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Mutations in zebrafish *pitx2* model congenital malformations in Axenfeld-Rieger syndrome but do not disrupt left-right placement of visceral organs

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ABSTRACT

Pitx2 is a conserved homeodomain transcription factor that has multiple functions during embryonic development. Mutations in human *PITX2* cause autosomal dominant Axenfeld-Rieger syndrome (ARS), characterized by congenital eye and tooth malformations. *Pitx2*$^{-/-}$ knockout mouse models recapitulate aspects of ARS, but are embryonic lethal. To date, ARS treatments remain limited to managing individual symptoms due to an incomplete understanding of PITX2 function. In addition to regulating eye and tooth development, Pitx2 is a target of a conserved Nodal (TGFβ) signaling pathway that mediates left-right (LR) asymmetry of visceral organs. Based on its highly conserved asymmetric expression domain, the Nodal-Pitx2 axis has long been considered a common denominator of LR development in vertebrate embryos. However, functions of Pitx2 during asymmetric organ morphogenesis are not well understood. To gain new insight into Pitx2 function we used genome editing to create mutations in the zebrafish *pitx2* gene. Mutations in the *pitx2* homeodomain caused phenotypes reminiscent of ARS, including aberrant development of the cornea and anterior chamber of the eye and reduced or absent teeth. Intriguingly, LR asymmetric looping of the heart and gut was normal in *pitx2* mutants. These results suggest conserved roles for Pitx2 in eye and tooth development and indicate Pitx2 is not required for asymmetric looping of zebrafish visceral organs. This work establishes zebrafish *pitx2* mutants as a new animal model for investigating mechanisms underlying congenital malformations in ARS and high-throughput drug screening for ARS therapeutics. Additionally, *pitx2* mutants present a unique opportunity to identify new genes involved in vertebrate LR patterning. We show Nodal signaling—independent of Pitx2—controls asymmetric expression of the fatty acid elongase *elovl6* in zebrafish, pointing to a potential novel pathway during LR organogenesis.

1. Introduction

Pitx2 (Paired-like homeodomain 2) is a member of the bicoid class of homeodomain transcription factors that has been highly conserved during evolution. Vertebrate *Pitx2* genes encode multiple protein isoforms that have different N-termini but share a common C-terminal homeodomain that mediates DNA binding (Chaney et al., 2005). Loss-of-function mutations in the homeodomain of human *PITX2* cause autosomal dominant Axenfeld-Rieger syndrome (ARS). PITX2 haploinsufficiency in ARS patients disrupts development of the anterior segment of the eye and tooth morphogenesis (Footz et al., 2009). Ocular defects found in ARS patients include malformation of the anterior chamber angle, central corneal thickness and iris atrophy with corectopia. A persistence of the endothelial layer on the angular structures and an anterior iris root insertion result in aqueous outflow defects. As a consequence, patients often have high intraocular pressure and are at risk for developing glaucoma that can lead to blindness (Chang et al., 2012; Shields et al., 1985). Dental abnormalities caused by *PITX2* mutations include microdontia, hypodontia and enamel hypoplasia (Jena and Kharbanda, 2005). Additional craniofacial malformations such as hypertelorism, a broad flat nasal bridge and maxillary defects are associated with ARS (Antevil et al., 2009). ARS patients generally have a normal life span and treatment options are currently limited to managing individual symptoms, which require complex multidisciplinary approaches.

Animal models have played an important role in our understanding of Pitx2 function and underlying causes of ARS malformations. Targeting the *Pitx2* homeodomain in knockout mouse models eliminated the activity of all Pitx2 isoforms and caused eye and tooth developmental defects in *Pitx2*$^{-/-}$ embryos that are consistent with ARS (Gage et al., 1999; Kitamura et al., 1999; Lin

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et al., 1999; Lu et al., 1999). However, global loss of *Pitx2* resulted in additional developmental malformations in the brain, visceral organs and body wall and was embryonic lethal. Most heterozygous *Pitx2* knockout mice developed into normal adults, but ~10% developed ARS phenotypes that included small eyes, tooth defects and reduced body size (Gage et al., 1999). Additional mutant alleles have been used to study *Pitx2* isoforms and gene dosage effects in mouse (Liu et al., 2001, 2002, 2003) and conditional alleles have helped define tissue-specific roles, such as the requirement for *Pitx2* in neural crest cells for normal eye development (Evans and Gage, 2005). However, fundamental gaps in our understanding of *Pitx2* functions and target genes have made it difficult to conceptualize therapeutic approaches for ARS patients. Thus, it will be critical to exploit existing knockout mouse models and develop new animal models to fully understand molecular functions of *PITX2* and identify effective treatments for ARS.

One of the roles of Pitx2 during embryonic development is to function as a downstream effector of an ancient Nodal signaling pathway associated with generating morphological asymmetries in cnidarians (Watanabe et al., 2014), echinoderms (Duboc et al., 2005) and chordates (Boorman and Shimeld, 2002). In vertebrates, the secreted TGFβ-related ligand Nodal triggers asymmetric expression of a specific *Pitx2* isoform, *Pitx2c*, in left lateral plate mesoderm during left-right (LR) patterning of the embryo (Hamada et al., 2002). Unlike Nodal, asymmetric *Pitx2c* expression was found to persist on left side of the developing heart and gut, where it is thought to regulate genes that mediate asymmetric morphogenesis of these organs. In chicken and frog embryos, misexpression of Pitx2 or Nodal in right lateral plate mesoderm reversed heart and gut looping (Levin et al., 1997; Ryan et al., 1998; Sampath et al., 1997) and blocking Pitx2 or Nodal function randomized looping direction (Toyoizumi et al., 2005; Yu et al., 2001). Further analyses in chick embryos revealed Pitx2c induces cellular condensations during gut looping (Davis et al., 2008; Welsh et al., 2013). Together, these findings suggested Nodal-Pitx2 signaling controls direction of asymmetric organ development. Consistent with this model, Nodal mouse mutants showed randomized heart and gut looping (Brennan et al., 2002). However, the initial looping of the heart and gut occurred normally in *Pitx2* knockout mice (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). Abnormalities during subsequent steps of asymmetric organ morphogenesis in Pitx2 knockout mice, and mice specifically lacking Pitx2c expression (Liu et al., 2001; Shiratori et al., 2006), resulted in organ-specific LR defects that included lung isomerism, abnormal looping of the duodenum and cardiac malformations. Cardiac outflow tract defects were subsequently linked to Pitx2 functions in the second heart field that contributes to outflow tract development (Ai et al., 2006). These studies revealed that the roles of Pitx2 downstream of Nodal in LR patterning remain unclear and may not be completely conserved.

The zebrafish provides a useful model system to investigate gene function and evolution, and the small size and external development of the zebrafish embryo makes it ideal for high-throughput chemical screening (Zon and Peterson, 2005). Since *pitx2* mutations have not been identified in genetic screens, we generated the first zebrafish *pitx2* mutants via genome editing to gain insight into Pitx2 functions and develop a new ARS animal model. We found that mutations that truncate the zebrafish homeodomain, which is 100% identical to human PITX2, caused eye and tooth developmental defects consistent with phenotypes observed in ARS patients. Importantly, homozygous *pitx2*$^{-/-}$ zebrafish grow to adulthood and thereby provide a new model of adult ARS. Interestingly, analysis of the heart and gut looping in mutant embryos revealed Pitx2 is dispensable for correct LR asymmetric orientation of these organs. These results identify functions for Pitx2 in eye and tooth development that appear conserved from fish to mammals, and indicate Pitx2 is not required for LR asymmetric looping of visceral organs in zebrafish. Mutant *pitx2* zebrafish are an important new disease model that can be used in chemical screens for therapeutic treatments of ARS and can be leveraged to discover new pathways that guide LR morphogenesis. To begin to identify new candidate molecules that may regulate LR development, we show asymmetric expression of the *ELOVL fatty acid elongase 6* (*elovl6*) gene is controlled by LR Nodal signaling and is independent of Pitx2 function in zebrafish.

2. Material and methods

2.1. Zebrafish

Wild-type TAB zebrafish, obtained from Zebrafish International Resource Center, were used to generate *pitx2* mutants and for microinjections. *cftr*^{pd1048} mutants were provided by the Bagnat lab (Duke University). All animal studies were approved by the Institutional Animal Care and Use Committee at SUNY Upstate Medical University.

2.2. Generation of pitx2 mutant zebrafish

pitx2HD and *Pitx2c* TALENs were designed using TAL Effector-Nucleotide Targeter 2.0 and assembled using golden gate assembly (Cermak et al., 2011). TALEN mRNAs were synthesized using the Ambion mMessage Machine kit and purified by LiCl precipitation. 150–200 pg of each TALEN mRNA was injected into wild-type zebrafish embryos at the one cell stage. Injected embryos were raised to adulthood and then outcrossed with wild-type fish to identify founders that transmitted mutations through the germ line. Mutations were identified via digestion of PCR products with SalI (*pitx2HD* TALEN) or BamHI (*pitx2c* TALEN) and confirmed by sequencing. The PCR primers used for the pitx2HD alleles were forward primer 5′-TGAAGCTTGTTCCTCTGC-3′ and Reverse primer 5′-AAAATTTAGGGTTATATCACATA-3′ and for the pitx2c allele were forward primer 5′ GGAGTG TCGCTTTTAGTGG-3′ and reverse primer 5′-ACTAGAGGCCATCGAAAGC-3′.

2.3.. mRNA synthesis and microinjection

For cloning of *pitx2* cDNAs, total RNA was extracted from pooled embryos of *pitx2HD*^{sny15} intercross at 6 dpf and RT-PCR was used to amplify full-length *pitx2c* (primers: 5′-agatctatgacctctatgaaggatcc 3′ and 5′-tctagattacaccggtctatccactg-3′). The product was inserted into a Topo vector (Invitrogen) and confirmed by sequencing. Confirmed *pitx2c* cDNAs were transferred into pCS2 vectors and then linearized for mRNA synthesis using the Sp6 mMessage mMachine kit (Ambion). 50 pg of wild-type or mutant *pitx2c* mRNA was injected into wild-type embryos at the 1-cell stage. Full-length *elovl6* was amplified from total RNA from 1 dpf wild type embryos (primers: 5′-cgcggatccatgtcggtgctggcattg-3′ and ccgcctcgagttattggcttttcttggctgc-3′) and cloned using a pCS2 vector. mRNA was prepared the same as *pitx2c* and 300 pg was injected into 1-cell stage embryos.

2.4. Eye sectioning

5 day old zebrafish were euthanized and flash frozen in O.C.T (Sakura) and cryosectioned as described (Uribe and Gross, 2007). Sections for every 5 μm or 10 μm were collected for 5 dpf and 3 month fish respectively. Slides were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet (Sigma) 5 min at room temperature. Stained sections were

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