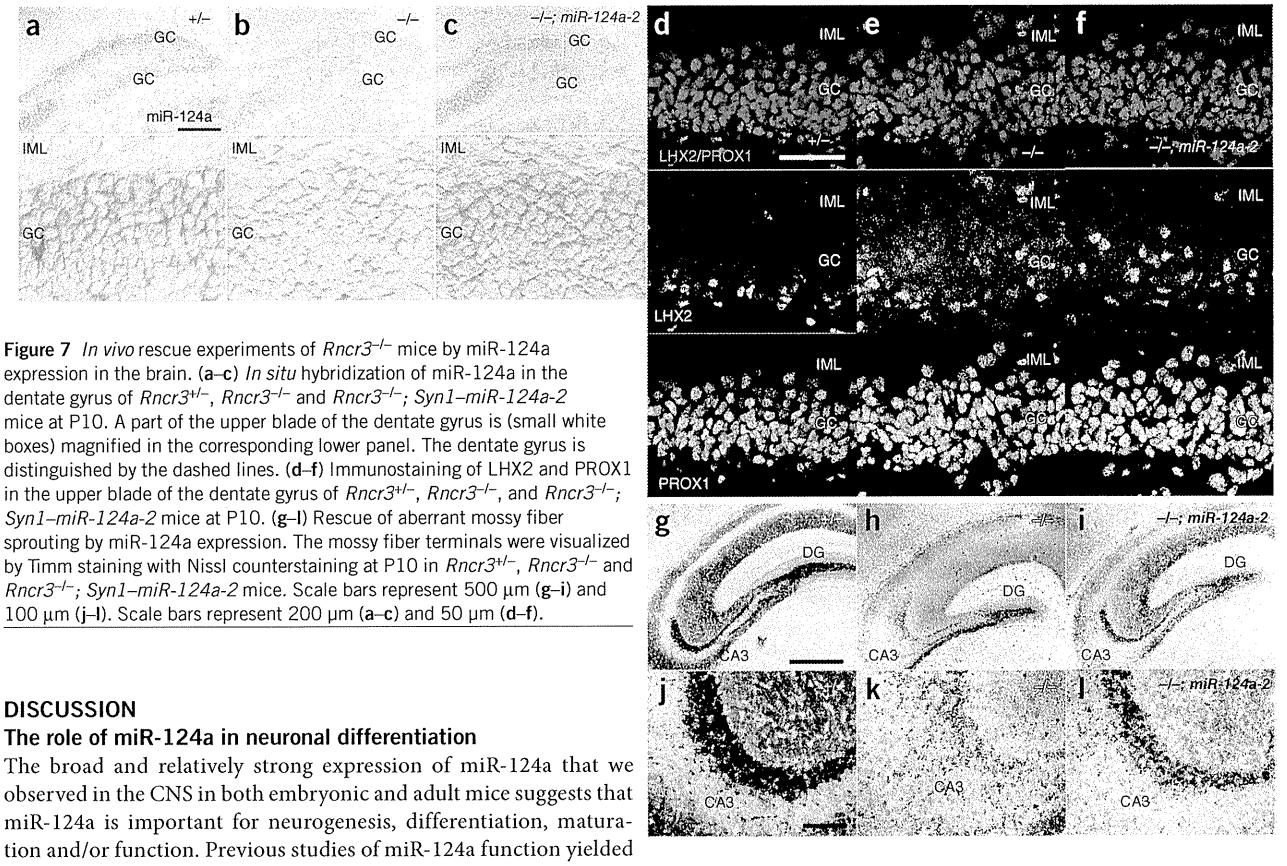

Figure 7 *In vivo* rescue experiments of *Rncr3*$^{-/-}$ mice by miR-124a expression in the brain. (**a–c**) *In situ* hybridization of miR-124a in the dentate gyrus of *Rncr3*$^{+/-}$, *Rncr3*$^{-/-}$ and *Rncr3*$^{-/-}$; *Syn1–miR-124a-2* mice at P10. A part of the upper blade of the dentate gyrus is (small white boxes) magnified in the corresponding lower panel. The dentate gyrus is distinguished by the dashed lines. (**d–f**) Immunostaining of LHX2 and PROX1 in the upper blade of the dentate gyrus of *Rncr3*$^{+/-}$, *Rncr3*$^{-/-}$, and *Rncr3*$^{-/-}$; *Syn1–miR-124a-2* mice at P10. (**g–l**) Rescue of aberrant mossy fiber sprouting by miR-124a expression. The mossy fiber terminals were visualized by Timm staining with Nissl counterstaining at P10 in *Rncr3*$^{+/-}$, *Rncr3*$^{-/-}$ and *Rncr3*$^{-/-}$; *Syn1–miR-124a-2* mice. Scale bars represent 500 μm (**g–i**) and 100 μm (**j–l**). Scale bars represent 200 μm (**a–c**) and 50 μm (**d–f**).

DISCUSSION

The role of miR-124a in neuronal differentiation

The broad and relatively strong expression of miR-124a that we observed in the CNS in both embryonic and adult mice suggests that miR-124a is important for neurogenesis, differentiation, maturation and/or function. Previous studies of miR-124a function yielded inconsistent results. A previous study found that miR-124a did not act as a determinant of neuronal generation through both knockdown and overexpression experiments of miR-124a in the developing chick neural tube[7]. In contrast, another study found that miR-124a is required for neuronal determination in the developing chick neural tube[4]. Studies using *Dicer* conditional knockout mice have shown that miRNA is not necessary for neuronal determination[8,9]. In *Xenopus*, a knockdown experiment by micro-injection of locked nucleic acid (LNA)–modified anti–miR-124a oligonucleotides into the eight-cell stage revealed no obvious effect[27]. We observed normal neurogenesis in the developing photoreceptor layer and the dentate gyrus in *Rncr3*$^{-/-}$ mice in which miR-124a expression is mostly eliminated. These results are consistent with previous reports that miR-124a is expressed in the Ki67-negative postmitotic cells and regulated by the REST complex[6,28], suggesting that miR-124a functions in neuronal maturation and maintenance, rather than neuronal determination (**Supplementary Fig. 12**). It will be important to examine the role of miR-124a in neuronal determination and maturation in the future,

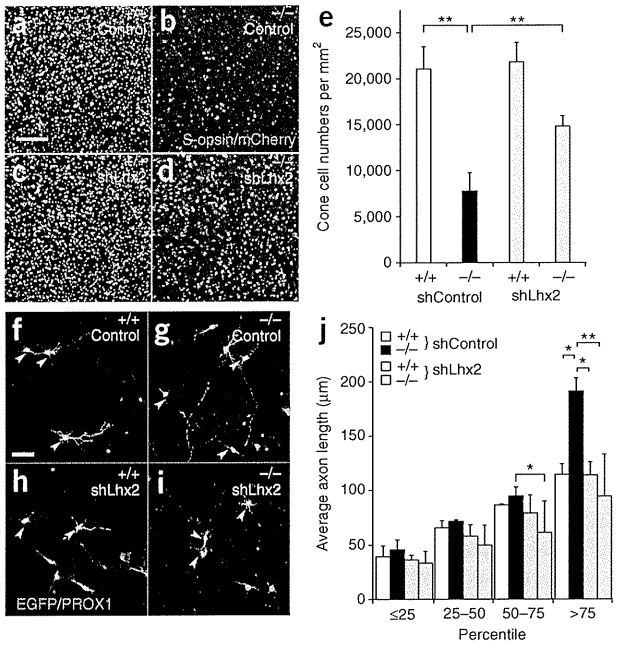

Figure 8 Rescue of *Rncr3*$^{-/-}$ mice by pre-miR-124a-2 expression. (**a–e**) Flat-mount immunostaining of S-opsin in the 5 DIV cultured retina. P0 wild-type (**a**,**c**) and *Rncr3*$^{-/-}$ retinas (**b**,**d**) were dissected and transfected with *U6* promoter–driven *shControl* (**a**,**b**) or *shLhx2* (**c**,**d**) with bicistronic expression of *CMV-mCherry* using AAV5 (**a–d**). Scale bar represents 50 μm. We counted the numbers of S-opsin–positive cone cell (**$P < 0.01$, **e**). Error bars represent s.d. from the means of $n = 4$. (**f–j**) Axon length of dentate granule cells after 70 h culture. P0 hippocampus of wild-type and *Rncr3*$^{-/-}$ mice were isolated and dissociated, then transfected with a *pBAsi-shControl* or *pBAsi-shLhx2-3* plasmid together with a *pCAG-egfp* plasmid. After immunostaining for PROX1 and EGFP, confocal fluorescence images were obtained. Scale bar represents 50 μm (**f–i**). The average axon length were calculated in 0–25th, 25–50th, 50–75th and 75–100th percentile groups (*$P < 0.05$ and **$P < 0.01$, **j**). Error bars represent s.d. from the means of $n = 3$. A total of 243 neurons were measured.

and analysis of pre-miR-124a-1 and pre-miR-124a-2 double knockout or miR-124a-1, miR-124a-2 and miR-124a-3 triple knockout mice will be necessary to draw a definitive conclusion.

Although the number of cone cells was significantly reduced in *Rncr3*$^{-/-}$ mice ($P < 0.001$), rod photoreceptor cells did not seem to be significantly affected ($P > 0.65$; **Fig. 3c,d** and **Supplementary Fig. 4a**). When we expressed a pre-miR-124a-2 transgene in the developing photoreceptors of *Rncr3*$^{-/-}$ mice, the number of cone cells was increased (**Fig. 6d–i**), suggesting that rod cells and other retinal neurons compensate for miR-124a loss with pre-miR-124a-2. Previous studies of the *Dicer* conditional knockout mouse in the retina suggested that photoreceptor differentiation at late developmental stages requires miRNAs[9] and that, of the retinal cell types, photoreceptors are the most sensitive to an imbalance of miRNAs[29]. Our results suggest that miR-124a is one of the main miRNAs functioning in photoreceptor cell maturation.

In vivo miR-124a target mRNAs

Several groups identified target mRNAs of miR-124a in the CNS using translation assay and *in vivo* knockdown analysis. It was recently suggested that CREB1 and *Actl6a* (*BAF53a*) are miR-124a target mRNAs in *Aplysia* and mouse, respectively[30,31]. *Sox9* was also reported to be a miR-124a target in SVZ adult neurogenesis[6]. *Ptbp1* and *Ctdsp1* were reported to be targets of miR-124a and are involved in neuronal gene regulation in cultured cells[4,5]. The pairing mechanism between miRNA and target mRNA is well established as a Watson-Crick pairing known as the miRNA seed match (especially nucleotides 2–7)[32–34]. Furthermore, it was recently found that guanidine:uridine wobble base-pairing between miRNA seed region and mRNA interferes with targeting activity[35]. Taking advantage of recently updated database information, we re-examined reported miR-124a targets using TargetScan 5.1 and found *Lhx2*, a miR-124a target mRNA candidate in both humans and mice, as well as *Ptbp1*, and *Ctdsp1*. Although *Lhx2* or *Neurod1* were predicted to be miR-124a target mRNAs by a miR-124a overexpression experiment in *Xenopus*[27,36], precursor miRNA overexpression may mistakenly produce a phenotype as a result of an effect on the miRNA synthesis mechanism or off-target effects. In fact, we did not detect a significant change in NEUROD1 protein and *Otx2* transcript expression ($P > 0.79$; **Fig. 2i–l** and **Supplementary Fig. 5g,k,n**), both of which were affected by pre-miR-124a overexpression in the *Xenopus* study[27,36]. Here, we found that *Lhx2* is a target of miR-124a in retinal cones and in the dentate gyrus in *Rncr3*$^{-/-}$ mice (**Fig. 5** and **Supplementary Fig. 8a–d**). Furthermore, LHX2 protein localization did not overlap with *Lhx2* mRNA distribution in regions in which *Rncr3* was expressed (**Supplementary Fig. 8f–o**). These observations suggest that LHX2 protein expression is affected by miR-124a by translational inhibition. We also performed both a luciferase assay and qPCR of luciferase mRNA (**Supplementary Fig. 8c,d**). Notably, although the luciferase activity of the construct containing a miR-124a target sequence in its 3′ UTR was decreased by miR-124a expression, the level of luciferase mRNA was unchanged. Our *Lhx2* overexpression assay revealed that cell death was increased in the developing retina, suggesting that inhibition of *Lhx2* translation by miR-124a is one of the essential mechanisms for down-regulating *Lhx2* in the normal development of retinal cells (**Supplementary Fig. 12**).

Lhx2 functions as a selector gene for forebrain and eye development[37]. *Lhx2*-expressing normal dorsal telencephalon cells show a marked tendency to form aggregation clusters[24], implying that LHX2 contributes to the regionalization of the cortical hem (LHX2 negative) and the non–cortical hem hippocampal region (LHX2 positive)[24]. In the *Rncr3*$^{-/-}$ brain, elevation of LHX2 protein levels may lead to a higher density of dentate neurons by promoting cell aggregation (**Fig. 5i,j,l**). Furthermore, we observed aberrant sprouting of dentate gyrus axons, mossy fibers, into the CA3 region in the *Rncr3*$^{-/-}$ brain (**Fig. 4e–h**), and found that the LHX2 level affects axonal outgrowth in primary cultured hippocampal dentate neurons (**Figs. 5m–u** and **8f–j**). It was recently shown that commissural neurons, which express LHX2 protein, extend their axons, but fail to cross the midline in *Lhx2* and *Lhx9* double knockout mice, suggesting that *Lhx2*-defective commissural neurons do not respond well to guidance cues[38]. In contrast, in the CA3 region of the *Rncr3*$^{-/-}$ hippocampus, we hypothesize that dentate gyrus granule cells may have an axon elongation capability and may over-respond to guidance cues, resulting in the mis-sprouting of granule cells to the CA3 region. Although elevated LHX2 may induce the aberrant axonal elongation phenotype, it should be noted that increased cell density and aberrant sprouting of dentate neurons in the hippocampus have been observed in seizure-induced rats[39]. Thus, understanding the precise mechanism that underlies these phenotypes awaits future study. Taken together, our findings suggest that LHX2 protein level regulation by miR-124a is critical for dentate gyrus maturation and survival (**Supplementary Fig. 12**).

Implication of miR-124a in human diseases

In humans, pre-miR-124a-1 is located on chromosome 8p23.1. Notably, chromosomal duplication, deletion or mutation of the 8p23.1 region have been reported to be involved in cerebral development and neuropsychiatric disorders, including autism, bipolar disorder, schizophrenia, learning difficulties, epilepsy and microcephaly[2,40,41]. In individuals with temporal lobe epilepsy and model animals, aberrantly sprouting mossy fibers are often observed[42]. However, the molecular mechanisms remain unknown. We found that a substantial reduction or loss of mature miR-124a (by 60–80%) or loss of miR-124a-1 resulted in a small brain, neuronal dysfunction and aberrant axonal sprouting in the hippocampus (**Fig. 4**), suggesting that dis-regulation of the miR-124a expression level is involved in developmental neuropsychiatric disorders and temporal lobe epilepsy in humans with chromosome 8p abnormalities.

A recent study of FMRP, a protein that is involved in a fragile X syndrome and a RISC component interacting with Argonaute, suggested that miR-124a interacts with the FMRP protein, which is present at synapses[43,44]. It was recently reported that miR-124a is bound and regulated by dFMR1, a FMRP protein in *Drosophila*[45]. Another study found that miR-124a is present in sensory-motor synapses and is involved in synaptic plasticity through CREB in *Aplysia*[30]. Thus, it was suggested that miR-124a is important for synaptic function. In contrast, it should be noted that another study using rat hippocampal neurons found that mature miR-124a is not enriched in synaptosomes[46]. Further analysis will be needed to fully understand the role of miR-124a in synaptic functions.

There are three pre-miR-124a loci (pre-miR-124a-1, pre-miR-124a-2 and pre-miR-124a-3) in the mouse and human genomes. Our findings strongly suggest that *Rncr3* is the primary source of miR-124a. Production and analysis of miR-124a double and/or triple knockout mice, if the mutations are not lethal, will further clarify *in vivo* miR-124a function and target mRNAs in other parts of the CNS.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

R.S. and T.F. designed the project. R.S., C.K., S.W., S.I. and T.F carried out the molecular and *in situ* hybridization experiments. R.S. and A.O. performed *in vivo* electroporation, virus infection and knockdown experiments in retinal and hippocampal neurons, and immunohistochemistry. S.U., T.K., M.K. and R.S. carried out the ERG experiments. R.S., Y.M. and T.F. produced the knockout and transgenic mice. R.S., R. Muramatsu and T.Y. carried out hippocampal tissue experiments. R.S., R. Matsui and D.W. produced lentivirus. R.S., Y.C. and Y.U. produced adeno-associated virus. R.S. and T.F. wrote the manuscript. T.F. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal care. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute. Mice were housed in a temperature-controlled room at 22 °C with a 12 h light/dark cycle. Fresh water and rodent diet were available at all times.

Generation of *Rncr3*$^{-/-}$ mice. We obtained *Rncr3* genomic clones from a screen of the 129/SvEv mouse genomic DNA library (Stratagene). We subcloned a 6.5-kb SalI-EcoRI fragment and a 4.4-kb SalI-SalI fragment from *Rncr3* genomic clones into a modified *pPNT* vector, and transfected the linearized targeting construct into the TC1 embryonic stem cell line. Genomic DNA from the liver was digested with BamHI or EcoRV, and hybridized with 5′ and 3′ probes, respectively.

Northern blot analysis. Northern blot analysis was performed as described previously[47]. An approximately 2.2-kb fragment (the BglII-BglII fragment) of mouse *Rncr3* cDNA (GenBank #BC096449) was used to synthesize the DNA probe.

PAGE northern for miR-124a. Total RNAs from mouse tissues were isolated by Trizol (Invitrogen). We denatured 20 μm of total RNAs in 5 mM EDTA containing formamide at 80 °C for 5 min, then separated them on 15% denaturing (7 M urea) polyacrylamide gels. RNAs were transferred to a nylon membrane (Pall Corporation Biodyne) at a constant current (3.3 mA cm^{-2}) for 35 min. The filter was baked for 1 h at 80 °C. LNA-modified anti–miR-124a (EXIQON, 20 pmol) was end-labeled with γ-^{32}P-ATP (3,000 Ci mmol^{-1}, Muromachi Yakuhin) using T4 polynucleotide kinase (Takara) and purified on spin columns (GE Healthcare Micro SpinTM G-25). The nylon filters were hybridized with the labeled probe in salmon sperm–containing hybridization solution (120 mM sodium phosphate (pH 7.2), 250 mM sodium chloride, 7% SDS (wt/vol) and 50% formamide (vol/vol) at 43 °C overnight, and washed twice with 0.1% SDS containing 2× SSC at 25 °C for 5 min. The filters were then exposed to X-ray film.

***In situ* hybridization.** *In situ* hybridization was performed as described previously[47]. Digoxigenin-labeled riboprobes were synthesized by T7, SP6, or T3 RNA polymerase using the *Rncr3* (same as the 3′ probe region in northern blots), *pri-miR-124a-2* (**Supplementary Table 1**), *Lhx2* (ref. 48, **Supplementary Table 1**), *Ngn2* (an EcoRI-*Xba*I fragment of cDNA AK143190), *Crx* or *Otx2* (ref. 19) cDNA as a template in the presence of 11-digoxigenin UTPs (Roche). For miR-124a detection, we used anti–miR-124a modified with a digoxigenin-labeled LNA probe (EXIQON).

Immunostaining. For immunohistochemistry, 14-μm retina and brain sections were washed twice in phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 (wt/vol) in PBS, then incubated with PBS containing 4% donkey serum (vol/vol) for 1 h to block samples. The samples were incubated with a primary antibody (**Supplementary Table 2**) at 4 °C overnight. After PBS-washing, these samples were incubated with secondary antibodies at 25 °C for 1 h.

For whole-mount immunostaining of the retina, each retina was gently peeled off from the sclera, rinsed in PBS and fixed with 4% paraformaldehyde (wt/vol) in PBS for 2 h. The retinas were permeabilized by incubation in 0.1% Triton X-100 in PBS for 30 min. After washing in PBS, samples were blocked with 4% donkey serum and 0.02% Triton X-100 in PBS for 3 h. The retinas were then immunostained with primary antibodies to M-opsin and S-opsin (**Supplementary Table 2**) at 4 °C overnight. Reactions with secondary antibodies were performed overnight at 4 °C.

Western blot analysis. Western blot analysis was performed as described previously[47]. The membrane was incubated with mouse antibody to Flag (1:5,000, Sigma) or goat antibody to LHX2 (3:500; Abcam). The membrane was then incubated with a horseradish peroxidase–conjugated donkey antibody to mouse IgG (1:10,000, Jackson) or rabbit antibody to goat IgG (1:10,000, Zymed). For secondary immunoreaction, the PVDF membrane was incubated with WB Stripping Solution (Nacalai Tesque) to remove antibodies, and blocked again with 5% skim milk (wt/vol) in TBS. Further immunoblots were performed using rat antibody to GFP (1:2,000, Nacalai Tesque) or mouse antibody to β-actin (ACTB, 1:5,000, Sigma). The signals were measured using ImageJ (US National Institutes of Health).

ERG. Electroretinographic recordings were performed as described in detail[47]. In brief, ERGs were picked-up with a gold-wire loop electrode placed on the cornea. The mice were placed in a Ganzfeld bowl and stimulated with four levels of stroboscopic stimuli ranging from −5.0 to 1.0 log cd s m^{-2} to elicit scotopic ERGs, and four levels of stimuli ranging from −0.5 to 1.0 log cd s m^{-2} for the photopic ERGs. The photopic ERGs were recorded on a rod-suppressing white background of 1.3 log cd m^{-2}.

Plasmid constructs. The *Lhx2* cDNA fragment was amplified by PCR (**Supplementary Table 1**) using PrimeStar (Takara), and cloned into the *pGEM-TEasy* vector (Promega). *Lhx2* 3′ UTR–containing fragments were also amplified and cloned into *pGEM-TEasy*. Mutations in the seed match region were introduced by PCR primers (**Supplementary Table 1**). The fragments of *Lhx2* cDNA and *Lhx2* 3′ UTR were ligated into the *pCAGGS* vector. To perform the luciferase assay, we constructed a miR-124a expression plasmid. Pre-miR-124-1, pre-miR-124-2 and pre-miR-124-3 were amplified by PCR (**Supplementary Table 1**) using ExTaq polymerase (Takara), and each PCR-amplified fragment was cloned into *pCRII* plasmids (Invitrogen). After verifying the sequence, we subcloned them into *pBAsi-mU6* (Takara). The *Lhx2* 3′ UTR were ligated into *pmirGLO* (Promega) to generate *pmirGLO–Lhx2-Nat* and *pmirGLO–Lhx2-Mut*. For *Lhx2* knockdown, *pBAsi-mU6* was used for DNA vector–based shRNA synthesis. Three target sequences, shLhx2-1, shLhx2-2 and shLhx2-3 (**Supplementary Table 1**), were selected from different positions in the mouse *Lhx2* open reading frame and subcloned into the *pBAsi-mU6* vector. The inhibition abilities of shLhx2-1, shLhx2-2 and shLhx2-3 were tested by western blot analysis using cultured cells (**Supplementary Fig. 11a,b**). The strongest inhibitor of Lhx2, shLhx2-3, was used.

Generation of miR-124a-2 transgenic mice. The *pCrx2k-Cre* plasmid[19] was digested with XhoI and BamHI to remove the *Cre* gene (*pCrx2k*). *RIP-miR-124a-2* (a gift from T. Maniatis, Harvard University)[5] is a pre-miR-124a-2 expression vector that encodes pre-miR-124a-2 in an intron of the *Ds-Red* gene. We digested *RIP-miR-124a-2* and ligated it into the *pCrx2k* plasmid (*pCrx2k-Ds-Red-124a-2*; **Supplementary Fig. 9a**). To construct a transgene vector of miR-124a-2 to rescue the hippocampal phenotype, we ligated the *pUC18-4.3Syn-CAT* plasmid (a gift from M. Kilimann, Ruhr-Universität Bochum)[26] containing a rat 4.3-kb *Syn1* promoter into Ds-red-124a-2 (*pSynI4.3k-Ds-Red-124a-2*; **Supplementary Fig. 10a**). The purified construct was injected into the pronuclei of fertilized one-cell eggs of B6C3F1 mice (Oriental Bio Service) followed by implantation into pseudopregnant foster mothers (ICR mice, Japan SLC).

Luciferase assay. We transfected 0.5 μg of the reporter plasmid DNA (*pmirGLO*, *pmirGLO–Lhx2-3′ UTR-Nat* or *pmirGLO–Lhx2-3′ UTR-Mut*) and 2 μg of miR-124a expression vector DNA (*pBAsi-mU6*, *pBAsi-mU6–pre-miR-124a-1*, *pBAsi-mU6–pre-miR-124a-2* or *pBAsi-mU6–pre-miR-124a-3*) per well into HEK 293T cells in a 6-well plate using the calcium phosphate method. After transfection, the cells were incubated for 48 h and lysed with Reporter Lysis Buffer (Promega). P0 hippocampal cells (approximately 2 × 10^5) were transfected with *pmirGLO–Lhx2-3′ UTR-Nat*, or *pBAsi-mU6–pre-miR-124a-1-Mut* (250 ng each) by electroporation (Amaxa Nucleofector). After transfection, the cells were incubated for 72 h, washed with PBS, and lysed with Reporter Lysis Buffer. The lysates were used for luciferase assays. Luciferase activity was measured with the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol using a Wallac 1420 Multilabel Counter (Wallac). Firefly luciferase activities were determined by three independent transfections and normalized by comparison with the Renilla luciferase activities of the internal control.

***In vivo* electroporation.** *In vivo* electroporation was performed on the P0 mouse retina as described previously[47]. The *pCAGGS*, *pCAGGS–Lhx2-Nat* or *pCAGGS–Lhx2-Mut* vectors were co-electroporated with the *pCAGGS-EGFP* vector. We used *pCAGGS* vector concentrations of 0, 2 and 3 μg μl^{-1}, *pCAGGS-Lhx2* (native or mutated) concentrations of 4, 2 and 1 μg μl^{-1}, and a *pCAGGS-EGFP* concentration of 1 μg μl^{-1} to make a 5 μg μl^{-1} DNA solution mix. The electroporated retinas were harvested at P4.

TUNEL assay. Fresh frozen retinas were sectioned to a thickness of 14 μm and fixed with 4% paraformaldehyde in PBS for 1 min. The TUNEL assay was performed according to the manufacturer's protocols.

RT-PCR and qPCR analysis. Total RNA was extracted Trizol reagent (Invitrogen), and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) with random hexamers. Quantitative PCR was performed using a SYBR GreenER qPCR SuperMix Universal (Invitrogen). Nucleotide sequences of primers are shown in **Supplementary Table 1**. To detect mature miR-124a, we isolated total RNA using the miRNeasy Mini Kit (Qiagen), and reverse transcribed using the miScript Reverse Transcription Kit (Qiagen). Real-time qRT-PCR was performed using the miScript SYBR Green PCR kit with miScript Universal primer and the miScript Primer assay (Qiagen). For the pri-miR-124a expression assay, total RNA isolated using the miRNeasy Mini Kit was reverse transcribed to cDNA using the TaqMan reverse transcription reagent kit and following the manufacturer's protocol (Applied Biosystems). Real-time qRT-PCR was performed using TaqMan Universal PCR Master Mix and specific TaqMan Pri-miRNA Assays for *Mus musculus* miR-124-1, miR-124-2 and miR-124-3 (Applied Biosystems).

Behavior test. *Rncr3*$^{-/-}$ and wild-type mice (4–5 months old) were suspended by their tails for 3 min, and clasping duration time was measured.

Timm staining and Nissl staining. Coronal sections, 14 μm thick, from P10 frozen mouse brains were stained by the neo-Timm's method[49]. After washing in de-ionized water, sections were counterstained by 0.1%(w/v) cresyl violet (wt/vol) for 5 min, washed in 100% ethanol, and incubated in xylene. Slides were coverslipped with Permount (Fisher Scientific).

Axon outgrowth assay. Neurons from P0 mouse hippocampus were dissociated using Nerve Cell Dissociation Medium (SUMILON) according to the manufacturer's protocol. The hippocampal cells (the approximate numbers are 4×10^5) were transfected with *pCAGGS*, *pCAGGS-Lhx2-Nat* or *pCAGGS-Lhx2-Mut* (250 ng each) together with the transfected cell marker plasmid, 125 ng of *pCAGGS-FGFP* by electroporation (Amaxa Nucleofector). Then, cells were cultured on 3.5-cm poly-D-lysine–coated dishes in Nerve-Cell Culture Medium (SUMILON). For Lhx2 knockdown experiments, hippocampal cells (the approximate numbers are 2×10^5) were transfected with *pBAsi-shControl*[50] or *pBAsi-shLhx2-3* (250 ng each), together with a transfected cell marker plasmid, *pCAGGS-FGFP*. At 72 h, the cells were fixed with 4% paraformaldehyde, 4% sucrose (wt/vol), and 0.02% Triton X-100 in PBS for 30 min, then washed with PBS. After blocking with 4% donkey serum in PBS, cells were incubated with primary antibodies to PROX1 and EGFP (**Supplementary Table 2**). Following PBS washes, Alexa 488–conjugated antibody to rat IgG and Cy3-conjugated antibody to rabbit IgG were used as secondary antibodies. Images of these cells were obtained using a confocal microscope LSM700 (Zeiss), and PROX1-positive axons (longest neurite) were measured using the LSM image browser (Zeiss).

Hippocampal slice culture. A P6 SD rat brain was dissected and sliced (300 μm thick). The hippocampal slices were placed onto membranes of Millicell-CM culture inserts (Millipore) and infected with *Camk2a* promoter–driven *Lhx2-IRES-mCherry* or *mock (control)-IRES-mCherry* expression lentivirus (approximately 10^{10} PFU ml^{-1} titer) into three loci of the dentate molecular layer using a micromanipulator. Nutrition medium was composed of 25% heat-inactivated horse serum, 25% Hank's balanced salt solution, and 50% MEM. The medium was changed every 2 d. After 5 DIV, the cultured slice was fixed with 4% paraformaldehyde, 4% sucrose and 0.02% Triton X-100 in PBS for 3 h, then permeabilized with PBS, and 0.1% Triton X-100 for 30 min. The sliced section was further immunostained.

Retinal explant culture and AAV infection. The P0 eyes were enucleated, and the choroid, sclera and cornea were removed. The eyecups (retinas with lens and vitreous) were incubated with fresh DMEM/F12 media containing AAV5 (approximately 1×10^{12} particles per ml titer), which bicistronically expresses *shControl* or *shLhx2* driven by the *U6* promoter and *mCherry* driven by the *CMV* promoter (*U6-shControl-CMV-mCherry* or *U6-shLhx2- CMV-mCherry*) for 1 h at 25 °C. The lens and vitreous were removed, and the retinas were explanted with the photoreceptor side up. After a 30-min incubation, 5 μl of AAV5 vector (approximately 1×10^{12} particles) was dropped to cover the surface of the explants. The explants cultured for 5 d were immunostained with an antibody to S-opsin.

Statistical analysis. Statistical comparison of datasets were performed with Student's *t*-test. For multiple comparison, we performed one-way ANOVA with Tukey-Kramer test or Kruskal-Wallis non-parametric ANOVA with Steel-Dwass multiple comparison test. Complete statistical information is described in the **Supplementary Statistical Analysis**.

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REBOUND OF MACULAR EDEMA AFTER INTRAVITREAL BEVACIZUMAB THERAPY IN EYES WITH MACULAR EDEMA SECONDARY TO BRANCH RETINAL VEIN OCCLUSION

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Purpose: To determine the incidence of rebound macular edema after intravitreal bevacizumab in eyes with macular edema secondary to branch retinal vein occlusion and to identify the pretreatment factors that were significantly associated with the rebound.

Methods: The changes in the foveal thickness after the intravitreal bevacizumab (1.25 mg/0.05 mL) were studied in 65 eyes of 65 patients with macular edema secondary to branch retinal vein occlusion. A rebound of macular edema was defined as a ≥110% increase in the foveal thickness or a foveal thickness ratio of ≥110% (foveal thickness at the recurrence/foveal thickness at the baseline × 100). Multivariate logistic regression analyses and subgroup analyses were performed to determine which pretreatment factors were associated with the rebound.

Results: Seven of 65 eyes (10.8%) showed a rebound (≥110% of baseline thickness). Subgroup analyses showed that a thinner pretreatment fovea and a shorter interval between symptom onset to the initiation of the intravitreal bevacizumab were significantly associated with a rebound of macular edema ($P < 0.01$). The interval from symptoms onset to the initiation of treatment was <8 weeks in all 7 eyes with a rebound macular edema.

Conclusion: These results suggest that a rebound of macular edema in eyes with branch retinal vein occlusion was more likely to occur when the intravitreal bevacizumab therapy is initiated before the macular edema reaches the maximum level. Rebound of macular edema may be effectively avoided by waiting at least 8 weeks after the onset of symptoms to begin the intravitreal bevacizumab.

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Macular edema is one of the most common complications and major cause of visual decrease in eyes with branch retinal vein occlusion (BRVO).[1–3] The Branch Vein Occlusion Study Group reported on the long-term visual prognosis of 35 untreated patients with macular edema after BRVO and a decrease of visual acuity to ≤20/40. They found that two thirds of these eyes had a visual acuity <20/40 after 3 years.[4] Although macular grid laser photocoagulation[4–6] is still the gold standard treatment for macular edema secondary to BRVO, other treatment methods have been advocated including intravitreal injections of steroids[7–10] and vitrectomy with or without sheathotomy.[11–14]

Recently, an intravitreal injection of bevacizumab (Avastin; Genentech, Inc, South San Francisco, CA), a full-length recombinant monoclonal antibody against human vascular endothelial growth factor (VEGF), has been used to treat macular edema secondary to BRVO.[15–18] This therapy is widely accepted because it is known that the VEGF plays an important role in the pathogenesis of macular edema.[19–22] Long-term follow-up studies[23–26] also suggested that intravitreal

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bevacizumab (IVB) therapy is an effective treatment for macular edema secondary to BRVO.

In 2007, Matsumoto et al[27] reported on 3 patients with macular edema secondary to retinal vein occlusion whose edema initially responded to bevacizumab, but then showed a rebound of the macular edema. In these three patients, the degree of macular edema was greater than that before the initial bevacizumab administration. However, very little is known about the exact incidence of this rebound phenomenon and which pretreatment factors are related to this unique phenomenon.

Thus, the purpose of this study was to determine the incidence of rebound macular edema in eyes that received an IVB for macular edema secondary to BRVO. We also wanted to identify the pretreatment factors that were significantly associated with the rebound.

Subjects and Methods

Subjects

We reviewed the medical records of all patients with macular edema secondary to BRVO who had received IVB therapy at the Nagoya University Hospital from July of 2006 to April of 2009 and were followed-up for more than 6 months. Eyes that had received other treatments, for example, vitrectomy, grid laser photocoagulation, or drug injections including triamcinolone acetonide, were excluded.

The procedures used conformed to the tenets of the World Medical Association's Declaration of Helsinki. An informed consent for the IVB therapy was obtained from each of the patients before the IVB, and afterward, they were provided sufficient information on the procedures to be used. The Nagoya University Hospital Ethics Review Board approved (#09-28) this retrospective analysis of the patients' data.

Bevacizumab Injection

The eyes were anesthetized with 1% topical tetracaine, and the fornices of the eyes were irrigated with 10% povidone–iodine. Each patient received an intravitreal injection of 1.25 mg/0.05 mL bevacizumab using a 30-gauge needle inserted 3.5 mm from the limbus. Antibiotics drops were given for 3 days after the IVB.

All patients received a single intravitreal injection of bevacizumab, and the effects were evaluated monthly by the best-corrected visual acuity (BCVA) and the foveal thickness determined by optical coherence tomography (OCT).

Best-Corrected Visual Acuity

The BCVA was measured by a standard Japanese decimal visual acuity chart at 5 m. The decimal values were converted to the logarithm of the minimum angle of resolution units for statistical analyses.

Foveal Thickness

The foveal thickness was determined by OCT (Stratus or Cirrus model; Carl Zeiss Meditec, Dublin, CA). The same OCT machine was used on the same patient. After the patients' pupils were fully dilated with 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen Co, Osaka, Japan), 6 mm vertical and horizontal scans were made through the fovea. The average foveal thickness of the vertical and horizontal scans was used as the foveal thickness. We used a manual method to place the cursors on the OCT images to measure the foveal thickness[28,29] because it has been reported that the automatic measurements of the foveal thickness often failed to identify the outer border of the neural retina, especially when the Stratus model of OCT was used.[30] We have also found that our manual method is more useful when two different OCT systems were used in the same study.[31]

Definition of Effectiveness, Recurrence, and Rebound of Macular Edema

A treatment was defined as effective when the OCT-determined foveal thickness had decreased by >30% after the initial bevacizumab injection. A recurrence of macular edema was defined as an increase of foveal thickness of >30% after an initial decrease of foveal thickness. A rebound of macular edema was defined as when the recurrence foveal thickness ratio (foveal thickness at the recurrence/foveal thickness at the baseline × 100) became ≥110% after an initial decrease of foveal thickness (Figure 1).

According to these definitions, we classified our 65 eyes into 4 groups (Figure 1). Group 1 included 3 eyes in which the initial bevacizumab injection was not effective. Group 2 included 21 eyes in which the initial bevacizumab injection was effective without any recurrence. Group 3 included 34 eyes in which the initial bevacizumab injection was effective, then a recurrence occurred with the recurrence ratio <110%. And Group 4 included 7 eyes in which the initial bevacizumab injection was effective, then a recurrence occurred with the recurrence ratio ≥110%, that is, a rebound.

Additional injections of bevacizumab were given only when a recurrence of macular edema or a worsening of the BCVA by ≥0.2 logarithm of the minimum angle of resolution units developed or the results of the initial injection did not reach the level considered to be effective.

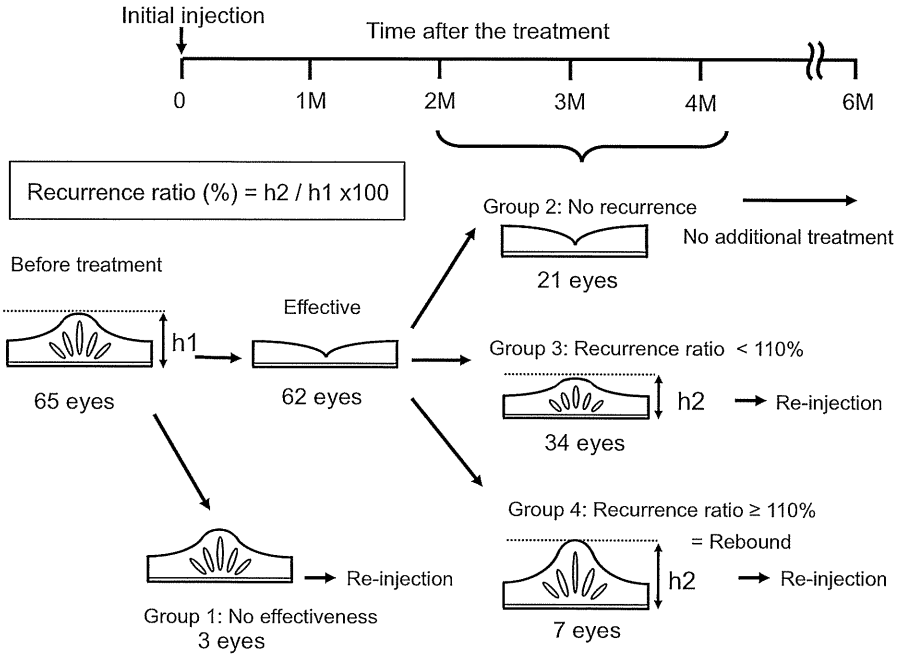

Fig. 1. Classification of macular edema according to the course of macular morphology obtained by OCT after IVB. Group 1 included the eyes in which the initial bevacizumab injection was not effective. Group 2 included the eyes in which the initial bevacizumab injection was effective without any recurrence thereafter. Group 3 included the eyes in which the initial bevacizumab injection was effective, and then a recurrence occurred with the recurrence ratio <110%. Group 4 included the eyes in which the initial bevacizumab injection was effective, and then a recurrence occurred with the recurrence ratio ≥110%, that is, a rebound.

Statistical Analyses

To identify the pretreatment factors that might influence the rebound of macular edema after the initial IVB, multivariate logistic regression analyses were performed with rebound macular edema as the dependent variable. The independent variables included patient's age, gender, presence of systemic complications, for example, hypertension, hypercholesterolemia, diabetes mellitus, pretreatment logarithm of the minimum angle of resolution visual acuity, pretreatment foveal thickness, and period from symptoms onset to the IVB.

We also performed subgroup analyses comparing the pretreatment factors presented above between Group 3 and Group 4. Differences in the patient's age, pretreatment logarithm of the minimum angle of resolution visual acuity, pretreatment foveal thickness, and period from symptoms onset to the IVB were compared using the nonparametric Mann–Whitney *U*-test. The significance of differences in gender and presence of hypertension, hypercholesterolemia, and diabetes mellitus were determined by chi-square tests.

The SPSS version 17.0J for Windows (SPSS, Inc, Chicago, IL) was used for all these statistical analyses. A P value <0.05 was considered significant.

Results

Intravitreal bevacizumab therapy was performed on 65 eyes of 65 consecutive patients (35 men and 30 women) whose mean ± SD age was 62.3 ± 8.6 years (range, 39–85 years). Forty-four of these patients had systemic hypertension, seven patients had diabetes mellitus without diabetic retinopathy, and six patients had hypercholesterolemia. The decimal BCVA at baseline ranged from 0.01 to 0.6, and the mean BCVA was 0.64 ± 0.33 logarithm of the minimum angle of resolution units. The mean foveal thickness was 585 ± 177 μm with a range from 244 μm to 1106 μm. The mean interval between the onset of symptoms and the IVB was 10.0 ± 8.3 weeks with a range of 2 weeks to 52 weeks. No serious systemic or local bevacizumab-related adverse events were observed in our 65 patients.

The course of macular edema after the initial IVB therapy in all 65 eyes is summarized in Figure 1. The initial injection of bevacizumab was effective in 62 of 65 eyes and was not effective in 3 eyes (4.6%; Group 1). Twenty-one eyes (32.3%) had no recurrence of macular edema after the initial reduction of the macular edema (Group 2). Thirty-four eyes (52.3%) had a recurrence of macular edema after the initial reduction of the macular edema but with a recurrence ratio <110% (Group 3). Seven eyes (10.8%) had a recurrence of macular edema after the initial reduction of the macula, and the recurrence ratio was ≥110% (Group 4). For patients in Groups 1, 3, and 4 (44 eyes, 67.7%), a second injection of IVB was performed as soon as appropriate unless the patient did not agree to a second injection.

Horizontal OCT images through the fixation point at baseline (left), at 4 weeks after the initial IVB

(middle), and at the recurrence of macular edema (right) for all 7 eyes of Group 4 are shown in Figure 2. All these 7 eyes had had an initial resolution of the macular edema at 4 weeks after the injection, but had a recurrence of the macular edema at 8 weeks to 12 weeks (mean, 9.1 weeks). The recurrence ratio (foveal thickness at the rebound/foveal thickness at the baseline × 100) ranged from 110% to 149%, and the mean was 124.6% for these 7 eyes. However, the recurrence ratio ranged from 39% to 102%, and the mean degree of recurrence was 74.7% for the 34 eyes of Group 3.

To identify the potential pretreatment factors that were associated with the rebound of macular edema, multivariate logistic regression analyses were performed on all 65 eyes, with rebound macular edema (recurrence rate ≥110%) as the dependent variable (Table 1). The results showed that a thinner pretreatment fovea (odds ratio, 0.98; 95% confidence interval, 0.96–1.00, $P = 0.063$) and a shorter interval from symptom onset to the initial injection (odds ratio, 0.47; 95% confidence interval, 0.21–1.05; $P = 0.067$) showed strong trends to be associated with the rebound of macular edema.

We next performed subgroup analyses comparing each pretreatment factor between Group 3 (recurrence rate <110%, n = 34) and Group 4 (recurrence rate ≥110, n = 7, Table 2). We found that the pretreatment fovea was significantly thinner ($P = 0.004$) in Group 4 (451 ± 70 μm) than in Group 3 (651 ± 179 μm). In addition, the interval from the symptom onset to the initiation of treatment in Group 4 (4.9 ± 2.2 weeks) was significantly shorter ($P = 0.007$) than that in Group 3 (9.1 ± 4.6 weeks). There was no significant difference in other pretreatment factors between the two groups.

To investigate the relationship between the recurrence ratio and the 2 pretreatment factors that were found to be associated with a rebound macular edema, we also plotted the recurrence ratio against the foveal

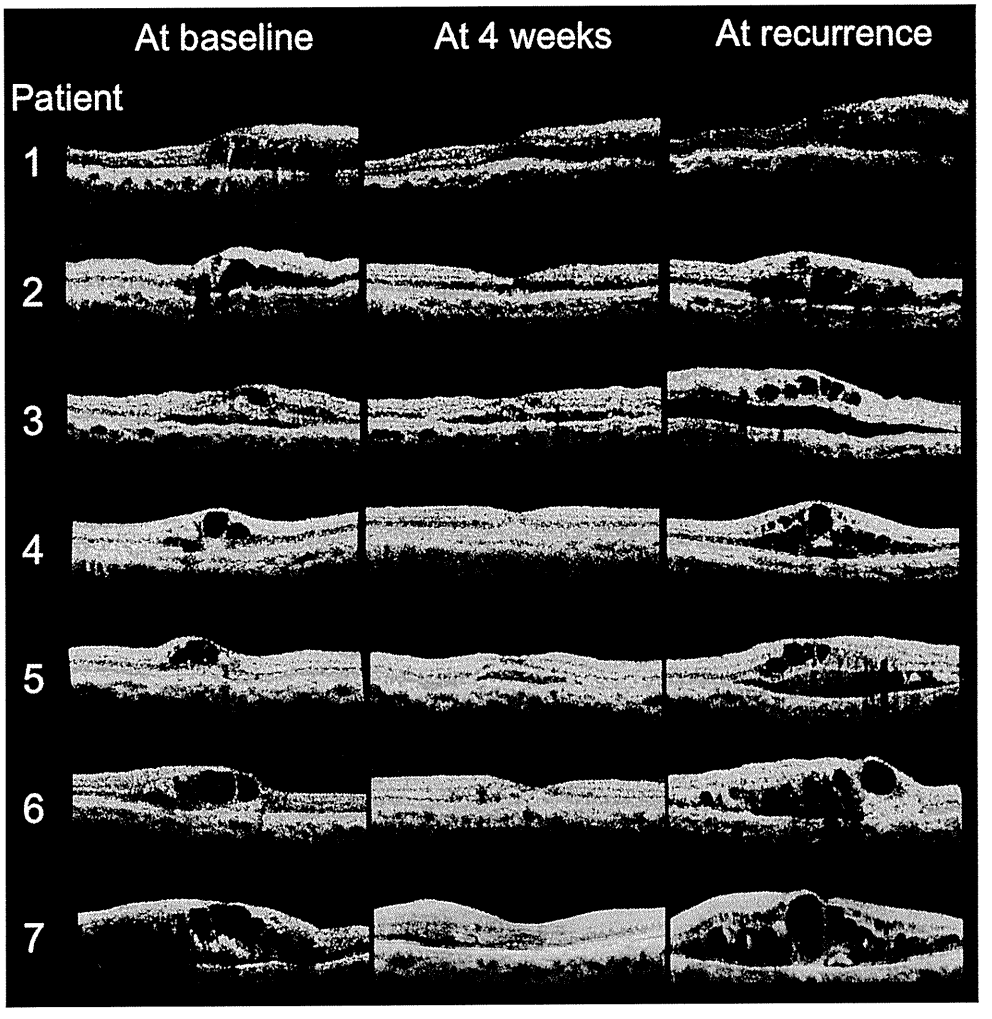

Fig. 2. Horizontal OCT images through the fixation point at baseline (left), at 4 weeks after initial bevacizumab injection (middle), and at the recurrence of macular edema (right) for all 7 eyes of Group 4 (recurrence rate ≥110%). These 7 eyes had an initial resolution of macular edema at 4 weeks after the injection but showed a recurrence of macular edema at 8 weeks or 12 weeks (mean, 9.1 weeks). At this time, their foveal thickness was ≥110% of the pretreatment thickness.

Table 1. Multivariate Logistic Regression Analysis of Pretreatment Factors Associated with the Rebound of Macular Edema Secondary to BRVO

Factor	Odds Ratio (95% CI)	*P*
Age (per year)	0.87 (0.68–1.12)	0.285
Gender (men vs. women)	30.09 (0.6401–1417.04)	0.083
Diabetes mellitus	20.04 (0.06–6268.55)	0.306
Hypertension	0.35 (0.07–17.89)	0.601
Hypercholesterolemia	<0.0001	0.999
Pretreatment visual acuity (logarithm of the minimum angle of resolution)	0.268 (0.01–143.60)	0.681
Pretreatment foveal thickness (μm)	0.98 (0.96–1.00)	0.063
Period from symptom onset to injection (weeks)	0.47 (0.21–1.05)	0.067

CI, confidence interval.

thickness in micrometers at the baseline (Figure 3A), and the interval from the symptom onset to the initiation of treatment in weeks (Figure 3B). In these figures, only the data of Groups 3 and 4 are plotted, because the eyes in Groups 1 and 2 did not show a recurrence of the macular edema. We found that the baseline foveal thickness for all 7 eyes with rebound (recurrence rate ≥110%) was ≤560 μm, and the interval from the symptom onset to the injection was ≤8 weeks for all 7 eyes. The intervals between the onset of symptoms and treatment in Groups 1 and 2 were 12.7 ± 10.3 and 12.5 ± 12.6 weeks, respectively.

Finally, to determine whether the rebound of macular edema resulted in a poorer post-IVB outcome, we compared the BCVA and foveal thickness at 6 months after the IVB and the total number of injections during the 6 months after the initial injection between Groups 3 and 4. The differences in these values between the 2 groups were not significant (Table 3). The reason for the low total number of injections during the 6 months in these 2 groups was that there were 14 patients (12 of Group 3 and 2 of Group 4) who did not want to receive a second injection mainly because their visual acuities were relatively maintained even though a recurrence of macular edema had occurred.

Discussion

Our results showed that the incidence of a rebound of macular edema (recurrence ratio ≥110%) after an initial resolution after IVB was 10.8% (7 of 65 eyes). The degree of recurrence for these 7 eyes ranged from 110% to 149%, and 4 eyes had a recurrence ratio of ≥120% (Figure 3). These results indicated that the rebound of macular edema is not a rare phenomenon, and clinicians should be aware that this phenomenon can occur during the IVB therapy for macular edema associated with BRVO.

Because the rebound of macular edema is an unfavorable finding for both patients and clinicians, it is important to know what pretreatment factors were associated with the rebound. Our results using subgroup analyses demonstrated that a thinner pretreatment foveal thickness and a shorter interval from the symptom onset to the initiation of IVB were

Table 2. Comparison of Various Pretreatment Factors Between Group 3 and Group 4

Factor	Group 3	Group 4	*P*
Number of eyes	34	7	
Age (years)*	62.0 ± 7.4	57.7 ± 8.8	0.22
Gender (men/women)	19/15	5/2	0.37†
Diabetes mellitus	4	2	0.25†
Hypertension	25	4	0.39†
Hypercholesterolemia	5	0	0.28†
Pretreatment visual acuity (logarithm of the minimum angle of resolution)*	0.55 ± 0.24	0.68 ± 0.18	0.1
Pretreatment foveal thickness (μm)*	651 ± 179	451 ± 70	0.004
Period from symptom onset to injection (weeks)*	9.1 ± 4.6	4.9 ± 2.2	

Group 3 = eyes with recurrence ratio <110%. Group 4 = eyes with recurrence ratio. Differences between two groups were analyzed using a nonparametric Mann–Whitney *U*-test.

*Data are expressed as the mean ± SD.

†Differences between the two groups were analyzed using a chi-square test.

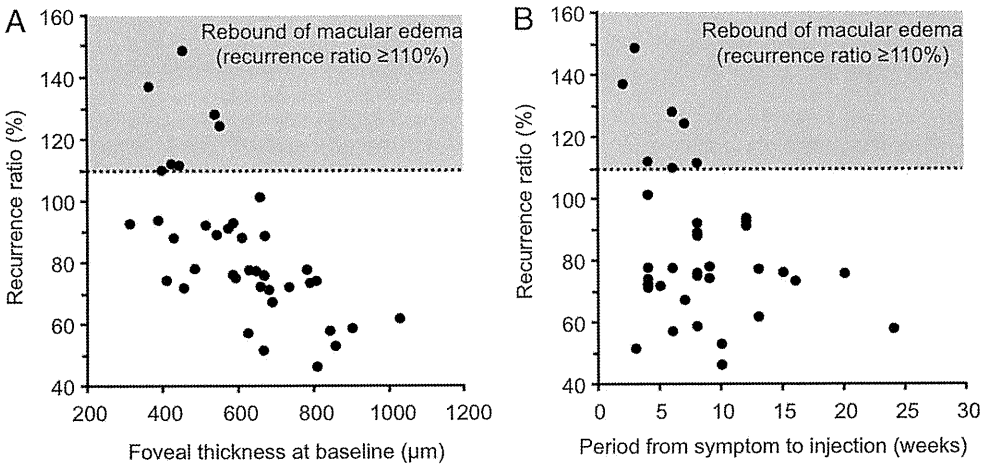

Fig. 3. Relationship between the recurrence ratio (foveal thickness at the rebound/ foveal thickness at the baseline × 100) and 2 pretreatment factors for the 41 eyes of Groups 3 and 4. **A.** The recurrence ratio plotted against the foveal thickness at the baseline (in micrometers). **B.** The recurrence ratio plotted against the period from symptom onset to the initiation of treatment (weeks). Gray areas show the eyes with recurrence ratio ≥110%, that is, a rebound.

significantly associated with a rebound of macular edema. Interestingly, all 7 eyes that had a rebound of macular edema received the IVB therapy within 8 weeks of the onset of the symptoms (Figure 3B). These results suggest that the rebound of macular edema was more likely to occur when the IVB therapy was initiated at a relatively early stage of the macular edema before the edema had reached the maximum degree of edema in eyes with a BRVO. In other words, the rebound of macular edema may be more likely to occur in eyes in which the macular edema might have worsened if they had not received the IVB therapy during its natural course.

However, other factors may be involved in the mechanism for the rebound of macular edema after the IVB therapy, because 1 of 3 patients with rebound macular edema reported by Matsumoto et al[27] had received the IVB therapy 22 months after the diagnosis. They hypothesized that the inhibition of the VEGF pathway by IVB may upregulate VEGF receptors within the retina of the patients, and this upregulation may make the endothelial cells more sensitive to the VEGF that are already upregulated because of the underlying ischemic state.[27] Quantitative analysis of the changes in the expression of the VEGF receptors at various times after IVB therapy in an animal model of BRVO may answer this question.

We also studied whether there was any difference in the post-IVB outcomes between the eyes with and without rebound (Group 3 vs. Group 4), and we found that there was no significant difference in any of the post-IVB outcome values (Table 3). These findings suggest that the eyes with rebound macular edema do not necessarily result in poorer visual outcome than eyes without a rebound. However, we hesitate to draw this conclusion based on our results, because of the few eyes in Group 4 and the high withdrawal rate for additional injections in Groups 3 and 4. The reason for the high withdraw rate was because the decision to give additional injections was done not only by the data of foveal

Table 3. Comparison of Posttreatment Values Between Group 3 and Group 4

Posttreatment Values	Group 3	Group 4	*P*
BCVA at recurrence (logarithm of the minimum angle of resolution)	0.27 ± 0.21	0.44 ± 0.30	0.15
Changes in BCVA between baseline and recurrence (logarithm of the minimum angle of resolution)	0.33 ± 0.26	0.28 ± 0.19	0.85
Number of injections within 6 months	1.8 ± 0.7	2.0 ± 0.8	0.50
BCVA at 6 months after initial injection (logarithm of the minimum angle of resolution)	0.22 ± 0.21	0.35 ± 0.25	0.16
Changes in BCVA between baseline and 6 months after initial injection (logarithm of the minimum angle of resolution)	0.35 ± 0.27	0.33 ± 0.14	0.89
Foveal thickness at 6 months after initial injection (μm)	400 ± 150	375 ± 170	0.59

Data are expressed as mean ± SD. Differences between two groups were analyzed using a nonparametric Mann–Whitney *U*-test.
VA, visual acuity.

thickness and visual acuity but also by the patient's approval. Thus, few eyes and high withdrawal rate for additional injections were limitations of our study.

The incidence of rebound macular edema in BRVO was 10.8%. However, this percentage may change because of the interval of the follow-up period. We followed-up our patients monthly and performed a second injection immediately when a recurrence was detected. It is possible that if our follow-up interval was shorter, for example, every week or every 2 weeks, then we might have detected a recurrence earlier and performed a second injection at an earlier stage of recurrence, resulting in lower incidence of rebound macular edema. The incidence of rebound macular edema can also change because of the period from symptom onset to the injection. If we initiated the IVB therapy after a 2- to 3-month observational period, then the incidence of rebound macular edema might have been lower than what we found (Figure 3B). In addition, the incidence of rebound macular edema can also change if multiple injections (e.g., three times monthly) had been adopted at the initial injection.

Finally, there is still discussion about when the IVB therapy should be initiated in eyes with macular edema secondary to BRVO. It is widely recommended that any invasive treatments for macular edema secondary to BRVO should be initiated at least 2 months to 3 months after the symptom onset because spontaneous resolution of macular edema can occur mostly within this period.[4,23,24] We did not set any observational window before the initiation of IVB. However, based on our results, we now believe that it is reasonable to wait at least 2 months after the onset to begin the IVB, because in addition to excluding eyes with spontaneous resolution, a rebound of macular edema may be avoided by waiting 2 months after the symptom onset.

Key words: branch retinal vein occlusion, macular edema, bevacizumab, vascular endothelial growth factor, rebound.

Acknowledgments

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A case of aniridia with unilateral Peters anomaly

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Aniridia is an autosomal-dominant, panocular, congenital anomaly transmitted with high penetrance and largely caused by mutations in the *PAX6* gene. Although Peters anomaly may also be caused by mutations in *PAX6*, there has not to our knowledge been a report of aniridia associated with lens displacement into the anterior chamber and lenticular-corneal attachment. We report a child with aniridia and Peters anomaly associated with a *PAX6* gene mutation.

Case Report

A 9-day-old boy was referred to the Department of Ophthalmology, Hamamatsu University Hospital, for an opacity noted in the left eye. He had been born via vacuum-assisted vaginal delivery with a birth weight of 2,222 g. An examination under anesthesia was performed with oral triclofos sodium sedation. Slit-lamp examination revealed bilateral aniridia. The left lens was dislocated into the anterior chamber (Figure 1A) and adherent to the cornea, with corneal opacification at the area of contact. IOP was 12 mm Hg in the right eye and 10 mm Hg in the left by Tono-Pen (Reichert Technologies, Depew, NY). Fundus examination revealed macular hypoplasia. Ultrasound biomicroscopy (TOMEY UD-6000, Tokyo, Japan) confirmed that the left lens was dislocated into the anterior chamber, thicker, and more spherical than the right lens and adherent to the cornea (Figure 1B and 1C). Slit-lamp examination of his parents' eyes revealed no abnormality.

On examination at 2 months of age, bilateral macular hypoplasia was confirmed, and horizontal pendular nystagmus was detected. At 4 months of age, visual acuity was 0.65 cycles/degree in the right eye at 38 cm with the use of Teller Acuity Cards (Stereo Optical Co., Chicago, IL), with strong resistance to occlusion of the right eye.

At age 2.5 years, the corneal opacity of the patient's left eye had not changed, but the lens opacity had worsened, affecting cosmesis. Lensectomy was performed by a 2-port procedure under general anesthesia. At surgery, the lens was found to be firmly attached to the cornea,

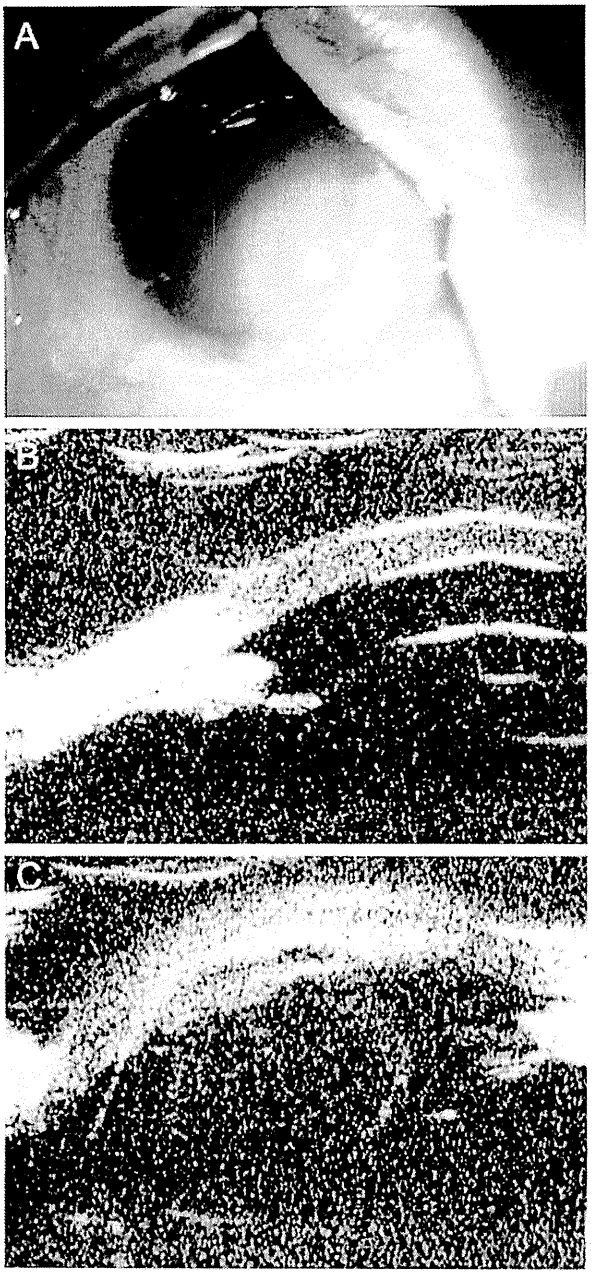

FIG 1. Clinical photographs and ultrasound of a 9-day-old boy with bilateral aniridia and left lens dislocation. A, Photograph of the anterior segment of the left eye: the lens appears to be adherent to the cornea, which is opacified at the site of attachment. Ultrasound biomicroscopic images of the right (B) and left (C) eyes showing absence of most of the iris in both: the left lens is thicker and more spherical than the right. Corneal density appears higher in the area of attachment.

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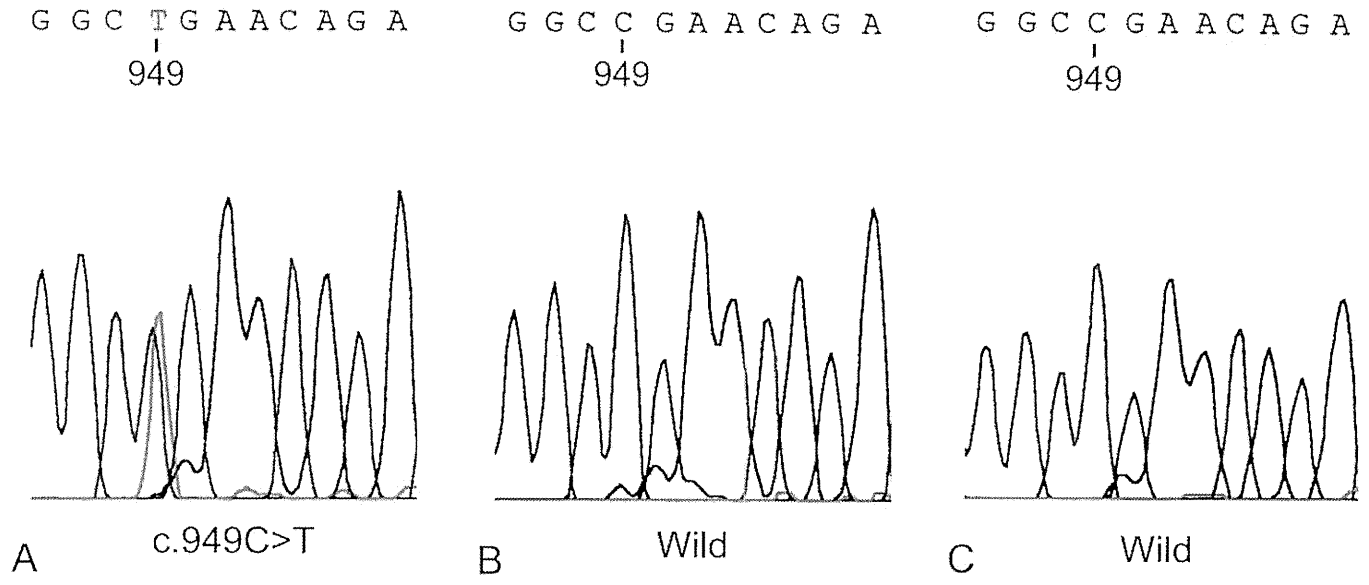

FIG 2. The patient had a nonsense mutation, C949T (R317X), in exon 11 of the *PAX6* gene (A). His parents, father (B), mother (C), did not have the mutation in exon 11 of the *PAX6* gene.

and the anterior capsule could not be distinguished from Descemet's membrane. Postoperatively, the corneal opacity increased slightly, and the IOP remained less than 20 mm Hg. At the age of 2 11/12 years, the patient recognized Landolt C optotypes with visual angle of 0.1° at 1.5 m (20/667) with his right eye but not with his uncorrected left eye. Amblyopia therapy was not initiated because of poor prognosis and infeasibility of contact lens wear.

After informed consent was obtained, genetic testing was performed, with the procedures described in detail elsewhere.[1] In brief, the 13 exons and surrounding intronic areas of the *PAX6* gene were amplified by polymerase chain reaction and directly sequenced. The patient had a nonsense mutation, C949T (R317X), in exon 11 of the *PAX6* gene (Figure 2A). The mutation was not detected in his parents (Figure 2B and 2C).

Discussion

Aniridia is most often caused by mutations in the PAX6 gene.[2] The R317X mutation of *PAX6* in our patient has also been reported in white, East Indian, and Chinese patients.[3-5] The absence of both the ocular abnormalities and the mutation in his parents suggested a de novo mutation. The parents were therefore informed that the risk of the aniridia in a future child was very low.

Aniridia may be accompanied by various ocular changes, including congenital cataract, subluxated lenses, glaucoma, and macular hypoplasia.[6] Various corneal opacities, including possible Peters anomaly accompanied with aniridia, have been reported.[7] The most common congenital cataract in aniridia is anterior pyramidal, which is thought to be a forme fruste of Peters. The pyramid can even be attached to the cornea. Lens subluxation in aniridia is rare[8]; we found no reports of unilateral Peters anomaly associated with aniridia or of unilateral lens dislocation into the anterior chamber and lenticular-corneal attachment associated with aniridia.

The total lens malposition may be caused by an incomplete separation of the lens from the surface ectoderm at an early stage of development or a secondary adhesion of a lens dislocated lens into the anterior chamber. Embryologically, the detachment of the lens vesicle from the surface ectoderm is the initial event leading to the formation of the chambers of the eye.

Because haploinsufficiency of the *PAX6* gene can cause congenital cataracts and Peters anomaly, which possibly result from faulty keratolenticular separation, the mutation may also cause total lens malposition by an incomplete keratolenticular separation. It is also possible that a dislocated lens that was initially located in the posterior chamber moved into the anterior chamber and attached to the cornea because the iris barrier between the anterior and posterior chamber was absent. If this were the mechanism, however, then anterior lens dislocation would be far more commonly observed in patients with aniridia. Although secondary adhesion of the lens may have possibly occurred, the intraoperative findings were most consistent with the hypothesis that lens malposition was caused by an incomplete separation of the lens from the surface ectoderm.

Literature Search

PubMed was searched for the following terms: *aniridia* AND *Peters anomaly* and combinations of *aniridia*, *subluxated lens*, *malposition*, and *PAX6* or *Peters*.

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総説

間欠性外斜視の評価と分類

佐藤美保

〔要　約〕

間欠性外斜視は，アジア人に最も多い斜視の一つである。小児では，整容面，立体視や視力といった視機能が問題となり，成人では，整容面と複視が問題となる。遠見と近見の眼位の差で分類し，術式の選択に役立てる。手術は4歳以降で行うことが勧められ，外直筋後転術，内直筋前転術の単独あるいは組み合わせが行われる。整容的な治癒をえるためには，斜視角が15プリズム以内に収まることが重要であるが，斜視角だけでなく，コントロール状態が良好であることが必要である。コントロール状態を評価するために，Newcastle Control Scoreを用いると良い。

はじめに

間欠性外斜視（intermittent exotropia: 通常X（T）と記載する。（表1）[1]）とは，外斜視と外斜位をくりかえすものでアジア人では最も頻度の高い斜視である。斜位のときには，両眼視機能がよいが，斜視のときには抑制がかかるか複視を自覚する。手術によって眼位も両眼視機能も改善することが多いが，術後の再発がしばしばみられることから，治療のタイミングや治療方法が議論となる。大切なことは，正しく病態の評価を行ったうえで治療することであり，そうすれば高い患者満足度が得られる。

表1　一般的な斜視の記載方法

	斜位		斜視		間欠性斜視	
	近見	遠見	近見	遠見	近見	遠見
内方偏位	E′	E	ET′	ET	E（T）′	E（T）
外方偏位	X′	X	XT′	XT	X（T）′	X（T）

E: Esophoria 内斜位，ET: Esotropia 内斜視
X: Exophoria 外斜位，XT: Exotropia 外斜視
「′」は近見眼位を意味する
（　）でくくると間欠性を意味する

Ⅰ．間欠性外斜視の症状

小児期に発症した間欠性外斜視では，外斜視のときには片眼が抑制されて複視を自覚しないが，斜位のときには抑制はかからず良好な立体視を示すことが多い。そのため多くの場合，見え方に関する症状を自覚することはなく，家族が外見から斜視に気づき来院することが多い。また戸外の明るい所に行くと，片目を閉じてしまい見づらそうにすることがある。本人が斜視に気づく年齢に達し，鏡や写真で自分を見たり，他人から指摘されたりして斜視を自覚するようになると，意識して両眼で見ようとすることが多い。一方，近見で斜視になり自分でコントロールができない場合，複視や眼精疲労を自覚することがある。遠方視で目立つ斜視だと，対人関係に自信をなくしたり，就職で不利な評価をうけたりして社会生活上の問題となる。患者のなかには，整容的に気にしていることは恥ずかしいと感じる人もあり，訴えが曖昧なことがある。その点に配慮したうえで診療に当たることが必要である。

佐藤　美保：浜松医科大学眼科

【禁忌（次の患者には投与しないこと）】
(1)気管支喘息、又はその既往歴のある患者、気管支痙攣、重篤な慢性閉塞性肺疾患のある患者［β遮断による気管支平滑筋収縮作用により、喘息発作の誘発・増悪がみられるおそれがある。］
(2)コントロール不十分な心不全、洞性徐脈、房室ブロック（Ⅱ、Ⅲ度）、心原性ショックのある患者［β遮断による陰性変時・変力作用により、これらの症状を増悪させるおそれがある。］
(3)本剤の成分に対して過敏症の既往歴のある患者

【効能・効果】
緑内障、高眼圧症

【効能・効果に関連する使用上の注意】
原則として、単剤での治療を優先すること。

【用法・用量】
1回1滴、1日1回点眼する。

【用法・用量に関連する使用上の注意】
頻回投与により眼圧下降作用が減弱する可能性があるので、1日1回を超えて投与しないこと。

【使用上の注意】
1. 慎重投与（次の患者には慎重に投与すること）
(1)肺高血圧による右心不全のある患者［β遮断による陰性変時・変力作用により、症状を増悪させるおそれがある。］
(2)うっ血性心不全のある患者［β遮断による陰性変時・変力作用により、症状を増悪させるおそれがある。］
(3)糖尿病性ケトアシドーシス及び代謝性アシドーシスのある患者［アシドーシスにより心筋収縮力の抑制を増強するおそれがある。］
(4)コントロール不十分な糖尿病のある患者［低血糖症状をマスクすることがあるので血糖値に注意すること。］
(5)無水晶体眼又は眼内レンズ挿入眼の患者［ラタノプロスト投与により嚢胞様黄斑浮腫を含む黄斑浮腫、及びそれに伴う視力低下を起こすとの報告がある。］
(6)眼内炎（虹彩炎、ぶどう膜炎）のある患者［ラタノプロスト投与により眼圧上昇がみられたとの報告がある。］
(7)ヘルペスウイルスが潜在している可能性のある患者［ラタノプロスト投与により角膜ヘルペスがみられたとの報告がある。］
(8)妊婦、産婦、授乳婦等［「妊婦、産婦、授乳婦等への投与」の項参照］
2. 重要な基本的注意
(1)本剤は1mL中にラタノプロスト50μg及びチモロールマレイン酸塩6.83mg（チモロールとして5mg）を含む配合点眼液であり、ラタノプロストとチモロールマレイン酸塩双方の副作用が発現するおそれがあるため、適切に本剤の使用を検討すること。
(2)本剤は、全身的に吸収される可能性があり、β遮断薬全身投与時と同様の副作用があらわれることがあるので、留意すること。
(3)本剤の投与により、虹彩色素沈着（メラニンの増加）があらわれることがある。投与に際しては虹彩色素沈着及び色調変化について患者に十分説明しておくこと。ラタノプロスト投与による色素沈着は投与により徐々に増加し、投与中止により停止するが、投与中止後消失しないことが報告されている。また、虹彩色素沈着による色調変化があらわれる可能性があり、特に片眼治療の場合、左右眼で虹彩の色調に差が生じる可能性がある。褐色を基調とする虹彩の患者において、虹彩色素沈着が多く報告されているが、虹彩の変色が軽度であり、臨床所見によって発見されないことが多い。［「重大な副作用」の項参照］
(4)本剤投与中に角膜上皮障害（点状表層角膜炎、糸状角膜炎、角膜びらん）があらわれることがあるので、しみる、そう痒感、眼痛等の自覚症状が持続する場合には、直ちに受診するよう患者に十分指導すること。
(5)本剤を閉塞隅角緑内障患者に投与する場合は、使用経験がないことから慎重に投与することが望ましい。
(6)縮瞳薬からチモロールマレイン酸塩製剤に切り替えた場合、縮瞳作用の消失に伴い、屈折調整を必要とすることがあることから、本剤投与の際も注意すること。
(7)本剤の点眼後、一時的に霧視があらわれることがあるため、症状が回復するまで機械類の操作や自動車等の運転には従事させないよう注意すること。
3. 相互作用
併用注意（併用に注意すること）
・アドレナリン、ジピベフリン塩酸塩 ・カテコールアミン枯渇薬（レセルピン等） ・β遮断薬（アテノロール、プロプラノロール塩酸塩、メトプロロール酒石酸塩等） ・カルシウム拮抗薬（ベラパミル塩酸塩、ジルチアゼム塩酸塩等） ・ジギタリス製剤（ジゴキシン、ジギトキシン） ・CYP2D6阻害作用を有する薬剤（キニジン硫酸塩水和物、選択的セロトニン再取り込み阻害薬等） ・プロスタグランジン系点眼薬（イソプロピルウノプロストン、ビマトプロスト等）
4. 副作用
国内で実施された臨床試験において、副作用（臨床検査値異常を含む）が報告されたのは201例中51例（25.4%）であった。主な副作用は眼刺激32例（15.9%）、点状表層角膜炎6例（3.0%）、結膜充血4例（2.0%）、角膜炎3例（1.5%）及びALT（GPT）上昇2例（1.0%）であった（承認時までの調査の集計）。

外国で実施された臨床試験において、副作用（臨床検査値異常を含む）が報告されたのは1536例中121例（7.9%）であった。主な副作用は眼刺激40例（2.6%）、結膜充血19例（1.2%）、眼痛17例（1.1%）であった（承認時までの調査の集計）
(1)重大な副作用
1)虹彩色素沈着（頻度不明）[注]：虹彩色素沈着があらわれることがあるので、患者を定期的に観察し、虹彩色素沈着があらわれた場合には臨床状態に応じて投与を中止すること。［「重要な基本的注意」の項参照］
2)眼類天疱瘡（頻度不明）[注]：眼類天疱瘡があらわれることがあるので、結膜充血、角膜上皮障害、乾性角結膜炎、結膜萎縮、睫毛内反、眼瞼眼球癒着等の症状があらわれた場合には投与を中止し、適切な処置を行うこと。
3)気管支痙攣、呼吸困難、呼吸不全（いずれも頻度不明）[注]：気管支痙攣、呼吸困難、呼吸不全があらわれることがあるので、症状があらわれた場合には投与を中止し、適切な処置を行うこと。
4)心ブロック、心不全、心停止、脳虚血、脳血管障害（いずれも頻度不明）[注]：心ブロック、心不全、心停止、脳虚血、脳血管障害があらわれることがあるので、症状があらわれた場合には投与を中止し、適切な処置を行うこと。
5)全身性エリテマトーデス（頻度不明）[注]：全身性エリテマトーデスがあらわれることがあるので、症状があらわれた場合には投与を中止し、適切な処置を行うこと。
注：外国で報告がある、又はラタノプロスト若しくはチモロールマレイン酸塩において報告がある副作用

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Ⅱ．間欠性外斜視の診断

間欠性外斜視には，斜位のときと斜視のときがなくてはならない。斜位とは両眼開放時には眼位ずれがなく，片眼を遮閉したときのみに眼位ずれがみられる状態である。近見時は常に外斜視であるが遠見時は常に斜位である場合，その逆の場合も間欠性外斜視という。斜視になりにくい場合には，長く遮閉してから遮眼子をはずすようにする。それでも診察室のなかでは，斜視があきらかにならないこともある。間欠性外斜視は明るい場所にでると出現しやすいが，暗室のような薄暗いところでは顕性になりにくいからである。どうしても斜視が顕性になりにくいときには，気になる写真を持参してもらうと良い。

Ⅲ．間欠性外斜視の頻度と自然経過

間欠性外斜視はアジア人に多い斜視であることが知られている。白人で最も多い斜視は調節性内斜視であるのに対し，アジア人では間欠性外斜視が最も多い。その理由はあきらかではないが，白人に遠視性屈折異常が多いのに対して，アジア人では近視が多いことから屈折異常との関連が深いと考えられている。間欠性外斜視の自然経過は，眼位の変化とコントロール状態の変化で評価を行うとわかりやすい。PEDIG（Pediatric Eye Disease Investigator Group）の研究によると，約50%は5年の間に斜視角は変化せず，約1/4は悪化，約1/4は改善するとされた。さらにコントロール状態（斜位の保ちやすさ）でも，ほぼ50%が不変で約1/4が悪化，1/4が改善していた[2]。

Ⅳ．間欠性外斜視の分類

間欠性外斜視の分類は，遠見と近見の眼位の差によって基礎型，輻湊不全型，開散過多型に分類され，手術方針決定の指針となる。その他，上方視と下方視の眼位の差によって，A型やV型と判断される。遠見と近見の眼位から3つの型にわけるためには，正確に眼位を測定する必要がある。間欠性外斜視の眼位は，診察する時間，患者の疲労，診察場所の明るさ，見ている画像や背景によって異なることを認識しておく。

遠見斜視角（XT）と近見斜視角（XT′）から間欠性外斜視を分類する。開散過多型の分類で注意が必要なものに偽開散過多型がある。本当はtenacious fusion（頑強な融像：実際は基礎型）か高AC/A比に分類すべきものが混在するからである。偽開散過多型の診断の方法を図に示す。まずtenacious fusionを除外するために，片眼遮閉を30分以上行い融像を除去する。近見斜視角が増加すれば基礎型となる。眼位の変化がなければ，+3Dのレンズを装用させる。近見斜視角が増加すれば，高AC/A比であり遠見斜視角をもとに手術をすると術後，近見内斜視となり遠近両用眼鏡が必要になる。もし，+3Dを加入しても近見眼位が遠見眼位より明らかに大きい場合に真の開散過多型と診断する。

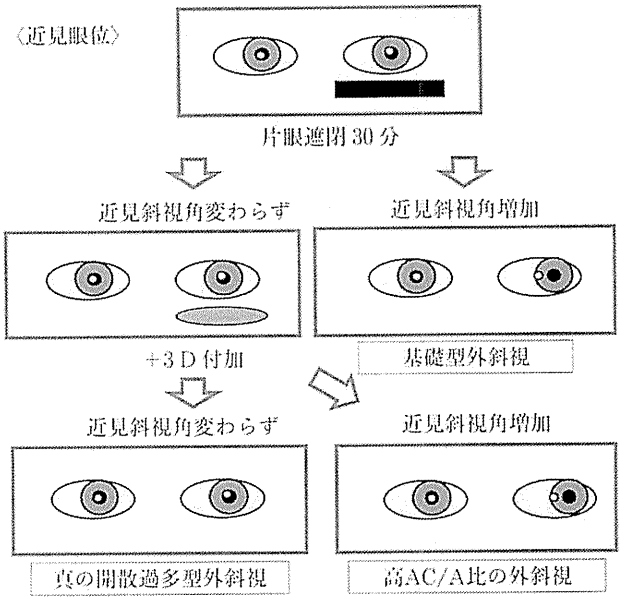

図　近見眼位＜遠見眼位の場合

Ⅴ．間欠性外斜視の検査

1．視力検査

間欠性外斜視でも，眼科一般検査は重要である。まず視力，屈折検査を行う。両眼あるいは片眼の視力低下があると廃用性外斜視になるので，視力検査ができない場合や，矯正視力が不良な場合には，視力不良の原因をさぐるために前眼部検査や眼底検査を行う。また，成人の斜視のなかには，片眼で見ているときには，軽度の近視であるにも関わらず，両眼視をさせると強い近視となり眼鏡処方に困難を要することがある。これは，外斜視をコントロールするために，調節性輻湊を行っているからで，両眼視時に近視化するために起きる現象である（斜位近視）。「両眼でみるとよく見えない」などと訴えて来院す

るため，片眼視力だけでなく，両眼開放視力を測定することがポイントで，斜視手術の良い適応である。くれぐれも過矯正の眼鏡を処方しないようにする。

2. 眼位・眼球運動検査

眼位検査には，両眼での動き（むき運動）と単眼での動き（ひき運動），さらに輻湊，開散（よせ運動）をみる。斜視角が大きい場合には，むき運動検査だけでは斜視眼の内転が制限されているように見えることがあるため，かならず片眼をかくしてひき運動検査で十分に内転できるかどうか確認する必要がある。

むき運動は，左右，上下，斜めを含む8方向でみる。斜め方向の動きをみるときは，内転眼が過剰に上転したり，過剰に下転したりしないか確認する。習慣的に内転時の上転過剰を下斜筋過動，内転時の下転過剰を上斜筋過動と呼んでいるが，実際にそれらの筋が肥厚したり過剰なインパルスをうけていることを示したデータはなく，あくまでも相対的な表現であることを理解しておく。

3. 斜視角の測定

遠見での斜視角測定には，はっきり見える固視目標をもちいて，正面（第一眼位），上下左右（第二眼位），斜め上下（第三眼位）を確認する。眼位検査は調節状態を理解しながら行う。調節をさせずに最大斜視角を引き出したいときには，ペンライトのように調節できない視表を用い，日常生活での眼位コントロール状態を見たいときには，調節しやすい絵柄のついた視表を用いる。大切なことは調節状態を検者がコントロールしながら行うことである。輻湊は，融像させた状態で眼前どこまで近づけるかを測定する。

斜視角測定にはプリズムを用いて交代遮閉試験を行う。プリズムは基底を内方にむけて目標を固視させながら交代遮閉試験を行う。間欠性外斜視は，検査時によって斜視角が変化することは珍しくない。常に同じ斜視角を測定するためには，最大斜視角を引き出すよう，努力するのがよい。そのためには，十分に遮閉してから遮閉を外す。また，30分以上の片眼遮閉を行ったり，5メートル以上はなれた固視目標を用いたりして斜視角測定をするとよい。

4. コントロール状態の評価

顕性になるかどうかは，診察室内および家族への質問で評価するのがよい。外斜視は明るいところで顕性になりやすいため，薄暗い眼科の診察室内でだけ見ていたのでは，斜視を見逃すことになる。特に手術前の診察では，最大斜視角を引き出すために十分に明るい室内で検査をすることが必要である。コントロール状態を数値化するためにNewcastle Control Score（NCS）などを用いるとよい（表2）[3]。我々が実際に斜視手術を進めているのは，NCSが4点以上であることが明らかになった[4]。

表2　Newcastle Control Score

1）家族による観察
　0：全く気づかない
　1：ときどき気づく
　2：しばしば気づく
　3：斜視／片眼つむりに近見でも気づく
2）診察室での観察（遠見）
　0：遮閉で顕性になるが，瞬きせずに戻る
　1：遮閉で顕性になるが，瞬きや刺激で戻る
　2：遮閉で顕性になり，戻らない
　3：自然に顕性になり，戻らない
3）診察室での観察（近見）
　0：遮閉で顕性になるが，瞬きせずに戻る
　1：遮閉で顕性になるが，瞬きや刺激で戻る
　2：遮閉で顕性になり，戻らない
　3：自然に顕性になり，戻らない

1），2），3）の点数の合計　　点

5. 両眼視機能

先天性の恒常性斜視と異なり，両眼視ができていて良好な立体視を獲得していることが多い。いったん立体視が低下しても，手術によって眼位が改善すれば良好な立体視を取り戻すことができると思われている。しかし，中には斜位を保っていても立体視が不良な症例や，手術で眼位が改善しても両眼視が不良なままのこともあり，術前から微少斜視であることが考えられる。

網膜対応検査は術後の複視の出現を予測するために行うが，検査方法によって結果が異なること，特殊な視環境の検査であることを知っておく。自然視に近い状態で検査するほど異常対応となることが多い。例として40プリズムの外斜視なのに30プリズム相当のプリズムで補正した場合，外斜視が残存するにもかかわらず非交叉性複視を自覚するような場合である。しかし，そのような場合でも，弱いプリズムから徐々に度数を上げながらプリズムを長時間