

NR layers was in a back match with that of the endogenous NR, corresponding to the direction of optic cup layers.

Compared with the Pax6-induced large-scale phenotypic changes and uniform expression of NR-specific markers in correct layers of the ectopic NR, GFP expression was restricted in a small number of cells (Figs 2C and 3C). It is likely that GFP faded out in cells that had rapidly proliferated and differentiated but still stayed in cells that had slowly proliferated. There is another possibility that Pax6 may induce the ectopic NR tissue in a cell non-autonomous manner. Pax6 may do so by activating the transcription of a diffusible factor that triggers NR tissue formation. The former idea is consistent with a study in *Xenopus laevis*. The cell autonomous activity of Pax6 misexpressed in *Xenopus* is thought to cause ectopic eye formation and ectopic expression of genes that relate to eye development including *Rx*, *Otx2*, *Six3* and endogenous Pax6 (11).

An opposite finding of the present study has been reported: combination of loss-of-function of Pax6 and Pax2 in the optic vesicle results in transdifferentiation of presumptive RPE to NR (46). The finding physiologically places Pax6 upstream of MITF and as a pro-RPE factor. This does not conflict with our data, however, because we transduced Pax6 into under-maturing RPE, in which endogenous Pax6 had been already downregulated. Probably, there may be differences in Pax6 function depending on timing.

Ectopic eye- or NR-like architecture is also induced by the misexpression of other homeobox transcription factors. Ectopic expression of Six3, a vertebrate functional orthologue of the *Drosophila* gene *sine oculis*, or Six6 that is closely related to Six3 (47) induces the formation of ectopic optic vesicle- or NR-like architectures in the brains of the fish, *Xenopus* and mouse embryos (15–17). Ectopic Six6 expression in embryonic or mature chicken RPE cells also results in a neuronal morphology and expression of markers characteristic of developing NR (18). *Xenopus* embryos injected with synthetic *Rx* RNA develop ectopic retinal tissue (20). However, fully structured NR, as induced by Pax6 misexpression, has not been yet obtained. Expression of endogenous Six3 and *Rx* in ectopic NR at the early phases post-Pax6 transduction, as shown in Figure 1, suggests that Pax6 regulates Six3 and *Rx* in the field of NR transdifferentiation, as in Pax6-induced ectopic eye formation in *Xenopus* embryo (11). Pax6 may be critical to induce a set of transcription factors that form NR laminar structure because of very high incidence of fully structured NR induced by the gene transduction.

The Pax6 protein has two DNA-binding domains, PD and HD (48–50). In PD, two structurally distinct subdomains, NTS and CTS, bind respective consensus sequences (23,24), and an insertion of additional 14 amino acid residues encoded by exon 5a in the NTS abolishes the NTS function and enhances the transactivation activity via CTS (25,26). Thus, exon 5a probably functions as a molecular switch to select specific targets. Recently, we found functional differences of the two isoforms in NR development: Pax6(–5a) is expressed in the entire NR, whereas Pax6(+5a) is especially in the NR portion where visual cells accumulate during eye development. Pax6(+5a) promotes the NR growth and, when overexpressed, induces an excessive well-differentiated

NR-like architecture, whereas Pax6(–5a) shows much weaker effect (51). In the present *in ovo* misexpression study, however, no difference was seen between two Pax6 isoforms with respect to their abilities to trigger NR transdifferentiation. One explanation for this is that the two isoforms may initiate the same genetic cascade via distinct pathways, possibly through control of partially overlapping target genes. Another explanation is based on the evidence of feedback regulation of Pax6 expression. Transcription of the Pax6 gene is intricately regulated via three promoters and a number of tissue-specific enhancers. Recently, several short sequences that closely match the Pax6 binding consensus (P6CON) were identified in *Drosophila* and vertebrate enhancers that drive Pax6 expression in the nervous system and eye, and it was suggested that these evolutionarily conserved P6CON sites may mediate the auto-activation of Pax6 by Pax6(–5a) (52). If so, both isoforms would be expressed after transduction of Pax6(–5a). Such a mechanism may account for similar phenotypic manifestation after transduction of Pax6(–5a) or Pax6(+5a). Although binding consensus sequences of the PD have been studied, little is known about its target genes, especially those recognized by CTS. This issue needs to be addressed to understand the mechanism of NR transdifferentiation by Pax6.

It has been considered that RPE is necessary for correct morphogenesis of NR in early stages and for organization of its layers by end of gestation, although signaling molecules emanating from RPE are not elucidated. Data obtained from organ culture suggest that RPE organizes the laminar structure of the differentiated NR (53). Transgenic mice expressing attenuated diphtheria toxin-A in RPE exhibit malformed RPE and disorganized NR (54). In contrast, our studies indicate that fully structured NR can be formed endogenously and ectopically, even though RPE is absent in areas of NR transdifferentiation from RPE. This suggests that RPE is not involved in the NR layers formation, but rather controls nutrition supply and/or cell proliferation at later stages. Compatible with this, ectopic NR is thinner than the normal NR, yet the laminar structure is clearly formed (Fig. 2).

Because primitive RPE and NR are contiguous in the optic vesicle, RPE cells has been considered as a possible candidate for a source of stem cells required for NR transdifferentiation (55). The retinogenic potential may be still preserved in RPE cells even in adult eyes, because RPE of chicken or other eye tissues, such as pigmented ciliary margin cells of mice and iris tissues of rats, generate immature NR-specific cells (3,4,18). In contrast, it has been thought that fully structured NR is generated from RPE only at early stages of development except for in amphibian eyes (5–8). However, our studies showed that RPE has the potential even at late stages. Pax6 induces the complete conversion from RPE to NR even at HH stage 40, whereas FGFs are able to transform RPE only before stage 24. As ectopic NR can be formed in broad and numerous spots at early stages (Fig. 2A), retinogenic RPE cells appear to be distributed widely throughout the RPE layer. In contrast, NR transdifferentiation was seen as small spotted areas at later stages, although expression of the exogenous gene monitored by GFP were detected in wider areas. This suggests that areas of NR transdifferentiation decrease not by inefficiency of gene transfer in late-stage-embryos. Retinogenic stem cells

may decrease in number as the RPE matures, as observed in mammalian brains (56), but be preserved widely even in late stages.

Transdifferentiation of NR from RPE by FGF treatment is a well-known phenomenon (5–8). Transcription factors or signaling cascade components that lie downstream of FGFs have been clarified recently. Switching of RPE to a neuronal fate by FGF8 is coupled with the induction of NR genes such as *Rx*, *Sgx-1* and *Fgf-8* itself (7). Switching of RPE to a neuronal fate by FGF9 is mediated by the Ras-Raf-MAPK pathway (8). It is very likely for several reasons that transdifferentiation of NR from RPE by FGFs is also mediated by increased expression of Pax6. First, Pax6 is strongly induced in RPE cells by FGF treatment (Fig. 5A and B). Secondly, transdifferentiation of NR from RPE by FGF8 is significantly disturbed by co-expression of dominant-negative Pax6 (Fig. 5E and F). Thirdly, *in vitro* assays using P19 cells demonstrate the upregulation of Pax6 expression by FGFs (Fig. 6A and B). Finally, P6CON- and 5aCON-CAT reporters are activated by FGF treatment in a dose-dependent manner (Fig. 6C). Because CAT activities of P6CON- and 5aCON-CAT reporters did not significantly respond to even high concentrations of FGFs when a small amount of Pax6 was introduced exogenously (Fig. 6D), FGFs induce expression of the Pax6 gene, but do not affect the transactivation potential of its gene product. Pax6 activity is also known to be controlled by FGF8 in somitogenesis (57). In this case, however, expression of Pax6 is suppressed by FGF signaling and is induced at the anterior limit of FGF expression that regresses caudally. Hence, regulatory relationship between Pax6 and FGF signaling may be different in these tissues.

The present study clarified roles of the Pax6 gene in ectopic NR formation, by itself and under a control of FGFs signaling. Further investigation using the mouse and rat eyes is under way, and Pax6-dependent NR transdifferentiation from RPE cells also has been preliminarily detected (data not shown). Our studies provide a new cue to regenerate functional NR in the eye with congenital anomalies or acquired degenerations by transfer of the Pax6 gene. Clinically, the RPE in the anterior portion of eye can be obtained easily by surgical procedures of peripheral iridectomy. NR reproduced from the retinogenic stem cells obtained from perinatal eyes would be a new therapeutic tool for reproduction and transplantation of functional NR tissues. Further steps to induce projection to a suitable portion in CNS are necessary to obtain useful vision. However, advanced surgical technique of experimental and clinical NR transplantation recently is achieving successful survival of the donor NR and visual improvement (58,59). Thus, reproduction of functional NR by use of Pax6 and RPE cells may be at least contribute to resurrect light sensation and visual field in patients who suffer from damaged NR and blindness.

## MATERIALS AND METHODS

### Expression and suppression plasmids

Expression plasmids ([pCAGGS–Pax6(-5a) and pCAGGS–Pax6(+5a)] to produce the entire human Pax6 coding region with or without exon 5a, under the control of a cytomegalo-

virus enhancer and a chicken  $\beta$ -actin promoter, were previously described (25,26). The mutant forms of Pax6 expression plasmid were generated by PCR-based *in vitro* mutagenesis (25–27). To produce a Pax6 suppression plasmid, a fragment carrying *En* repression domain (40) was connected to the N-terminal fragment of mouse Pax6 cDNA (*Bam*HI–*Acc*II sites that contains 1–928 nucleotides) (41) and inserted into the *Bgl*II–*Xho*I sites of pCAGGS. Expression plasmid (pCAGGS–Fgf-8) to produce the entire *Fgf-8* coding region was generated by inserting chicken *Fgf-8* cDNA cloned by RT–PCR into pCAGGS.

### In ovo electroporation

Each Pax6 expression or suppression plasmid cited above was electroporated into a chick embryo at stage 8–40 together with the pCAGGS–GFP plasmid to monitor incorporation of DNA (22,28). For electroporation, a CUY 21 electroporator (BEX) with platinum electrodes was used. A small window was opened on the stage 12 fertilized eggs for access, and embryos were allowed to develop in humidified incubators after sealing the window. At stage 12, 18, 24, 30, 35 and 40 (we used 100 embryos for each stage), the window of eggshells was unsealed and phosphate buffered saline was poured over the embryo to obtain the appropriate resistance. After injecting DNA solution into the outer coat of the eye with a sharp glass pipette, the head of the embryo was placed between the electrodes and electric pulses were applied (25–40 V, 90 ms, 1–6 times). The eggshells were sealed again and embryos were allowed to develop in humidified incubators. Eyes were incised 1–10 days after electroporation (stage 18–45) and fixed in 4% paraformaldehyde. Eight micrometer frozen sections were prepared for immunohistochemistry and *in situ* hybridization.

### In ovo injection of FGFs

FGF2 and FGF8 recombinant proteins were purchased from Genzyme. Fertilized eggs were purchased from Nisseizai (Tokyo). A small window was opened for access, then phosphate buffered saline was poured over the embryo to preserve humidity. Each FGF at a concentration of 10–100 ng/ml was injected into the mesenchymes around the eyes of HH12–40 chick embryos with a sharp glass pipette. The eggshells were sealed and embryos were allowed to develop in humidified incubators.

### In situ hybridization and immunohistochemistry

Section *in situ* hybridization was performed as described (60). Probes were prepared from plasmids containing chick *Notch1* (*Spe*I, T7 polymerase), *Musashi* (*Eco*RI, T7), *Six3* (*Hind*III, T3) and *Rx* (*Hind*III, T3). A monoclonal antibody against Pax6 protein was gifted by Dr Fujisawa (43). A monoclonal antibody against Islet1 protein was purchased from DSHB, which against Chx10 protein from Exalpha Biologicals, that against glutamate transporter 1 from Affinity BioReagents, that against parvalbumin from Sigma and that against glutamine synthetase from BD Transduction Laboratories. Tissues from chick embryo were fixed in 4% paraformaldehyde.

Eight micrometer of frozen sections were stained immunohistochemically using a method described previously (61).

#### Cell culture and RNA detection by RT-PCR

Mouse embryonic carcinoma P19 cells were maintained in MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells at a density of  $1 \times 10^5$  cells per 35 mm Petri dish were maintained in MEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. For each dish, 1, 10 or 100 ng/ml of either FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. After 3 days, total RNA was isolated from cells in each dish using an RNA easy Mini Kit (Qiagen) and converted to cDNA by a standard procedure using SuperScript II RNase H- reverse transcriptase and adaptor primers (GibcoBRL) (62). DNA segments for mouse *Pax6* and  $\beta$ -*actin* were amplified in 30 and 19 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min with the following primers: mouse *Pax6*-forward primer 5'-CACAGCGGAGTGAATCAGCTTG-3' and reverse primer 5'-CCAGAATTTTACTCACACAACCGT-3' [respective product size:160 bp for *Pax6(-5a)* and 202 bp for *Pax6(+5a)*];  $\beta$ -*actin*-forward primer 5'-GTGGGCCGCC TAGGCACCA and reverse primer 5'-CTCTTTGATGTC ACGCACGATTTTC (product size:540 bp).

#### Reporter plasmid

To obtain clones carrying the promoter region of the *Pax6* gene, we first screened the human BAC Library (Research Genetics) and detected one clone (32H10). A *HindIII*-*PshAI* fragment carrying ~2 kb *Pax6* promoter region (1285–3381 nucleotides in GenBank accession no. U63833) was excised and inserted into the *HindIII*-*SalI* sites of pCAT Basic (Promega). The insert was verified by sequencing as having the reported sequence. CAT reporter constructs carrying six copies of P6CON or two copies of 5aCON were reported previously (23–25).

#### Transient transfection and CAT assay

P19 cells at a density of  $5 \times 10^5$  cells per 60 mm petri dish were transfected with 0.5  $\mu$ g of reporter plasmid (*Pax6* promoter, P6CON or 5aCON) and 0.05  $\mu$ g of pSV $\beta$ gal (Promega) as an internal control coated with polycationic liposome (Lipofectoamine Plus, Life Technology) according to the manufacturer's instruction. For each dish, 10, 30 or 100 ng/ml of FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. Cell extracts were prepared after 72 h and assayed for CAT activities using FAST CAT Green Reagent (Molecular Probes) according to the standard procedure (62). The CAT activity was quantified by measurement with a phosphor-imager (Molecular Dynamics) and illustrated in a fold-activation compared with the condition without application of FGF.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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# Transdifferentiation of the retinal pigment epithelia to the neural retina by transfer of the Pax6 transcriptional factor

Noriyuki Azuma<sup>1,2,\*</sup>, Keiko Tadokoro<sup>2</sup>, Astuko Asaka<sup>2</sup>, Masao Yamada<sup>2</sup>, Yuki Yamaguchi<sup>3</sup>, Hiroshi Handa<sup>3</sup>, Satsuki Matsushima<sup>4</sup>, Takashi Watanabe<sup>4</sup>, Yasuyuki Kida<sup>5</sup>, Toshihiko Ogura<sup>5</sup>, Kenji Shimamura<sup>6,7</sup> and Masato Nakafuku<sup>6,8</sup>

<sup>1</sup>Department of Ophthalmology, National Center for Child Health and Development, Tokyo 157-8535, Japan, <sup>2</sup>Department of Genetics, National Research Institute for Child Health and Development, Tokyo 154-8567, Japan, <sup>3</sup>Department of Biological Information, Tokyo Institute of Technology, Graduate School of Bioscience and Biotechnology, Yokohama, 226-8501, Japan, <sup>4</sup>Department of Clinical Research Medicine, Kyorin University School of Medicine, Tokyo 181-8611, Japan, <sup>5</sup>Department of Developmental Neurobiology, Institute of Development, Aging and Cancer, Sendai 980-8575, Japan, <sup>6</sup>Department of Neuroscience, University of Tokyo Graduate School of Medicine, Tokyo 113-0033, Japan, <sup>7</sup>Division of Morphogenesis, Department of Embryogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan and <sup>8</sup>Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH 45229, USA

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The *Pax6* gene plays an important role in eye morphogenesis throughout the animal kingdom. The *Pax6* gene and its homologue could form ectopic eyes by targeted expression in *Drosophila* and *Xenopus*. Thus, this gene is a master gene for the eye morphogenesis at least in these animals. In the early development of the vertebrate eye, *Pax6* is required for the instruction of multipotential progenitor cells of the neural retina (NR). Primitive retinal pigment epithelial (RPE) cells are able to switch their phenotype and differentiate into NR under exogenous intervention, including treatment with fibroblast growth factors (FGFs), and surgical removal of endogenous NR. However, the molecular basis of phenotypic switching is still controversial. Here, we show that *Pax6* alone is sufficient to induce transdifferentiation of ectopic NR from RPE cells without addition of FGFs or surgical manipulation. *Pax6*-mediated transdifferentiation can be induced even at later stages of development. Both *in vivo* and *in vitro* studies show that the *Pax6* lies downstream of FGF signaling, highlighting the central roles of *Pax6* in NR transdifferentiation. Our results provide an evidence of retinogenic potential of nearly mature RPE and a cue for new therapeutic approaches to regenerate functional NR in patients with a visual loss.

## INTRODUCTION

Once the neural retina (NR) is damaged by developmental malformation or age-related degeneration, it is unable to regenerate, therefore resulting in a significant visual loss. Regeneration of well-defined NR has not been induced in human retinal tissues by previous trials. In contrast, in adult salamander eyes, fully functional NR regenerates from

retinal pigment epithelial (RPE) cells, when the endogenous NR is surgically removed (1). However, this regenerative event can be seen only in some amphibian eyes, but not in the eyes of other higher animals. Nonetheless, Muller cells in the postnatal chick NR de-differentiate and form NR neurons, in response to acute chemical damage (2). Pigmented ciliary margin cells in the adult mouse eye are able to form sphere colonies *in vitro* and differentiate into NR specific

\*To whom correspondence should be addressed at: Department of Ophthalmology, National Center for Child Health and Development, 2-10-1, Okura, Seatagaya-ku, Tokyo 157-8535, Japan. Tel: +81 334160181; Fax: 81 334162222; Email: azuma-n@ncchd.go.jp

cells, including photoreceptors (PR), bipolar cells and Muller cells (3). Iris tissues in the adult rat eye generate cells expressing rhodopsin, a specific antigen for rod PR (4). These observations suggest that, even in higher animals, regeneration of functional NR could be induced in some circumstances.

In embryonic eyes of chicks and mice, the primitive RPE (until embryonic day 4.5 in chick embryos, and E15 in rat embryos) is able to switch its phenotype and transdifferentiate into NR when treated with fibroblast growth factors (FGFs) (5–8). The two distinct functional components of the vertebrate retina, the inner NR and outer RPE, develop as a two-layered optic cup that is formed by folding the optic vesicle at an early stage of development. Because FGFs are expressed in the anterior parts of the primitive eye, they are considered to play roles for NR differentiation as well (6). Primitive RPE may still have retinogenic potential, but once it differentiates to mature one, it loses its potential to transdifferentiate to NR even by treatment with FGFs. Although several transcription factors and signaling cascade have been reported to act downstream of FGF signaling (7,8), nuclear events that control the differentiation competence of FGF signaling remain unsolved.

The *Pax6* gene, encoding a paired-class transcription factor, is critical for eye development (9). Target expression of the *eyeless* gene, a *Pax6* homologue of *Drosophila melanogaster*, results in ectopic formation of functional compound eyes on the wings, legs and antennae (10). The *Pax6* can also induce ectopic eyes in frog *Xenopus laevis* (11), indicating that the gene can initiate the regulatory cascade for eye formation in both invertebrates and vertebrates. Ectopic eyes in frogs contain all major components of eye, but not the full architecture. Ectopic eye architectures have been also induced by misexpression of other transcription factors, *eyes absent* (12–14), *sine oculis/Six* (14–18), *dachshund* (13,19), *Rx* (20) and *teashirt* (21), that lie downstream of or cooperate with *eyeless/Pax6* in the eye morphogenesis, in *Drosophila* (12–14,19,21) and vertebrates (15–18,20). However, such ectopic eye architectures are far smaller and more immature compared with those induced by *eyeless/Pax6* misexpression. Thus, *Pax6* could be a useful tool for the regeneration of eye tissues in vertebrates. We transduced the human *Pax6* gene into avian RPE cells *in vivo*, and elucidate here a direct role of the *Pax6* gene in transdifferentiation of fully structured NR from nearly mature RPE cells and also a functional relationship between FGF signaling and this gene.

## RESULTS

### *In ovo* misexpression of the *Pax6* gene induces fully structured NR from RPE cells

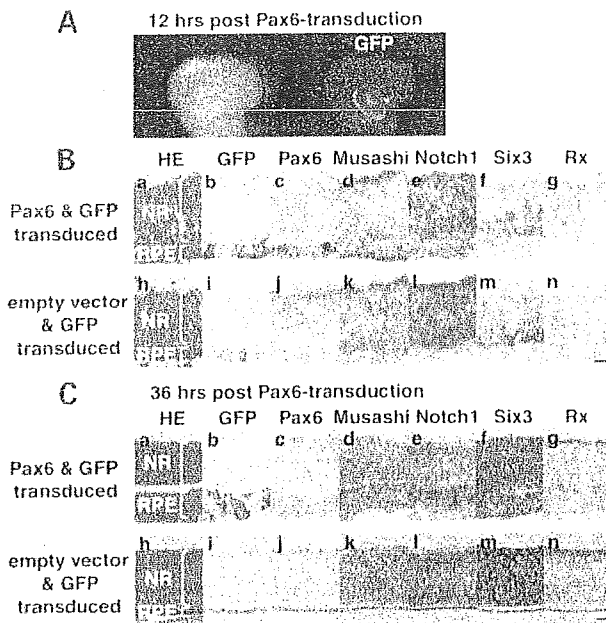
To analyze the effect of *Pax6* on RPE, expression plasmids that carry the human *Pax6* cDNAs were misexpressed in the RPE of chick embryos by *in ovo* electroporation (22). The *Pax6* gene produces two isoforms by alternative splicing: one with exon 5a and another without this exon. The variant 5a form has an additional 14 amino acid residues inserted into the DNA-binding domain, paired domain (PD) (23,24). We generated two plasmids carrying each isoform [pCAGGS-*Pax6*(–5a) or pCAGGS-*Pax6*(+5a)] (25–27).

Areas expressing the exogenous gene were monitored by signals of green fluorescence protein (GFP) by co-electroporating pCAGGS–GFP (Fig. 1A) (28).

When *Pax6*(–5a) or *Pax6*(+5a) was misexpressed in the RPE at stage 12–40, RPE cells were found to lose their intracellular pigments and form a thick cell layer 1–2 days after electroporation, whereas the control RPE, in which empty plasmid (pCAGGS) alone, pCAGGS–GFP or both constructs were electroporated, showed the normal morphology. Immunohistochemical analyses using anti-GFP and anti-*Pax6* antibodies detected distinct staining in the thickened RPE layer. Cross sections were subjected to *in situ* hybridization with probes specific for transcription factors or signaling molecules that regulate the proliferation of retinal progenitor cells and the specification of cell fate. *Musashi*, which encodes a neural RNA-binding protein, highly enriched in neural precursor cells (29). *Notch1*, which encodes a receptor for a signaling pathway, regulates neurogenesis (30). *Six3*, a homologue of *Drosophila* homeobox gene *sine oculis*, is early on expressed in the optic vesicle, turns off in the future pigment epithelium and becomes restricted to the prospective NR and to the lens placode. In the NR development, *Six3* is expressed in the entire undifferentiated neuroepithelium, then in differentiating cell layers including the inner and outer nuclear layer, and ganglion cell layer (31). *Rx*, a paired-class homeobox gene, is expressed early on in the optic vesicle and later on in the inner on nuclear layer, presumably bipolar cells of the developing NR (20). *In situ* hybridization detected signals for *Musashi*, *Notch1* and *Rx* 12 h after electroporation (Fig. 1B), then that for *Six3* 36 h after electroporation (Fig. 1C), suggesting that they transdifferentiate to NR. Signals for *Rx* in endogenous and ectopic retinas transiently decreased at a stage when bipolar cells do not yet differentiate. At these early phases post-*Pax6* transduction, immunohistochemical staining with antibodies against retinal cell markers cited below was yet undetectable (data not shown). When electroporation was performed even in stage 40 embryos, RPE cells were still found to transdifferentiate to NR. GFP fluorescence is no longer detectable 5–7 days after electroporation, as expression of GFP was terminated or faded out owing to cell growth. In a serial section of each eye at early phases after electroporation, NR transdifferentiation was seen only within the areas showing GFP fluorescence. Electroporation of the pCAGGS alone, pCAGGS–GFP or both constructs failed to induce transdifferentiation, suggesting that the *Pax6* gene alone is able to transdifferentiate NR from RPE cells without addition of FGFs or surgical manipulation. Embryos were unable to survive or hatch, when electroporation was performed at later than stage 40.

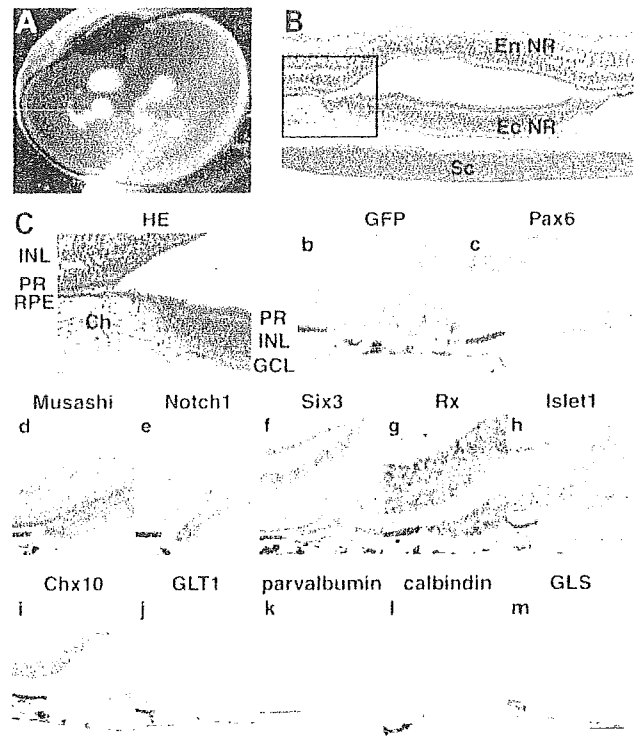
Four to five days after electroporation, formation of ectopic NR occurred as a wide sheet, but later in spotted areas, which scattered in the whole fundus (Fig. 2A). Sections showed that the ectopic NR is well differentiated, and the vertical direction of the transdifferentiated NR layers was reversed with PR inside and ganglion cells outside (Fig. 2B), similar to the FGF-treated eyes (5–8). Cross sections were subjected to *in situ* hybridization with probes specific for *Musashi*, *Notch1*, *Six3* and *Rx*. Sections also were subjected to immunohistochemistry with antibodies against retinal cell markers: *Islet1*, a homeodomain-containing transcription factor that is





**Figure 1.** Early phases of NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene. The *Pax6* and *GFP* genes were misexpressed into the outer layer of the optic cup of stage 18 chick embryos. Eyes were examined 12 h (A and B) and 36 h (C) after electroporation. (A) Expression of GFP in the outer layer of the right eye was examined using fluorescence microscopy. (B and C) Light microscopy [a, h; hematoxylin and eosin (HE) staining]. Immunohistochemistry with antibodies for GFP (b, i) and Pax6 (c, j). *In situ* hybridization with probes specific for *Musashi* (d, k), *Notch1* (e, l), *Six3* (f, m) and *Rx* (g, n). Eyes misexpressed with Pax6 (a–g) and controls (h–n). Pax6 in the thickened RPE layer are exogenous, whereas that in NR may be endogenous (c). Bars, 20  $\mu$ m.

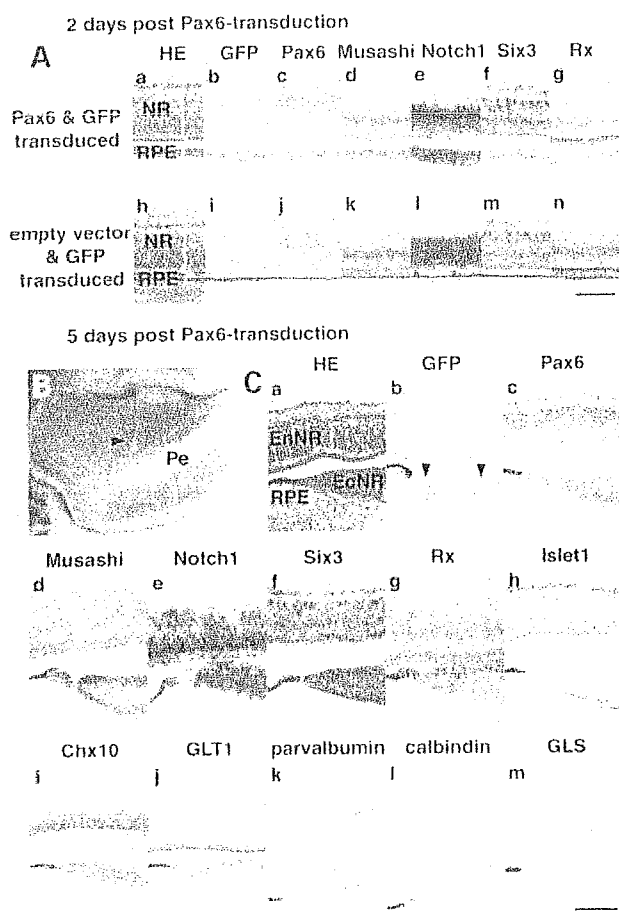
expressed in the ganglion cells in the developing retina (32); Chx10, a paired-type homeobox-containing transcription factor that is expressed in bipolar cells (33); glutamate transporter 1 that removes glutamine from the synaptic cleft and is expressed in bipolar cells and terminals of PR (34); parvalbumin, a low molecular weight calcium-binding protein that is expressed in amacrine cells (35); calbindin, a calcium binding protein involved in calcium transport that is expressed in horizontal cells (35,36) and glutamine synthetase that catalyzes the amination of glutamic acid to form glutamine and is highly enriched in Muller glial cells (37). The *in situ* hybridization and immunohistochemical staining resulted in distinct staining of each type of NR neuronal and glial cells, including PR, bipolar cells, amacrine cells, horizontal cells, ganglion cells and Muller cells, at correct layers (Fig. 2C), suggesting that they were well-differentiated NR as observed in the endogenous NR. The endogenous NR attaching to the ectopic NR is slightly thinner than that attaching to the intact RPE. Fully structured NR was formed through the fundus, albeit in small spotted areas, when Pax6 was misexpressed until stage 40 (Fig. 3B). Nearly mature RPE cells lose their intracellular pigments and form a thick NR layer, in which the neuronal cell-specific genes were expressed, when Pax6(-5a) or Pax6(+5a) was misexpressed in the RPE even at stage 35–40 (Fig. 3A). The *in situ* hybridization and immunohistochemistry also showed that the ectopic NR is



**Figure 2.** Fully structured NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene. The *Pax6* gene was misexpressed into the RPE layer of stage 24 chick embryos, and eyes were examined at stage 40. (A) In a half of the eyeball, patched areas of white swelling tissue are scattered. At this time, GFP is no longer detectable by fluorescence microscopy (data not shown). (B) Light microscopy [hematoxylin and eosin (HE) staining] of the white swelling tissue in (A). EnNR, the ectopic NR transdifferentiated from RPE; EnNR, the endogenous NR; Sc, the sclera. (C) Light microscopy (a; HE staining), immunohistochemistry with antibodies for GFP (b), Pax6 (c), Islet1 (h), Chx10 (i), glutamate transporter 1 (GLT1, j), parvalbumin (k), calbindin (l) and glutamine synthetase (GLS, m), and *in situ* hybridization against *Musashi* (d), *Notch1* (e), *Six3* (f) and *Rx* (g) in magnified fields of the boxed area in (B). GCL, the ganglion cells layer; INL, the inner nuclear layer; PR, photoreceptors and Ch, the choroid. Immunoproducts for GFP was detectable in few cells in the ectopic NR. Bar, 100  $\mu$ m. The results shown are representative of more than 200 independent experiments.

relatively well differentiated, which forms irregular laminar structure but contains each type of NR neuronal and glial cells (Fig. 3C).

Ectopic NR was identified histologically in 83% ( $n = 393$ ) of the eyes transduced with *Pax6* at stage 12–24 and in 68% ( $n = 196$ ) of eyes treated at stage 30–40. Fully structured ectopic NR was identified in 77% ( $n = 250$ ) of morphologically altered eyes treated at stage 12–24 and in 46% ( $n = 134$ ) of altered eyes treated at stage 30–40. Further details on the incidence of the Pax6-dependent eye architectural changes at each stage are available in Supplementary Material, Table S1. No difference was seen between two *Pax6* isoforms (either -5a or +5a) by the *in situ* hybridization and immunohistochemical analysis. Transduction of *Pax6* using an adenoviral vector or electroporation using lower dose of plasmid constructs caused similar, although somewhat weak, phenotypic changes (data not shown).



**Figure 3.** NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene at a late stage. The *Pax6* and *GFP* genes were misexpressed into the RPE layer at stage 35. Eyes were examined 2 days (at stage 37) (A) and 5 days (at stage 40) (B and C) post electroporation. (A) Light microscopy [a, h; hematoxylin and eosin (HE) staining], immunohistochemistry with antibodies for GFP (b, i) and Pax6 (c, j), and *in situ* hybridization with probes specific for *Musashi* (d, k), *Notch1* (e, l), *Six3* (f, m) and *Rx* (g, n). Pax6 in the thickened RPE layer are exogenous, whereas that in NR may be endogenous (c). (B) In a half of the eyeball, small areas of white swelling tissue (arrowhead) are detected. At this time, GFP is no longer detectable by fluorescence microscopy. Pe, the pecten. (C) Light microscopy (a; HE staining), immunohistochemistry for GFP (b), Pax6 (c), Islet1 (h), Chx10 (i), glutamate transporter 1 (GLT1, j), parvalbumin (k), calbindin (l) and glutamine synthetase (GLS, m), and *in situ* hybridization for *Musashi* (d), *Notch1* (e), *Six3* (f) and *Rx* (g). EcNR, the ectopic NR transdifferentiated from RPE; EnNR, the endogenous NR. GFP in the ectopic NR is expressed partially and weakly (b, arrowheads), whereas Pax6 is widely but in mottle (c). Bars in each, 100  $\mu$ m. The results shown are representative of more than 50 independent experiments.

#### Effect of missense mutations or repression of the *Pax6* gene in NR transdifferentiation

To identify the critical domains in the *Pax6* for the ectopic NR induction, we transduced several mutations into the *Pax6* gene and misexpressed them in the RPE of stage 12–40 embryos. For this purpose, we generated expression plasmids carrying several *Pax6* mutants, in which an amino acid is substituted in either the PD or the homeodomain (HD). Namely, (a)

F258S mutant with substitution in HD found in optic nerve anomaly (27) (Fig. 4A, (2)), (b) R26G mutant with an amino acid substitution in the N-terminal subdomain (NTS) of PD found in patients with anterior segment eye anomaly (38) [Fig. 4A, (3)] and (c) R128C mutant with amino acid substitution in the C-terminal subdomain (CTS) of PD found in foveal hypoplasia (39) [Fig. 4A, (4)]. Repression by these mutations of DNA-binding to respective binding-consensus motifs was already confirmed by an *in vitro* functional assay (25–27). When these mutants were misexpressed, only the F258S mutant, either with or without exon 5a, induced the RPE to NR conversion, yet with an incomplete layers structure (Fig. 4B and C). Other mutants failed to induce ectopic NR formation in more than 200 eyes we examined.

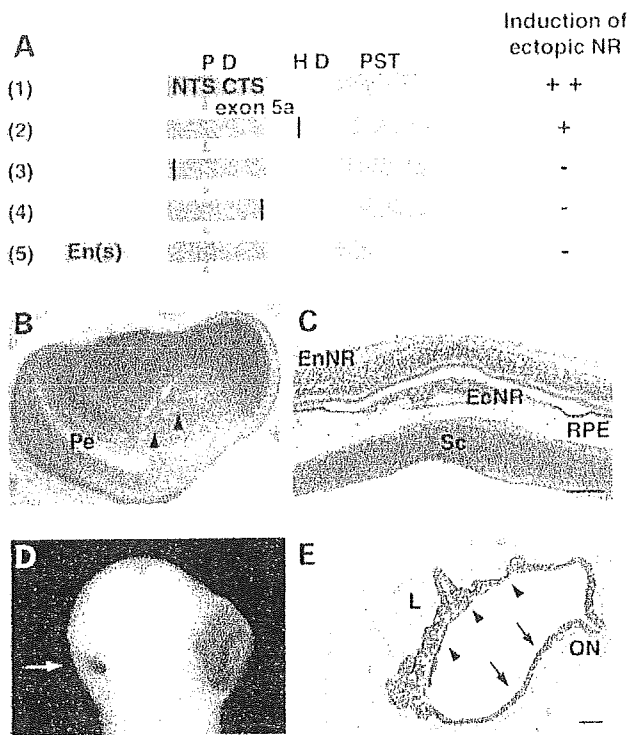
To analyze the effects induced by repression of the endogenous *Pax6* function in the development of NR and RPE, we next expressed a dominant-negative form of the gene into the early developing eye. For this purpose, we fused an *Engrailed* (*En*) repressor domain to Pax6delC+, in which the C-terminal proline–serine–threonine rich transactivation domain was deleted [En(s)–Pax6delC+, Fig. 4A, (5)] (40,41). When this mutant was expressed in the optic vesicle at stage 8–10, eye formation was totally disturbed, consequently resulting in anophthalmos (data not shown). In contrast, when this plasmid was electroporated in the optic cup at stage 12–18, microphthalmos was induced with relatively normal RPE, but with scarce, malformed NR (Fig. 4D and E). Consistent with previous results, these findings indicate that endogenous *Pax6* is important and pivotal for correct NR differentiation, but not for RPE development. The incidence of eye architectural changes by the transduction of each mutant at each developmental stage is available in Supplementary Material, Table S1.

These findings indicated that the ectopic retina was formed not as an artifact by electroporation procedure, but by function of misexpressed *Pax6*, and that PD, but not HD, is required for retinal transdifferentiation and ectopic NR formation.

#### *Pax6* is expressed in the ectopic NR transdifferentiated from RPE by FGFs treatment

According to previous protocols (5–8), we injected FGF2 or FGF8 protein or electroporated *Fgf-8* cDNA into mesenchymal tissue surrounding the eye of stage 12–40 chick embryos. In both cases, NR was transdifferentiated from RPE, and the vertical direction of its layers was again reversed (Fig. 5A–D) (data on FGF8 protein not shown), as observed in *Pax6* misexpression (Figs 1–3) and previous reports (5–8). Ectopic NR was identified histologically in 85% ( $n = 177$ ) and 67% ( $n = 159$ ) in FGF2 and FGF8 protein-treated eyes and 67% ( $n = 92$ ) in *Fgf-8* cDNA introduced eyes, respectively, and fully structured NR layers were found in 45% ( $n = 151$ ), 27% ( $n = 107$ ) and 25% ( $n = 63$ ) in morphologically altered eyes, only when FGF treatment was carried out before stage 24, whereas *Pax6*-mediated transdifferentiation can be induced until much later stages. In other cases, a mixture of various NR architectures including cell aggregation and rosettes was observed. We examined endogenous *Pax6* expression in ectopic NR by immunohistochemistry and confirmed that *Pax6* expression is induced in RPE cells 6–12 h

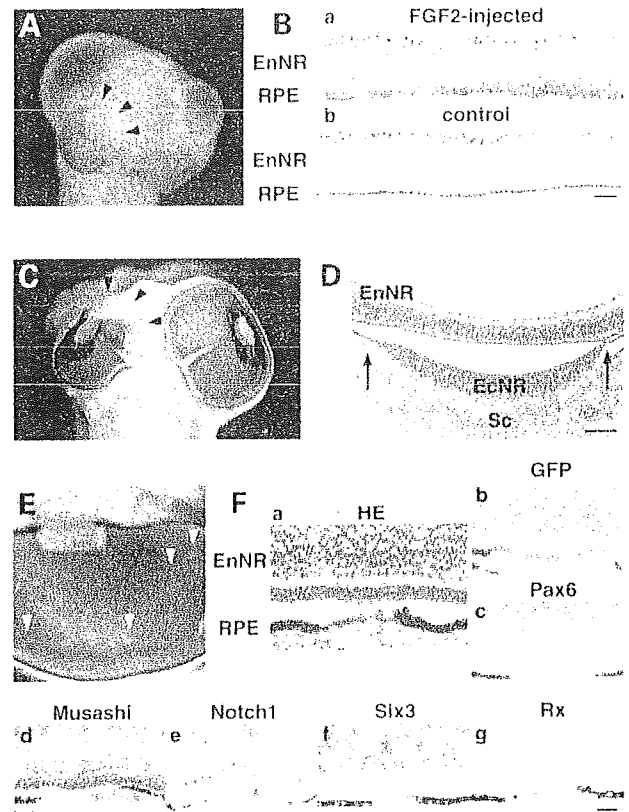




**Figure 4.** Effect of missense mutations or repression of the Pax6 gene in NR transdifferentiation. (A) Structure of the Pax6 cDNA (1), Pax6 mutants [F258S (2), R26G (3) and R128C (4)] and En(s)-Pax6delC+ (5) used in these studies. The effects of the mutants and repression on NR transdifferentiation are also summarized. PD, paired domain [red, N-terminal subdomain (NTS); purple, C-terminal subdomain (CTS); red triangle, exon 5a]; HD, homeodomain; PST, proline-serine-threonine rich transactivating domain; En(s), En repression domain. (B and C) A stage 40 chick embryo, in which a Pax6 mutant F258S was misexpressed in RPE at stage 24. (B) A half of the eyeball shows linear areas of white tissue (arrowheads) were scattered. Pe, the pecten. (C) Light microscopy [hematoxylin and eosin (HE) staining] shows the ectopic NR (EcNR) that contains rosettes was transdifferentiated from RPE. EnNR, the endogenous NR; Sc, the sclera. Bar, 100  $\mu$ m. (D and E) The Pax6 suppressant, pCAGGS-En(s)-Pax6delC+, was misexpressed by electroporation into the right eye of stage 18 chick embryos, and the resulting morphology was examined at stage 28. (D) The right eye developed microphthalmos (arrow) as evident in comparison with the normally developed eye on the other side. (E) The endogenous NR retina is absent, while in contrast, development of RPE (arrows) and the ciliary body (arrowheads) are less disturbed (HE staining). L, the lens; ON, the presumable optic nerve. Bar, 50  $\mu$ m. Each result shown is representative of more than 50 independent experiments.

after FGFs treatment, at which the cells began to switch their phenotype (Fig. 5A and B).

Next, we co-electroporated two expression plasmids that contain *Fgf-8* cDNA and dominant-negative Pax6 (En(s)-Pax6delC+) into the developing eye. In this case, only a few small spots of white tissue were formed (Fig. 5E). Histological analysis showed immature NR formation. *In situ* hybridization signals for *Musashi* and *Notch1* were distinctly positive and those for *Six3* and *Rx* were faint (Fig. 5F), whereas immunohistochemical staining with antibodies against retinal cell markers was not detectable (data not shown), indicating that NR differentiation of RPE was premature and incomplete. These findings suggest that Pax6 mediates ectopic NR formation by FGFs treatment. The

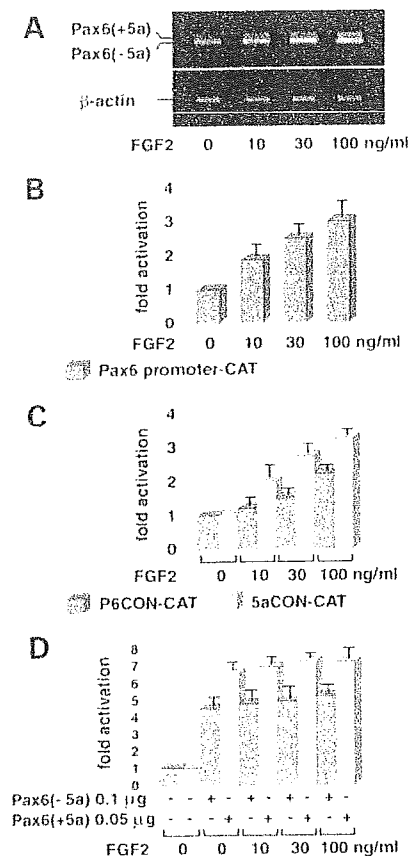


**Figure 5.** Expression of Pax6 in the ectopic NR transdifferentiated from RPE by FGFs treatment. (A and B) A stage 20 embryo, in which FGF2 was injected 12 h before (stage 18). (A) In the anterior half of the eye, the RPE layers lose pigments (arrowheads). (B) Immunohistochemistry shows expression of Pax6 in the endogenous NR (EnNR) and RPE of the eye treated with FGF2 that begins to transdifferentiate (a), but only in EnNR in the control tissue (b). Bar, 20  $\mu$ m. (C and D) A stage 30 chick embryo, in which *Fgf-8* DNA was electroporated into RPE at stage 18. (C) A half of the eyeball shows that the anterior portion of RPE transdifferentiates to NR (arrowheads). (D) Light microscopy [hematoxylin and eosin (HE) staining] shows the layers of the ectopic NR (EcNR) in the back match with those of the endogenous NR (EnNR). Sc, the sclera; arrows, transition portion of RPE and the ectopic NR. Bar, 100  $\mu$ m. (E and F) A stage 30 chick embryo, in which *Fgf-8* expression plasmid, a Pax6 dominant-negative form expression plasmid [pCAGGS-En(s)-Pax6delC+] and GFP expression plasmid were co-electroporated into RPE at stage 18. (E) Small spots of white tissue (arrowheads) scattered in the fundus were formed. GFP is undetectable by fluorescence microscopy (data not shown). (F) Light microscopy (a; HE staining) shows loss of pigments and morphological change in RPE cells. Immunohistochemistry for anti-GFP (b) and anti-Pax6 (c) antibodies and *in situ* hybridization against *Musashi* (d), *Notch1* (e), *Six3* (f) and *Rx* (g). Compared with number of GFP-positive cells, Pax6 is expressed rarely and weakly in morphologically altered RPE cells, although the anti-Pax6 antibody detects both endogenous Pax6 and exogenous En(s)-Pax6delC+. Bar, 20  $\mu$ m. Each result shown is representative of more than 10 independent experiments.

incidence of the FGFs-dependent eye architectural changes at each stage is available in Supplementary Material, Table S1.

#### FGFs upregulates Pax6 in mouse embryonic carcinoma P19 cells

To investigate the effects of FGF signaling on Pax6 expression, we performed an *in vitro* functional assay using



**Figure 6.** Effect of FGFs on Pax6 expression by functional assay. (A) Semi-quantitative analyses for expression levels of endogenous Pax6 by RT-PCR in P19 cells treated with FGF2. The results shown are representative of three independent experiments. (B and C) CAT activities in P19 cells after transfection of a Pax6-promoter (B), P6CON or 5aCON reporter construct (C) and treatment with FGF2. (D) CAT activities in P19 cells after co-transfection of a small amount of Pax6 [0.1 μg of Pax6(-5a) or 0.05 μg of Pax6(+5a)] and P6CON- or 5aCON-CAT reporter plasmids. The results shown are an average of three independent experiments.

mouse embryonic carcinoma P19 cells that are frequently used for functional analysis of the Pax6 gene. From P19 cells cultured for 3 days in a medium containing FGF2 or FGF8 protein, total RNA was isolated and reverse-transcribed to cDNA. This cDNA mixture was then amplified for semi-quantitative PCR using specific primers for mouse Pax6. When cells were cultured with an increasing amount of FGF2 or FGF8, PCR products corresponding to both the Pax6(+5a) and the Pax6(-5a) increased in a dose-dependent manner (Fig. 6A) (data on FGF8 not shown), indicating that FGF signaling upregulates expression of endogenous Pax6 in this system. Using a chloramphenicol acetyltransferase (CAT) reporter construct carrying ~2 kb genomic DNA upstream of the Pax6 initiation codon, in which various control elements were found (42), activity of Pax6 promoter was quantified after addition of FGF2 or FGF8 protein. When an increasing amount of FGFs was added into the medium, the CAT activities increased in a dose-dependent manner (Fig. 6B), indicating that FGF signaling stimulates the transcription of the Pax6 gene.

To see whether FGFs induce the production of functionally active Pax6 proteins, we next transfected CAT reporter plasmids carrying six copies of P6CON or two copies of 5aCON, the consensus binding sequences of the NTS or CTS of Pax6 PD (24–26), respectively. As mentioned earlier, two Pax6 isoforms were produced by alternative splicing [Pax6(+5a) or Pax6(-5a)] (23). These structural differences affect DNA-binding configuration, namely, the NTS mainly functions as a DNA-binding domain in Pax6(-5a) and the CTS in Pax6(+5a) (24,25). Hence, Pax6(+5a) binds to 5aCON, whereas Pax6(-5a) binds to P6CON. When cells were cultured with an increasing amount of each FGF, both P6CON- and 5aCON-CAT activities increased in a dose-dependent manner (Fig. 6C), compatible with the idea that FGFs stimulate Pax6-dependent transcription. To explore the possibility that FGFs may also regulate Pax6 activity at a post-transcriptional level, small amounts of pCAGGS-Pax6(-5a) or pCAGGS-Pax6(+5a) were co-transfected along with P6CON- or 5aCON-CAT reporter plasmids, respectively. CAT activities were several folds higher and were not activated significantly by further addition of FGFs at various concentrations (Fig. 6D), suggesting that the stimulatory effect of FGFs on Pax6 is mainly at the transcriptional level. Overall, these data indicate that Pax6 is one of downstream targets of FGF signaling.

## DISCUSSION

Our studies clearly showed that Pax6 alone is sufficient to induce transdifferentiation of ectopic NR from RPE. Reflecting evolutionary conservation of the amino acid sequence of the Pax6 protein, the human Pax6 acts well in chicken cells. At an early stage of eye development (e.g. stage 10–12 of a chick embryo and 4–5 weeks human gestation), Pax6 is expressed in both inner and outer layers of the optic cup, the respective future NR and RPE. Then, this gene is widely expressed in multipotential progenitor cells in the primitive NR, although its expression disappears rapidly from RPE (43,44). Transduction of the dominant-negative Pax6 in the optic cup induced premature and scarce NR, yet leaving RPE layer relatively normal. These findings suggest that Pax6 is required for the specification of NR and RPE and for the maturation of NR, but not for the maturation of RPE (45). RPE cells differentiate and mature at earlier stages than NR. Nonetheless, as we have shown, even nearly mature RPE cells can lose their phenotype and re-differentiate to complete NR when Pax6 was misexpressed. It remains to be elucidated whether Pax6 *per se* triggers de-differentiation of RPE and converts its fate to re-differentiate to NR cells or whether this gene initiates genetic cascade for NR formation by repressing that for RPE formation. In either case, once initiated by Pax6, a set of endogenous genes begins to start the pathway of NR formation. *In situ* hybridization showed ectopic expression of some transcription factors or signaling molecules that regulate the proliferation of NR progenitors and the specification of cell fate. Immunohistochemistry with antibodies against retinal cell markers identified each type of neuronal and glial cells at correct layers in the ectopic NR, although the vertical direction of the ectopic

NR layers was in a back match with that of the endogenous NR, corresponding to the direction of optic cup layers.

Compared with the Pax6-induced large-scale phenotypic changes and uniform expression of NR-specific markers in correct layers of the ectopic NR, GFP expression was restricted in a small number of cells (Figs 2C and 3C). It is likely that GFP faded out in cells that had rapidly proliferated and differentiated but still stayed in cells that had slowly proliferated. There is another possibility that Pax6 may induce the ectopic NR tissue in a cell non-autonomous manner. Pax6 may do so by activating the transcription of a diffusible factor that triggers NR tissue formation. The former idea is consistent with a study in *Xenopus laevis*. The cell autonomous activity of Pax6 misexpressed in *Xenopus* is thought to cause ectopic eye formation and ectopic expression of genes that relate to eye development including *Rx*, *Otx2*, *Six3* and endogenous Pax6 (11).

An opposite finding of the present study has been reported: combination of loss-of-function of Pax6 and Pax2 in the optic vesicle results in transdifferentiation of presumptive RPE to NR (46). The finding physiologically places Pax6 upstream of MITF and as a pro-RPE factor. This does not conflict with our data, however, because we transduced Pax6 into under-maturing RPE, in which endogenous Pax6 had been already downregulated. Probably, there may be differences in Pax6 function depending on timing.

Ectopic eye- or NR-like architecture is also induced by the misexpression of other homeobox transcription factors. Ectopic expression of Six3, a vertebrate functional orthologue of the *Drosophila* gene *sine oculis*, or Six6 that is closely related to Six3 (47) induces the formation of ectopic optic vesicle- or NR-like architectures in the brains of the fish, *Xenopus* and mouse embryos (15–17). Ectopic Six6 expression in embryonic or mature chicken RPE cells also results in a neuronal morphology and expression of markers characteristic of developing NR (18). *Xenopus* embryos injected with synthetic *Rx* RNA develop ectopic retinal tissue (20). However, fully structured NR, as induced by Pax6 misexpression, has not been yet obtained. Expression of endogenous Six3 and *Rx* in ectopic NR at the early phases post-Pax6 transduction, as shown in Figure 1, suggests that Pax6 regulates Six3 and *Rx* in the field of NR transdifferentiation, as in Pax6-induced ectopic eye formation in *Xenopus* embryo (11). Pax6 may be critical to induce a set of transcription factors that form NR laminar structure because of very high incidence of fully structured NR induced by the gene transduction.

The Pax6 protein has two DNA-binding domains, PD and HD (48–50). In PD, two structurally distinct subdomains, NTS and CTS, bind respective consensus sequences (23,24), and an insertion of additional 14 amino acid residues encoded by exon 5a in the NTS abolishes the NTS function and enhances the transactivation activity via CTS (25,26). Thus, exon 5a probably functions as a molecular switch to select specific targets. Recently, we found functional differences of the two isoforms in NR development: Pax6(-5a) is expressed in the entire NR, whereas Pax6(+5a) is especially in the NR portion where visual cells accumulate during eye development. Pax6(+5a) promotes the NR growth and, when overexpressed, induces an excessive well-differentiated

NR-like architecture, whereas Pax6(-5a) shows much weaker effect (51). In the present *in ovo* misexpression study, however, no difference was seen between two Pax6 isoforms with respect to their abilities to trigger NR transdifferentiation. One explanation for this is that the two isoforms may initiate the same genetic cascade via distinct pathways, possibly through control of partially overlapping target genes. Another explanation is based on the evidence of feedback regulation of Pax6 expression. Transcription of the Pax6 gene is intricately regulated via three promoters and a number of tissue-specific enhancers. Recently, several short sequences that closely match the Pax6 binding consensus (P6CON) were identified in *Drosophila* and vertebrate enhancers that drive Pax6 expression in the nervous system and eye, and it was suggested that these evolutionarily conserved P6CON sites may mediate the auto-activation of Pax6 by Pax6(-5a) (52). If so, both isoforms would be expressed after transduction of Pax6(-5a). Such a mechanism may account for similar phenotypic manifestation after transduction of Pax6(-5a) or Pax6(+5a). Although binding consensus sequences of the PD have been studied, little is known about its target genes, especially those recognized by CTS. This issue needs to be addressed to understand the mechanism of NR transdifferentiation by Pax6.

It has been considered that RPE is necessary for correct morphogenesis of NR in early stages and for organization of its layers by end of gestation, although signaling molecules emanating from RPE are not elucidated. Data obtained from organ culture suggest that RPE organizes the laminar structure of the differentiated NR (53). Transgenic mice expressing attenuated diphtheria toxin-A in RPE exhibit malformed RPE and disorganized NR (54). In contrast, our studies indicate that fully structured NR can be formed endogenously and ectopically, even though RPE is absent in areas of NR transdifferentiation from RPE. This suggests that RPE is not involved in the NR layers formation, but rather controls nutrition supply and/or cell proliferation at later stages. Compatible with this, ectopic NR is thinner than the normal NR, yet the laminar structure is clearly formed (Fig. 2).

Because primitive RPE and NR are contiguous in the optic vesicle, RPE cells has been considered as a possible candidate for a source of stem cells required for NR transdifferentiation (55). The retinogenic potential may be still preserved in RPE cells even in adult eyes, because RPE of chicken or other eye tissues, such as pigmented ciliary margin cells of mice and iris tissues of rats, generate immature NR-specific cells (3,4,18). In contrast, it has been thought that fully structured NR is generated from RPE only at early stages of development except for in amphibian eyes (5–8). However, our studies showed that RPE has the potential even at late stages. Pax6 induces the complete conversion from RPE to NR even at HH stage 40, whereas FGFs are able to transform RPE only before stage 24. As ectopic NR can be formed in broad and numerous spots at early stages (Fig. 2A), retinogenic RPE cells appear to be distributed widely throughout the RPE layer. In contrast, NR transdifferentiation was seen as small spotted areas at later stages, although expression of the exogenous gene monitored by GFP were detected in wider areas. This suggests that areas of NR transdifferentiation decrease not by inefficiency of gene transfer in late-stage-embryos. Retinogenic stem cells

may decrease in number as the RPE matures, as observed in mammalian brains (56), but be preserved widely even in late stages.

Transdifferentiation of NR from RPE by FGF treatment is a well-known phenomenon (5–8). Transcription factors or signaling cascade components that lie downstream of FGFs have been clarified recently. Switching of RPE to a neuronal fate by FGF8 is coupled with the induction of NR genes such as *Rx*, *Sgx-1* and *Fgf-8* itself (7). Switching of RPE to a neuronal fate by FGF9 is mediated by the Ras-Raf-MAPK pathway (8). It is very likely for several reasons that transdifferentiation of NR from RPE by FGFs is also mediated by increased expression of Pax6. First, Pax6 is strongly induced in RPE cells by FGF treatment (Fig. 5A and B). Secondly, transdifferentiation of NR from RPE by FGF8 is significantly disturbed by co-expression of dominant-negative Pax6 (Fig. 5E and F). Thirdly, *in vitro* assays using P19 cells demonstrate the upregulation of Pax6 expression by FGFs (Fig. 6A and B). Finally, P6CON- and 5aCON-CAT reporters are activated by FGF treatment in a dose-dependent manner (Fig. 6C). Because CAT activities of P6CON- and 5aCON-CAT reporters did not significantly respond to even high concentrations of FGFs when a small amount of Pax6 was introduced exogenously (Fig. 6D), FGFs induce expression of the Pax6 gene, but do not affect the transactivation potential of its gene product. Pax6 activity is also known to be controlled by FGF8 in somitogenesis (57). In this case, however, expression of Pax6 is suppressed by FGF signaling and is induced at the anterior limit of FGF expression that regresses caudally. Hence, regulatory relationship between Pax6 and FGF signaling may be different in these tissues.

The present study clarified roles of the Pax6 gene in ectopic NR formation, by itself and under a control of FGFs signaling. Further investigation using the mouse and rat eyes is under way, and Pax6-dependent NR transdifferentiation from RPE cells also has been preliminarily detected (data not shown). Our studies provide a new cue to regenerate functional NR in the eye with congenital anomalies or acquired degenerations by transfer of the Pax6 gene. Clinically, the RPE in the anterior portion of eye can be obtained easily by surgical procedures of peripheral iridectomy. NR reproduced from the retinogenic stem cells obtained from perinatal eyes would be a new therapeutic tool for reproduction and transplantation of functional NR tissues. Further steps to induce projection to a suitable portion in CNS are necessary to obtain useful vision. However, advanced surgical technique of experimental and clinical NR transplantation recently is achieving successful survival of the donor NR and visual improvement (58,59). Thus, reproduction of functional NR by use of Pax6 and RPE cells may be at least contribute to resurrect light sensation and visual field in patients who suffer from damaged NR and blindness.

## MATERIALS AND METHODS

### Expression and suppression plasmids

Expression plasmids ([pCAGGS-Pax6(-5a) and pCAGGS-Pax6(+5a)] to produce the entire human Pax6 coding region with or without exon 5a, under the control of a cytomegalo-

virus enhancer and a chicken  $\beta$ -actin promoter, were previously described (25,26). The mutant forms of Pax6 expression plasmid were generated by PCR-based *in vitro* mutagenesis (25–27). To produce a Pax6 suppression plasmid, a fragment carrying *En* repression domain (40) was connected to the N-terminal fragment of mouse Pax6 cDNA (*Bam*HI–*Acc*II sites that contains 1–928 nucleotides) (41) and inserted into the *Bgl*II–*Xho*I sites of pCAGGS. Expression plasmid (pCAGGS-Fgf-8) to produce the entire *Fgf-8* coding region was generated by inserting chicken *Fgf-8* cDNA cloned by RT-PCR into pCAGGS.

### In ovo electroporation

Each Pax6 expression or suppression plasmid cited above was electroporated into a chick embryo at stage 8–40 together with the pCAGGS-GFP plasmid to monitor incorporation of DNA (22,28). For electroporation, a CUY 21 electroporator (BEX) with platinum electrodes was used. A small window was opened on the stage 12 fertilized eggs for access, and embryos were allowed to develop in humidified incubators after sealing the window. At stage 12, 18, 24, 30, 35 and 40 (we used 100 embryos for each stage), the window of eggshells was unsealed and phosphate buffered saline was poured over the embryo to obtain the appropriate resistance. After injecting DNA solution into the outer coat of the eye with a sharp glass pipette, the head of the embryo was placed between the electrodes and electric pulses were applied (25–40 V, 90 ms, 1–6 times). The eggshells were sealed again and embryos were allowed to develop in humidified incubators. Eyes were incised 1–10 days after electroporation (stage 18–45) and fixed in 4% paraformaldehyde. Eight micrometer frozen sections were prepared for immunohistochemistry and *in situ* hybridization.

### In ovo injection of FGFs

FGF2 and FGF8 recombinant proteins were purchased from Genzyme. Fertilized eggs were purchased from Nisseizai (Tokyo). A small window was opened for access, then phosphate buffered saline was poured over the embryo to preserve humidity. Each FGF at a concentration of 10–100 ng/ml was injected into the mesenchymes around the eyes of HH12–40 chick embryos with a sharp glass pipette. The eggshells were sealed and embryos were allowed to develop in humidified incubators.

### In situ hybridization and immunohistochemistry

Section *in situ* hybridization was performed as described (60). Probes were prepared from plasmids containing chick *Notch1* (*Spe*I, T7 polymerase), *Musashi* (*Eco*R1, T7), *Six3* (*Hind*III, T3) and *Rx* (*Hind*III, T3). A monoclonal antibody against Pax6 protein was gifted by Dr Fujisawa (43). A monoclonal antibody against Islet1 protein was purchased from DSHB, which against Chx10 protein from Exalpha Biologicals, that against glutamate transporter 1 from Affinity BioReagents, that against parvalbumin from Sigma and that against glutamine synthetase from BD Transduction Laboratories. Tissues from chick embryo were fixed in 4% paraformaldehyde.

Eight micrometer of frozen sections were stained immunohistochemically using a method described previously (61).

#### Cell culture and RNA detection by RT-PCR

Mouse embryonic carcinoma P19 cells were maintained in MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells at a density of  $1 \times 10^5$  cells per 35 mm Petri dish were maintained in MEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. For each dish, 1, 10 or 100 ng/ml of either FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. After 3 days, total RNA was isolated from cells in each dish using an RNA easy Mini Kit (Qiagen) and converted to cDNA by a standard procedure using SuperScript II RNase H- reverse transcriptase and adaptor primers (GibcoBRL) (62). DNA segments for mouse *Pax6* and  $\beta$ -*actin* were amplified in 30 and 19 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min with the following primers: mouse *Pax6*-forward primer 5'-CACAGCGGAGTGAATCAGCTTG-3' and reverse primer 5'-CCAGAATTTTACTCACACAACCGT-3' [respective product size:160 bp for *Pax6*(-5a) and 202 bp for *Pax6*(+5a)];  $\beta$ -*actin*-forward primer 5'-GTGGGCCGCC TAGGCACCA and reverse primer 5'-CTCTTTGATGTC ACGCAGGATTC (product size:540 bp).

#### Reporter plasmid

To obtain clones carrying the promoter region of the *Pax6* gene, we first screened the human BAC Library (Research Genetics) and detected one clone (32H10). A *Hind*III-*Psh*AI fragment carrying ~2 kb *Pax6* promoter region (1285–3381 nucleotides in GenBank accession no. U63833) was excised and inserted into the *Hind*III-*Sal*I sites of pCAT Basic (Promega). The insert was verified by sequencing as having the reported sequence. CAT reporter constructs carrying six copies of P6CON or two copies of 5aCON were reported previously (23–25).

#### Transient transfection and CAT assay

P19 cells at a density of  $5 \times 10^5$  cells per 60 mm petri dish were transfected with 0.5  $\mu$ g of reporter plasmid (*Pax6* promoter, P6CON or 5aCON) and 0.05  $\mu$ g of pSV $\beta$ gal (Promega) as an internal control coated with polycationic liposome (Lipofectoamine Plus, Life Technology) according to the manufacturer's instruction. For each dish, 10, 30 or 100 ng/ml of FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. Cell extracts were prepared after 72 h and assayed for CAT activities using FAST CAT Green Reagent (Molecular Probes) according to the standard procedure (62). The CAT activity was quantified by measurement with a phosphor-imager (Molecular Dynamics) and illustrated in a fold-activation compared with the condition without application of FGF.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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BRIEF COMMUNICATION

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## Infantile Case of Occlusive Microvascular Retinopathy After Bone Marrow Transplantation

Eriko Kawase<sup>1</sup>, Noriyuki Azuma<sup>1</sup>, Yoko Shioda<sup>2</sup>, and Masaaki Kumagai<sup>2</sup>

<sup>1</sup>Department of Ophthalmology, National Center for Child Health and Development, Tokyo, Japan; <sup>2</sup>Department of Pediatric Oncology, National Center for Child Health and Development, Tokyo, Japan

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### Abstract

**Background:** A variety of anterior and posterior segment ocular complications following bone marrow transplantation (BMT) have been well documented in adults and children, but retinal complications after BMT in infants have rarely been reported.

**Case:** A 6-month-old male infant developed occlusive microvascular retinopathy after BMT to treat acute lymphocytic leukemia.

**Observations:** Four months after the transplantation, retinal edema, hemorrhage, soft exudates, and neovascularization were found in the posterior pole fundus of the right eye and in the peripheral fundus of the left eye. After oral prednisolone was administered, the retinal lesions regressed and cicatrices with chorioretinal atrophy and fibrous tissue formed.

**Conclusions:** Neovascularization following occlusive microvascular retinopathy after BMT in infant eyes responds well to oral prednisolone. The visual prognosis depends on the foveal involvement of the retinopathy. *Jpn J Ophthalmol* 2005;49:318-320 © Japanese Ophthalmological Society 2005

**Key Words:** bone marrow transplantation, graft-versus-host disease, infant, occlusive microvascular retinopathy

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### Introduction

Bone marrow transplantation (BMT) has been used to successfully treat a variety of hematologic malignancies and severe aplastic anemia. However, anterior and posterior ocular complications associated with BMT have been reported both in adult and pediatric patients. The anterior complications, including keratoconjunctivitis sicca, corneal infections, and cataract, frequently occur. The use of high-dose anticancer agents, steroids, and total-body irradiation (TBI) are suggested to be the principal causative factors of the complications. Graft-versus-host disease (GVHD) also causes these complications. Following BMT, the patients are

predisposed to develop GVHD, because the donor graft mounts an immunologic response against the immunocompromised host. The posterior segment is also affected by BMT-related complications such as retinal occlusive microvasculopathy;<sup>1-4</sup> however, this is very rare in pediatric patients.<sup>4</sup> We report a case of an infant with occlusive microvascular retinopathy and neovascularization after BMT.

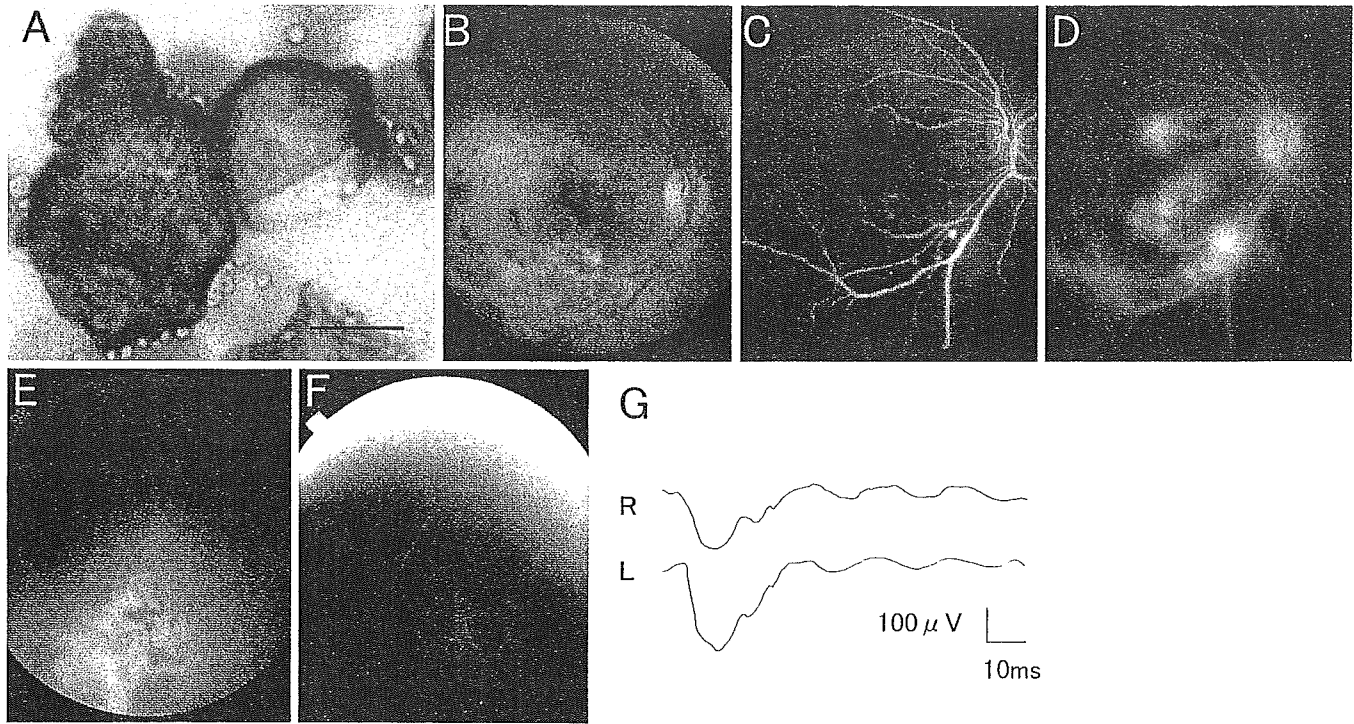
### Case Report

A 1-month-old male infant was brought to our hospital with loss of appetite and anemia, found at a screening examination. Laboratory analysis of the peripheral blood showed that the white blood cell count was 27 700/ $\mu$ l, the hemoglobin was 7.5 g/dl, and the blood platelet count was 50 000/ $\mu$ l during the first examination at our Department of Pediatric Oncology. The infant was diagnosed with acute lymphocytic

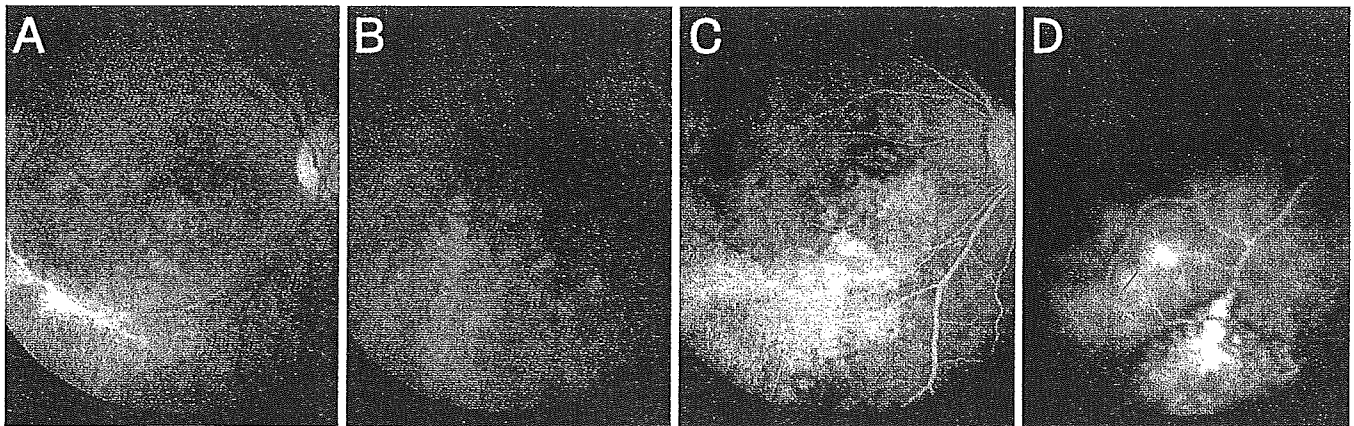
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Correspondence and reprint requests to: Noriyuki Azuma, Department of Ophthalmology, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan  
e-mail: kawase@eye-center.org



**Figure 1.** **A** Leukemic blasts obtained from bone marrow (May-Giemsa staining). Bar = 5  $\mu$ m. **B** Fundus photography of the right eye 4 months after BMT. Retinal edema, intraretinal hemorrhage, soft exudates, and lipid deposits are mainly in the central retina. **C** Early-stage fluorescein angiography of the right eye shows fluorescein dye leakage from the retinal vessels and microvascular abnormalities. **D** Late-stage fluorescein angiography of the right eye shows expansion of dye leakage indicating neovascularization. **E** Fundus photography of the left eye 4 months after BMT. The same findings as in the right eye are seen locally in the nasal periphery. **F** Fluorescein angiography of the left eye shows the same findings as in the right eye at the nasal periphery. **G** Electroretinography with a single flash stimulus of 25 J with a red filter shows normal responses from the left eye and reduced A and B wave amplitudes from the right eye. (100  $\mu$ V  $\times$  10ms).



**Figure 2.** **A** Fundus photography of the right eye 3 months after prednisolone treatment. The chorioretinal atrophy remains in the posterior fundus and inferior branched arcade vessel sheathing. **B** Fundus photography of the left eye 3 months after prednisolone treatment shows chorioretinal atrophy is in the nasal periphery. **C** Fluorescein angiography of the right eye 3 months after treatment with oral prednisolone shows fluorescein staining in the chorioretinal atrophy area and branched vessel infarction. **D** Fluorescein angiography of the left eye 3 months after treatment with oral prednisolone shows fluorescein staining in the chorioretinal atrophy area and branched vessel infarction in the nasal periphery.

leukemia (ALL). By bone marrow cytological analysis, the blast cells showed the rearrangement of the mixed lineage leukemia (*MLL*) gene (Fig. 1A). Immunohistochemical analysis showed that the blast cells were negative for CD10 but positive for CD19 and HLA-DR. Chromosomal analysis identified translocation of chromosomes 5 to 11 [t(5:11)(q31;q23)]. The patient initially underwent three sessions of chemotherapy with etoposide, cytosine arabinoside, and mitoxantrone, according to the protocol of the MLL98 study.<sup>5</sup> After pretreatment with whole-body irradiation (12 Gy) and administration of etoposide and cyclophosphamide, BMT was performed when the patient was 6 months old. Despite administration of tacrolimus and methotrexate to prevent GVHD, acute GVHD developed in the liver and skin 6 days after BMT. The GVHD gradually improved after administration of intravenous methylprednisolone. Careful examinations were performed by pediatricians, dermatologists, and ophthalmologists.

Four months after BMT, edema, hemorrhage, and soft exudates appeared widely in the posterior retina of the right eye (Fig. 1B) and locally in the periphery of the left eye (Fig. 1E); the anterior segment was normal. Fluorescein angiography showed some areas of nonperfusion of the capillary vessels and dye leakage, which indicated neovascularization of the retinal vessels (Fig. 1C, D, F). Electroretinography showed normal responses in the left eye and reduced A and B wave amplitudes in the right eye (Fig. 1G). Bone marrow aspiration indicated that the donor cells had survived and there was no recurrence of ALL; however, mild liver damage from the GVHD was still observed. Seven months after BMT, inflammation of the skin developed on the trunk, legs, arms, and face. Biopsy showed invasion of inflammatory cells into the subcutaneous tissue, suggesting chronic GVHD. Following administration of oral prednisolone (5 mg/kg per day), the retinopathy cicatrized to fibrous tissue and chorioretinal atrophy (Fig. 2A, B). The retinopathy gradually improved, and the dye leakage on fluorescein angiography stopped (Fig. 2C, D). The following fixation in the right eye was poor, and the visual evoked potential (obtained by flash stimulus of 1.2 J in a dark room) was also poor, while the responses of the right eye were normal. At 2 years of age, the patient's corrected visual acuity was 0.01 in the right eye and 0.5 in the left eye.

## Discussion

Posterior segment complications have rarely been reported in pediatric patients after BMT. Suh et al.<sup>4</sup> surveyed ocular findings in 104 pediatric patients after BMT. In 14 of the 104 patients, the posterior segment had radiation retinopathy, disc edema, cytomegalovirus retinitis, and nocardia retinitis. Microvascular occlusive retinopathy developed in only four patients, aged 10 to 18 years, and there were no infants. In the 14 patients, telangiectasia, hemorrhages, cotton-wool spots, lipid exudates in the retina, macular edema, papillitis,

and optic atrophy were seen, as is common in adults, but neovascularization, capillary nonperfusion, vitreous hemorrhage, tractional retinal detachment, and neovascular glaucoma did not develop.

Microvascular retinopathy after BMT occurs as the result of various mechanisms, including GVHD, total-body irradiation, immunosuppression, and administration of high-dose anticancer agents.<sup>3</sup> Our patient underwent total-body irradiation, received immunosuppressant and high-dose anticancer agents, and developed GVHD. Because the GVHD and occlusive microvascular retinopathy occurred simultaneously, the retinopathy in our patient was inferred to be mainly caused by the GVHD. Cyclosporine may worsen occlusive microvascular retinopathy because of its toxic effects, and total-body irradiation may modify the retinopathy, but it takes about 1 year for these changes to occur.<sup>3</sup> Thus, early occurrence of retinopathy in our patient also supports the theory that the retinopathy originated from the GVHD.

As a mechanism of chronic GVHD, the endothelia of the retinal vessels can be damaged by a lymphocyte-mediated immunologic attack that results in capillary occlusive retinopathy.<sup>2,3</sup> In our patient, occlusive microvascular retinopathy after BMT might have resulted from injury of the immature vessels in the neonatal and infantile eyes. Neovascularization was minimized not by the use of photocoagulation but by administration of oral prednisolone, because the occlusion of the capillary vessels occurred in a small area.

The visual prognosis after occlusive microvascular retinopathy after BMT is usually excellent, because lesions in the posterior retina are small and the fovea is rarely affected. However, the development of extensive neovascularization that covers the posterior retina is serious and vision threatening. As BMT has become an increasingly successful treatment and its indication is expanding in infantile patients, ophthalmologic evaluation followed by careful management is important for improving the visual prognosis after retinopathy.

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Yumi Suzuki  
Eriko Kawase  
Sachiko Nishina  
Noriyuki Azuma

## Two patients with different features of congenital optic disc anomalies in the two eyes

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Y. Suzuki · E. Kawase · S. Nishina ·  
N. Azuma (✉)  
Department of Ophthalmology,  
National Center for Child Health and  
Development, 2-10-1,  
Okura, Setagaya-ku,  
Tokyo, 157-8535, Japan  
e-mail: azuma-n@ncchd.go.jp  
Tel.: +81-3-34160181  
Fax: +81-3-34162222

**Abstract Purpose:** Description of two patients, each with different features of congenital optic disc anomalies in the two eyes.

**Methods:** Case report **Results:** Patient 1, a 3-month-old girl, showed retinochoroidal coloboma involving the optic nerve in the right eye and optic nerve hypoplasia in the left eye. Patient 2, a 5-month-old boy, showed retinal fold extending inferiorly in the right eye and optic disc coloboma in the left eye. **Conclusions:** Since in both cases coloboma was seen in one

eye, the optic nerve hypoplasia or retinal fold in the fellow eye of these two patients may have been related to the timing of embryonic fissure opening or closing.

**Keywords** Congenital optic disc anomalies · Coloboma · Optic nerve hypoplasia · Retinal fold

### Introduction

Numerous developmental events contribute to optic disc/nerve formation, including transient formation of embryonic fissure, hyaloid artery, and Bergmeister's papilla, and projection of nerve fibers. Thus, optic disc/nerve malformations occur when these developmental events transiently or spatially arrest and may present a variety of fundus features. Bilateral anomalies usually show the same phenotype, because of the same genetic background, intrauterine circumstances, or timing of a causative intervention. We report two patients with bilateral optic disc/nerve anomalies that were different clinical entities in the two eyes.

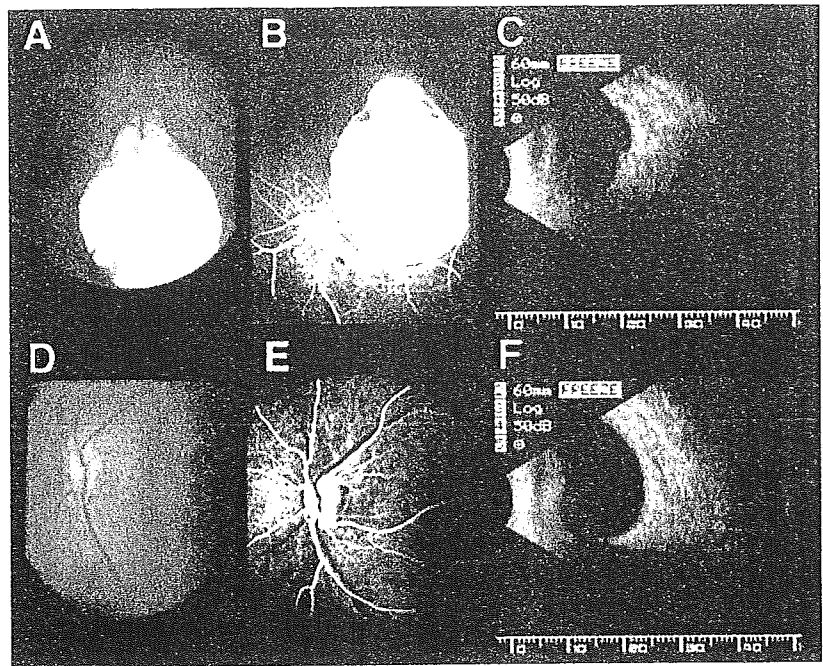
### Case report

Patient 1, a 3-month-old girl, presented with nystagmus in both eyes. Ocular examinations showed normal anterior segments, large retinochoroidal coloboma involving the optic nerve in the right eye, and hypoplasia of the optic nerve with a small optic disc and surrounding depigmented ring

(double-ring sign) in the left eye. Computed tomography (CT) identified hypoplasia of the cerebellar vermis, a callosal defect, ventricular enlargement, and extrusion of the posterior portion of the eyeball in the right eye and a thin optic nerve in the left eye (Fig. 1). The patient, now 3 years old and mentally challenged, has normal growth and no systemic abnormalities.

Patient 2, a 5-month-old boy, presented with nystagmus in both eyes. A retinal fold was seen extending from the optic disc and connected to fibrous tissue on the inferior portion of the posterior lens surface in the right eye. The left anterior segment was normal, although the fundus had a classic optic disc coloboma (Fig. 2). CT was normal, except for the retinal fold in the right eye and eye wall ectasia of the optic nerve region in the left eye. The patient, now 7 years old and mentally challenged, has normal growth and no systemic abnormalities. Other family members of each patient were apparently normal, thus indicating sporadic onset. Each patient was the product of a full-term pregnancy, and careful pediatric examination failed to identify any history of infectious disease.

**Fig. 1** Fundus photography (a, d), fluorescein angiography (b, e), and echography (c, f) of patient 1 show large retinocho-roidal coloboma that involves the optic disc OD (a–c) and optic hypoplasia OS (d–f)



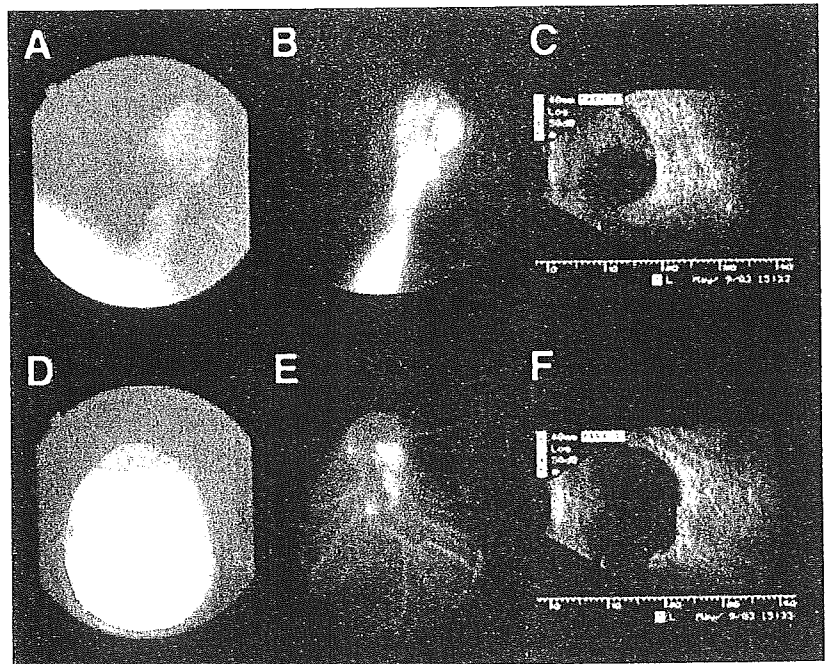
**Discussion**

Among events related to optic disc/nerve formation, opening and closing of the embryonic fissure at 5–6 weeks gestation, when transiently arrested, are the most common pathogenetic factors for malformations associated with peripapillary excavation, including coloboma, peripapillary staphyloma, and morning glory disc anomaly. Slightly

different manifestations of optic disc/nerve anomalies (coloboma, optic disc pit) were reported bilaterally in the affected members of a pedigree showing inherited defects, suggesting that the difference depended on the degree of peripapillary excavation and that both anomalies are in the same spectrum [9].

In contrast, the fundus features in the two eyes of each of our patients markedly differed. Optic nerve hypoplasia is

**Fig. 2** Fundus photography (a, d), fluorescein angiography (b, e), and echography (c, f) of patient 2 show fibrous tissue on the inferior retinal periphery and retinal fold OD (a–c) and coloboma that involves the optic disc OS (d–f)



rarely associated with coloboma in the same patient [1, 3]. The case of a patient with hemifacial microsomia showing optic nerve hypoplasia in the ipsilateral eye and optic nerve coloboma in the contralateral eye has been reported [5]. Optic disc/nerve hypoplasia arises from insufficient growth of retinal ganglion cells and nerve fibers [4], or retrograde nerve fiber degeneration secondary to central nervous system abnormalities [6], while excessive closure of the embryonic fissure may disturb nerve fiber projections in the optic nerve, resulting in optic disc/nerve hypoplasia [3]. Retinal folds and tractional retinal detachments caused by vascular or mesenchymal proliferation in the developing vitreous and retina occur in eyes with persistent fetal vasculature (PFV), familial exudative vitreoretinopathy, and retinopathy of prematurity. Because fibrous proliferations in patient 2 were in the inferior peripheral vitreous cavity, which coincides with part of the embryonic fissure, the tissue may be PFV with excessive migration of mesen-

chymal cells through the fissure. Thus, each anomaly might result from abnormalities in closing of the embryonic fissure.

Mutations of the *PAX2* or *PAX6* gene have been identified in a variety of optic disc/nerve anomalies [2, 7]. *PAX2* plays a crucial role in the development of the optic stalk, and *PAX6* in that of the optic cup [8]. The affected members of a pedigree showed a variety of phenotypes when these genes were mutated, while there was not much difference in the phenotypes between the two eyes in each affected member, suggesting that downstream *PAX2* or *PAX6* genes modify phenotypic expression. However, differences in phenotypes between the two eyes also occurred, albeit in few cases [2]. Although no mutation of these genes was identified in our patients, stochastic effects on developmental events may modify ocular cell growth and differentiation, resulting in different phenotypic manifestations.

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