**MIP/Aquaporin 0 Represents a Direct Transcriptional Target of PITX3 in the Developing Lens**

Elena A. Sorokina¹, Sanaa Muheisen¹, Nevin Mlodik¹, Elena V. Semina¹,²,⁎

¹Department of Pediatrics and Children’s Research Institute, Medical College of Wisconsin and Children’s Hospital of Wisconsin, Milwaukee, Wisconsin, United States of America, ²Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America

**Abstract**

The PITX3 *bicoid*-type homeodomain transcription factor plays an important role in lens development in vertebrates. PITX3 deficiency results in a spectrum of phenotypes from isolated cataracts to microphthalmia in humans, and lens degeneration in mice and zebrafish. While identification of downstream targets of PITX3 is vital for understanding the mechanisms of normal ocular development and human disease, these targets remain largely unknown. To isolate genes that are directly regulated by PITX3, we performed a search for genomic sequences that contain evolutionarily conserved *bicoid*/PITX3 binding sites and are located in the proximity of known genes. Two *bicoid* sites that are conserved from zebrafish to human were identified within the human promoter of the major intrinsic protein of lens fiber, MIP/AQP0. MIP/AQP0 deficiency was previously shown to be associated with lens defects in humans and mice. We demonstrate by both chromatin immunoprecipitation and electrophoretic mobility shift assay that PITX3 binds to MIP/AQP0 promoter region in vivo and is able to interact with both *bicoid* sites in vitro. In addition, we show that wild-type PITX3 is able to activate the MIP/AQP0 promoter via interaction with the proximal *bicoid* site in cotransfection experiments and that the introduction of mutations disrupting binding to this site abolishes this activation. Furthermore, mutant forms of PITX3 fail to produce the same levels of transactivation as wild-type when cotransfected with the MIP/AQP0 reporter. Finally, knockdown of *pitx3* in zebrafish affects formation of a DNA-protein complex associated with *mip1* promoter sequences; and examination of expression in *pitx3* morphant and control zebrafish revealed a delay in and reduction of *mip1* expression in *pitx3*-deficient embryos. Therefore, our data suggest that PITX3 is involved in direct regulation of MIP/AQP0 expression and that the alteration of MIP/AQP0 expression is likely to contribute to the lens phenotype in cataract patients with PITX3 mutations.

**Introduction**

The PITX3 *bicoid*-related homeodomain transcription factor represents an important regulator of lens development in vertebrates. Mutations in *PITX3* result in congenital cataracts, anterior segment mesenchymal dysgenesis (ASMD), Peter’s anomaly, and microphthalmia in humans [1–6]. Deletions within the *Pitx3* promoter region in mice produce the *aphakia* phenotype, which is characterized by small eyes lacking a lens [7,8]. In lower vertebrates (zebrafish and frog), *pitx3* was shown to be essential to normal lens and retina formation [9–13]. Knockdown of *pitx3* protein in zebrafish embryos via translational morpholino results in small eyes, lens degeneration, misshapen head and reduced jaw and fins [9,10,12]. In vertebrates, expression of *Pitx3/pitx3* is first detected in the lens placode and then the lens vesicle; early expression is observed in the lens epithelial cells and primary fibers while later expression is restricted to the equator regions of the developing lens [1,14].

Despite its vital importance for eye development, little is currently known about the ocular function of *PITX3/Pitx3* and its downstream targets. Expression of several genes/proteins was found to be altered in the lenses of *Pitx3*-deficient mice. Some early reports demonstrated that expression of β- and γ-crystallins is completely absent at developmental stages 10–18 days as well as in newborn *aphakia* mice [15–17]. Two recent publications provided additional data on this matter; Ho and colleagues detected precocious activation of both β- and γ-crystallins in the eyes of 10.5–11.5-dpc *Pitx3*–knockout mice [18] while Medina-Martinez and coauthors reported deregulation of crystallin expression in *aphakia* mice with α- and β-crystallin expression being reduced at both transcript and protein levels and γ-crystallin expression being downregulated at the protein level [19]. In addition to crystallins, expression of the transcription factors *Foxe3* [18–20] and *Prox1* [19] as well as the cell cycle regulator *p57Kip2* [19] were found to be affected in *Pitx3*-deficient animals, which seems more likely to be related to the overall abnormal lens development in *aphakia* mice rather than direct involvement of *Pitx3* in transcriptional regulation of these genes [19].

*PITX3* belongs to the *PITX* family of *bicoid*-type homeodomain-containing proteins that regulate expression of other genes during development and, possibly, in adult organisms. Other members of this family were shown to be involved in developmental disorders such as idiopathic clubfoot [PITX1; 21] and Axenfeld-Rieger syndrome [PITX2; 22]. PITX factors are known to interact with...
bicoid-type DNA sequences and to regulate downstream gene expression through these interactions [23–28]. PITX factors are primarily known as activators of transcription, though they may also act as repressors [29,30]. Several transcriptional targets of PITX homeoproteins have been identified and bicoid sequences located in the regulatory regions of these downstream genes were shown to mediate these interactions; two or more bicoid sites were found in some promoters [25,26,20,31,32], although a single bicoid element was demonstrated to be sufficient in several other cases [24,33–35]. Interspecies conservation of bicoid sequences has been reported for some promoters [26,32]. Preservation of regulatory sequences is frequently observed for developmental genes which demonstrate a conserved expression pattern; therefore, identification of regulatory sequences represents a useful tool in uncovering genetic pathways [36,37].

In order to isolate downstream targets of the PITX3 homeodomain transcription factor we performed a search for evolutionarily conserved non-coding sequences containing bicoid sites and located in proximity to known genes, therefore potentially interacting with PITX3 to regulate expression of that gene. As a result, we identified two bicoid sites located in the promoter of Major Intrinsic Protein of lens fiber (MIP) or Aquaporin 0 (AQP0) that are conserved between human, mouse, zebrafish and several other species. We further demonstrated that PITX3 is able to specifically interact with the identified sequences both in vitro and in vivo and to transactivate gene expression as a result of this interaction. In addition to this, expression of mip1 was found to be altered in pitx3 deficient zebrafish morphants. Our data suggest that PITX3 is involved in direct regulation of MIP/AQP0 expression and provide new insight into the PITX3 pathway as well as mechanisms of lens development.

Materials and Methods

Ethics statement

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 silico analysis

ECR Browser web-based tool (http://ecrbrowser.dcode.org) was used to identify conserved paired bicoid sites in the promoters/intronic regions of genes with known expression/function. Paired comparison of human and mouse genomes was performed using the following parameters: presence of two conserved bicoid sites with distance between the sites not to exceed 650-bp. Secondary analysis of identified regions for sequence conservation was performed using the UCSC Genome Browser multiple alignment module (http://genome.ucsc.edu) as well as the BLAST tool (http://blast.ncbi.nlm.nih.gov), including examination of the corresponding genes in lower vertebrates when available.

Cell culture

Human lens epithelial cells (B3) and human embryonic kidney cells (293HEK) were obtained from ATCC (Manassas, VA). B3 cells were cultured in MEM medium (Invitrogen; Carlsbad, CA) supplemented with heat-inactivated 20% fetal calf serum (FBS), glutamine, sodium pyruvate to a final concentration of 1 mM, non-essential amino acids and antibiotic-antimycotic (Invitrogen; Carlsbad, CA). 293HEK cells were maintained in DMEM medium containing 10% FBS, glutamine and antibiotic-antimycotic solution.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from B3 cells transiently transfected with PITX3-pcDNA3.1 vector with CellLytic NuCLEAR extraction kit (Sigma, St. Louis, MO). Cells were harvested after 48 hours with a cell scraper and the compact cellular pellet was re-suspended in 3 volumes of Hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl) with Protease inhibitor cocktail (Sigma; St. Louis, MO). After 15 minutes of incubation on ice, Igepal CA-630 was added to a final concentration 0.6%, then the cells were vortexed and spun down for 30 seconds at 10000 g. Crude nuclear pellet was extracted with about 2/3 of the original packed cell volume of Extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 0.2 mM EDTA, 1 mM DTTO and 25% Glycerol) in the presence of protease inhibitors for 30 minutes on ice. 32-mer 5’-GGA-GAAAGGCGTCTAATCGCTGGGAACCTAAAG oligonucleotide spanning region −537 to −502 from transcriptional start site (tss) of MIP/AQP0 promoter, 32-mer 5’-CTGCCCTCCTCCAGG-GATTCAAGTGCTCTTATAA corresponding to the promoter sequence −715 to −40 and their complement oligonucleotides as well as both sets of oligonucleotides with TAATCC (GGATTA) bicoid sites replaced by TAATTT (AAATTA) (see above) were labeled with Biotin 3’ End DNA Labeling kit (PIERCE) and annealed. Electrophoretic mobility shift assays (EMSA) were performed with LightShift Chemiluminescent EMSA kit (Pierce; Rockford, IL) in accordance with the manufacturer’s protocol and using 50 ng/μl of Poly(dI-dC), 20 fmol of labeled DNA and 2 μl of nuclear extracts. After 20 minutes of incubation at room temperature, reactions were either diluted with 5× Loading buffer or further incubated for 30 minutes in presence of 1 μg of polyclonal Pitx3 antibody for supershift assay. Binding reactions and free probe were run on 5% native polyacrylamide gel in 0.5× TBE buffer.

For EMSA experiments performed using whole zebrafish embryo nuclear extracts, the 32-mer and its compliment containing the region from −44 to −76 of zebrafish mip1 promoter were utilized: 5’-CAA TTG AGC AGG ATT ACA GTG TCA CAG AG. In addition to this, both sets of oligonucleotides were made with TAATCC (GGATTA) bicoid sites replaced by TAATTT (AAATTA) to be used as a control for bicoid site binding specificity. Nuclear extracts were generated from sixty 48-hpf zebrafish pitx3 morphant or wild-type embryos that demonstrated normal body length and morphology; the preparation was carried out as described above except for that the embryos were first grinded with glass tissue homogenizer equipped with type B pestle in hypotonic detergent-less lysis buffer to assist nuclei release. Binding reaction was performed in the presence of 25% glycerol, 5 mM MgCl2 and 0.05% NP-40 in addition to the buffer composition described above; 3 μl of extract was used in each binding reaction. Five embryos from each group were analyzed for pitx3 transcript presence to verify the degree of morpholino-mediated knockdown.

Chromatin immunoprecipitation (ChIP)

ChIP was performed with ChIP-IT enzymatic kit or ChIP-IT Express enzymatic kit (Active Motif; Carlsbad CA) according to manufacturer recommendations.

B3 human lens epithelial (HLE) cells were grown in 100 mm tissue culture dish to 90–95% confluence and utilized for ChIP assays; experiments were performed using native untransfected cells as well as cells transfected with PITX3 expression plasmids. Cells were transfected with 7.5–10 μg of pcDNA3.1_PITX3_FLAG or empty pcDNA3.1 plasmid and cross-linked after 48 hours with 1% formaldehyde for 10 minutes at room temperature with agitation. Following this, the monolayers were washed with 125 mM of...
glycine and lysed. The nuclear pellet was resuspended in digestion buffer and DNA was sheared with Enzymatic shearing cocktail for 10 minutes at 37 °C in a water bath. The resulting fragments ranged between 200- and 1000-bp in size. The quality of chromatin was verified in a control experiment of immunoprecipitation with the Polymerase II antibody followed by PCR with primers specific for the GADPH promoter. Only those chromatin preparations that demonstrated significant enrichment in these control experiments were used in further analysis. Immunoprecipitation was performed with 2 µg of Pitx3, FLAG or control IgG antibody overnight in a cold room and, after washing and de-crosslinking, the precipitated DNA was analyzed by PCR. Goat polyclonal PitX3 (N-20) and normal goat IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and FLAG-M2 mouse monoclonal antibody from Sigma (St. Louis, MO). For PCR amplification of MIP/AQP0 promoter, the following primers were utilized: set 1 (spanning region −110/+495) forward, 5'-GCCTGTAAGGGTTAAGAGG-3' and reverse 5'-GAGGGTGCGGAAAGAATCTCAG-3' and set 2 (spanning region −473/-275) forward, 5'-CTGAACCC-CACCTGCTACAGCA-3' and reverse, 5'- TCCTGCCCCTCTGTTGGTGC-3'. For control experiments, the following primers were used: GAPDH forward 5'-TACTAGCCGTGTTTACCG-GGCCG-3' and reverse 5'-TCAAAACAGGGAGGACGAGAG-CGGA-3', product = 166 bp (provided as positive control as part of ChIP-IT Express Enzymatic kit (Active Motif; Carlsbad, CA); forward 5'-ATGGTTGGCCTACTGGGATCT-3' and reverse 5'-TGGCCAAAAGCCTAGGGGAA-3', product = 174 bp (provided as negative control as part of ChIP-IT Express Enzymatic kit, Active Motif, Carlsbad CA). The PCR reactions were repeated at least three times using precipitated DNA from independent chromatin immunoprecipitation experiments. Quantification of ChIP PCR products was performed by densitometry using the ImageJ program developed by Dr. Rasband, NIH (http://rsweb.nih.gov/ij/). The measurements obtained for ChIP PCR results were normalized by input DNA and expressed as percent of its value. Data from at least three independent experiments were combined to calculate mean and standard deviation. Statistical significance was determined using the homoscedastic Student's t-test with a two-tailed distribution.

Expression and reporter plasmids

PITX3 wild type, PITX3-WT, and mutant expression constructs, PITX3-K111E, PITX3-S13N and PITX3- G219F, were previously described [27]. To produce the MIP656-bcd1,2 reporter plasmid, a 656-bp fragment containing 597-bp of upstream and 59-bp of downstream sequence from the transcriptional start site (tss) of MIP/AQP0 was amplified by PCR and cloned into the pCRII-TOPO vector (Invitrogen; Carlsbad, CA) and then subcloned into basic pG3 luciferase reporter vector (Promega, Madison, WI). The transcriptional start site (tss) of MIP/AQP0 was designated based on the ENST00000257979 entry in Ensemble Database. Site-directed mutagenesis was performed with Quick-Change II Site-Directed mutagenesis kit (Stratagene, La Jolla, CA), oligonucleotide 5'-CTCACGGCTGCGGGGGCTCGAGAAAT-TAAGGAGTCCCTTATAAA-3' and its complement for the proximal bicaudal site and oligonucleotide 5'-CTACGGCAATGGG-GAGAAAGGCTTCTAATTTCCTGGGAATT-3' and its complement for the distal site on MIP/AQP0 promoter. Therefore, in both bicaudal sites the consensus recognition sequence TAATCC was replaced by TAATT and three additional constructs were produced: MIP656-bcd1 (carrying mutant bcd1 site), MIP656-bcd2 (carrying mutant bcd2 site) and MIP656-bcd0 (carrying mutations in both bicaudal sites). All constructs were verified by sequencing.

Reporters assays

Human embryonic kidney cells (293HEK) were plated in 24-well plates and transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol. Equimolar amounts of basic pGL3 reporter plasmid (100 ng) and MIP656 wild-type and mutant reporters (114 ng) were used. Each cotransfection included 300 ng of eGFP (PITX3 wild type and mutant expression constructs) and 60 ng of β-galactosidase in pdNA3.1 vector (internal control for efficiency of transfection); the total DNA amount was kept the same in all transfections by adding empty pdNA3.1 vector when needed. Cells were harvested after 48 hours; luciferase and β-galactosidase activities were determined using Luciferase assay and Enzyme Assay systems (Promega, Madison, WI), respectively. Every experiment was performed at least three times in triplicate. Student's paired t-Test with a one-tailed distribution was utilized to determine the statistical significance of any differences in activity level.

Zebrafish care and morpholino injections

Zebrafish (Danio rerio) were maintained on a 14-hour light/10-hour dark cycle. The embryos were obtained by natural spawning and maintained at 28.5 °C. The pitx3 morpholino, 5'-AGGT-TAAAATCCATCACCTGTACCC-3', that was previously reported [9] or control morpholino (Gene Tools, Philomath, OR) were suspended at 250 µM in injection buffer [0.1% (w/v) phenol red (Sigma) in 0.3× Daniecu buffer (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO4, 1.8 mM Ca(NO3)2 and 1.5 mM HEPES, pH 7.6) and 19.2 ng was injected into zebrafish embryos immediately after fertilization at the 1–2 cell stage. Microinjections were performed using the Nanoject II injector (Drummond Scientific, Broomall, PA). Embryos were incubated at 28.5 °C in 0.2 mM 1-phenyl-2-thiourea (PTU) to inhibit pigment formation and anesthetized with 0.05% Tricane before imaging. The developmental stage was determined by time (hours post fertilization [hpf]) and by morphological criteria [38]. Nikon SMZ 1500 and Zeiss M2 Discovery microscopes were utilized for embryo imaging.

RNA isolation, RT-PCR and in situ hybridization

For RNA isolation, the embryos were homogenized in TRI reagent (Sigma) in the presence of glycogen and processed using a standard extraction protocol. The cDNA was generated using equal amounts of RNA for every sample and SuperScript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer recommendations. Semi-quantitative PCR was performed using gene-specific oligonucleotides for mmp1, exon_1F, 5'- CTCCCAGATGTCCCTGTTC-3', and exon_2R, 5'- CATACTTGCCAGGCTCA-3', (PCR product = 148 bp); for pitx3, exon_1F, 5'-CTCGACTAGACCGGGATTCA-3', and exon_3R, 5'-AAAGTTGCGCTTCAAGG-3', (PCR product = 276 bp); and for β-actin, exon2F, 5'- GAGAAGATCTGG- CATCAGAC-3', and exon_3R, 5'- ATCGAGGCTTCAAGG-3', (PCR product = 523 bp). The PCR conditions were as follows: initial denaturation at 94 °C for 3 min followed by 23–32 cycles of 94 °C for 20 seconds, 59 °C for 30 seconds and 72 °C for 30 seconds and final extension at 72 °C for 7 min. The number of cycles was optimized to maintain PCR reaction in linear range.

To construct an antisense riboprobe for in situ hybridization experiments, a 638-bp fragment specific to zebrafish mmp1 transcript was generated using the following primers, forward, 5'-CTGGCAGAGATGTCGTAC-3' and reverse, 5'-GGTGCTGAAAATGTTCAAGA-3', and inserted into pCRII-TOPO plasmid vector. An
antisense RNA probe was generated using DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN) following manufacturer recommendation; in situ hybridization was performed as previously described [39].

Results

Genome search for regulatory regions containing conserved PITX3 binding sites identifies MIP/AQP0 promoter

Examination of the ECR Browser web-based tool for clusters of PITX3 binding sites conserved between different species yielded a total of 976 genomic regions: 511 sequences were found inside of intergenic regions, 454 elements were located within genes (309 in intronic, 90 in coding and 55 in untranslated regions), and only 11 were positioned within 1500 bp from a transcriptional start site. The eleven identified promoter regions were subjected to a secondary analysis of sequence conservation that included examination of the corresponding genes in lower vertebrates when available. One sequence demonstrated the strongest level of conservation of bcd sites across multiple species: the promoter region of the gene encoding for the major intrinsic protein of lens fiber or aquaporin 0 (MIP/AQP0).

The MIP/AQP0 promoter region contains two bcd sites separated by 456 base pairs at positions −58 (bcd1) and −520 (bcd2) from the transcriptional start site. Alignment of MIP/AQP0 promoters from different species demonstrates high conservation of both bcd sites in nine mammalian/vertebrate species from human to zebrafish (Figure 1). Zebrafish (Danio rerio) has two orthologs of the human MIP/AQP0 gene designated mip1 and mip2 [40]. The promoter sequence/structure of zebrafish mip1 appears to be more similar to mammalian species showing conservation for both bcd sites (positions −59 (bcd1) and −442 (bcd2)) and surrounding sequence (Figure 1).

Conservation of the bcd sites in MIP promoters of different species points to the potential importance of these sequences in the regulation of MIP/AQP0 expression.

The conserved bcdoid sequences within the MIP/AQP0 promoter are capable of binding PITX3

We first performed electrophoretic mobility shift assay (EMSA) to examine whether these putative bcd sites are capable of bind PITX3 in vitro. Nuclear extracts were isolated from human lens epithelial cells transiently transfected with either a PITX3 expression plasmid or an empty pcDNA vector and incubated with labeled oligonucleotides containing the TAATCC motif and 13-bp of flanking sequences on either side of each bcd site. The samples derived from PITX3-enriched nuclear extracts produced clearly visible shifts with both probes which were not observed with samples prepared from mock-transfected cells (Figure 2). The PITX3-DNA complexes were further verified by addition of PITX3 polyclonal antibody, which resulted in reduction of the intensities of the shifted bands and formation of supershifts (Figure 2). In addition to this, the specificity of binding was confirmed by EMSA analysis using modified oligonucleotides carrying a 2-nt mutation within the bcd sites: the TAATCC motif was replaced with TAATTT in both probes to abolish PITX3 binding [27]. Mutations in the bcd sites resulted in the disappearance of protein-DNA complexes, confirming that these bands are the product of specific PITX3-DNA interactions (Figure 2).

These results demonstrate that PITX3 is capable of binding to both bcd sites located in the human MIP/AQP0 promoter in vitro.

PITX3 interacts with MIP/AQP0 promoter in human lens epithelial cells in vivo

To investigate whether PITX3 interacts with the MIP/AQP0 promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays. Native untransfected human lens epithelial (HLE) cells or HLE cells following transfection with pcDNA3.1_PITX3-MIP/AQP0 FLAG expression plasmid or pcDNA3.1 empty vector were used in these experiments.

Immunoprecipitations with PITX3 antibody that used nuclear extracts from native untransfected HLE cells resulted in enrichment of MIP/AQP0 promoter sequences in the precipitated DNA in comparison to ChIP samples produced with control antibody (IgG) (Figure 3A). Immunoprecipitation with FLAG antibodies that used nuclear extracts from pcDNA3.1_PITX3-MIP/AQP0 transfected cells resulted in enrichment of MIP/AQP0 promoter sequences in the precipitated DNA in comparison to ChIP samples produced with control antibody (IgG)/same nuclear extracts as well as precipitations that utilized the same antibody (FLAG) but employed mock-transfected cells (Figure 3B). This enrichment for MIP/AQP0 promoter sequences in the precipitated DNA was demonstrated by semi-quantitative PCR using specific MIP/AQP0 and control primers (described in Materials and Methods) and calculated to be ~2.2 times in experiments performed in native untransfected HLE cells and ~4.5 times in assays that used transfected HLE cells; the observed differences were found to be statistically significant with P<0.05 based on t-test (Figure 3C and D). The chromatin immunoprecipitation and PCR-based enrichment analysis was repeated eight times using independently transfected cells with consistent results.

These experiments demonstrated the specific association of PITX3 with the MIP/AQP0 promoter region in vivo.

Mutations in bcdoid sites located in the MIP/AQP0 promoter affect the activity of the promoter

To examine whether the conserved bcd sites within the MIP/AQP0 promoter are involved in regulation of its activity, we created several reporter constructs: MIP656-bcd1,2, which contained a 656-bp fragment of the MIP/AQP0 wild-type promoter encompassing both bcd sites and nucleotides from positions −597 to +59 in relation to the MIP/AQP0 transcriptional start site inserted into a basic pG5L3 plasmid containing the luciferase reporter gene; MIP636-bcd1, which contained a mutation in the bcd2 site that changed the 5′-TAATCC-3′ sequence to 5′-TAATTT-3′, thus abolishing its interaction with wild-type PITX3 [27]; MIP656-bcd2, which contained a similar mutation in the bcd1 site changing the 5′-GGATTA-3′ sequence into 5′-AAATTA-3′; and MIP656-bcd0, which included both of the above described mutations.

Reporter assays demonstrated a 5.2-fold upregulation of luciferase expression in the presence of the MIP656-bcd1,2 promoter fragment in comparison to the empty vector in human embryonic kidney cells (Figure 4A). Mutations in either the bcd1 or bcd2 sites resulted in a decrease in MIP/AQP0 promoter activity compared to the wild-type promoter: to 3.5-fold (67% of MIP656-bcd1,2 activity; P<0.001) when the bcd1 site was mutated, to 4.3-fold (83%; P = 0.014) when the bcd2 site was abolished and to 3.4-fold (65%; P<0.001) when both sites were disrupted.

Based upon these results, both bcd sites appear to be involved in regulation of MIP/AQP0 expression with the proximal site, bcd1, playing a more significant role in its activation.
PITX3 is capable of transactivating the MIP/AQP0 promoter via the bcd1 bicoid site

In order to investigate the effect of PITX3 on the transcriptional activity of the MIP/AQP0 promoter, we performed cotransfection assays using the above described MIP656-bcd1,2, MIP656-bcd1, MIP656-bcd2 and MIP656-bcd0 reporter constructs and a PITX3 expression plasmid.

Cotransfection of the PITX3-WT expression plasmid with the MIP656-bcd1,2 luciferase reporter into human embryonic kidney cells resulted in a ~4.8-fold normalized activation in comparison to the cotransfection of the PITX3-WT plasmid with the promoter-less reporter (Figure 4B). In contrast, cotransfection of the MIP656-bcd1,2 luciferase reporter with an expression plasmid carrying PITX3 mutants produced a ~2-fold increase over the same control for the G219f mutant (42% of wild-type activity; P < 0.001), ~3.1-fold for S13N (65%; P = 0.015) and no transactivation was observed for the K111E mutant. These results are consistent with the previously reported data on the residual activities of the corresponding mutant PITX3 forms [27].

We next examined the two bicoid sites present in the MIP/AQP0 promoter for their role in this observed transactivation. The PITX3-WT expression plasmid and mutant MIP/AQP0 promoter constructs, MIP656-bcd1 (carrying mutant bcd2 site), MIP656-bcd2 (carrying mutant bcd1 site) and MIP656-bcd0 (carrying mutations in both bicoid sites) were cotransfected into human embryonic kidney cells and the resultant luciferase activities were compared to values observed in experiments involving cotransfection of wild-type MIP/AQP0 promoter (MIP656-bcd1,2) and PITX3-WT. The
transactivation of the MIP656-bcd1,2 promoter by PITX3-WT decreased to ~2-fold (42% of PITX3 induced wild-type promoter transactivation) when the mutation in the proximal bicoid site, bcd1, was introduced; increased to ~6-fold (125%; P = 0.001) when the distal bicoid site, bcd2, was mutated; and produced ~2.2-folds (46%; P < 0.001) when both bicoid sites were disrupted (Figure 4B).

These data suggest that PITX3 is involved in activation of the MIP/AQP0 promoter via its proximal bicoid site, bcd1.
Knockdown of pitx3 affects formation of a DNA-protein complex associated with mip1 promoter sequences

In order to efficiently disrupt pitx3 gene expression in zebrafish and to be able to tightly monitor residual activity/knockdown level, we tested several splicing morpholinos that were designed against pitx3 intron-exon junctions. Unfortunately, none of these morpholinos produced the desired outcome, resulting in either no effect on pitx3 splicing or highly abnormal phenotype due to toxicity/non-specific defects. Then we tested the previously reported translational morpholinos [9,12] and discovered that the antisense morpholino reported by Dutta and coauthors [9] and designed against the sequence containing the translation initiation codon located in exon 2 of pitx3 results in abnormal splicing of the pitx3 transcript due to exon 2 skipping (Figure 5). This pitx3 morpholino [9] matches the nucleotide sequence at positions +8 to +32 of exon 2 and therefore is located only 7-nt upstream of the intron 1/exon 2 acceptor site. In our experiments, we found this morpholino to be highly efficient in blocking normal splicing with the abnormal 148-bp product lacking exon 2 generated in pitx3-mo injected embryos versus the normal 276-bp product containing exon 2 seen in control-mo injected embryos (Figure 5B, C). The first potential initiation codon (for methionine) in the pitx3-mo transcript is located at position 28 of the pitx3 homeodomain and, as a result, the translation of this transcript will produce an abnormal protein lacking the N-terminal region and 45% of its homeodomain and, therefore, predicted to be nonfunctional.

An abnormal phenotype was detected in ~95% of pitx3-mo injected embryos; early lethality (before 20-hpf) was observed.
20% of pitx3-mo and control-mo injected embryos. The pitx3-mo displayed a misshapen smaller head, jaw abnormalities and reduction in eye size due to progressive lens degeneration and retinal defects leading to a complete loss of lens by 7-dpf consistent with the previous reports [9,12] (Figure 5D–G). Robust expression of pitx3 is seen in lens vesicle at 24-hpf and it continues to be highly expressed during all stages of lens development [9–13; Figure 5H and I]. In addition to the strong lens expression, pitx3 transcripts are also detected in the developing brain, craniofacial region and trunk musculature as previously described [9,12,13,41,42].

Since we demonstrated above that PITX3 is capable of binding human MIP/AQP0 promoter in vivo, further experiments were performed to establish if knockdown of pitx3 would affect formation of protein-mip1 promoter complexes in zebrafish. We injected zebrafish embryos with above described pitx3 morpholino oligonucleotides that result in abnormal splicing of pitx3 transcript. Embryos were harvested at 48-hpf and nuclear extracts from wild-type embryos and pitx3 morphants were tested for their ability to bind a biotinylated DNA fragment containing the proximal bicoid site and corresponding to zebrafish −44/−76 mip1 promoter region (Figure 6). The experiments were performed using nuclear protein extracts isolated from the upper trunk/head region of the 48-hpf wild-type and pitx3 morphant embryos that displayed normal body length and morphology (Figure 6A) and demonstrated a normal presence (wild-type) or a significant reduction (pitx3-mo) in normal pitx3 transcript based on RT-PCR analysis performed using RNA extracted from the lower trunk region of the same embryos (Figure 6B). For positive control, aliquots of nuclear extracts from wild-type and pitx3 morphants were analyzed on 10% polyacrylamide gel followed by Coomassie Blue R-250 staining to assure equal protein concentration in both samples (Figure 6C). Two apparent slow-migrating complexes were formed that were evident at the top of the gel when EMSA was performed with nuclear extracts obtained from wild-type embryos. The formation of these
complexes was abolished by introduction of a mutation into the bicoid site contained within the −44/−76 mip1 promoter, which suggests that pitx3 is directly involved in DNA-binding of this fragment (Figure 6D). In addition to this, the slow-migrating complexes were significantly diminished when nuclear extract obtained from pitx3 morphants were utilized (Figure 6D). Therefore the observed reduction in the intensity of the slow-migrating DNA-protein complexes correlates well with the residual amount of normal pitx3 transcript in morphants in this experiment. These data support our previous findings which demonstrate that pitx3 is a part of large complex occupying the mip1 promoter in the developing zebrafish embryo.
Knockdown of pitx3 affects mip1 expression in zebrafish embryos

Examination of mip1 expression by in situ hybridization identified a specific and robust expression pattern in 100% of control-mo injected embryos (15/15), while a complete absence (9/14 or 64.3%) or a very low level (5/14 or 35.7%) of mip1 expression was seen in pitx3-mo injected embryos at 29-hpf (Figure 7A–C, F, I). mip1 expression is clearly observed in both control and pitx3-mo injected embryos at later stages but appears to be somewhat reduced in pitx3 morphants (Figure 7 D, E, G, H, J, K).

Semi-quantitative RT-PCR analysis of mip1 expression in the pitx3 morphants and control-injected embryos confirmed a specific delay and decrease in mip1 expression in pitx3 morphants. The expression was initiated at ~22-hpf in control injected embryos consistent with the start of lens fiber cell differentiation. The expression increased at later stages with the highest levels being detected in 48-hpf embryos and decreased levels by 72-hpf. In the pitx3-mo embryos, the first expression was observed in 26-hpf embryos with expression levels being noticeably lower in comparison to the control-mo injected larvae. The mip1 expression in 28–34-hpf pitx3 morphants continued to be reduced in comparison to control-injected larvae but reached similar expression levels by 72-hpf (Figure 7L). Examination of beta-actin (loading control) demonstrated similar levels of expression between pitx3-mo and control-mo injected embryos (Figure 7L).

These experiments revealed a specific delay and decrease in expression of an important lens factor, mip1, in response to pitx3 deficiency.

Discussion

PITX3 is a homeodomain transcription factor that is essential to normal eye development in vertebrates. Yet, its direct downstream targets and mechanism of action are poorly understood. In this manuscript, we present identification of the first direct target of PITX3 during lens development, the major intrinsic protein of lens fibers, MIP/AQP0.

The MIP/AQP0 promoter was identified via scanning of the human genome for regions containing conserved clusters of bicoid sequences and located in the proximity of known genes. Since the sequence, expression and function of PITX3 are conserved in vertebrates and bicoid sites are known to mediate its interaction with DNA, the conserved presence of these elements in a gene’s promoter/regulatory region suggests that it may be regulated by PITX3. In addition to this, MIP/AQP0 represents a logical downstream target of PITX3 because of its known role in lens development/function.

MIP/AQP0 is one of the most abundant proteins found in lens fibers where it acts as a water channel and adhesion molecule [43–46]. Mouse Pitx3 and Mip/Aqp0 display overlapping expression patterns during eye development as both genes are expressed in the developing primary and secondary lens fibers, with continued expression in adult organisms [1,14,47,48]; expression of zebrafish pitx3 preceeds mip1 in the developing lens [9–13; this manuscript] consistent with its proposed role in activation of mip1 expression. Mutations in both PITX3 and MIP/AQP0 are implicated in congenital cataracts in humans [1–6,49] and result in lens phenotypes in mice [7,8,18,30–52]. Gene expression patterns as well as phenotypic abnormalities observed in mutant animals suggest an earlier appearance of Pitx3 in comparison to Mip/Aqp0, which would also be consistent with Mip/Aqp0 being a downstream target of Pitx3. Expression of the Mip/Aqp0 transcript and protein is first detectable at mouse embryonic stage E11.25 in the ventro-temporal half of the lens vesicle concurrent with the initial stages of primary fiber cell differentiation and continues to be restricted to the lens differentiating primary and secondary fiber cells throughout adulthood [47,48].

Transcriptional regulation of the Mip/Aqp0 expression pattern is not yet well understood with several potential players discussed in the literature. Previous studies have shown that the human MIP/AQP0 promoter is regulated by FGF-2 [57]. In this manuscript, we present evidence that the two evolutionarily conserved sequences containing bicoid sites within the MIP/AQP0 promoter are capable of binding specifically to PITX3. Moreover, through chromatin immunoprecipitation assays we demonstrated that PITX3 is bound to the MIP/AQP0 promoter in vivo in human lens epithelial cells. Analysis of the functional significance of this binding using luciferase reporter assays demonstrated that wild-type PITX3 is able to transactivate the MIP/AQP0 promoter while mutant PITX3 forms showed reduced or absent transactivation ability. Further functional analysis utilizing site-specific mutations revealed the importance of the proximal bicoid site for the observed transactivation. The obliteration of the proximal bicoid site resulted in a statistically significant reduction of MIP/AQP0 promoter activity as well as a decreased level of transactivation by PITX3. Finally, analyses performed in zebrafish embryos suggested that pitx3 is bound to the mip1 promoter sequences during embryonic development and that mip1 expression is altered in zebrafish pitx3 morphants. At later stages of development (48–72-hpf), the expression of mip1 in pitx3 morphants appears to be largely unaffected, suggesting that regulation of mip1 activity at these stages may be mainly controlled by other, pitx3 pathway-independent, factors; pitx3 may also contribute to the recovery of mip1 expression since increasing amounts of normal pitx3 transcript can be observed in zebrafish embryos starting at 48-hpf due to weakening of the effects of morpholino injections (Figure 5C). Identification/development of permanent pitx3 mutant lines is needed to allow more careful evaluation of the relationship between these factors.

Mutations in the human MIP/AQP0 gene were shown to underlie various dominant forms of cataracts [49,58–65]. To the best of our knowledge, only two of the reported MIP/AQP0 mutations were explored for functional defects and a dominant-negative mechanism was suggested [58]. The dominant nature of MIP/AQP0 mutations may also be explained by haploinsufficiency which would suggest that lens development is highly sensitive to dosage/timely expression of MIP/AQP0. The later possibility is further supported by the phenotype reported in the mouse.
Figure 7. Analysis of *mip1* expression in pitx3-mo and control embryos via *in situ* hybridization and RT-PCR. A, D, F-H. Normal *mip1* expression in control-injected embryos at 29-, 34- and 48-hpf. B, C, E, I–K. Altered *mip1* expression is observed in pitx3 morphants at 29-hpf with 64% of embryos demonstrating a complete absence of *mip1* expression (B) and the remaining larvae showing markedly reduced *mip1* expression (C and I). Reduced *mip1* expression is also observed in 34- and 48-hpf embryos (E, J, K). Red arrows show sites of expected *mip1* expression. Scale bars: A–E: 100 μM; F–L: 20 μM. L. Results of semi-quantitative RT-PCR showing reduced expression of *mip1* in pitx3 morphants at early stages of development (red arrow).
doi:10.1371/journal.pone.0021122.g007
carrying a null allele of Aqp0 [34]. Deletion of mouse Aqp0 was shown to result in cataracts at 3 weeks of age and at 24 weeks of age in homozygous and heterozygous mice, respectively. In heterozygous animals, the lens osmotic water permeability value was reduced to around 46% and the lens focusing power was significantly decreased in comparison to wild-type [52]. These findings demonstrated that a loss of one Aqp0 allele, which presumably leads to reduced Aqp0 expression, can be associated with lens abnormalities. Therefore, since MIP/AQP0 represents an apparent transcriptional target of PITX3, the alteration of the MIP/AQP0 expression in patients affected with PITX3 mutations is likely to contribute to the lens phenotype observed in these individuals.

Further studies of the MIP/AQP0 promoter will not only yield important insight into the transcriptional regulation of this critical lens differentiation factor but will also provide better understanding of the function of PITX3 and its interacting partners and allow for more specific identification of additional downstream targets of this ocular factor. Also, genetic screening of both PITX3 and MIP/AQP0 in human patients affected with ocular conditions may lead to an identification of synergistic or compensatory mutations/variants that may help to explain the considerable intra- and inter-familial phenotypic variability associated with mutations in either gene.

Acknowledgments

The authors would like to thank Rebecca C. Tyler and Gary Gardner for assistance with experiments involving DNA sequencing and zebrafish maintenance, and Linda M. Reis for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: EAS EVS. Performed the experiments: EAS SM NAM. Analyzed the data: EAS SM NAM EVS. Wrote the paper: EAS EVS.

References

1. Semina EV, Ferrell RE, Mintz-Hittner HA, Bisoum P, Abward WL, et al. (1998) A novel homeo gene PITX3 is mutated in families with autosomal-dominant cataracts and ASMD. Nat Genet 19(2): 167–170.
2. Berry V, Yang Z, Addison PK, Francis PJ, Iousida A, et al. (2004) Recurrent 17 bp duplication in PITX3 is primarily associated with posterior polar cataract (PPC1). J Med Genet 41(6): e109.
3. BidMost C, Matsumoto M, Chung D, Salem N, Zhang K, et al. (2006) Heterozygous and homozygous mutations in PITX3 in a large Lebanese family with posterior polar cataracts and neurodevelopmental abnormalities. Invest Ophthalmol Vis Sci 47(4): 1274–1280.
4. Finzi S, Li A, Mitchell TN, Farr A, Masonneau IH, et al. (2005) Posterior polar cataract: genetic analysis of a large family. Ophthalmic Genet 26(3): 125–130.
5. Burdon KP, McKay JD, Wirth MG, Russell-Eggit IM, Bhatti S, et al. (2006) The PITX3 gene in posterior polar congenital cataract in Australia. Mol Vis 12: 367–371.
6. Summers KM, Wuthers SJ, Gole GA, Piras S, Taylor PJ (2008) Anterior segment mesenchymal dysgenesis in a large Australian family is associated with the recurrent 17 bp duplication in PITX3. Mol Vis 14: 2010–2015.
7. Semina EV, Murray JC, Reiter R, Horoka RF, Grav J (2000) Deletion in the promoter region and altered expression of Pitx3 homoebox gene in aphakia mice. Hum Mol Genet 9(11): 1575–1585.
8. Rieger DK, Reichenberger E, McLean W, Sidrow A, Olsen BR (2001) A double-deletion mutation in the Pitx3 gene causes arrested lens development in aphakia mice. Genomics 72(1): 61–72.
9. Dutt S, Dietrich JE, Aspock G, Burdine RD, Schier A, et al. (2005) Pitx3 defines an equivalence domain for lens and anterior pituitary placode. Development 132: 1579–1590.
10. Khosrowshahian F, Wolanski M, Chang WY, Fujiki K, Jacobs L, et al. (2005) Lens and retina formation require expression of Pitx3 in Xenopus pre-lens ectoderm. Dev Dyn 234(3): 577–589.
11. Pommerenke D, Piefer T, Hollermann TX (2001) Pitx1, a member of the Rieger/Pitx gene family expressed during pituitary and lens formation in Xenopus laevis. Mech Dev 102(1–2): 255–257.
12. Shi X, Bosenkov DV, Zinkевич NS, Foley S, Hyde DR, et al. (2005) Zebrafish pitx3 is necessary for normal lens and retinal development. Mech Dev 122(4): 513–527.
13. Zilinski CA, Shah R, Lane ME, Janmargi M (2005) Modulation of zebrafish pitx3 expression in the preoridia of the pituitary, lens, olfactory epithelium and cranial ganglia by hedgehog and nodal signaling. Genesis 41(1): 33–40.
14. Semina EV, Reiter RS, Murray JC (1997) Isolation of a new homeobox gene belonging to the Pitx/Rieg family: expression during lens development and mapping to the aphakia region on mouse chromosome 19. Hum Mol Genet 6(12): 2109–2116.
15. Malinina NA, Konikovkh BV (1981) Action of mutant genes belonging to the Pitx/Rieg family: expression during lens development and mapping to the aphakia region on mouse chromosome 19. Hum Mol Genet 6(12): 2109–2116.
16. Webster EH, Jr, Zwaan J, Cooper PJ (1986) Abnormal accumulation of sulfated materials in lens tissue of mice with the aphakia mutation. Embryol Exp Morphol 92: 82–101.
17. Zwaan J (1975) Immunofluorescent studies on aphyklia, a mutation of a gene involved in the control of lens differentiation in the mouse embryo. Dev Biol 44(2): 306–312.
18. Ho HY, Chang KH, Nichol J, Li M (2009) Homoeodomain protein Pitx3 maintains the mitotic activity of lens epithelial cells. Mech Dev 126(1–2): 10–29.
19. Medina-Martinez O, Shah R, Janmargi M (2009) Pitx3 controls multiple aspects of lens development. Dev Dyn 238(9): 2193–2201.
39. Zinkevich NS, Bosenko DV, Liak BA, Semina EV (2006) laminin alpha 1 gene is essential for normal lens development in zebrafish. BMC Dev Biol 6: 13.

40. Vihtelic TS, Fadool JM, Gao J, Thornton KA, Hyde DR, et al. (2005) Expressed sequence tag analysis of zebrafish eye tissues for NEIBank. Mol Vis 11: 1093–1100.

41. Qiu HY, Guo C, Cheng XW, Huang Y, Xiong ZQ, Ding YQ (2008) Pitx3-CreER mice showing restricted Cre expression in developing ocular lens and skeletal muscles. Genesis 46(6): 524–529.

42. L' Honoré A, Coulon V, Marciel A, Lebel M, Lafrance-Vanasse J, et al. (2007) Sequential expression and redundancy of Pitx2 and Pitx3 genes during muscle development. Dev Biol 307(2): 421–433.

43. Chepelinsky AB (2009) Structural Function of MIP/Aquaporin 0 in the Eye Lens; Genetic Defects Lead to Congenital Inherited Cataracts. Handb Exp Pharmacol (190): 265–297.

44. Dunia I, Recouvreur M, Nicolas P, Kumar N, Bloemendal H, et al. (1998) Assembly of connexins and MP26 in lens fiber plasma membranes studied by SDS-fracture immunolabeling. J Cell Sci 111(15): 2109–2120.

45. Mulders SM, Preston GM, Deen PM, Guggino WB, van Os CH, et al. (1995) Water channel properties of major intrinsic protein of lens. J Biol Chem 270(15): 9010–9016.

46. Pisano MM, Chepelinsky AB (1991) Genomic cloning, complete nucleotide sequence, and structure of the human gene encoding the major intrinsic protein (MIP) of the lens. Genomics 11(4): 981–990.

47. Varadaraj K, Kumari SS, Mathias RT (2007) Functional expression of aquaporins in embryonic, postnatal, and adult mouse lenses. Dev Dyn 236(5): 1319–1328.

48. Zhou L, Chen T, Church RL (2002) Temporal expression of three mouse lens fiber cell membrane protein genes during early development. Mol Vis 8: 143–148.

49. Berry V, Francis P, Kaushal S, Moore A, Bhattacharya S (2000) Missense mutations in MIP underlie autosomal dominant 'polymorphic' and lamellar cataracts linked to 12q. Nat Genet 25(1): 15–17.

50. Sidjanin DJ, Parker-Wilson DM, Neuhauer-Klaus A, Pretsch W, Favor J, et al. (2001) A 76-bp deletion in the Mip gene causes autosomal dominant cataract in Hit mice. Genomics 74(3): 313–319.

51. Shiel A, Basnett S, Varadaraj K, Mathias R, Al-Ghoul K, et al. (2001) Optical dysfunction of the crystalline lens in aquaporin-0-deficient mice. Physiol Genomics 7(2): 179–186.

52. Wang XY, Ohtaka-Maryama C, Pisano MM, Jaworski CJ, Chepelinsky AB (1995) Isolation and characterization of the 5′-flanking sequence of the human ocular lens MIP gene. Gene 167(1–2): 321–325.

53. Ohtaka-Maryama C, Wang X, Ge H, Chepelinsky AB (1998) Overlapping Sp1 and AP2 binding sites in a promoter element of the lens-specific MIP gene. Nucleic Acids Res 26(2): 407–414.

54. West-Mays JA, Zhang J, Nottoli T, Hagepian-Donaldson S, Libby D, et al. (1999) AP-2alpha transcription factor is required for early morphogenesis of the lens vesicle. Dev Biol 209(1): 46–62.

55. West-Mays JA, Coyle BM, Piatigorsky J, Papagiotas S, Libby D (2002) Ectopic expression of AP-2alpha transcription factor in the lens disrupts fiber cell differentiation. Dev Biol 245(1): 13–27.

56. Golestaneh N, Fan J, Farzin RN, Le WK, Zelenka PS, et al. (2004) Lens major intrinsic protein (MIP/aquaporin 0 expression in rat lens epithelia explants requires fibroblast growth factor-induced ERK and JNK signaling. J Biol Chem 279(30): 31013–31022.

57. Francis P, Chang JJ, Yasui M, Berry V, Moore A, et al. (2000) Functional impairment of lens aquaporin in two families with dominantly inherited cataracts. Hum Mol Genet 9(15): 2329–2334.

58. Francis P, Berry V, Bhattacharya S, Moore A (2000) Congenital progressive polymorphic cataract caused by a mutation in the major intrinsic protein of the lens, MIP (AQP0). Br J Ophthalmol 84(12): 1376–1379.

59. Geyer DD, Spence MA, Johannes M, Flodman P, Clancy KP, et al. (2006) Novel single-base deletional mutation in major intrinsic protein (MIP) in autosomal dominant cataract. Am J Ophthalmol 141(4): 761–763.

60. Gu F, Zhai H, Li D, Zhao L, Li C, et al. (2007) A novel mutation in major intrinsic protein of the lens gene (MIP) underlies autosomal dominant cataract in a chinese family. Mol Vis 13: 1651–1656.

61. Jiang J, Jin C, Wang W, Tang X, Shenma X, et al. (2009) Identification of a novel splice-site mutation in MIP in a chinese congenital cataract family. Mol Vis 15: 38–44.

62. Lin H, Heitjanck CJ, Qi Y (2007) A substitution of arginine to lysine at the COOH-terminus of MIP caused a different binocular phenotype in a congenital cataract family. Mol Vis 13: 1822–1827.

63. Wang KJ, Li SS, Yun B, Ma WX, Jiang TG, et al. (2011) A novel mutation in MIP associated with congenital nuclear cataract in a chinese family. Mol Vis 17: 70–77.

64. Wang W, Jiang J, Zhu Y, Li J, Jin C, et al. (2010) A novel mutation in the major intrinsic protein (MIP) associated with autosomal dominant congenital cataracts in a Chinese family. Mol Vis 16: 534–539.