harvesting embryos, noon on the day of finding a vaginal plug was designated as E0.5. CcrkKO is a null allele generated from E2a Cre- mediated recombination of loxP sites flanking the first two exons of the gene, and this allele was genotyped as previously described [12].

Mouse embryonic lung explants

Control and Ccrk mutant embryo lungs were harvested at E12.5. Lungs were placed on a Nucleopore Trak-Etch membrane (Whatman; 8 µm) and cultured at the air/liquid interface in DMEM-F12 and 10% fetal bovine serum. To activate SHH signaling, SAG was added at a final concentration of 200 or 500 nM. DMSO was used as a diluent control. Lungs were cultured at 37°C in 5% CO2 for 48 h before harvest and analysis.

Histology

Embryos were dissected on the indicated days and fixed in 4% (vol/vol) paraformaldehyde (PFA) overnight. Embryos were then embedded in Tissue-Tek OCT compound, frozen, and sectioned at 12 µm. Sections were stained with hematoxylin and eosin (H&E) using a standard protocol.

Lac Z staining

To detect the presence of β-galactosidase for whole-mount staining, dissected lung was fixed in 4% PFA at 4°C for 1 hour. After washing in PBS for three times, tissues were incubated with X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% NP-40 and 2 mM MgCl2) containing 1 mg/ml 5- bromo-4-chloro-3-indolyl-β-D-galactosidase and applied to tissues for 24 h at 37°C.
**Immunostaining**

E14.5-E17.5 embryo lungs were dissected in ice-cold 1× PBS, fixed in 4% PFA, and prepared for cryosectioning using standard protocols. Cryosections were stained with the indicated primary antibodies and fluorophore-conjugated secondary antibodies, followed by DAPI counterstaining. Primary antibodies were goat anti-SOX2 (1:100, Santa Cruz Biotechnology), rabbit anti-SOX9 (1:500, Millipore), rabbit anti-Ki67 (1:500, Millipore), rabbit anti-ARL13B (1:1,000), anti-γ-tubulin (γ-TUB, 1:500, Sigma), anti-acetylated alpha tubulin (Ac-TUB, 1:1,000, Sigma), and mouse anti-beta tubulin type 4 (β-TUB4, 1:1,000, Novus). The secondary antibodies used were species-specific antibodies conjugated with AlexaFluor 488 or 594 (1:500; Jackson Immunoresearch). EdU was injected 24 h prior to sample preparation, which was performed at E13.5 and E15.5 d after plug check. EdU staining was performed following the manufacturer’s protocol using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen).

Primary cilia within the focal plane were measured from the base of the cilium where it intersected the centrosome to the tip of the ARL13B staining. Length and frequency were measured using a C1 (Nikon) and NIS Software. Statistical analysis was performed using Student’s t-tests (two-tailed, equal variance).

**Quantification of relative gene expression**

Total RNA was isolated from E14.5 and E17.5 embryo lungs using the TRI reagent (Invitrogen), and 1 μg of total RNA was reverse transcribed using an oligo- dT primer and a First-Strand cDNA Synthesis kit (Takara). Real-time PCR was performed using the Rotor Gene Q instrument (Qiagen) with SYBR (Takara). Beta-actin was used as an internal control. Results were analyzed with the Rotor Gene Q series software to calculate the relative mRNA levels using the $2^{-\Delta\Delta CT}$ method.

**In situ hybridization**

Sectioned lung tissue in situ hybridization was performed as previously described. Briefly, specimens were fixed overnight in 4% (vol/vol) PFA in PBS, dehydrated in graded methanol, and permeabilized by proteinase K. Specimens were then hybridized with digoxigenin (Dig)-labeled antisense RNA probes followed by incubation with anti-Dig antibody conjugated with alkaline phosphatase. Colorimetric reactions were performed using NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) as the substrate. Riboprobes for Shh, Gli1, Ptch1, and Bmp4 were prepared as previously described.
Western blotting

Whole-embryo protein lysates for GLI3 immunoblotting were obtained by clarifying E14.5 embryo lungs in modified RIPA buffer (150 mM NaCl, 50 mM Tris- HCl [pH 7.4], 1 mM EDTA, 1% NP40, 0.25% Na-deoxycholate) with a protease inhibitor cocktail (Roche) for 15 min at 17,000 × g at 4°C. Protein concentration was determined with the BCA Protein Assay (Pierce). Equal amounts of protein (10 μg) were loaded for electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes (Millipore) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were washed with Tris-buffered saline solution containing 0.2% Tween 20 (TBST) and blocked for 1 h in TBST containing 5% skim milk. Membranes were then incubated overnight at 4°C with a mouse monoclonal anti-GLI3 antibody (kindly provided by Dr. Susie Scales, Genentech, CA) or anti-β-actin antibody (Cell Signaling Technology). After washing, membranes were incubated for 2 h at room temperature (or overnight at 4°C) with the appropriate horseradish peroxidase-labeled secondary antibodies. Signals were detected with a chemiluminescent reagent (Millipore) following the manufacturer’s instructions.

Quantification and statistical analysis

Quantitative real-time PCR data were normalized by dividing each value by the mean of the control. We presented all data as means with standard errors. Data analysis was performed using SigmaStat3.5, and P-values <0.05 were considered statistically significant (*P < 0.05; **P < 0.01, ***P < 0.001).
Figure S1. *Ccrk* is highly expressed in mouse trachea and bronchioles.
Whole-mount X-gal staining of E15.5 heterozygote *Ccrk*+/tm1a embryos was performed to visualize the tissue-specific distribution of *Ccrk* gene expression in embryonic trachea and pulmonary bronchioles at E15.5. Scale bars = 50 μm and 100 μm.
Figure S2. *Ccrk*<sup>KO</sup> mutant lungs exhibit reduced epithelial cell differentiation. The epithelial cell differentiation markers Clara cell secretory protein (*Ccsp*, secretory cells), *Foxj1* (motile multi-ciliated cells), aquaporin 5 (*Aqp5*, alveolar type 1), podoplanin (*Pdpn1*, alveolar type 1), surfactant protein A (*SP-A*, alveolar type 2), and surfactant protein B (*SP-B*, alveolar type 2) were decreased in *Ccrk*<sup>KO</sup> mutant developing lungs, but surfactant protein C (*SP-C*, alveolar type 2) and *Muc5ac* (mucosal cells) were not significantly changed. *P*-values from Student's *t*-tests: *** *P* < 0.005, ** *P* < 0.01, * *P* < 0.05, n.s., not significant.
Figure S3. CCRK regulates multiple motile ciliogenesis.

(A-F) Representative images show immunostaining for ciliary membrane (ARL3B)/ciliary axoneme (Ac-TUB), motile cilia specific markers (β-TUB IV)/ciliary membrane (ARL13B), and ciliary membrane (ARL3B)/basal body (γ-TUB) in motile multi-cilia in the airway ciliated cells in CcrkKO mutants compared to wild-type littermates. Nuclei are counterstained with DAPI. Scale bars = 100 μm.
Figure S4. CCRK regulates transcript levels of ciliogenic transcription factor and ciliary genes in canalicular stage.

The expression of ciliogenic transcription factors (Foxj1, RFX2 and RFX3) and ciliary genes (IFT172, IFT144, IFT140, IFT122, IFT88, Dnah9, Dnah11, Dyn2h1 and Dyn2li1) were analyzed by quantitative real-time RT-PCR analysis in pseudoglandular and canalicular stage. *P*-values from Student’s *t*-tests: *** *P* < 0.005, ** *P* < 0.01, * *P* < 0.05, n.s., not significant.