**Abstract**

Human PITX2 mutations are associated with Axenfeld-Rieger syndrome, an autosomal-dominant developmental disorder that involves ocular anterior segment defects, dental hypoplasia, craniofacial dysmorphism and umbilical abnormalities. Characterization of the PITX2 pathway and identification of the mechanisms underlying the anomalies associated with PITX2 deficiency is important for better understanding of normal development and disease; studies of pitx2 function in animal models can facilitate these analyses. A knockdown of pitx2 in zebrafish was generated using a morpholino that targeted all known alternative transcripts of the pitx2 gene; morphant embryos generated with the pitx2ex4/5 splicing-blocking oligomer produced abnormal transcripts predicted to encode truncated pitx2 proteins lacking the third (recognition) helix of the DNA-binding homeodomain. The morphological phenotype of pitx2ex4/5 morphants included small head and eyes, jaw abnormalities and pericardial edema; lethality was observed at ~6–8 dpf. Cartilage staining revealed a reduction in size and an abnormal shape/position of the elements of the mandibular and hyoid pharyngeal arches; the ceratobranchial arches were also decreased in size. Histological and marker analyses of the misshapen eyes of the pitx2ex4/5 morphants identified anterior segment dysgenesis and disordered hyaloid vasculature. In summary, we demonstrate that pitx2 is essential for proper eye and craniofacial development in zebrafish and, therefore, that PITX2/pitx2 function is conserved in vertebrates.

**Introduction**

**PITX2** is a member of the *boid*-like homeodomain transcription factor family which, when mutated, is responsible for Axenfeld-Rieger syndrome (MIM ID #180500), an autosomal-dominant developmental disorder characterized by ocular anterior chamber anomalies, increased risk for glaucoma, dental hypoplasia, craniofacial dysmorphism and umbilical region abnormalities [1].

The majority of PITX2 mutations are located in the homeobox region encoding the homeodomain and affect all known PITX2 isoforms (see below). These mutations typically generate a null-allele, which supports PITX2 haploinsufficiency as a mechanism for Axenfeld-Rieger syndrome, consistent with reports of gene deletion in some patients; PITX2 mutations that appear to retain limited wild-type activities are usually associated with milder phenotypes [2]. Identification of increased transactivation activity of two mutant PITX2 proteins associated with human disease suggests that elevated PITX2 activity may also be disruptive [2,3]. Expression studies demonstrate that Pitx2 is expressed in neural crest derived cells that contribute to the ocular and craniofacial tissues affected in Axenfeld-Rieger syndrome [1], as well as during brain, heart, lung, stomach, gut and gonad development [4–11].

Several PITX2 isoforms have been reported with four transcripts, PITX2a–d, identified in humans [12], three, Pitx2a–c, in mice and frogs [13] and two, pitx2a and pitx2c, described in chickens [14], zebrafish [15] and even ascidians [16]; the isoforms have different N-terminal sequences but share exons encoding for the homeodomain and C-terminal region. Although some variations were observed, the Pitx2 isoforms demonstrate largely overlapping expression patterns [13–15,17]; similarly, functional assays demonstrate comparable DNA-binding and transactivation activities for the main isoforms with minor differences revealed under certain conditions [12,18].

In the mouse, complete knockout of Pitx2 results in embryonic lethality and ocular, craniofacial, dental, brain, heart, lung, body wall and other systemic defects; various conditional Pitx2-deficient animals were generated to study specific aspects of its function [7,9,10,17,19–21]. Ocular anomalies are observed in the Pitx2+/− animals but not in Pitx2+/− heterozygous animals and include arrest in anterior segment development, thickening of the mesothelial layer of the cornea resulting in enophthalmos, dysgenesis of the extraocular muscle and other defects. In terms of craniofacial anomalies, Pitx2+/− animals display arrested tooth development at the placode (for maxillary teeth) or bud (for mandibular teeth) stage. The combination of ocular and craniofacial defects observed in Pitx2-deficient mice shows similarity to Axenfeld-Rieger syndrome in humans, suggesting conservation of function for this gene during embryonic development.

Zebrafish offer several advantages compared to mammalian models including rapid early development, optical clarity, availability of gene manipulation techniques (including the
recently developed gene knockout technology via zinc-finger nucleases, and large number of progeny, therefore providing powerful genetic screening opportunities [22,23]. Zebrafish have been utilized to demonstrate the effects of pitx2 misexpression on heart development [5,15] but the overall effects of pitx2-deficiency in zebrafish have not yet been fully investigated. In this manuscript, we demonstrate that the function of pitx2 is conserved in vertebrates and that zebrafish pitx2 is essential for proper eye and craniofacial development.

Materials and Methods

Animals

Zebrafish (Danio rerio) adult fish were maintained on a 14-hour light/10-hour dark cycle in system water; the embryos were acquired by natural spawning and kept at 28.5°C. The developmental stage was determined by hours post fertilization (hpf) or days post fertilization (dpf) as well as by morphological criteria [24]. The Tg (flh:3 Pf) Tg (goll:3 Pf) and p53-/- zebrafish lines used in this study have been previously described [25-27]. The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin (protocol number AUA00000352).

In situ hybridization

Plasmids for dix2a, dix4a, fox3 and bax1b were obtained from Open Biosystems (Thermo Fisher Scientific Inc, Huntsville, AL); ptax6a was kindly shared by Dr. Link (Medical College of Wisconsin, Milwaukee, WI); ptx3 probes have been previously described [29]; ptxs-exon 5, foxa1 and dbk2 plasmids were constructed by PCR amplification and cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA) using with the followingprimers: zptx2ac-1345r, 5‘-CAAGATTTGGGATTGACGACCACT-3’ and zptx2ac-1345f, 5‘-TTGGATGTTGAAAAACGAAA-3’ (to produce a 1345-bp probe that detects both ptxs isoforms); zfoxa1-273F and 5‘-GCCAACCCTCCGTACGACTAC-3’, zfoxa1-273R, 5‘-AAAAAGCTGCGTTCTCATA-3’ (to produce a 273-bp probe); zdbk2-ex1f305, 5‘-CACACTTGCCAGAGGAGATCA-3’ and zdbk2-ex3r503: TCCGTGTGTTTGTGA-3’ (to produce a 503-bp probe). Digoxigenin-labeled RNA probes were synthesized by in vitro transcription and in situ hybridization was performed as previously described [28].

Morpholino injections

Morpholino oligonucleotides were synthesized by Gene Tools (Gene Tools, Philomath, OR). Antisense morpholino oligonucleotides were designed to target translation initiation sites of the pitx2a, 5‘-CAAGTTTTGCGGACTTGGAGTGATCC-3’ (pitx2atrcf), and pitx2a, CCTTTGATAGGTCAAGGATAGAC (pitx2artcR), isoforms as well as the pitx2 exon 4 donor site, 5‘-TTATTTAGCATTTGGGACTGCGACTG-3’ (pitx24orf5), and a standard control morpholino, 5‘-CTTCTTTACCTCACTGTAATACTTATA-3’ (control-MO), was used. The morpholinos were solubilized in water and diluted to 500–500 μM with 0.1% phenol red for microinjections into embryos. The morpholino oligomers (4–14 ng) were injected into 1–4 cell-stage embryos as previously described [28]. The efficacy of morpholino injections was evaluated by semi-quantitative RT-PCR using RNA extracted from injected embryos.

Nucleic acid extractions and amplification

RNA was extracted using zebrafish embryos harvested in Trizol reagent (Invitrogen, Carlsbad, CA) and standard procedures. cDNA was synthesized from 1 μg of total RNA with SuperScript III (Invitrogen, Carlsbad, CA) and random hexamers. Semi-quantitative PCR was performed using cDNA prepared from whole embryos, unjected wild-type, pitx2+/− and control-MO injected embryos, and using two sets of pitx2 primers (a combination of pitx2 primers 1 and 2 (399-bp product) and a combination of primers 2 and 3 (637-bp product to amplify isoforms pitx2a and pitx2b, respectively) and a control set of primers for β-actin (329-bp product). Oligonucleotide sequences are as follows: pitx2 forward primer 1 (zpitx2fP): 5‘-AAGACTGGCAGCGGTGGCAAA-3’, pitx2 forward primer 2 (zpitx2fP): 5‘-CTTCGTAGATCCGTTGTCT-3’ and pitx2 reverse primer 2 (zpitx2rP): 5‘-ACAGTTGATCGTACCG-3’, and control forward and reverse primers: β-actin-F, 5‘-GAGAGATCTCGGAC-3’, β-actin-R, 5‘-ATCAGGTTGCTGT-CAGGTC-3’. The DNA sequence of PCR products was determined by direct automated DNA sequencing. DNA sequencing was performed to confirm the identity of PCR products by comparison with database records; no novel sequences were reported.

Histological analysis, alcian blue and alkaline phosphatase staining

Histological analysis was performed using standard protocols. Briefly, embryos were fixed in 4% paraformaldehyde at 4°C, washed three times in PBS (pH 7.4), dehydrated in ascending grades of ethyl alcohol (25%, 50%, 75%, 100%) and infiltrated overnight in the dark at 4°C. The embryos were placed in a mold cup tray capped by an EBH-2 Block Holder (Electron Microscopy Sciences, West Chester, PA), embedded in a fresh mixture of embedding solution and polymerized at 4°C overnight according to the manufacturer’s directions. Polymerized blocks were cut at 1-2 mm using a Leica RM2255 microtome (Leica Microsystems, Vienna, Austria) with glass knives made from 7.0 mm glass strips on a Leica EMKMR2 Knifemaker (Leica Microsystems, Vienna, Austria); sections were collected onto charged glass slides on a Leica cryostat. 7.0 mm sections were prepared, mounted on charged glass slides and dried on the heater at 150°C. Hematoxylin and eosin staining was performed using standard protocols. Alcian blue staining was performed according to Barrallo-Gimeno et al. [29]; cartilage elements were identified as previously described [30]. The alkaline phosphatase staining of hyaloid vessels was performed according to Alvarez et al. [31]. Images were obtained using an AxioImager Zeiss microscope (Zeiss, Thornwood, NY).

Immunohistochemistry

Immunohistochemistry was performed with the following primary serum: mouse anti-Keratin Sulfate Proteoglycan antibody (Chemicon International, Temecula, CA), mouse anti-Keratan sulfate monoclonal antibody (Millipore, Billerica, MA), rabbit anti-crystallin αA and βB1 antibodies (a generous gift from Dr. Thomas C. Vihtelic); and the following secondary antibodies, Alexa Fluor 568 donkey anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA), Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) and FITC conjugated AffiniPure donkey anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories Inc, West Grove, PA). Embryos were fixed in 4% paraformaldehyde/PBS and infiltrated with 30% sucrose and then OCT, embedded in cryomolds and frozen. 7-μm sections were prepared, mounted on charged glass slides and dehydrated for 30 min at 37°C. Sections were incubated in blocking solution (1×
PBS/10% normal donkey serum /0.1% Tween 20) for 1 hour at room temperature and then overnight at 4°C in primary antibody/blocking solution. The sections were washed in 1× PBS/0.1% Tween 20 and incubated for 1 hour at room temperature in blocking solution and then exposed to secondary antibody. Immunoreactivity was captured with a AxioImager Zeiss microscope (Zeiss, Thornwood, NY).

Results

Generation of a complete knockdown of pitx2 in zebrafish by blocking translation or mRNA splicing

The zebrafish pitx2 gene generates two transcripts, pitx2a and pitx2c, produced by alternative promoter usage similar to the human PTTX2 gene [1,15] (Figure 1A). Both pitx2 transcripts are expressed embryonically at various sites including the developing pharyngeal arches, tissues surrounding the oral cavity and anterior segment of the eye [15,32]. To achieve a complete knockdown of pitx2, the following antisense morpholino oligomers were designed: pitx2aATG and pitx2aATG (targeting the translation initiation sites of the pitx2a and pitx2c isoforms, respectively) and pitx2ex4/5 (targeting the donor site of exon 4 and predicted to affect normal splicing of exons 4 and 5 that are common to both pitx2a and pitx2c transcripts and, therefore, to simultaneously knockdown both pitx2 isoforms) (Figure 1A). Injection of either a combination of pitx2aATG/ pitx2cATG oligomers or the single pitx2ex4/5 morpholino resulted in an abnormal phenotype, described below. RT-PCR analysis of mRNA extracted from pitx2ex4/5 morphants identified an acute reduction (in embryos injected with 4.7 ng of pitx2ex4/5 morpholino) or a complete absence (in larvae injected with 7 ng of morpholino) of normal pitx2 transcripts along with the presence of an aberrant PCR product at 24–48-hpf (Figure 1B); the quantity of normal transcript continued to be significantly diminished in 72–96-hpf pitx2ex4/5 morphants and reached normal levels in 120-hpf embryos (Figure 1B). Sequence analysis of the aberrant RT-PCR product revealed the presence of a 902-bp fragment corresponding to intron 4 (Figure 1C). The abnormal transcript was predicted to encode truncated pitx2a and pitx2c proteins lacking the third (recognition) helix of the DNA-binding homeodomain with pitx2a sequence truncated at 92 amino acids (34% of normal length) and pitx2c at 137 amino acids (44% of normal length). Knockdown of pitx2 disrupts embryonic development

Embryos injected with either a combination of pitx2aATG and pitx2cATG oligomers or a single pitx2ex4/5 morpholino demonstrated similar abnormal phenotypes, indicating an essential role for pitx2 in normal zebrafish development; pitx2ex4/5 morpholino was selected for further studies as it provided the ability to easily verify/quantify injection outcomes by RT-PCR (please see above; Figure 1).

Figure 1. zebrafish pitx2 knockdown and associated phenotype. A. Schematic drawing of pitx2 genomic structure. Exons are shown as numbered boxes, sizes are indicated at the top (for exons) or at the bottom (for introns). The positions of primers to amplify pitx2 transcripts are shown and numbered 1–3; primers 1 and 3 are used for pitx2a and 2 and 3 for pitx2c. The position of antisense morpholino oligonucleotides, pitx2aATG, pitx2cATG and pitx2ex4/5 are shown with red lines. B. RT-PCR of pitx2 expression in pitx2ex4/5 morphants. Please note a complete absence of pitx2 transcripts along with the presence of abnormal large PCR product (indicated with red arrowheads) in mRNA extracted from pitx2ex4/5 embryos at 24–48-hpf, the presence of both normal (diminished) and abnormal products at 72–96-hpf pitx2ex4/5 and normal levels of pitx2 by 120-hpf due to weakening of morpholino effects. C. DNA sequencing of the abnormal PCR product observed in pitx2ex4/5 morphant embryos identified the presence of the 902-bp intron 4 in the pitx2ex4/5 transcript consistent with aberrant splicing (forward sequence is shown and the beginning of the intron is indicated with a red arrow; exon 4 sequence is shown in upper case while intron 4 is in lower case letters; the exon-intron junction sequence corresponding to the pitx2ex4/5 antisense oligomer is indicated in red). Therefore, the pitx2ex4/5 protein is predicted to contain partial pitx2 sequence (lacking amino acids encoded by exon 5) followed by 10 erroneous amino acids (pitx2ex4/5 stop codon is indicated with red box). D. Representative images of control and pitx2ex4/5 embryos with specific phenotype. E. pitx2ex4/5 phenotype in p53−/− and wild-type zebrafish embryos.
Gross morphological comparison of pitx2ex4/5 morphant and control embryos was performed using 150–300 embryos in each experiment: at 24-hpf, the first embryos displaying smaller head, eyes and body length were detected with the number of abnormal embryos increasing sharply by 48-hpf; most of these embryos survived until 6–8-dpf and, in addition to the small eye, head, and trunk, displayed pericardial edema (starting at ~60-hpf) and jaw abnormalities (from ~72-hpf) (Figure 1D). Other phenotypic classes included severely malformed fish (displaying twisted shortened trunk, small malformed head, massive heart edema, and associated with lethality at ~72-hpf in most fish), “curved body” fish, and embryos with normal appearance. The proportions of phenotypes were as follows: specific head and trunk and severe phenotypes were observed in 70–82% of pitx2ex4/5 morphants and not observed in embryos injected with control morpholinio or uninjected larvae; the non-specific “curved body” phenotype was detected in ~12% of pitx2ex4/5, 14–16% of control morpholinio-injected larvae, and not observed in uninjected embryos; normal phenotype was seen in 6–16% of pitx2ex4/5 morphants, 84–86% of control morpholinio-injected fish and ~100% of uninjected embryos. Since the “curved body” fish were present at a similar rate in pitx2ex4/5 morphants and control embryos, this phenotype was classified as non-specific and further studies focused on morphants with specific head/trunk or severely malformed phenotypes.

To further investigate the specificity of the observed pitx2ex4/5 phenotypes, injections into p53-deficient zebrafish embryos were performed to identify features possibly associated with off-target effects which may accompany morpholinio injections due to activation of p53-mediated apoptosis [27]. The distribution of observed phenotypes following pitx2ex4/5 injections was similar at the 96-hpf stage while at 48-hpf a lower percentage of “curved body” (4% versus 12%) and severe phenotypes (4.3% versus 38.4%) was detected in p53−/− versus wild-type backgrounds (Figure 1E). These data suggest that both the “curved body” and severe phenotypes detected at earlier stages may be related to off-target effects of morpholinio injections while the specific head and trunk phenotype observed at both earlier and later stages is caused by pitx2 deficiency. Further analyses of morphology, histology and alcin blue staining were performed using zebrafish that displayed the specific phenotype in wild-type and p53−/− backgrounds with no apparent difference in features observed in these two backgrounds.

pitx2 ex4/sp morphants demonstrate craniofacial and ocular defects

To investigate the craniofacial and ocular anomalies associated with pitx2 deficiency, pitx2ex4/5 morphant and control embryos were assayed by alcin blue staining and histological analysis. The cartilage elements of the pitx2ex4/5 pharyngeal arches were reduced in size, malformed and displaced (Figure 2A-H). This phenotype was clearly visible starting at 72-hpf; no noticeable abnormalities were detected in morphant embryos at earlier stages of development using morphological and histological analyses. The position of the palatoquadrate of the mandibular arch (or the first pharyngeal arch) appeared to be normal while the Meckel’s cartilage component of the mandibular arch was positioned perpendicular to the palatoquadrate and pointing towards the upper jaw in 72-hpf embryos (Figure 2A-D). The ceratohyal cartilage of the hyoid arch (or the second pharyngeal arch) was positioned perpendicular to the upper jaw and pointing ventrally. The ceratobranchial arches 1 through 5 (pharyngeal arches 3-7) as well as the basihyal and basibranchial components of the pharyngeal arches appeared to be underdeveloped. At 120-hpf (Figure 2E-H), the mandibular and the hyoid pharyngeal arches were pointed ventrally, giving the appearance of an “open mouth”. In addition to this, a visible reduction in size and irregular shape of the elements of the mandibular arch (Meckel’s and palatoquadrate cartilages) was noted. The ceratobranchial arches could be observed at this stage but were reduced in size. At the same time, the ethmoid plate cartilage of the upper jaw appeared to be largely unaffected. The described anomalies were observed in 100% of 72, 120-hpf pitx2ex4/5 morphants (n = 35 (72–96-hpf), n = 20 (120-hpf) embryos).

Analysis of histological sections at the eye level revealed defects in ocular development in pitx2ex4/5 morphants (Figure 2I-T). At 72-hpf, the developing eye appeared to be largely unaffected with the exception of an increased number of cells observed in the anterior segment in some embryos (40%, n = 30; Figure 2I,J, O, P). At 120-hpf and 8-dpf, the pitx2ex4/5 eyes seemed small/missshapen and appeared to lack developed anterior segment region structures (93%, n = 30 (120-hpf) and 100%, n = 10 (8-dpf); Figure 2K-N and Q-T).

Since pitx2ex4/5 morphant embryos express abnormally spliced pitx2 transcript that includes exon 5, we were able to use the pitx2-exon 5 antisense riboprobe to detect cells with active pitx2 expression in morphant and control embryos and to compare these patterns: in early stage (24–48-hpf) pitx2ex4/5 embryos, we expected to primarily observe abnormal transcript while at later stages (72–120-hpf) we were likely to detect a mixture of abnormal and normal pitx2 transcripts due to weakening of the effects of morpholinio in the maturing pitx2ex4/5 embryos. At 24-hpf, pitx2-positive cells were detected in morphants in a pattern similar to normal pitx2 expression in control embryos in the brain, developing pharyngeal arches and around the eye (Figure 3A-D). At 48-hpf, the overall pattern continued to be similar but increased pitx2 staining was observed in the anterior segment of the eye on sections (80% of morphants, n = 10; Figure 3E-I) and by 72-hpf, an abnormal accumulation/pattern of pitx2 positive cells was detected by both whole mount and section in situ hybridization (100% of morphants, n = 17; Figure 3J-P). Clusters of pitx2 positive cells in the anterior segment of the eye were seen as late as 120-hpf (100% of pitx2ex4/5 embryos, n = 10; Figure 3Q-W); an increased staining behind the lens, in the developing hyaloid vasculature was also observed (Figure 3W). Similarly, robust expression of pitx2 in the craniofacial region of morphant embryos was observed at 72-hpf and 120-hpf in contrast to downregulation of normal pitx2 expression in control embryos (100% of morphants, n = 17 (72-hpf), n = 10 (120-hpf); Figure 3J-W).

Patterns of craniofacial and ocular gene expression are affected in pitx2ex4/5 morphants

In vertebrate embryos, the pharyngeal arches receive a significant contribution from the cranial neural crest cells that migrate into the craniofacial region. Forkhead transcription factor foxd3 is required for maintenance of neural crest population [33,34], fih1 is expressed in neural crest derived lineages [33] and the distal-less homeobox gene family plays an important role in early as well as late stages of craniofacial development [35,36]. We analyzed expression of foxd3, dlb2a and dlb2a, and Tgf001 (gfp) in pitx2ex4/5 morphants and control embryos and observed a number of differences.

Expression of foxd3 in 24-hpf pitx2ex4/5 embryos appeared to be mostly unaffected with similar levels of transcripts seen in the migrating neural crest cells marked with foxd3 (Figure 4A/A' and
Expression of *paixa* was detected in the retinal ganglion cells and inner nuclear layer neurons in *pitx2* morphant and control 100–120-hpf eyes (Figure 5A–D). Expression of *pitx3* in the developing lens also seemed to be mostly unaffected, although morphant lenses appeared to be smaller than control lenses (n = 6; Figure 5E–H); in addition to the lens, *pitx3* is expressed in the developing pharyngeal arches in a pattern similar to *pitx2* and this expression continued to be observed in the abnormally developing craniofacial structures (Figure 5F and H).

The most significant changes were detected in the expression of genes associated with the developing anterior segment structures. Analysis of *dkk2* expression in *pitx2* morphants revealed a sharp reduction in the amount of *dkk2* transcript in the anterior segment region at 72-hpf (67% of *pitx2* embryos; n = 12) (Figure 5I–L). Dual immunohistochemistry of keratan sulfate proteoglycan and crystallin (*αA* and *βB1*) in control and *pitx2* morphants revealed a complete absence in *pitx2* morphants while highly reduced/absent staining for keratan sulfate proteoglycan was observed in 75% of *pitx2* embryos (n = 16) while highly reduced/absent staining for keratan sulfate proteoglycan was observed in 75% of *pitx2* embryos (n = 16; Figure 5M–P). Examination of GFP expression in *Tg(fli1:gfp)* zebrafish embryos injected with control or *pitx2* morpholino identified clearly detectable patches of GFP positive cells in the iridocorneal angle and the developing annular ligament in 72–96-hpf eyes followed by strong expression in all 120-hpf control embryos (n = 10 for every stage) while a significant reduction (80%) or a complete absence (20%) of ocular GFP expression was observed in 70% of *pitx2* morphants (n = 10) (Figure 5Q–T).

Finally, the development of the ocular vasculature in *pitx2* morphants was evaluated using the *Tg(fli1:gfpi)* transgenic line [25]; in this line, the *fli1* promoter drives the expression of green fluorescent protein (gfp) in all blood vessels throughout embryogenesis. In 100% of 72–120-hpf *pitx2* morphants, the ocular...
similar to 48- and 72-hpf morphants demonstrate an accumulation of patterns during ocular and craniofacial development in comparison to abnormal pitx2 for whole mount and sections from two different

and sections (\(\text{pitx2}\)) are shown for embryonic stages of 24-hpf (A–D), 48-hpf (E–I), 72-hpf (J–P) and 120-hpf (Q–W). Please note abnormal \(\text{pitx2}\) transcripts around the developing eye (arrowheads in B, D) and pharyngeal arches (arrows in B) in morphants at 24-hpf, which is similar to \(\text{pitx2}\) expression in control embryos (A, C). Staining for \(\text{pitx2}\) positive cells in morphant embryos at 48-, 72- and 120-hpf identifies abnormal patterns during ocular and craniofacial development in comparison to \(\text{pitx2}\) expression in control embryos (E–W). In terms of ocular patterns, some 48- and 72-hpf morphants demonstrate an accumulation of \(\text{pitx2}\) transcriptionally active cells in the anterior segment of the eye (arrowheads; images for whole mount and sections from two different \(\text{pitx2}\) morphant embryos at 48-hpf (F, H, I) and 72-hpf (K, M, O, P) in comparison to control 48-hpf (E, G) and 72-hpf (J, L, N) are shown). In 120-hpf eyes, a disorganized pattern of \(\text{pitx2}\) positive cells continues to be observed in \(\text{pitx2}\) morphant embryos (arrowheads in T, U, two different morphant embryos are shown, and W) in comparison to \(\text{pitx2}\) expression in control embryos (S, V); in addition to the abnormal pattern in the anterior structures, an increased signal behind the lens corresponding to the hyaloid vasculature is also observed (asterisks in W). With regards to craniofacial development, in 72-hpf morphant embryos, strong staining is observed around the malformed oral cavity and arches with the level of expression similar to control \(\text{pitx2}\) expression (arrows in J–M); at 120-hpf, \(\text{pitx2}\) transcripts continue to be strongly expressed in the malformed pharyngeal arches (arrows in R and W) while expression of wild-type \(\text{pitx2}\) is downregulated in control 120-hpf embryos (arrows in Q and V).

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blood vessels appeared to be disorganized and misdirected based on fluorescence detection (\(n=15\); Figure 5U, V) and in situ analysis with the \(\text{gfp}\) probe (data not shown). To further investigate the hyaloid vasculature patterning in control and \(\text{pitx2}\) morphants, alkaline phosphatase staining was performed as described [31]. Consistent with the \(\text{Tg(fli1;gfp)}\) data, \(\text{pitx2}\) hyaloid vessels demonstrated abnormal branching with extra interconnections, appeared to be thinner, were increased in number and attached loosely to the smaller lens in comparison to control lenses (Figure 5W, X). The size of the \(\text{pitx2}\) lens was measured and found to be significantly smaller than the control lens (\(p=0.034\) for 72-hpf and \(p=0.007\) for 120-hpf).

**Discussion**

In this manuscript we describe the effects of \(\text{pitx2}\) knockdown in zebrafish. The functional disruption generated with \(\text{pitx2}\) splice-blocking morpholino is highly similar to the effects of human \(\text{PTX2}\) mutations, which are clustered in the homeodomain region of the protein with many of them resulting in truncation of normal \(\text{PTX2}\) sequence and lack of the recognition helix of the homeodomain [1–3]. Deficiency of \(\text{pitx2}\) led to a number of abnormalities in zebrafish including craniofacial and ocular defects consistent with the classic features of Axenfeld-Rieger syndrome. These data, together with the previously reported preservation of \(\text{PTX2/pitx2}\) expression pattern and transcriptional regulation [32] suggests a high level of conservation of \(\text{PTX2/pitx2}\) function in vertebrates.

The craniofacial abnormalities observed in \(\text{pitx2}\) morphants appear to be similar to what was reported in \(\text{foxd3}\) and \(\text{dlx}\) mutants and morphants [34,36]. We observed no significant differences in the expression pattern of \(\text{foxd3}\) in \(\text{pitx2}\) morphants; in general, expression of \(\text{foxd3}\) appears to precede \(\text{pitx2}\) expression in the pharyngeal arches’ primordial region and, therefore, \(\text{foxd3}\) may be acting upstream of \(\text{pitx2}\) in the developmental pathway. \(\text{PTX2}\) and \(\text{DLX2}\) have been previously reported to be acting in the same pathway during tooth development in mammals; \(\text{PTX2}\) was shown to bind to bicoil-like sites in the \(\text{DLX2}\) promoter resulting in a 30-fold activation of reporter expression in transfected cells [43].

Ocular developmental phenotypes observed in \(\text{pitx2}\) zebrafish such as accumulation of \(\text{pitx2}\)-transcriptionally active cells in the front of the developing eye followed by lack of differentiation of anterior segment structures are highly similar to the features previously reported in \(\text{Pitx2}\) knockout mice [8,19] and point to possible defects in migration patterns and differentiation of neural-crest derived periocular mesenchymal cells. The expression pattern of several ocular markers was found to be affected in \(\text{pitx2}\) morphants consistent with the abnormal development of anterior segment structures and overall ocular dysgenesis. In addition to anterior segment dysgenesis and vasculature defects in \(\text{pitx2}\) deficient zebrafish, a misshapen and small retina and lens were observed. A small and misshapen eye retina has been previously reported in \(\text{Pitx2}\)–/– deficient mice [19]. At this point it is unclear whether \(\text{pitx2}\) plays a direct role in the development of posterior ocular structures or if maldevelopment of the anterior segment and/or hyaloid vasculature affects the formation of other
parts of the eye including the retina; the second possibility seems to be more likely since *pitx2* is not expressed in the developing retina.

Zebrafish *pitx2* deficiency was also found to result in an abnormal patterning of the hyaloid vasculature with an increased number of thin and disorganized vessels observed in the *pitx2* ex4/5 morphant eyes. Recently, Rutland et al demonstrated that defects in the development of the hyaloid vasculature can lead to microphthalmia and lens anomalies in mice [44]. A similar mechanism may be implicated in the small eye/lens phenotype as even partial loss-of-function mutations can result in ocular abnormalities in human or mouse mutants as well as in association with mutations in the human *LAMB2* [59], *NDP* [60] and *FZD2* [61] genes. Interest-ingly, Cited2 has been previously reported to directly control Pitx2c expression during cardiovascular development [62]. Pitx2c expression in the left lateral plate mesoderm was absent in the *Cited2(−/−) mice; Cited2 and Tfap2 were found to be bound to the Pitx2c promoter in embryonic heart tissues and to activate Pitx2c transcription in transient transfection assays. Therefore, it is possible that a similar pathway is acting during ocular vasculature development.

The Axenfeld-Rieger malformation spectrum represents a complex group of conditions showing significant intrafamilial and interfamilial variability [2,3]. Ocular abnormalities appear to be the most penetrant feature of *PITX2*-deficient phenotypes as even partial loss-of-function mutations can result in ocular manifestations [2,3]. The mechanisms of these ocular defects and, in particular, the developmental glaucoma seen in *pitx2/PITX2* mutants as well as in association with mutations in the human *LAMB2* [59], *NDP* [60] and *FZD2* [61] genes. Interestingly, Cited2 has been previously reported to directly control Pitx2c expression during cardiovascular development [62]. Pitx2c expression in the left lateral plate mesoderm was absent in the *Cited2(−/−) mice; Cited2 and Tfap2 were found to be bound to the Pitx2c promoter in embryonic heart tissues and to activate Pitx2c transcription in transient transfection assays. Therefore, it is possible that a similar pathway is acting during ocular vasculature development.

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Figure 4. Craniofacial development and gene expression in *pitx2* ex4/5 morphants. Developmental patterns of foxd3, dlx2a and dlx4a, and *Tg(fli1: gfp)* expression in *pitx2* ex4/5 embryos. In situ hybridization was performed with foxd3, dlx2a, dlx4a or gfp-specific antisense riboprobe in control (A–P) or morphant (A′–P′) zebrafish embryos. Please note similar expression patterns in migrating neural crest cells (foxd3) and pharyngeal arch primordial regions (dlx2a) at 24-hpf (arrows in control (A–C) and morphant (A′–C) embryos). Starting at 32-hpf, expression of dlx2a, dlx4a and *Tg(fli1: gfp)* in the posterior arches appears to be reduced in *pitx2* ex4/5 fish (arrowheads in control (D–G) and morphant (D′–G′) embryos) while expression in the anterior arches (arrows in control (D–G) and morphant (D′–G′) embryos) is not significantly affected. In 48–72-hpf embryos, an increased expression of dlx4a in the anterior arches is detected (arrows in control (H–N) and morphant (H′–N′) embryos) while expression in the posterior arches remains reduced (arrowheads in control (H–N) and morphant (H′–N′) embryos); dlx4a expression around the oral cavity in 48-hpf embryos (arrow with asterisk in K and K′) as well as a frontward extension of the anterior arches at 72-hpf (arrows in L–N for controls and L′–N′ for morphants) are not observed in *pitx2* ex4/5 fish. Analysis of sections at 72-hpf also shows broadened expression of dlx4a in the craniofacial region of morphant embryos (O′, P′) in comparison to controls (O, P). doi:10.1371/journal.pone.0030896.g004

Developmental patterns of foxd3, dlx2a and dlx4a, and *Tg(fli1: gfp)* expression in *pitx2* ex4/5 embryos. In situ hybridization was performed with foxd3, dlx2a, dlx4a or gfp-specific antisense riboprobe in control (A–P) or morphant (A′–P′) zebrafish embryos. Please note similar expression patterns in migrating neural crest cells (foxd3) and pharyngeal arch primordial regions (dlx2a) at 24-hpf (arrows in control (A–C) and morphant (A′–C) embryos). Starting at 32-hpf, expression of dlx2a, dlx4a and *Tg(fli1: gfp)* in the posterior arches appears to be reduced in *pitx2* ex4/5 fish (arrowheads in control (D–G) and morphant (D′–G′) embryos) while expression in the anterior arches (arrows in control (D–G) and morphant (D′–G′) embryos) is not significantly affected. In 48–72-hpf embryos, an increased expression of dlx4a in the anterior arches is detected (arrows in control (H–N) and morphant (H′–N′) embryos) while expression in the posterior arches remains reduced (arrowheads in control (H–N) and morphant (H′–N′) embryos); dlx4a expression around the oral cavity in 48-hpf embryos (arrow with asterisk in K and K′) as well as a frontward extension of the anterior arches at 72-hpf (arrows in L–N for controls and L′–N′ for morphants) are not observed in *pitx2* ex4/5 fish. Analysis of sections at 72-hpf also shows broadened expression of dlx4a in the craniofacial region of morphant embryos (O′, P′) in comparison to controls (O, P). doi:10.1371/journal.pone.0030896.g004
deficient zebrafish are likely to facilitate examination of the developmental roles of this important factor.

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Author Contributions

Conceived and designed the experiments: EVS. Performed the experiments: YL. Analyzed the data: YL EVS. Wrote the paper: YL EVS.

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Figure 5. Ocular development and marker expression in pitx2ex4/5 morphants. In situ hybridization (A–L), double immunohistochemistry (M–P), live gfp fluorescence images (Q–V) and alkaline phosphatase staining (W, X) for control and morphant embryos are shown and are labeled with corresponding developmental stages in the lower right corner; dorsal (A, B, I, J) and ventral (E–H) views of a zebrafish larval head as well as transverse sections at the eye level (C, D, M–P), all positioned anterior to the top, and lateral views of an embryonic eye (K, L) or head (Q–V), positioned anterior to the left, are shown. Please note expression of pax6a in the developing retina of the control and morphant embryos (arrowheads in A–D); similar pitx2 expression in the developing lens in 48–72-hpf control and morphant embryos (arrowheads in E–H); abnormally developing pharyngeal arches and oral cavity in 48–72-hpf embryos are marked with arrows); reduced expression of dkk2 in the anterior segment of the pitx2 morphants (arrowheads in I–L); decreased/absent expression of corneal keratan sulfate proteoglycan (CKS) in the developing morphant corneas (red fluorescence, arrowheads in M–P) but similar expression of crystallins αB (green fluorescence, arrows in M, N) and βB1 (green fluorescence, arrows in O, P) in control and morphant lenses; and a decreased/absent expression of the Tg(gsnl1:gfp) transgene in the developing indocarneal structures of pitx2ex4/5 morphants (arrowheads in R, T) in comparison to control embryos (Q, S). In addition to this, fluorescence (Tg(fft1:gfp) transgene) images (U, V) as well as alkaline phosphatase staining (W, X) demonstrate abnormal development of the hyaloid vasculature in pitx2ex4/5 morphants (arrowhead in V) in comparison to control embryos (U); increased number and disorganized appearance of pitx2ex4/5 hyaloid vessels (X) in comparison to controls (W) is revealed by alkaline phosphatase staining.

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