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Mouse Myocilin (*Myoc*) Gene Expression in Ocular Tissues[1]

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Human myocilin is identical to TIGR (trabecular meshwork inducible glucocorticoid response) which is responsible for the pathogenesis of juvenile-onset primary open angle glaucoma (GLC1A). We have isolated cDNA for mouse myocilin (*Myoc*) and investigated mouse myocilin gene expression in ocular tissues with *in situ* RNA hybridization. Hybridization signals were observed in the iris, ciliary body, trabecular meshwork, sclera, and retina in the mouse eye. The marked signals were seen in trabecular meshwork cells and the anterior portion of sclera. These findings suggest that myocilin mutation could affect the capacity of aqueous outflow and cause elevation of the intraocular pressure which is involved in the pathogenesis of glaucoma.

To understand and analyze inherited retinal disease, we have constructed a subtracted retina cDNA library and isolated several cDNA clones including "myocilin (MYOC)" (1). Human myocilin with homology to myosin and olfactomedin-related protein is localized preferentially in the ciliary rootlet and basal body of the connecting cilium of photoreceptors and skeletal muscle, and is thought to be a cytoskeletal protein. Recently, it was realized that human myocilin gene (MYOC) is identical to TIGR gene whose expression is induced by glucocorticoid treatment in cultured trabecular meshwork cells and is responsible for chromosome 1q-linked juvenile-onset primary open angle glaucoma (GLC1A) (2). Primary open angle glaucoma (POAG) is one of the major leading cause of blindness which comes from optic nerve atrophy. The elevated intraocular pressure is one of the risk factor, however, the molecular pathogenesis of the elevation of the intraocular pressure in POAG has not been understood in detail.

To further characterize myocilin, we have cloned cDNA for the mouse homolog of human myocilin and investigated mouse myocilin (*Myoc*) gene expression in murine ocular tissues with *in situ* hybridization. The study of mouse myocilin should contribute to clarify the role of human myocilin and the relation between the elevation of the intraocular pressure and myocilin gene mutation.

MATERIALS AND METHODS

Animals. Male and female adult BALB/cA Jcl and Jcl:ICR mice were used for this study. All animal procedures were performed in accordance with local ethics committee for animal experimentation.

Isolation of cDNA clone. A λgt10 BALB/c mouse skeletal muscle cDNA library (Clontech) was screened by plaque hybridization with ^{32}P-labeled 0.9-kb fragment BB8 (1), which was obtained by subtractive and differential cDNA cloning strategy and contains the coding region of human myocilin (DDBJ/GenBank/EMBL Accession No. D88214). Positive clones were selected and cDNA inserts were amplified by PCR using the oligonucleotide primers λgt10F1 (5′-GCT GGG TAG TCC CCA CCT TT-3′) and λgt10R1 (5′-CTT ATG AGT ATT TCT TCC AGG GTA-3′). The PCR products were purified using a Qiaquick Spin PCR purification kit (Qiagen).

DNA sequencing. The DNA sequences of PCR-amplified cDNAs were determined by the dideoxy chain termination method (3) using a 377 automated DNA sequencer (Applied Biosystems).

Rapid amplification of cDNA end (RACE). The 5′ end of mouse myocilin cDNA was amplified and sequenced using RACE procedure. Fifty picogram of mouse skeletal muscle Marathon-Ready cDNA

[1] The HUGO Nomenclature Committee approved symbol for the human myocilin gene is MYOC. Therefore, we designated mouse myocilin gene as *Myoc*. The DDBJ/EMBL/GenBank DNA Library Accession No. for mouse myocilin cDNA is AB013592.

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Abbreviations used: TIGR, trabecular meshwork-induced glucocorticoid response; POAG, primary open angle glaucoma; PCR, polymerase chain reaction.

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(Clontech) was used as template for PCR performed with gene-specific primers to the mouse myocilin corresponding to bases 112-131 (5′-GCA GGC CAG AAA CAG CAG AT-3′), adapter primers and AmpliTaq Gold DNA polymerase (Perkin Elmer). The PCR product was purified using a Qiaquick Spin PCR purification kit (Qiagen) and the DNA sequence was determined by the dideoxy chain termination method.

Sequence analysis. Nucleotide sequence information was analyzed by Geneworks 2.3.1 (Intelligenetics). Nucleotide and amino acid sequence similarity were searched against nucleotide and protein databases using the program BLAST and FASTA, respectively. The program MOTIF was used to search for amino acid sequence motif.

In situ RNA hybridization. For *in situ* RNA hybridization, the excised mouse eye was quickly embedded in OCT compound (Lab-Tek Product) and frozen in dry ice-ethanol. These tissue blocks were sliced into 5 μm sections by cryostat (Bright), and then these sections were air-dried on silan-coated slides. Before hybridization, sections were fixed in 4% paraformaldehyde in PBS and then pretreated with proteinase K (4). Transcripts were detected by *in situ* hybridization with digoxigenin (DIG)-labeled RNA probes. PCR amplified 2-kb mouse myocilin cDNA fragment was cut with *Eco*RI and subcloned into pBluescript II KS (-), designated pBSMyoc1. DIG-labeled antisense and sense RNA probes were generated by transcribing the linearized pBSMyoc1 DNA from T3 and T7 promoters respectively using DIG RNA labeling kit (Boehringer Mannheim). Subsequently, probes were hydrolyzed with alkaline to obtain RNA fragments with an average size of 300 nucleotides. *In situ* hybridization was carried out overnight at 50°C in a mixture containing 60% deionized formamide, 0.6 M NaCl, 1× Denhardt's solution, 10 mM Tris-HCl (pH 7.6), 1 mg/ml *E. coli* tRNA, 10% dextran sulfate, 2.5 mM EDTA, and 1∼2 μg/ml labeled probe. Following hybridization, tissue sections were washed twice in 2× SSC, 50% deionized formamide at 45°C for 30 min followed by washing with 10 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA for 10 min at room temperature. Signal was detected with anti-DIG antibody conjugated with alkaline phosphatase using the standard protocol (4). As a control, sections were hybridized with sense RNA probe.

RESULTS AND DISCUSSION

A λgt10 mouse skeletal muscle cDNA library (Clontech) was screened with a human myocilin cDNA clone BB8. Three clones, 1.0 kb, 1.4 kb, and 2.0 kb in size were isolated and sequenced. One clone, mMyoc1, contained a 2,004-bp cDNA sequence. This cDNA seemed to represent nearly full-length cDNA, because Northern blot analysis of mouse skeletal muscle RNA detected a single mRNA of 2.0 kb (data not shown). Furthermore, 5′ end of the clone mMyoc1 matched with the sequence of 5′-RACE product. Figures 1 through 3 show the nucleotide sequence of cDNA and the deduced amino acid sequence of mouse myocilin (Fig. 1), the schematic structure of mouse myocilin (Fig. 2), and the comparison of the amino acid sequence of the mouse myocilin with human myocilin (Fig. 3). We assigned the first ATG at nucleotide position 99 as an initiation codon based on the canonical sequence of mammalian translation initiation site described by Kozak (5). Alignment analysis showed that initiator methionine ATG of mouse myocilin is at 42 nt downstream of human initiator ATG, which also aligns the second ATG of human myocilin. However, CTG at nucleotide position 57 in mouse cDNA can also be the initiation codon (6, 7), which is at the same initiation site as human

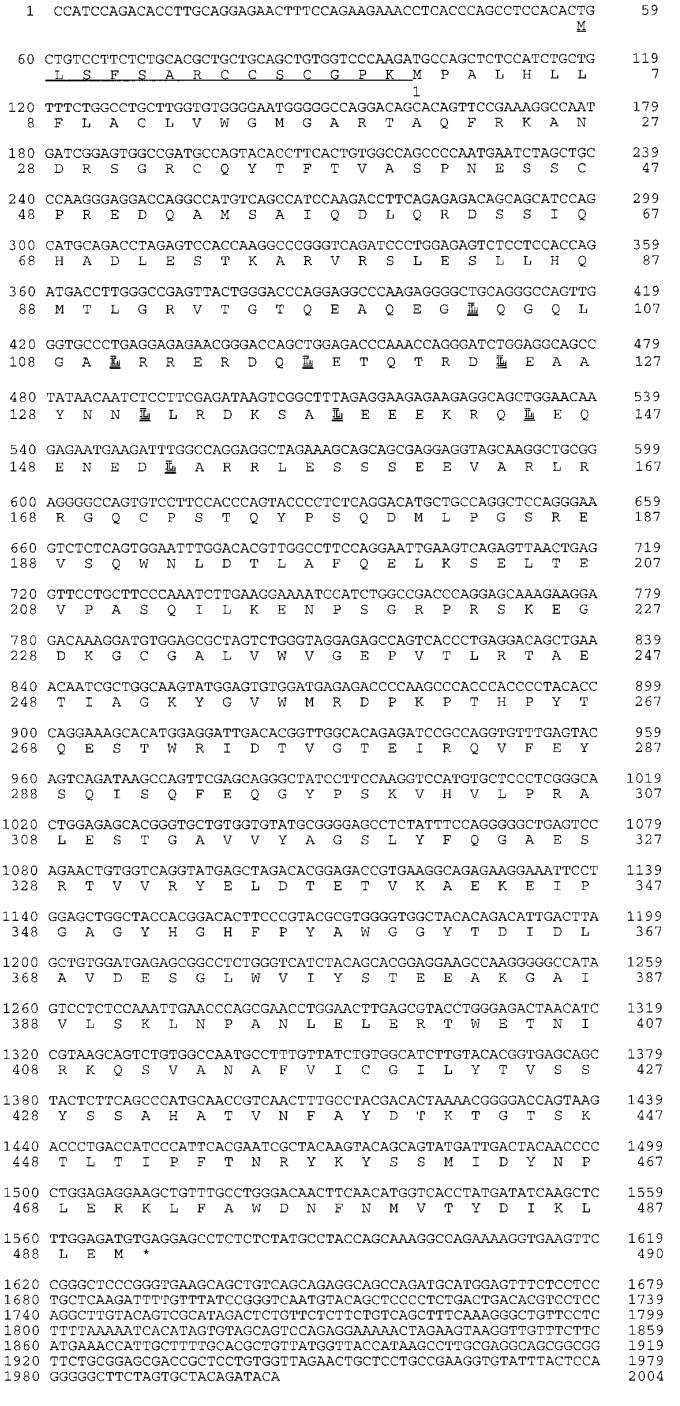

FIG. 1. Nucleotide sequence and deduced amino acid sequence of mouse myocilin. The coding region is defined by the positions of the initiation codon (ATG) and stop codon (TGA). CTG at nucleotide position 57 that corresponds to initiation ATG codon in human myocilin (Fig. 3) can also be an initiation codon and 14 predicted amino acids are also shown (underlined).

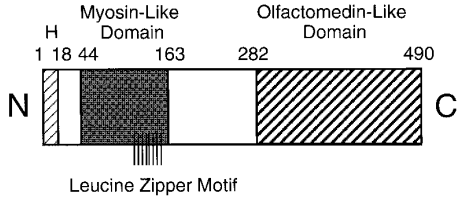

FIG. 2. Schematic diagram of mouse myocilin. This protein consists of an N-terminal hydrophobic sequence (H), a myosin-like domain, a leucine zipper, and an olfactomedin-like domain.

myocilin, and the amino acids coded between CTG and ATG are also homologous (Fig. 3). In this paper, we assigned the initiation site of mouse myocilin as ATG at nucleotide position 99 (Fig. 1).

The predicted open reading frame (ORF) of mouse myocilin encodes 490 amino acids with a molecular mass of 55 kDa. Mouse myocilin also contains a signal sequence at the N-terminus, and a leucine zipper motif ranging from 103 to 152 a.a. like human myocilin. The homology search indicated that mouse myocilin protein has 24% identity (31/125) and 45% similarity with myosin heavy chain of *Argopecten irradians* at from 44 to 163 a.a., and 43% identity (90/209 amino acids) and 63% similarity (132/209 amino acids) with olfactomedin related protein from 282 to 490 a.a. (Fig. 2). The overall homology of mouse myocilin to human myocilin was 81% identity and 90% similarity at the amino acid level. All the human myocilin amino acids at which amino acid alteration reported in POAG were the same as those in the mouse myocilin except for Gln368STOP mutation (Fig. 3).

Very recently, nucleotide sequence of mouse myocilin gene was reported by Fingert *et al.* (8) and Tomarev *et al.* (9). Our sequence perfectly matched with Tomarev's cDNA sequence in 2001-bp overlapping region. However, there are many differences between ours and that reported by Fingert *et al.* including base substitutions at positions 200 (G to A), 588 (G to A), 1409 (T to C), 1684 (C to A), and 1749 (C to T); a deletion of CTCT at 1581-1584; and a duplication of AGCT at 1713-1716. The substitution of G to A at position 588 causes amino acid change Ala164Thr. Tomarev *et al.* proposed that this polymorphism may contribute to the difference of the intraocular pressure among different strains of mice (9).

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                          1
mouse       MLSFSARCCSCGPKMPALHLLFLACLVWGMGARTAQFRKANDRSGRCQYTFTVASPNESS   46
            :  :.:::::: ::.:::..::.::::::..:::::.:::::.::::::::::::::::
human    1  MRFFCARCCSFGPEMPAVQLLLLACLVWDVGARTAQLRKANDQSGRCQYTFSVASPNESS   60

mouse   47  CPREDQAMSAIQDLQRDSSIQHADLESTKARVRSLESLLHQMTLGRVTGTQEAQEGLQGQ  106
            :: ..:::::.:..:::::::.:. :::.:::::.:::::::.::.... .::.:::::: .
human   61  CPEQSQAMSVIHNLQRDSSTQRLDLEATKARLSSLESLLHQLTLDQAARPQETQEGLQRE  120

mouse  107  LGALRRERDQLETQTRDLEAAYNNLLRDKSALEEEKRQLEQENEDLARRLESSSEEVARL  166
            ::.:::::::::::::::.::.::.::::::::::.:: :::::.:::::::::.:::::
human  121  LGTLRRERDQLETQTRELETAYSNLLRDKSVLEEEKKRLRQENENLARRLESSSQEVARL  180

mouse  167  RRGQCPSTQYPSQDMLPGSREVSQWNLDTLAFQELKSELTEVPASQILKENPSGRPRSKE  226
            :::::: :. ..... ::::::: :::::::::::::::::::::.::::.:::  :: :
human  181  RRGQCPQTRDTARAVPPGSREVSTWNLDTLAFQELKSELTEVPASRILKESPSGYLRSGE  240

mouse  227  GDKGCGALVWVGEPVTLRTAETIAGKYGVWMRDPKPTHPYTQESTWRIDTVGTEIRQVFE  286
            ::.:::.::::::::.::::::::::::::::.:::::.::::::::::::.:::::::
human  241  GDTGCGELVWVGEPLTLRTAETITGKYGVWMRDPKPTYPYTQETTWRIDTVGTDVRQVFE  300
                 *

mouse  287  YSQISQFEQGYPSKVHVLPRALESTGAVVYAGSFYFQGAESRTVVRYELDTETVKAEKEI  346
            :. :::: :::::::::.:::.::::::::::.::.::::::::::.:::::.::::::::
human  301  YDLISQFMQGYPSKVHILPRPLESTGAVVYSGSLYFQGAESRTVIRYELNTETVKAEKEI  360
                                                *                 *

mouse  347  PGAGYHGHFPYAWGGYTDIDLAVDESGLWVIYSTEEAKGAIVLSKLNPANLELERTWETN  406
            ::::::::.:::.:::::::::::::::.::::::::::::::::::::.:::::.:::::
human  361  PGAGYHGQFPYSWGGYTDIDLAVDEAGLWVIYSTDEAKGAIVLSKLNPENLELEQTWETN  420
            *  *  *# *      *                *

mouse  407  IRKQSVANAFVICGILYTVSSYSSAHATVNFAYDTKTGTSKTLTIPFTNRYKYSSMIDYN  466
            ::::::::::.:::.::::::::.::.::::::::: ::.::::::::: ::::::::::::
human  421  IRKQSVANAFIICGTLYTVSSYTSADATVNFAYDTGTGISKTLTIPFKNRYKYSSMIDYN  480
                   *     *                                          *  *

mouse  467  PLERKLFAWDNFNMVTYDIKLLEM  490
            ::: ::::::: ::::::::: ::
human  481  PLEKKLFAWDNLNMVTYDIKLSKM  504
                          *
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FIG. 3. Alignment of the predicted amino acid sequences of mouse and human myocilin. Dots indicate identical (:) and similar (.) amino acids. The alignment of the amino acids showed that all the human myocilin amino acids at which gene mutation with amino acid alteration reported in POAG (2, 12, 13, 14, 15, 16) were the same as those in the mouse myocilin (*) except for Gln368STOP mutation (#).

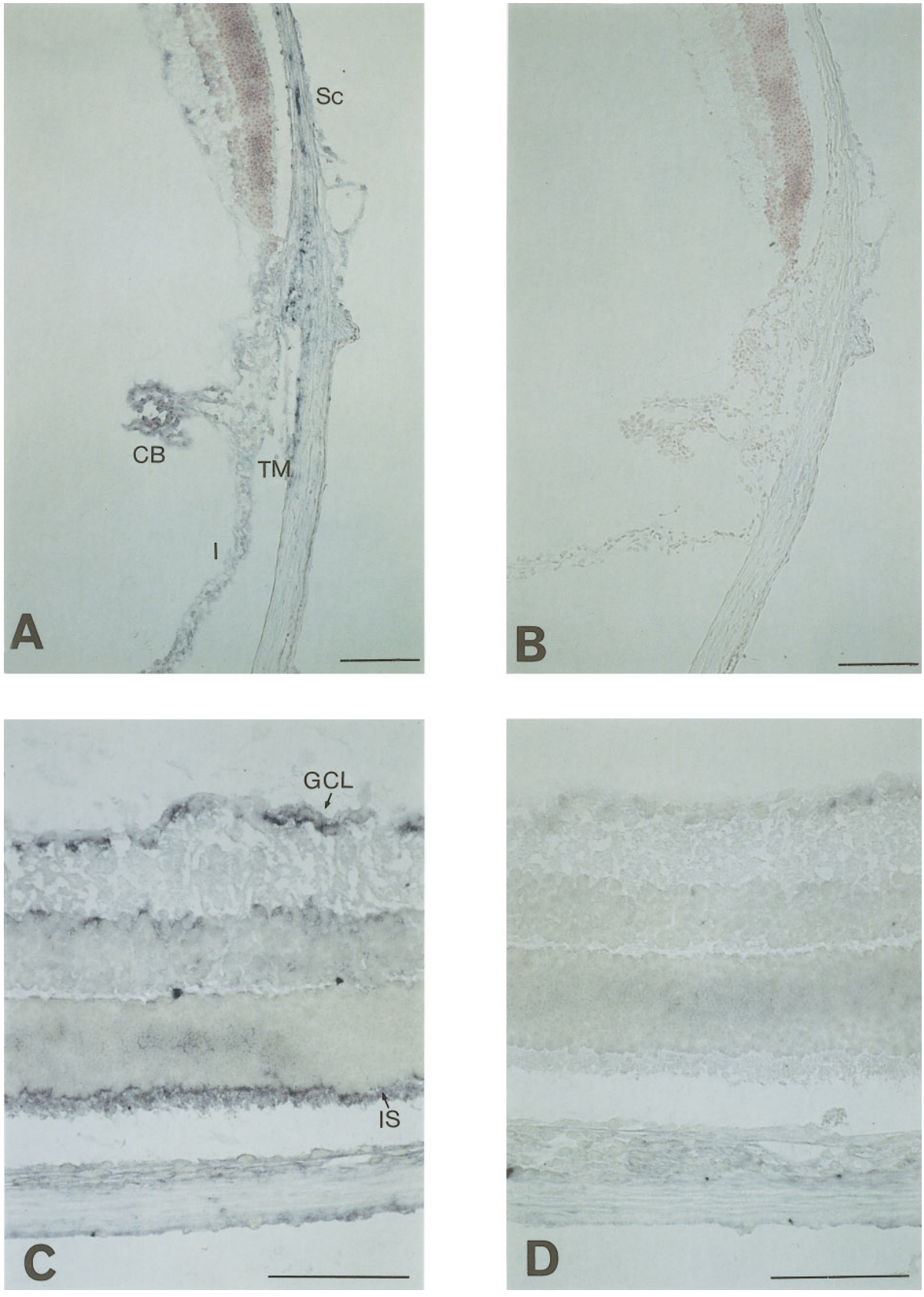

FIG. 4. *In situ* RNA hybridization analysis of mouse myocilin gene expression in the anterior portion (A, B) and retina (C, D) of mouse eye. Nuclei take a pink stain with safranin O in A and B. Hybridization signals were detected in trabecular meshwork (TM), ciliary body (CB), iris (I), sclera (Sc) in A, inner segment of photoreceptor cell (IS), and ganglion cell layer (GCL) in C. (B, D) No signal was detected with sense RNA probe.

In situ RNA hybridization analysis revealed that mRNA for myocilin was clearly expressed in various parts of the mouse eye including iris, ciliary body, trabecular meshwork, sclera, inner segment of photoreceptor, and ganglion cell layer of retina. No signal was detected with the control sense RNA probe (Fig. 4).

Human myocilin/TIGR cDNAs have been isolated from three ocular tissues including retina (1), cultured human trabecular meshwork cells (2, 10), and ciliary body (11), and gene expression has been reported in various kinds of human ocular tissues such as cultured trabecular meshwork cells (12), iris (11), ciliary body (11, 12), choroid (12), sclera (12), and retina (1) by Northern blot analysis. Our analysis using *in situ* hybridization revealed that mouse myocilin is expressed alike in the tissues of mouse and human.

It is now established that myocilin is a responsible gene for chromosome 1q-linked primary open angle glaucoma (GLC1A) (2, 12–16). Primary open angle glaucoma (POAG) is one of the major leading cause of blindness which comes from optic nerve atrophy. The elevated intraocular pressure is one of the risk factor, however, the molecular pathogenesis of the elevation of the intraocular pressure in POAG has not been understood. Polansky *et al.* proposed that myocilin/TIGR is one of the extracellular matrix proteins distributing in the trabecular meshwork and induced under the influence of glucocorticoid, oxidative stress, and so on (10, 17). Furthermore, they also indicated that myocilin in trabecular meshwork increases in the case of POAG in comparison to normal eye (10), affecting the capacity of aqueous outflow in the same manner as hyaluronan and other glycosaminoglycans (18, 19).

Two main pathways of the aqueous outflow are corneoscleral outflow and uveoscleral outflow. In this study, marked expression of mouse myocilin was observed in trabecular meshwork cell and the cells in anterior portion of sclera and uvea (ciliary body and iris) with *in situ* RNA hybridization. These cells were distributed along the two main pathways of the aqueous outflow in the mouse eye, and hence they may contribute to the architecture of two pathways. Myocilin mutation and its dysfunction may change the structure of trabecular meshwork, sclera, and uvea, also affecting the capacity of aqueous outflow in the mouse eye. It should be noted that hybridization signal in the ciliary body was detected preferentially in the ciliary epithelium. Ciliary epithelium is considered to produce aqueous humor and therefore there is a possibility that myocilin may contribute to the inflow of the aqueous humor.

Our previous study indicated that myocilin is a cytoskeletal protein localized in the photoreceptor and skeletal muscle (1) having homology with myosin of *Argopecten irradians*. It is interesting that myocilin is expressed in iris and ciliary body which are rich in smooth muscle. Myocilin may contribute to the contraction or dilation of iris, ciliary body, photoreceptor, or the phagocytotic activity of trabecular meshwork cell (20).

Analysis of the promoter region of human myocilin gene revealed the presence of a steroid response element in the myocilin gene (12, 21, 22). It is consistent with inducible nature of myocilin/TIGR by glucocorticoid treatment (10). Polansky *et al.* proposed the possibility that myocilin/TIGR is related to steroid glaucoma (10). Further studies using steroid treated mice will provide valuable information on their effects *in vivo*.

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