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Evidence of a role of inositol polyphosphate 5-phosphatase INPP5E in cilia formation in zebrafish

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ABSTRACT

Inositol phosphatases are important regulators of cell signaling and membrane trafficking. Mutations in inositol polyphosphate 5-phosphatase, INPP5E, have been identified in Joubert syndrome, a rare congenital disorder characterized by midbrain malformation, retinitis pigmentosa, renal cysts, and polydactyly. Previous studies have implicated primary cilia abnormalities in Joubert syndrome, yet the role of INPP5E in cilia formation is not well understood. In this study, we examined the function of INPP5E in cilia development in zebrafish. Using specific antisense morpholino oligonucleotides to knockdown *Inpp5e* expression, we observed phenotypes of microphthalmia, pronephros cysts, pericardial effusion, and left–right body axis asymmetry. The *Inpp5e* morphant zebrafish exhibited shortened and decreased cilia formation in the Kupffer's vesicle and pronephric ducts as compared to controls. Epinephrine-stimulated melanosome trafficking was delayed in the *Inpp5e* zebrafish morphants. Expression of human INPP5E expression rescued the phenotypic defects in the *Inpp5e* morphants. Taken together, we showed that INPP5E is critical for the cilia development in zebrafish.

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1. Introduction

The primary cilium is an evolutionarily conserved subcellular structure that protrudes from nearly all post-mitotic eukaryotic cells (Rohatgi & Snell, 2010). By sensing changes in the extracellular environment, the primary cilium can coordinate signaling cascades that subsequently become amplified throughout the cell (Fisch & Dupuis-Williams, 2011). A highly specialized extension of the plasma membrane, the ciliary membrane is enriched with many signaling precursors, such as Patched1 (Ptc1) (Corbit et al., 2005; Rohatgi, Milenkovic, & Scott, 2007). Upon ligand binding of Sonic Hedgehog (Hg), Ptc1 is removed from the cilium and Smoothened is then accumulated within the ciliary membrane, allowing initiation of downstream signaling cascades. The ciliary membrane covers a microtubule-based axoneme, which is anchored by a basal body (Pearson, Culver, & Winey, 2007; Pearson & Winey, 2009). Within the ciliary membrane are phospholipids, including phosphoinositides that may serve as second messengers in signal transduction. Defects in cilia formation or maintenance have been found to underlie a wide range of human diseases, including retinitis pigmentosa, renal cysts, polydactyly, and developmental delays, which are collectively called ciliopathies (Jacoby et al., 2009; Novarino, Akizu, & Gleeson, 2011; Schurman & Scheinman, 2009).

Joubert syndrome, a rare form of autosomal recessive ciliopathy, is characterized by an underdevelopment of cerebellar vermis, with a distinctive "molar tooth sign" of cerebellar vermis hypoplasia on MRI (Bielas et al., 2009). The most common features of Joubert syndrome include retinitis pigmentosa, hypotonia, severe psychomotor delay, and ataxia (Lee & Gleeson, 2011). Other physical deformities may include polydactyly, cleft palate, renal cysts, and liver disease. A rapidly expanding number of genes have been implicated in Joubert syndrome, including NPHP1, NPHP6/CEP290, NPHP8, ARL13B and INPP5E (Arts et al., 2007; Bielas et al., 2009; Cantagrel et al., 2008; Kim, Krishnaswami, & Gleeson, 2008; McEwen et al., 2007; Travaglini et al., 2009; Valente et al., 2010).

INPP5E belongs to a family of inositol polyphosphate 5-phosphatases, which dephosphorylate the D5 position of the inositol ring (Asano et al., 1999; Kisseleva, Wilson, & Majerus, 2000; Kong et al., 2000). There are 10 mammalian members of the 5-phosphatase family, which play critical yet distinct roles in a number of

Abbreviations: RPE, retinal pigmented epithelium; NHF, normal human fibroblasts; MORM syndrome, mental retardation, truncal obesity, retinal dystrophy, and micropenis; RBD, RAB binding domain; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; IFT, intraflagellar transport; hTERT-RPE1, human telomerase transformed RPE cells; PFA, paraformaldehyde; BSA, bovine serum albumin; NGS, normal goat serum; PBS, phosphate buffered saline; FCS, fetal calf serum; PRD, proline rich domain; dpf, days post-fertilization; hpf, hours post-fertilization; KV, Kupffer's vesicle.

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biological processes, such as the regulation of insulin signaling, vesicular trafficking, synaptic vesicle formation, and hematopoietic cell proliferation (Ooms et al., 2009; Pirruccello & De Camilli, 2012). The members of inositol polyphosphate 5-phosphatase family share a common inositol phosphatase domain, but these individual 5-phosphatases have different protein–protein interaction domains that regulate their subcellular localization and function (Dyson et al., 2012). For instance, in response to growth factor stimulation, INPP5E regulates the intracellular levels of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ by controlling downstream AKT activation (Kisseleva, Cao, & Majerus, 2002). Overexpression of INPP5E also results in the hydrolysis of $PI(3,5)P_2$ to PI(3)P at the plasma membrane, and translocation of GLUT4 glucose transporter into the plasma membrane (Kong et al., 2006). Mutations in the INPP5E phosphatase domain have been identified in a series of patients with Joubert syndrome, thus highlighting the role of inositol phosphatases in cilia development (Bielas et al., 2009). In addition, a C-terminal deletion mutant of INPP5E has been reported in a family with MORM syndrome, a variant of the Bardet–Biedl group of syndromic ciliopathies (Jacoby et al., 2009). *In vitro* studies revealed that the INPP5E deletion mutant failed to localize to the cilia while retaining its inositol phosphatase activity, thus suggesting that spatial localization of INPP5E, as well as enzymatic activity, is critical to its function in the cilia (Jacoby et al., 2009).

Although INPP5E has been implicated in ciliogenesis, the functional role of inositol phosphatase in the cilia is not understood. The murine model of MORM syndrome yielded knockout animals that died shortly after birth (Jacoby et al., 2009). Thus, to seek a viable INPP5E model system, we examined the function of INPP5E in zebrafish cilia development by transient knockdown with morpholino anti-sense oligonucleotides specific for *Inpp5e*.

2. Materials and methods

2.1. Reagents and DNA constructs

Anti-acetylated α-tubulin and anti-Myc monoclonal antibodies were purchased from Sigma (St. Louis, MO). Mouse antibody against INPP5E antibody was obtained from Abcam (Cambridge, MA). Secondary antibodies were AlexaFluor 488 and 546-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-mouse IgG, and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). IRDye goat anti-mouse and anti-rabbit (680 and 800) were obtained from Li-Cor Bioscience (Lincoln, NB). Myc-tagged INPP5E was previously described (Kisseleva, Cao, & Majerus, 2002). Site-directed mutagenesis for INPP5E R378C and R435Q mutants was performed using QuikChange II (Aglient, Santa Clara, CA).

2.2. Immunoblot analysis

Cell lysates were subjected to SDS–PAGE followed by immunoblot analysis with the indicated antibodies. Equal amounts of protein were resolved on 10–12% polyacrylamide gels, and protein bands were transferred to nitrocellulose membranes (BioRad, Hercules, CA), which were blocked with 5% non-fat dried milk in PBST; and incubated with the primary and then secondary antibodies as indicated. Odyssey imaging system (Li-Cor Bioscience, Lincoln, NE) was used to analyze the immunoblots.

2.3. Zebrafish immunohistochemistry and cilia measurements

Zebrafish (wildtype strain: AB *tevbigan*) (gift of Dr. Ryan Anderson, Indiana University, Indianapolis, IN) were raised and maintained at the Laboratory Animal Resource Center of Indiana University. All animal procedures were subject to the Institutional Animal Care and Use Committee of Indiana University approved protocols. The phenotypes of morphants were photographed with Leica DFC310 FX. Eye size was determined by the longest diameter of eye area. The diameter was measured using Leica Application Suite V4.1 and NIH Image J v1.46.

Embryos were fixed overnight at 4 °C in 4% PFA and 1% sucrose in PBS. Embryos were dechorionated and washed with PBST for 6–8 times 10 min each. Following 1 h of blocking with 10% NGS and 0.5% BSA, immunostaining was performed with 1:200 anti-acetylated α-tubulin monoclonal antibody and later with 1:500 Alexa Fluor 546 goat anti-mouse conjugated IgG at 4 °C overnight. KV cilia measurements were performed as described (Luo et al., 2012). Cresyl violet staining was performed as described (Luo et al., 2012).

2.4. Morpholino (MO) antisense oligonucleotides knockdown and mRNA rescue in zebrafish

Antisense MOs were designed and purchased from Gene Tools, Inc. (Gene Tools, Philomath, OR). The *Inpp5e* ATG initiation codon sequence is GCTCACTCATCCTATTGGCGGGCTT. A mismatch morpholino MO: sequence ATGCGAAATCAAGGTTCGATCATCA served as a negative control. We also used a *p53* ATG morpholino: GCGCCATTGCTTTGCAAGAATTG to test for off-target effects. Morpholino stocks were dissolved at 1 mM in water and 2 or 4 nl of injection solution (0.25% phenol red) containing 125–500 μM morpholino was injected into fertilized eggs at the one- to two-cell stage using a pressure injector, Pressure System IIe (Toohey Company, Fairfield, NJ). Synthetic mRNA was prepared from linearized human INPP5E-pcDNA3.1 DNA with Ambion mMessage mMachine® high-yield Capped RNA transcription kit, and purified with phenol–chloroform; mRNA was co-injected for rescue experiments.

2.5. Retrograde melanosome transport assay

The melanosome transport assay was performed as described (Yen et al., 2006). Briefly, zebrafish 5 dpf larvae were exposed to epinephrine (50 mg/ml, Sigma) in the final concentration of 2 mg/ml in a dark room, and melanosome retractions were observed under the brightfield microscope Leica DFC310 FX. The end of melanosome transport was marked when all melanosomes in the head were perinuclear.

2.6. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL) and the p value less than 0.05 was considered significant. An unpaired t-test was carried out to analyze if there was a significant difference between the cilia length or percentage of cilia formation as described above. ANOVA test was performed to analyze the difference observed in different groups of hTERT-RPE1 cells transfected with INPP5E constructs.

3. Results

3.1. Inpp5e knockdown results in cilia-dependent phenotypes

Inositol metabolism has been explored in a number of model organisms, including zebrafish (*Danio rerio*) (Sarmah et al., 2007). We have examined zebrafish orthologs of known inositol polyphosphate 5-phosphatases and identified the orthologous zebrafish *Inpp5e*. Human INPP5E contains an N-terminal proline-rich domain (PRD), an inositol polyphosphate 5-phosphatase

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