A novel missense mutation of Mip causes semi-dominant cataracts in the Nat mouse

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Abstract: Major intrinsic protein of lens fiber (MIP) is one of the proteins essential for maintaining lens transparency while also contributing to dominant cataracts in humans. The Nodai cataract (Nat) mice harbor a spontaneous mutation in Mip and develop early-onset nuclear cataracts. The Nat mutation is a c.631G>A mutation (MipNat), resulting in a glycine-to-arginine substitution (p.Gly211Arg) in the sixth transmembrane domain. The MipNat/Nat homozygotes exhibit congenital cataracts caused by the degeneration of lens fiber cells. MIP normally localizes to the lens fiber cell membranes. However, the MipNat/Nat mice were found to lack an organelle-free zone, and the MIP was mislocalized to the nuclear membrane and perinuclear region. Furthermore, the MipNat/+ mice exhibited milder cataracts than MipNat/Nat mice due to the slight degeneration of the lens fiber cells. Although there were no differences in the localization of MIP to the membranes of lens fiber cells in MipNat/+ mice compared to that in wild-type mice, the protein levels of MIP were significantly reduced in the eyes. These findings suggest that cataractogenesis in MipNat mutants are caused by defects in MIP expression. Overall, the MipNat mice offer a novel model to better understand the phenotypes and mechanisms for the development of cataracts in patients that carry missense mutations in MIP.

Key words: congenital cataract, MIP, missense mutation, mouse, semi-dominant cataract

Introduction

The major intrinsic protein of lens fiber (MIP), also known as Aquaporin 0 (AQP0), is a member of the aquaporin family which is composed of at least 12 related proteins [33]. The aquaporin family of proteins has six transmembrane α-helices, forms homo-tetramers in the cell membranes, and plays a role in water channel activity in the cell plasma membrane in multiple organs [4, 28]. MIP is known as the lens-specific aquaporin protein [4, 8, 21], and is one of the most abundant proteins in the lens, which constitutes up to 60% of the membrane proteins found in the lens. MIP also acts to maintain transparency in the lens by playing a role in water channel activity and cell-cell gap junctions [4, 20].

Mutations in human MIP are known to be responsible for human dominant cataracts, and many types of mutations in MIP have been reported [3, 4, 7, 8, 10, 13, 16, 19, 20, 25, 26, 30, 36, 37, 40–43]. Berry et al. first reported two missense mutations (p.Glu134Gly and p. Thr138Arg) in MIP that caused human congenital cataracts. These mutations were in the fourth transmembrane region of the MIP protein, each resulting in different pathologies [3, 8]. The MIPp.Thr138Arg mutation resulted
in a progressive, bilateral, punctate lens opacity that was limited to the mid- and peripheral lamellae [8]. The $\text{MIP}^{\text{Glu134Gly}}$ mutation resulted in a fine, non-progressive congenital lamellar and structural opacification [8]. In addition, Gu et al. reported that a $\text{MIP}^{\text{Arg33Cys}}$ mutation resulted in total cataracts, which is characterized by bilateral, complete opacification of the fetal nucleus and cortex [13]. Other reports have also shown that mutations in $\text{MIP}$ exhibited polymorphic phenotypes, such as punctate and nuclear cataracts [18, 35]. Therefore, different mutations in $\text{MIP}$ can lead to varying degrees of lens opacity [3], and missense mutations in $\text{MIP}$ account for over half of the mutations reported in patients with cataracts [17].

In mice, four mutant alleles of $\text{Mip}$ ($\text{Mip}^{\text{Cat-Fr}}$ [27], $\text{Mip}^{\text{Cat-Lop}}$ [27], $\text{Mip}^{\text{Hb}}$ [29], and $\text{Mip}^{\text{Cat-Tohm}}$ [23]) have been reported to develop semi-dominant cataracts. These mouse models are important resources in understanding the detailed pathology of human cataracts that are caused by mutations in $\text{MIP}$. The $\text{Mip}^{\text{Cat-Lop}}$ and $\text{Mip}^{\text{Cat-Fr}}$ alleles were the first reported mouse mutant alleles of $\text{Mip}$. The $\text{Mip}^{\text{Cat-Lop}}$ allele contains a missense mutation (c.151G>A) which results in an alanine-to-proline (p. Ala51Pro) substitution [27]. The $\text{Mip}^{\text{Cat-Fr}}$ allele results in a transposon-induced splicing error that substitutes a long terminal repeat (LTR) sequence for the carboxy-terminus of $\text{MIP}$ [27]. The $\text{Mip}^{\text{Hb}}$ and $\text{Mip}^{\text{Cat-Tohm}}$ alleles contain in-frame mutations that result in the deletion of 55 and 4 amino acids in $\text{MIP}$, respectively [23, 29]. The $\text{Mip}^{\text{Cat-Fr}}$ allele was predicted to be a loss-of-function allele, whereas the $\text{Mip}^{\text{Cat-Lop}}$, $\text{Mip}^{\text{Hb}}$ and $\text{Mip}^{\text{Cat-Tohm}}$, alleles result in dominant-negative effects [23, 27–29]. In addition to these spontaneous mutations, a $\text{Mip}$ null mutant ($\text{Mip}^{-/-}$) mice have also been produced [28]. Overall, MIP haploinsufficiency leads to dominant cataract formation.

Recently, we isolated the Nodai cataract (Nat) mouse, a spontaneous mutant exhibiting lens opacity, from our SJL/J mouse strain colony. Here, we report that a novel missense mutation in $\text{Mip}$ is responsible for congenital cataracts in Nat mice, resulting in abnormal MIP expression in the perinuclear region of the lens fiber cells. The profound lens opacity and lens fiber degeneration were confirmed in the lens of Nat/Nat homozygotes at an early age, indicating that the mutant phenotype for Nat homozygous mice is similar to that of previously reported $\text{Mip}$ mutant mice. In contrast, Nat/+ heterozygous mice had mild lens fiber degeneration without grossly diagnosable lens opacity. The lens phenotypes in Nat/+ mice were also notably milder than that of other Mip mutants. Thus, the Nat mice constitute a potential novel model for studying the pathological features of patients with cataracts caused by missense mutations in $\text{Mip}$.

### Materials and Methods

#### Ethics statement

All of the procedures involving animals met the guidelines described in the Proper Conduct of Animal Experiments, as defined by the Science Council of Japan, and were approved by the Animal Care and Use Committee on the Ethics of the Tokyo University of Agriculture (Approval number: 270048).

#### Mouse husbandry

We used wild-type SJL/J mice (Charles River Laboratories Japan, Yokohama, Japan) in all experiments. The Nat mutant was first identified in a litter in the SJL/J colony. The founder mutant mouse was crossed to a SJL/J mouse, and the F₂ offspring with severe lens opacity were isolated and maintained by sibling matings at the Tokyo University of Agriculture. For all of the phenotypic and expression analyses, we used Nat/+ mouse that were generated by mating wild-type and Nat mice on the SJL/J background. For the genetic analysis, the Nat/Nat mice were crossed to the BALB/cAJcl (BALB/cA) strain (CLEA Japan, Tokyo, Japan) to generate back-cross progeny.

#### Gross diagnosis of the lens phenotype

The pupillary dilatation was conducted using Mydri-P (Santen Pharmaceutical, Osaka, Japan), and both eyes were observed after 5 min. After euthanasia, the eyeballs were excised, and the presence or absence of lens opacity was diagnosed by observations under dark-field microscopy using the Leica M60 stereomicroscope (Leica Microsystems, Wetzlar, Germany).

#### Histological analysis and immunohistochemistry

The eyeballs were excised from the mice after being euthanized by cervical dislocation and were fixed by Superfix (Kurabo, Osaka, Japan), dehydrated in methanol, embedded in paraffin, and sectioned (5 µm) as previously described [34, 35, 38]. After removing the paraffin, the sections were stained with haematoxylin and
eosin, and then were observed using a Leica DM2500 light microscope (Leica Microsystems). The eyeball paraffin sections were also used for immunohistochemistry as previously described [34, 35, 38]. The primary antibodies for AQP0 (1:300, Alpha Diagnostic International, catalog #AQP01-S-A-P) and CTNNB1 (1:500, BD Biosciences, catalog #610154) used in this study were obtained commercially and had been characterized in previous studies [38]. The fluorescent images were obtained using a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems), Zeiss LSM780 confocal microscope (Carl Zeiss, Jena, Germany), and BZX700 fluorescence microscope (Keyence, Osaka, Japan).

**Linkage analysis and mutation analysis**

To identify the Nat locus by linkage analysis, we generated backcross progeny by mating between SJL/J-Nat/ Nat mice with (BALBc/A x SJL/J-Nat/Nat) F1 mice. Genomic DNA was extracted from the livers and was genotyped by multiple microsatellite markers located throughout the mouse genome. PCR were performed with the KAPA2G Fast PCR Kit (Kapa Biosystems, Woburn, MA, USA) according to the manufacturer’s protocol. The PCR products were separated on a 4% agarose (3% Agarose KANTO HC, Kanto Chemical, Tokyo, Japan and 1% Agarose S, Nippon gene, Tokyo, Japan) gel as previously described [38].

The Nat mutation in Mip was confirmed by DNA sequencing of the PCR products. Four coding exons of Mip were amplified in wild-type, Nat/+, and Nat/Nat mice by AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the primer sets as shown in Table S1. PCR products were purified using the QIAniQuick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and were sequenced using a 3730 × 1 DNA analyzer (Thermo Fisher Scientific).

For genotyping the Nat allele, we performed PCR restriction fragment length polymorphisms (PCR-RFLP) analysis by using genomic DNAs from the Nat mutants, SJL/J, C57BL/6J, C3H/HeN, DBA/2J, NOD/Shi, BALB/cA, and KOR/Stm mice. Exon 4 of Mip was amplified via PCR with the following primer pair: exon4RFLP_F and exon4RFLP_R (Table S1). The resulting 575-bp DNA fragment was digested by the restriction enzyme BssHII (New England Biolabs, Ipswich, MA, USA). The PCR products were then separated on a 4% agarose gel [38].

**Bioinformatics analysis**

Alignments of the MIP were performed using Clustal X [18]. The effect of the Nat mutation was assessed using by S-VAR (http://p4d-info.nig.ac.jp/s-var/), which is provided the automatic tools, SIFT [22], PolyPhen-2 [1] and PROVEAN [6], for predicting the possible impact of the amino acid substitutions.

An electron crystallographic structure of MIP (PBD: 2B60) [11] was utilized as a template to model the Nat mutation. The ribbon and B-spline diagrams were created using SWISS-MODEL (http://swissmodel.expasy.org/) and Waals software (Altif Labs, Inc., Tokyo, Japan).

**Quantitative RT-PCR**

Total RNAs from postnatal day 0 (P0) eyes were extracted by using the RNeasy mini kit (Qiagen), and approximately 2 µg of total RNA treated by DNase I (Takara Bio, Kusatsu, Japan) was used for cDNA synthesis with the Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). The quantitative RT-PCR was performed using QuantiTect Primer Assays (Qiagen) and the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Biological replicates of the RNA samples were obtained from three individuals. The signal values were normalized to the Gapdh signals, and the geometric means of the target signals were calculated in triplicate. The wild-type expression level was assigned an arbitrary value of 1. The results were presented as the mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA with Turkey’s HSD test using R (https://www.r-project.org/) (**P<0.01).

**Immunoblotting**

The insoluble protein fraction from P1 eyes of wild-type, Nat/+, and Nat/Nat mice were extracted as previously described [37]. Approximately 2.5 µg of protein was separated on a 10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (GE Healthcare Japan, Tokyo, Japan). The MIP protein bands were detected using the anti-AQP0 antibody (1:1,000), followed by a HRP-anti-rabbit IgG secondary antibody (1:20,000). ECL Prime Western blotting detection regents (GE Healthcare Japan) were used for the enhanced chemiluminescent detection of the specifically bound antibody. CTNNB1 was used as an internal control, and was detected using the CTNNB1 (1:500) antibody and HRP-anti-mouse IgG secondary antibody (1:20,000). Three biological replicates of MIP were analyzed using western
blot and quantified via ImageJ (http://rsb.info.nih.gov/ij). The wild-type expression level was assigned an arbitrary value of 1. The results were presented as the mean ± SD, and statistical analysis was performed by Welch’s t test (**P<0.01).

Results

Cataract phenotypes of Nat mutant mice

All Nat/Nat homozygous mice exhibited severe lens opacity within one month after birth. The normal lens transparency found in wild-type mice is shown in Fig. 1A and 1A’. In contrast, the lens opacities in Nat/Nat homozygous mice are shown in Fig. 1B and 1B’. A comparison of the extirpated eyes and lenses between the wild-type and Nat/Nat mice confirmed the severe lens opacity in Nat/Nat mice (Figs. 1A’, 1A’’, 1B’ and 1B’’). Moreover, a remarkable size reduction in the Nat/Nat lens was observed (Fig. 1B’’). Meanwhile, Nat/+ heterozygous mice did not exhibit lens opacity by gross diagnosis until at least 12 months after birth (Figs. 1C, 1C’, 1D and 1D’). However, a slight degeneration and lens opacity were detected at 3 weeks and 12 months of age in Nat/+ heterozygous mouse under dark-field microscopy (Figs. 1C”, 1C’”, 1D” and 1D’”).

To investigate the histological defects in the lens of Nat mutants, we analyzed paraffin-embedded sections of the eye in the wild-type, Nat/Nat, and Nat/+ mice. At P0, the normal development of the lens fibers was observed in wild-type mice (Fig. 2A). In contrast, the Nat/Nat mice already displayed mild degeneration and swollen cells in the lens fiber at P0 (Fig. 2B). Although the normally aligned lens fiber cells were observed in wild-type mice at P30 (Fig. 2D), in comparison, Nat/Nat mice...
had severe degeneration, large swollen cells, and small vacuoles (Fig. 2E). Furthermore, in Nat/+ mice, normal lens fibers were observed at P0 (Fig. 2C). By P30, we detected a degeneration of the lens in all of the tested Nat/+ mice (n=3), but the phenotypes were milder than that of the Nat/Nat mice (Fig. 2F).

Identification of the Nat mutation

To identify the causative mutation for cataractogenesis in Nat mice, we produced 56 (BALB × SJL/J-Nat/Nat) F1 × SJL/J-Nat/Nat backcross progeny and diagnosed the lens phenotypes at P30. The affected and unaffected individuals were segregated in a 1:1 ratio (26:30, P>0.5). We performed linkage mapping with 58 microsatellite markers throughout the mouse chromosomes 1–19 on 19 backcross progeny mice. The analysis revealed a linkage association with the region between the telomere and 118.2 Mb on chromosome 10 (Fig. 3A). Although this genomic interval contains more than 190 protein coding genes (Ensembl: GRCm38.p5), we hypothesized that there was a mutation in Mip in the Nat mutants because mutation in Mip have been shown to be responsible for cataracts in humans [3, 4, 7, 8, 10, 13, 16, 19, 20, 25, 26, 30, 36, 37, 40–43], mice [23, 27–29], and rat [38]. We analyzed all the exon sequences of Mip that were amplified from genomic DNA of wild-type, Nat/+ and Nat/Nat mice, and identified a 1-bp substitution at nucleotide position 631 in exon 4 of Mip(c.631G>A) in the Nat mutants (Fig. 3B). Moreover, PCR-RFLP analysis using BsiI showed a homozygous c.631G genotype of Mip in all of the tested inbred strains. An undigested fragment representing the mutant allele was only observed in mice carrying the Nat mutation. In addition, we genotyped the (BALB × SJL/J-Nat/Nat) F1 × SJL/J-Nat/Nat backcross progeny using by PCR-RFLP analysis to ensure that the phenotypes of the progeny correlated with their genotypes. The results indicate that unaffected and affected phenotypes of 56 backcross progeny completely correlated with their heterozygous and homozygous genotypes, respectively (Fig. 3A and data not shown). The c.631G>A mutation is a missense mutation that results in a glycine-to-arginine substitution at position 211 (p.Gly211Arg) in the sixth transmembrane (H6) domain of MIP (Figs. 3B and 3D). Fig. 3D also shows the site and types of mutations identified in humans, mice, and rat. The MIPp.Asp213Valfs*46 [10], MIPp.Gly215Asp [7], and MIPp.Tyr219* [30] mutant alleles have also been found in the H6 domain in humans, and the MIPp.Val203fs*60 (MipCat-Fr) allele was identified in mice [27]. These mutations are frameshift mutations except for the MIPp.Gly215Asp allele; therefore, the Nat allele is the first reported case of a missense mutation affecting the H6 domain of the MIP protein in mice.

The Gly211 residue is highly conserved across species from fish to mammals, as shown by an alignment of MIP orthologs (Fig. 4A). Moreover, the p.Gly211Arg mutation was predicted to damage the H6 domain of MIP as analyzed by the SIFT (score=0), PolyPhen2 (score=1.0), and PROVEAN (score=−6.89) algorithms. Therefore, we constructed a mutant model using a high-resolution crystal structure of MIP (PBD: 2B6O) [11] (Fig. 4B) to predict the structural effects of the p.Gly211Arg mutation in Nat mice. The p.Gly211Arg mutation converts a neutral residue to a positively charged residue (Fig. 4B), suggesting that the p.Gly211Arg mutation may affect neighboring conformations of the MIP structure. We simulated the conformational changes by structural modeling. The analysis indicated that there is a probability for the Arg211 residue to charge and crash into the Glu134 residue and H4 domain because of the space constraints (Fig 4C and 4D). These results strongly suggested that Nat mice develop cataracts because of the p.Gly211Arg mutation in Mip and thus is a novel mutant allele of Mip.
Fig. 3. The Nat mutation in Mip. A. Mapping of the Nat locus. The diagram shows the fine-mapping results for the genomic interval between the telomere and 118.2 Mb region on chromosome 10 that was linked to the normal and cataract phenotypes observed in [(SJL/J-Nat/Nat × BALB/cA) F₁ × SJL/J-Nat/Nat] backcrossed mice. NL: normal lens. B. Sequence analysis of the wild-type, Nat⁺, and Nat⁻/⁻ mice revealing a c.631G>A substitution (arrows) in Mip, resulting in a p.Gly211Arg substitution. C. The c.631G>A introduces a BstI site, facilitating genotyping of the mice via PCR-RFLP analysis. M: size standard (100-bp ladder). D. Schematic diagram of the MIP secondary structure showing the locations of the mutations in humans (green), mice (blue), and rat (black). The six transmembrane domains (H₁, H₂, H₃, H₄, H₅, and H₆), two hemichannels (HB and HE), and the extracellular (LA, LC, and LE) and intracellular (LB and LD) loops are indicated.

Effects of the MipNat allele on Mip transcript and protein expression

To estimate the effect of the c.631G>A mutation on Mip transcription, we relatively quantified Mip mRNA in the eyes of wild-type, MipNat⁺, heterozygous, and MipNat⁻/⁻ homozygous mice. Although there were no differences in the expression levels between the wild-type and MipNat⁺ mice, Mip transcript levels in MipNat⁻/⁻ mice were significantly decreased compared with those of wild-type and MipNat⁺ mice (Fig. 5A). Next, we performed western blot analysis to investigate the effects of the p.Gly211Arg mutation on MIP protein expression.
in the eyes of the Mip^Nat^ mice. In wild-type and Mip^-^ mice, approximately 26 kDa bands were detected (Fig. 5B). However, the signal was significantly decreased in Mip^-^ mice (Figs. 5B and 5C). Moreover, no signals were obtained in the Mip^-^ mice (Figs. 5B and 5C). Thus, we confirmed that the c.631A>G mutation results in a reduction of the Mip transcript in Mip^-^ mice, and that the p.Gly211Arg mutation affects the expression levels of the MIP protein in Mip^-^ and Mip^-^ mice.

**Effects of the Nat mutation on MIP expression and localization in the lens**

The vertebrate lens is divided into two parts: the lens epithelium and lens fiber (Fig. 6A, left panel). The lens epithelial cells form a single cell layer on the anterior segment of the lens proliferate, which then migrate to the equator region, and then differentiate into fiber cells (Fig. 6A, middle panel) [4]. Moreover, organelles such as the cell nucleus and mitochondria are digested, the cell body is elongated, and the organelle-free zone forms in the nucleus of the lens during the differentiation of the lens fiber cells (Fig. 6A, right panel) [4]. In normal lens fiber cells, MIPs are widely expressed in the plasma membrane of the lens fiber cells (Fig. 6A). To estimate the effects of the p.Gly211Arg mutation on the MIP protein, we investigated its expression profile in the lens via immunohistochemistry. MIP localized to the immature lens fiber cell membrane throughout the equator and the organelle-free zone in mice at P0 (Figs. 6B and 6C). Furthermore, MIP co-localized with CTNNB1, which has been reported to be
expressed in the lens fiber cell membrane, and also showed consistent localization with MIP [38]. However, we observed a different localization pattern for MIP in the lens of Mip\textsuperscript{Nat/Nat} homozygous mice from that of the wild-type mice. The MIP fluorescent signals were detected in the perinuclear region of the immature lens fiber cells of Mip\textsuperscript{Nat/Nat} mice (Fig. 6D). Interestingly, even though CTNNB1 localized normally, no signals for MIP were observed in the organelle-free zone of the Mip\textsuperscript{Nat/Nat} mice (Fig. 6E). The normal expression patterns of MIP were observed in the lens of Mip\textsuperscript{Nat/+} heterozygous mice. MIP was abundantly expressed without localization of the mutant MIP protein in the lens fiber cell membranes in both the equator region and the organelle-free zone of the immature lens (Figs. 6F and 6G). In the mature lens of P30 mice, we did not detect any changes in expression and localization at P0 in wild-type (Figs. 6H and 6I), Mip\textsuperscript{Nat/Nat} (Figs. 6J and 6K), and Mip\textsuperscript{Nat/+} (Figs. 6L and 6M) mice. Thus, our data clearly showed that the p.Gly211Arg mutation results in the mislocalization of MIP to the equator region and a loss of MIP in the organelle-free zone in Mip\textsuperscript{Nat/Nat} mice. However, we did not observe any differences in MIP expression between the wild-type and Mip\textsuperscript{Nat/+} mice.

**Discussion**

In this study, we identified a new missense mutation in Mip resulting in congenital semi-dominant cataracts. Mip\textsuperscript{Nat/Nat} mice exhibited severe lens opacity, whereas only a mild phenotype was observed in Mip\textsuperscript{Nat/+} mice (Figs. 1 and 2). A p.Gly211Arg mutation was detected in the H6 domain of the MIP protein in the Mip\textsuperscript{Nat} mice (Figs. 3B and 3D). The Gly211 residue is conserved among representative vertebrates (Fig. 4A) and was shown via several bioinformatics analyses to have a high probability to be deleterious if this residue was mutated (Figs. 4B–4D). Moreover, we predicted that the Arg211 residue causes a disruption in the protein structure by crashing into the Glu134 residue in the H4 domain, which may inhibit tetramer formation and/or traffic to the plasma membrane (Figs. 4C and 4D). Therefore, we strongly suggest that the p.Gly211Arg mutation in MIP is a causative mutation for the development of cataracts in Mip\textsuperscript{Nat} mutants.

In the lens of the Mip\textsuperscript{Nat/Nat} homozygous mice, we were unable to detect MIP via western blot analysis (Fig. 5B). In the Mip\textsuperscript{H6} mutant mice, which lack 55 amino acids in the MIP protein as a result of a 76-bp deletion, the mutant protein was less stable [29]. In humans, the Mip\textsuperscript{219*} mutant allele also showed a reduction in mutant proteins levels in in vitro experiments [30]. Although mRNA transcripts could be detected for these mutant
alleles, the translated products were drastically reduced. This feature probably indicates that these missense mutations result in the destabilization of the translated protein. The $Mip^{H6}$ and $MIP^{p-219}$ mutant alleles affect the transmembrane region of the $H4–H5$ and $H6$ domains, respectively (Fig. 3D) [29, 30], suggesting that
mutations in the transmembrane region results in the loss of MIP protein stability. Furthermore, since the Arg211 residue in MipNat mice is located in the transmembrane domain, we predicted that the p.Gly211Arg mutation also decreases the stability of MIP (Figs. 5B and 5C). Moreover, we showed that a substitution at residue 211, an evolutionarily conserved amino acid, in the H6 domain of MIP results in an abnormal expression pattern in the perinuclear region on the lens fiber cells (Figs. 6D and 6J). Previous studies have shown that MIP is mislocalized to the perinuclear region in the homozygous mutants of the MipNat, MipNat-Er, MipNat-H6 and MipNat-Toh alleles [23, 27, 29]. Studies in vitro also confirmed that several MIP mutations in humans result in an abnormal localization of MIP [7, 8, 26, 30]. In addition, Zhou et al. recently reported that the lens fiber degeneration in mutant MIP lens is caused by cell-death via the endoplasmic reticulum (ER) stress in MipNat-Lop/+ mice [44]. Therefore, we hypothesized that the abnormal perinuclear expression of MIP results in it being digested in the ER, and which may then lead to the reduction of mutant MIP protein, such as that observed in MipNat/Nat mice.

Furthermore, we showed here that the MipNat/+ mice also develop cataracts (Figs. 1C and 1D). In theory of formal genetics, the onsets of phenotypes in heterozygous animals can be explained by a gain-of-function effect, dominant negative effect, or haploinsufficiency [31]. The dominant effects of MIP/Mip mutations of humans and mice are also suggested the cataractgenesis based on the genetic theories. For example, several studies are indicated that abnormal mislocalization of MIP to the perinuclear region results in the development of dominant cataracts via interference of the wild-type protein by the mutant protein [8, 30, 44]. These findings suggest that mutant MIP can be cytotoxic, implying that these mutants develop cataracts through a gain-of-function effect. However, there were no differences between the expression pattern of MIP in the lens of the wild-type and MipNat/+ mice (Fig. 6). Therefore, we predict that it is unlikely for MipNat/+ mice to develop cataracts by a mutant MIP gain-of-function effect of the mutant MIP protein. In contrast, other previous reports have suggested that mutant MIP may also have dominant-negative effects. Francis et al. performed injections of cRNAs encoding human 134G and 138R mutant MIP proteins into X. laevis oocytes, resulting in a reduction in water permeability and the localization of both mutant proteins to the cytoplasmic space [8]. This was shown in an experiment where both wild-type and mutant MIP proteins were co-expressed in X. laevis oocytes [8]. Furthermore, Song et al. also reported the development of cataracts by a dominant-negative effect as a result of a MIPp.Tyr219* mutation in MIP, which was observed in a Chinese family [30]. The mutant protein derived from the MIPp.Tyr219* co-localizes with wild-type MIP in the cytoplasm, suggesting that the mutant MIP functions to inhibit trafficking of wild-type MIP to the plasma membrane [30]. This may also be the case in the development of cataracts in MipNat/+ mice because MIP localization in the lens of MipNat/+ mice is similar to that of the wild-type mice (Fig. 6). Furthermore, MIP haploinsufficiency is likely to be a more acceptable genetic mechanism to describe the development of cataracts in MipNat/+ mice because we observed massive reduction of MIP in eye-derived protein by western blot analysis in MipNat mutants (Figs. 5B and 5C). The cataract phenotypes are milder than those of the MipNat/Nat mice (Figs. 1 and 2), which appear to be similar to MipNat/+ mice [28].

Although we could not completely explain the molecular mechanism underlying the massive reduction of MIP in MipNat mutants, we may have been unable to detect the mutant MIP via western blotting due to an aggregation of the mutant MIP into the ER without it being trafficked to the plasma membrane, or its rapid degradation in ER. The massive reduction of mutant MIP due to the complete loss of expression was also observed in the organelle-free zone of MipNat/Nat mice lens (Figs. 6E and 6K) because aggregated proteins in the ER can be extracted from the insoluble fraction of the eye. Generally, the mutant proteins such as those that misfolded, are degraded by cellular proteases via protein quality control system [39]. Many diverse mutant proteins are unable to progress beyond the ER to their correct sites of action, such as the plasma membrane or the outside the cell following secretion [39]. For aquaporin-2, one of the members of the aquaporin family, several mutant versions of this protein mislocalized to the ER where it was degraded [32]. However, the speculations mentioned above have a fatal logical flaw because it is inconsistent with the observations of reduced MIP in MipNat/+ mice. The MIP protein levels in the eyes of MipNat/+ mice were less than half of that of wild-type mice as shown via western blot analysis (Figs. 5B and 5C). However, there were no differences in MIP expression levels between the wild-type and MipNat/+ mice as shown via immuno-
histrochemistry of lens (Fig. 6). In addition, we cannot explain the significant reduction of Mip transcript in MipNat/Nat mice (Fig. 5A). We suggest that the reduction of Mip transcript in MipNat/Nat mice was not dependent on the missense mutation because no significant differences of Mip expression between wild-type and MipNat/+ mice were observed. Thus, our results provided a new interesting phenomenon concerning changes in gene and protein expression; however, we cannot fully explain the molecular mechanism as yet, as demonstrated in this study.

Cataracts are a profound and common eye disease in human populations [2, 9, 24], and many gene mutations related to the development of cataracts have been identified [5, 12, 14, 15]. The mutations in MIP are some of the most frequently observed causes of cataracts in human. The MipNat allele is the 2nd case of semi-dominant cataracts caused by a missense mutation, and is the first report of a missense mutation affecting the H6 domain of MIP. Therefore, we suggest that the MipNat mice may be a useful model for studying the role of MIP in maintaining lens transparency, as well as the detailed pathology of human cataracts as a result of missense mutations in the H6-encoding region of MIP.

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