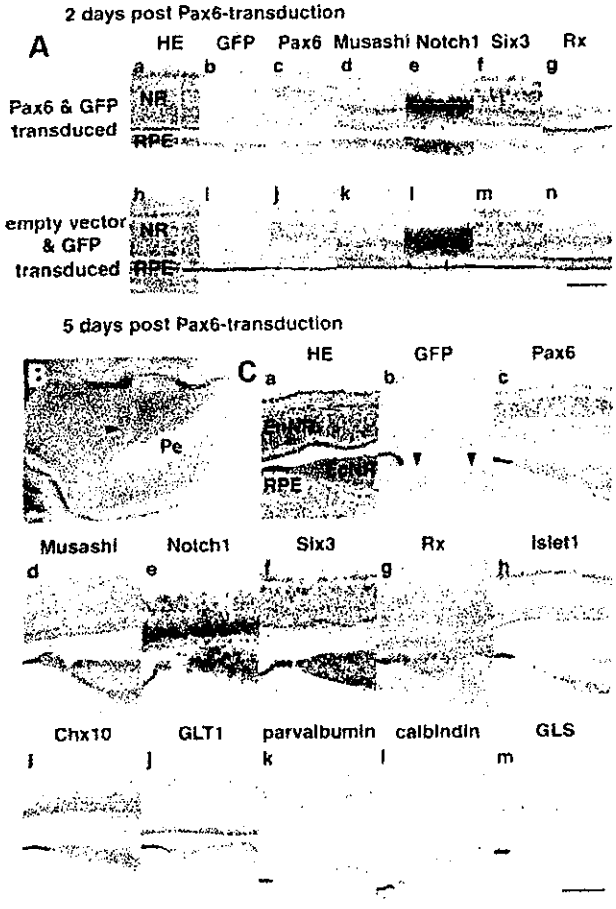

Figure 3. NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene at a late stage. The *Pax6* and *GFP* genes were misexpressed into the RPE layer at stage 35. Eyes were examined 2 days (at stage 37) (A) and 5 days (at stage 40) (B and C) post electroporation. (A) Light microscopy [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 μm. 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

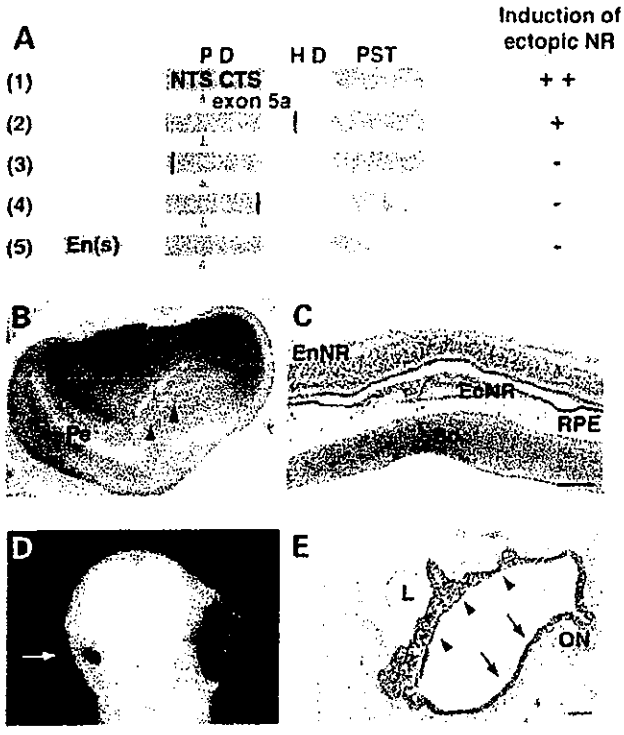

Figure 4. Effect of missense mutations or repression of the Pax6 gene in NR transdifferentiation. (**A**) Structure of the *Pax6* cDNA (1), Pax6 mutants [F258S (2), R26G (3) and R128C (4)] and En(s)–Pax6delC+ (5) used in these studies. The effects of the mutants and repression on NR transdifferentiation 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 μm. (**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 μm. Each result shown is representative of more than 50 independent experiments.

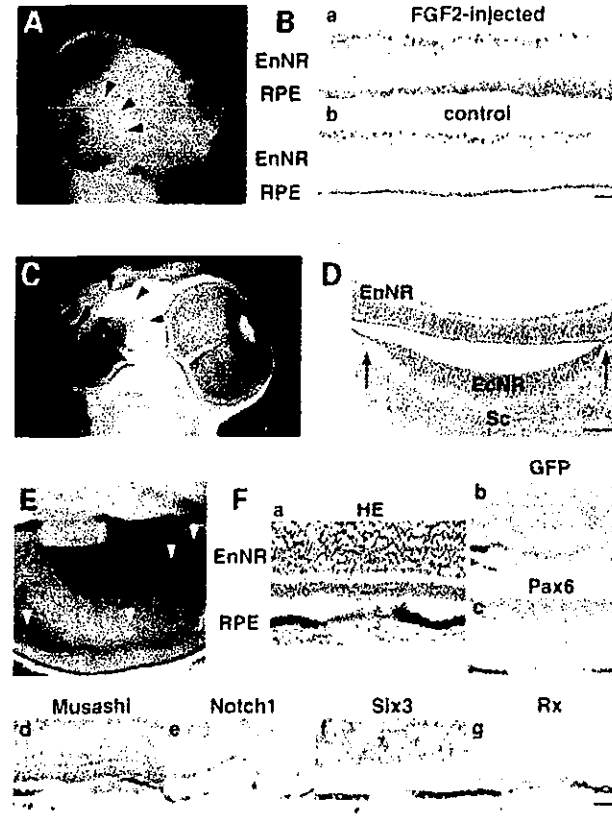

Figure 5. Expression of Pax6 in the ectopic NR transdifferentiated from RPE by FGFs treatment. (**A** and **B**) A stage 20 embryo, in which FGF2 was injected 12 h before (stage 18). (A) In the anterior half of the eye, the RPE layers lose pigments (arrowheads). (B) Immunohistochemistry shows expression 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 μm. (**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 μm. (**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 μm. Each result shown is representative of more than 10 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 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

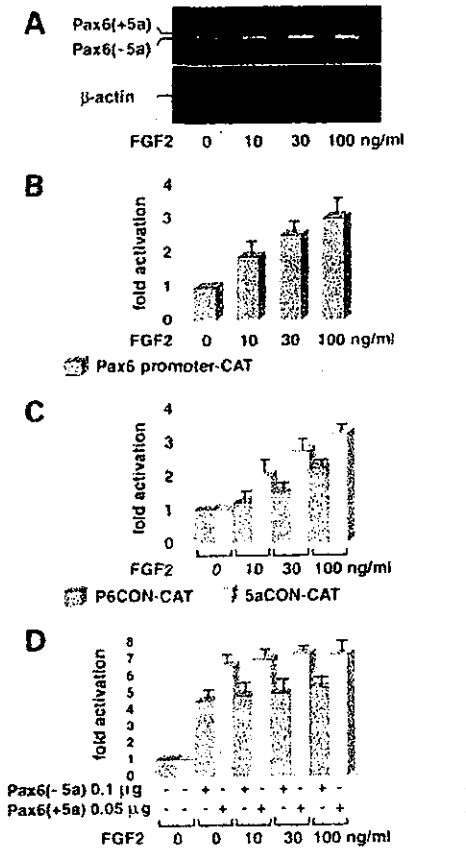

Figure 6. Effect of FGFs on Pax6 expression by functional assay. (**A**) Semi-quantitative analyses for expression levels of endogenous *Pax6* by RT–PCR in *P19 cells treated* with FGF2. The results shown are representative of three independent experiments. (**B** and **C**) CAT activities *in* P19 cells 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 larves*. 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-maturating 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 cytomegalovirus enhancer and a chicken β-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 egg-shells 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 egg-shells 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 egg-shells 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* (*EcoR*I, T7), *Six3* (*Hin*dIII, T3) and *Rx* (*Hin*dIII, 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_2. Cells at a density of 1×10^5 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_2. 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 β-*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*(−5*a*) and 202 bp for *Pax6*(+5*a*)]; β-*actin*-forward primer 5′-GTGGGCCGCCC TAGGCACCA and reverse primer 5′-CTCTTTGATGTC ACGCACGATTTC (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 *Hin*dIII–*Psh*AI fragment carrying ∼2 kb *Pax6* promoter region (1285–3381 nucleotides in GenBank accession no. U63833) was excised and inserted into the *Hin*dIII–*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 5×10^5 cells per 60 mm petri dish were transfected with 0.5 μg of reporter plasmid (*Pax6* promoter, P6CON or 5aCON) and 0.05 μg of pSVβgal (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 phosphofluor-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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REFERENCES

1. Mitashov, V.I. (1975) Proliferation of cells of pigmentary epithelium of retina in adult newts at late regeneration stages after retinectomy. *Sov. J. Dev. Biol.*, **5**, 70–73.
2. Fischer, A.J. and Reh, T.A. (2001) Muller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nat. Neurosci.*, **4**, 247–252.
3. Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R. and van der Kooy, D. (2000) Retinal stem cells in the adult mammalian eye. *Science*, **287**, 2032–2036.
4. Haruta, M., Kosaka, M., Kanegae, Y., Saito, I., Inoue, T., Kageyama, R., Nishida, A., Honda, Y. and Takahashi, M. (2001) Induction of photoreceptor-specific phenotypes in adult mammalian iris tissue. *Nat. Neurosci.*, **4**, 1163–1164.
5. Park, C.M. and Hollenberg, M.J. (1989) Basic fibroblast growth factor induces retinal regeneration *in vivo*. *Dev. Biol.*, **134**, 201–205.
6. Pittack, C., Grunwald G.B. and Reh A. (1997) Fibroblast growth factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos. *Development*, **124**, 805–816.
7. Vogel-Hopker, A. Momose, T., Rohrer, H., Yasuda, K., Ishihara, L. and Rapaport, D.H. (2000) Multiple functions of fibroblast growth factor-8 (FGF-8) in chick eye development. *Mech. Dev*, **94**, 25–36.
8. Zhao, S., Hung, F.C., Colvin, J.S., White, A., Dai, W., Lovicu, F.J., Ornitz, D.M. and Overbeek, P.A. (2001) Patterning the optic neuroepithelium by FGF signaling and Ras activation. *Development*, **128**, 5051–5060.
9. Gehring, W.J. (1996) The master control gene for morphogenesis and evolution of the eye. *Genes Cells*, **1**, 11–15.
10. Halder, G., Callaerts, P. and Gehring, W.J. (1995) Induction of ectopic eye by targeted expression of the eyeless gene in *Drosophila*. *Science*, **267**, 1788–1792.
11. Chow, R.L., Altmann, C.R., Lang, R.A. and Hemmati-Brivanlou, A. (1999) Pax6 induces ectopic eye in a vertebrate. *Development*, **126**, 4213–4222.
12. Bonini, N.M., Bui, Q.T., Gray-Board, G.L. and Warrick, J.M. (1997) *The Drosophila eyes absent* gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. *Development*, **124**, 4819–4826.
13. Chen, R., Amoui, M., Zhang, Z. and Mardon, G. (1997) Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. *Cell*, **91**, 893–903.
14. Pignoni, F., Hu, B., Zavitz, K.H., Xiao, J., Garrity, P.A. and Zipursky, S.L. (1997) The eye-specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. *Cell*, **91**, 881–891.
15. Loosli, F., Winkler, S. and Wittbrodt, J. (1999) Six3 overexpression initiates the formation of ectopic retina. *Genes Dev.*, **13**, 649–654.
16. Bernier, G., Panitz, F., Zhou, X., Hollemann, T. and Gruss, P., Pieler, T. (2000) Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. *Mech. Dev.*, **93**, 59–69.
17. Lagutin, O., Zhu, C.C., Furuta, Y., Rowitch, D.H., McMahon, A.P. and Oliver, G. (2001) Six3 promotes the formation of ectopic optic vesicle-like structures in mouse embryos. *Dev. Dyn.*, **221**, 342–349.

18. Toy, J., Yang, J.M., Leppert, G.S. and Sundin, O.H. (1998) The *optx2* homeobox gene is expressed in early precursors of the eye and activates retina-specific genes. *Proc. Natl Acad. Sci. USA*, **95**, 10643–10648.
19. Shen, W. and Mardon, G. (1997) Ectopic eye development in *Drosophila* induced by directed dachshund expression. *Development*, **124**, 45–52.
20. Mathers, P.H., Grinberg, A., Mahon, K.A. and Jamrich, M. (1997) The *Rx* homeobox gene is essential for vertebrate eye development. *Nature*, **387**, 603–607.
21. Pan, D., Rubin, G.M. (1998) Targeted expression of teashirt induces ectopic eyes in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **95**, 15508–15512.
22. Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999) 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.*, **1**, E203–E207.
23. Epstein, J.A., Cai, J., Glaser, T., Jepeal, L. and Maas, R.L. (1994) Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes. *J. Biol. Chem.*, **269**, 8355–8361.
24. Epstein, J.A. Glaser, T., Cai, J., Jepeal, L., Walton, D.S. and Maas, R.L. (1994) Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. *Genes Dev.*, **8**, 2022–2034.
25. Yamaguch, Y., Sawada, J., Yamada, M., Handa, H. and Azuma, N. (1997) Autoregulation of Pax6 transcriptional activation by two distinct DNA-binding subdomains of the paired domain. *Genes Cells*, **2**, 255–261.
26. Azuma, N., Yamaguchi Y., Handa, H., Hayakawa, M., Kanai, A. and Yamada, M. (1999) Missense mutation in the alternative splice region of the *PAX6* gene in eye anomalies. *Am. J. Hum. Genet.*, **65**, 656–663.
27. Azuma, N., Yamaguchi, Y., Handa, H., Tadokoro, K., Asaka, A., Kawase. E. and Yamada, M. (2003). Mutations of the *PAX6* gene detected in patients with a variety of optic nerve malformations. *Am. J. Hum. Genet.*, **72**, 1565–1570.
28. Niwa, H., Inoue, S., Hirano, T., Matsuo, T., Kojima, S., Kubota, M., Ohashi, M. and Tsuji, F.I. (1996) Chemical nature of the light emitter of the Aequorea green fluorescent protein. *Proc. Natl Acad. Sci. USA*, **93**, 13617–13622.
29. Sakakibara, S. and Okano, H. (1997) Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J. Neurosci.*, **17**, 8300–8312.
30. Bao, Z.Z. and Cepko, C.L. (1997) The expression and function of *Notch* pathway genes in the developing rat eye. *J. Neurosci.*, **17**, 1425–1434.
31. Bovolenta, P., Mallamaci, A., Puelles, L., Boncinelli, E. (1998) Expression pattern of cSix3, a member of the *Six/sine oculis* family of transcription factors. *Mech. Dev.*, **70**, 201–203.
32. Halfter, W. (1998) Disruption of the retinal basal lamina during early embryonic development leads to a retraction of vitreal end feet, an increased number of ganglion cells, and aberrant axonal outgrowth. *J. Comp. Neurol.*, **397**, 89–104.
33. Chen, C.M. and Cepko, C.L. (2000) Expression of Chx10 and Chx10–1 in the developing chicken retina. *Mech. Dev.*, **90**, 293–297.
34. Reye, P., Sullivan, R., Fletcher, E.L. and Pow, D.V. (2002) Distribution of two splice variants of the glutamate transporter GLT1 in the retinas of humans, monkeys, rabbits, rats, cats, and chickens. *J. Comp. Neurol.*, **445**, 1–12.
35. Hamano, K., Kiyama, H., Emson, P.C., Manabe, R., Nakauchi, M., Tohyama, M. (1990) Localization of two calcium binding proteins, calbindin (28 kD) and parvalbumin (12 kD), in the vertebrate retina. *J. Comp. Neurol.*, **302**, 417–424.
36. Mitchell, C.K., Rowe-Rendleman, C.L., Ashraf, S., Redburn, D.A. (1995) Calbindin immunoreactivity of horizontal cells in the developing rabbit retina. *Exp. Eye Res.*, **61**, 691–698.
37. Riepe, R.E. and Norenburg, M.D. (1977) Muller cell localisation of glutamine synthetase in rat retina. *Nature*, **268**, 654–655.
38. Hanson, I., Fletcher, J.M., Jordan, T., Brown, A., Taylor, D., Adams, R.J., Punnett, H.H. and van Heyningen, V. (1994) Mutations at the PAX6 locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nat. Genet.*, **6**, 168–173.
39. Azuma, N., Nishina, S., Okuyama, T., Yanagisawa, H. and Yamada, M. (1996) PAX6 missense mutation in isolated foveal hypoplasia. *Nat. Genet.*, **13**, 141–142.
40. Plaza, S., Langlois, M.C., Turque, N., LeCornet, S., Bailly, M., Begue, A., Quatannens, B., Dozier, C. and Saule, S. (1997) The homeobox-containing Engrailed (En-1) product downregulates the expression of Pax-6 through a DNA binding-independent mechanism. *Cell Growth Differ.*, **8**, 1115–1125.
41. Singh, S., Tang, H.K., Lee, J.K. and Saunders, G.F. (1998) Truncation mutations in the transactivation region of Pax6 result in dominant-negative mutants. *J. Biol. Chem.*, **273**, 21531–21541.
42. Xu, Z.P. and Saunders, G.F. (1997) Transcriptional regulation of the human *PAX6* gene promoter. *J. Biol. Chem.*, **272**, 3430–3436.
43. Kawakami, A., Kimura-Kawakami, M., Nomura, T. and Fujisawa, H. (1997) Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. *Mech. Dev.*, **66**, 119–130.
44. Walther, C. and Gruss, P. (1991) *Pax6*, a murine paired box gene, is expressed in the developing CNS. *Development*, **113**, 1435–1449.
45. Marquardt, T., Ashley-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001) Pax6 is required for the multipotent state of retinal progenitor cells. *Cell*, **105**, 43–55.
46. Baumer, N., Marquardt, T., Stoykova, A., Spieler, D., Treichel, D., Ashery-Padan, R. and Gruss, P. (2003) Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6. *Development*, **130**, 2903–2915.
47. Jean, D., Bernier, G. and Gruss, P. (1999) Six6 (*Optx2*) is a novel murine *Six3*-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk. *Mech. Dev.*, **84**, 31–40.
48. Czerny, T., Schaffner, G. and Busslinger, M. (1993) DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev.*, **7**, 2048–2061.
49. Xu, W., Rould, M.A., Jun, S., Despan, C. and Pabo, C.O. (1995) Crystal structure of a paired domain-DNA complex at 25A resolution reveals structural basis for Pax developmental mutations. *Cell*, **80**, 639–650.
50. Mishra, R., Gorlov, I.P., Chao, L.Y., Singh, S. and Saunders, F. (2002) PAX6, paired domain influences sequence recognition by the homeodomain. *J. Biol. Chem.*, **277**, 49488–49494.
51. Azuma, N., Tadokoro, K., Asaka, A., Yamada, M., Yamaguchi, Y., Handa, H., Matsushima, S., Watanabe, T., Kohsaka, S., Kida, Y. *et al.* (2005) The *Pax6* isoform bearing an alternative spliced exon promotes the development of the neural retinal structure. *Hum. Mol. Genet.*, in press. Q1
52. Morgan R. (2004) Conservation of sequence and function in the Pax6 regulatory elements. *Trends Genet.*, **20**, 283–287.
53. Layer, P.G. and Willbold, E. (1993) Histogenesis of the avian retina in reaggregation culture: from dissociated cells to laminar neuronal networks. *Int. Rev. Cytol.*, **146**, 1–47.
54. Raymond, S.M. and Jackson, I.J. (1995) The retinal pigmented epithelium is required for development and maintenance of the mouse neural retina. *Curr. Biol.*, **5**, 1286–1295.
55. Reh, T.A. and Levine, E.M. (1998) Multipotential stem cells and progenitors in the vertebrate retina. *J. Neurobiol.*, **36**, 206–220.
56. Maslov, A.Y., Barone, T.A., Plunkett, R.J. and Pruitt, S.C. (2004) Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J. Neurisci.*, **24**, 1726–1733.
57. Bertrand, N., Medevielle, F. and Pituello, F. (2000) FGF signalling controls the timing of Pax6 activation in the neural tube. *Development*, **127**, 4837–4843.
58. Arai, S., Thomas, B.B., Seiler, M.J., Aramant, R.B., Qiu, G., Mui, C., De Juan, E. and Sadda, S.R. (2004) Restoration of visual responses following transplantation of intact retinal sheets in rd mice. *Exp. Eye Res.*, **79**, 331–341.
59. Radtke, N.D., Aramant, R.B., Seiler, M.J., Petry, H.M. and Pidwell, D. (2004) Vision change after sheet transplant of fetal retina with retinal pigment epithelium to a patient with retinitis pigmentosa. *Arch. Ophthalmol.*, **122**, 1159–1165.
60. Koshiba-Takeuchi, K., Takeuchi, J.K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. *et al.* (2000) Tbx5 and the retinotectum projection. *Science*, **287**, 134–137.
61. Azuma, N., Hirakata, A., Hida, T. and Kohsaka, S. (1994) Histochemical and immunohistochemical studies on keratan sulfate in the anterior segment of the developing human eye. *Exp. Eye Res.*, **58**, 277–286.
62. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Siedman, J.G., Smith, J.A., and Struhl, K. (eds) (1995) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York, 9.0.1–9.5.5, 9.6.1–9.6.9.

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LETTERS

Surgical Treatment of Recurrent Juvenile Xanthogranuloma of the Eyelid

Juvenile xanthogranuloma, a benign histiocytic disorder, generally arises in the skin of infants and young children and spontaneously regresses. However, surgeons should be aware that multiple recurrences are possible after surgical excision of ocular lesions.[1,2] We report a case of a recurrent eyelid lesion successfully treated by surgical excision and grafting.

Case Report

A 7-year-old girl presented with a mass on the left upper eyelid that had recurred after three excisions. The initial lesion, a 6 × 7-mm solitary subcutaneous nodule, appeared when the patient was 1 year old. The anterior segment and fundus were unremarkable. Computed tomography revealed no orbital involvement; no systemic anomalies were found. Simple excision was initially performed, and histology showed nodular lesions consisting of foamy histiocytes and Touton giant cells (Fig. 1A). The immunoreactivities were $CD68^+$, $CD1a^-$, and $S100^-$, confirming the diagnosis of juvenile xanthogranuloma (Fig. 1B). When the child was 5 years old, the eyelid mass recurred. Simple excision was repeated; however, the mass recurred after 10 months and grew (Fig. 2A). The third operation consisted of a wide excision including the surrounding tissue, but the mass recurred subcutaneously after 3 months.

The fourth operation consisted of complete excision of the mass with surrounding cutaneous and subcutaneous tissue, including the orbital septum. A skin incision was made with esthetic considerations in mind.

Histologic examination confirmed that the surgical margin was lesion-free. Orbital fat tissue was placed on the levator muscle to form a recipient bed (Fig. 2B). To obtain better color and texture matches, a full-thickness skin graft was harvested from the preauricular area. The skin graft was secured with tie-over sutures for 6 days (Fig. 2C). A frost suture was placed for 8 days. The graft adapted well and there has been no recurrence for 2 years (Fig. 2D). In addition to the characteristic morphologic and immunohistochemical features of juvenile xanthogranuloma, histologic study revealed multiple nodular lesions in the dermis (Fig. 1C), in which prominent spindle-shaped cells were arranged in a storiform pattern (Fig. 1D). The remarkable arrangement of spindle cells was only partly seen in the initial lesion but increased in the recurrent lesions.

Comments

In a previous report of a recurrent eyelid lesion, six excisions were performed when the patient was between 5 and 12 years old, but the mass continued to recur. However, histopathologic differences between the original and recurrent lesions were not investigated in that case.[1] Recently, juvenile xanthogranuloma was reported to represent a disorder of the dermal dendrocytes.[3] Within this disease spectrum, solitary spindle-cell xanthogranuloma and progressive nodular histiocytosis, which seldom regress, are characterized by predominantly (>90%) spindle cells arranged in a storiform pattern.[4,5] Although the recurrent lesion in our case had the same immunophenotypic characteristics as the original, the increased number of spindle cells may suggest an evolutionary stage of xanthogranulomatous reaction and the tendency for local recurrence. Our case indicates that juvenile xanthogranuloma of the eyelid may include the refractory variant, and grafting, following complete excision confirmed by biopsy of each edge, may be effective to treat recurrent lesions.

Key Words: eyelid, juvenile xanthogranuloma, surgical treatment

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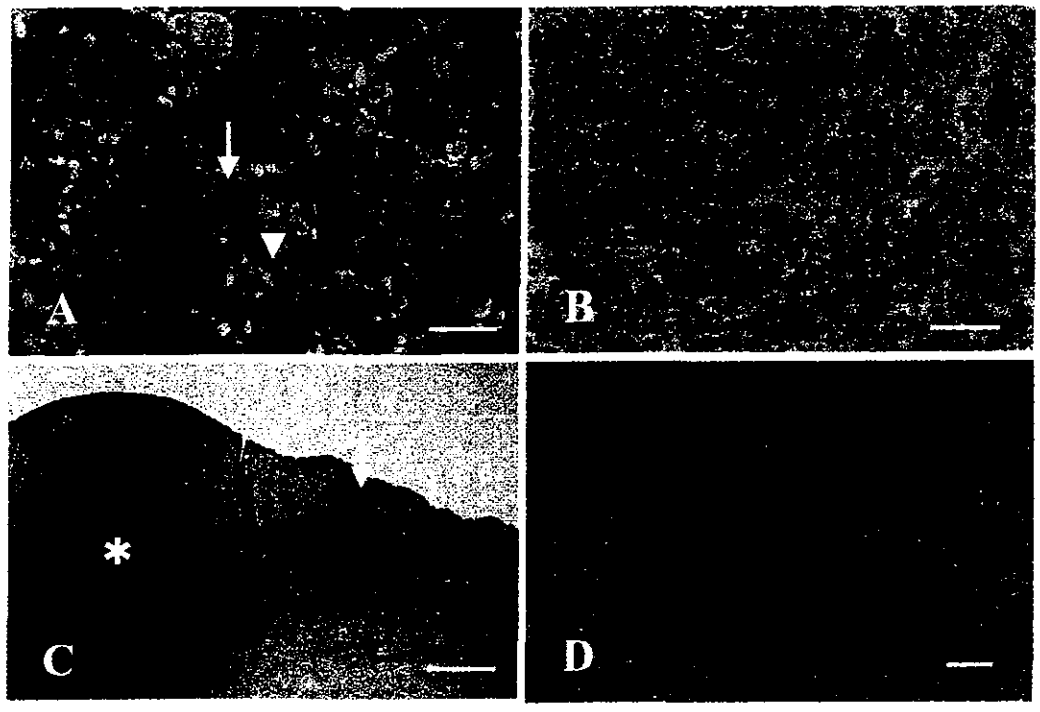

Figure 1. **A** Photomicrograph of the first eyelid excision shows lesion with foamy histiocytes (*arrowhead*) and characteristic Touton giant cells (*arrow*). H&E, bar = 50μm. **B** Immunolabeling for CD68 is positive in all sections. Bar = 100μm. **C** Low-power photomicrograph of the fourth eyelid excision shows the major dermal nodular lesion (*asterisk*) accompanied by satellite lesions (*arrow*). H&E, bar = 1000μm. **D** Prominent spindle-shaped cells are arranged in a storiform pattern. Bar = 100μm.

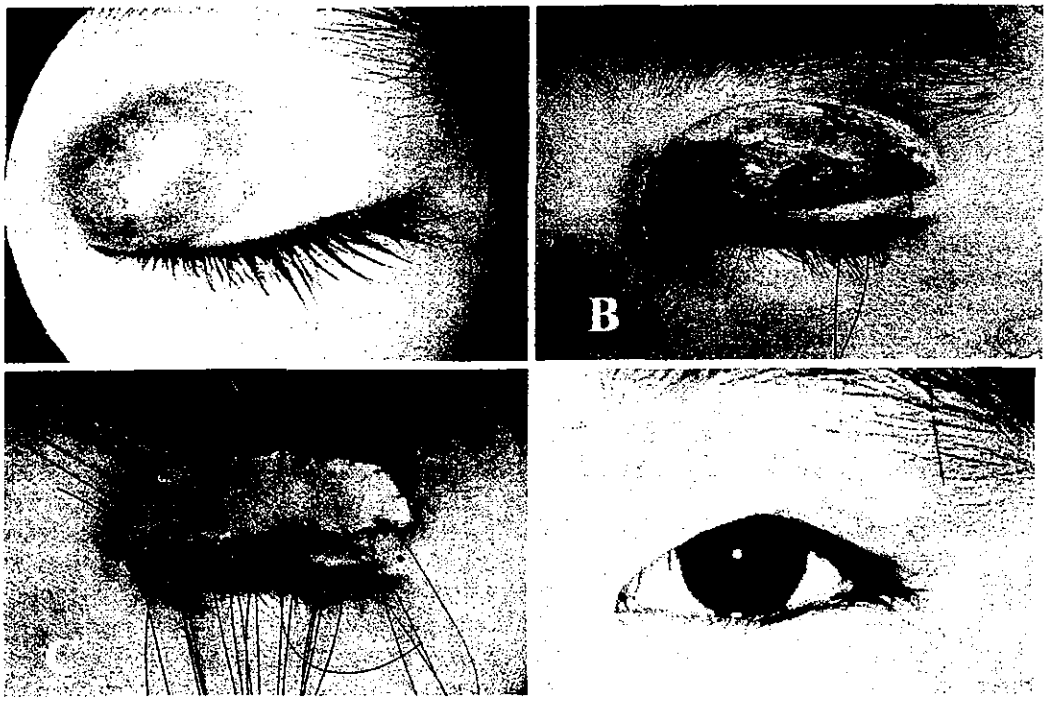

Figure 2. **A** The recurrent lesion has grown to a reddish, 14 × 19-mm mass. **B** The fourth operation: after complete excision of the mass with surrounding cutaneous tissue, orbital fat tissue is placed on the levator muscle to form a recipient bed. **C** The skin graft from the preauricular area is secured. **D** Postoperatively, the graft was well adapted, and there was no recurrence of the eyelid lesion.

References

1. Mansour AM, Traboulsi E, Frangieh G. Multiple recurrence of juvenile xanthogranuloma of the eyelid. J Pediatr Ophthalmol Strabismus 1985;22:156–157.
2. Collum LMT, Power WJ, Mullaney J, Farrell M. Limbal xanthogranuloma. J Pediatr Ophthalmol Strabismus 1991;28: 157–159.
3. Favara BE, Feller AC, Pauli M, et al. Contemporary classification of histiocytic disorders. Med Pediatr Oncol 1997;29:157–166.
4. Zelger BWH, Staudacher CH, Orchard G, Wilson-Jones E. Burgdorf WHC. Solitary and generalized variants of spindle cell xanthogranuloma (progressive nodular histiocytosis). Histopathology 1995;27:11–19.
5. DeStafeno JJ, Carlson JA, Meyer DR. Solitary spindle-cell xanthogranuloma of the eyelid. Ophthalmology 2002;109:258–261.

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Activation mechanism and physiological roles of stress-activated protein kinase/c-Jun NH_2-terminal kinase in mammalian cells

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ABSTRACT: Stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), which belongs to the family of mitogen-activated protein kinase (MAPK), is activated by many types of cellular stress or extracellular signals. Recent studies, including the analysis with knockout cells and mice, have led towards understanding the molecular mechanism of stress-induced SAPK/JNK activation and the physiological roles of SAPK/JNK in embryonic development and immune responses. Two SAPK/JNK activators, SEK1 and MKK7, are required for full activation of SAPK/JNK, which responds to various stimuli in an all-or-none manner in mouse embryonic stem (ES) cells. SAPK/JNK activation plays essential roles in organogenesis during mouse development by regulating cell proliferation, survival or apoptosis and in immune responses by regulating cytokine gene expression. Furthermore, SAPK/JNK is involved in regulation of mRNA stabilization, cell migration, and cytoskeletal integrity. Thus, SAPK/JNK has a wide range of functions in mammalian cells. (J Biol Regul Homeost Agents 2003; 17: 295-302)

KEY WORDS: *Stress, MAPK, SAPK/JNK, Apoptosis, Knock out, ES cells*

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MAP kinases (MAPKs) are evolutionary conserved signal-transducing enzymes involved in the regulation of many cellular events. Several MAPK groups have been identified in mammalian cells, including extracellular signal-regulated kinase (ERK), p38, ERK5, and SAPK/JNK. These MAPKs are activated by their specific MAPK kinases (MAPKKs): ERK by MEK1 and MEK2, p38 by MKK3 and MKK6, ERK5 by MEK5, and SAPK/JNK by SEK1 (also known as MKK4) and MKK7 (SEK2). These MAPKKs are also activated by various MAPKK kinases (MAPKKKs) such as Raf, MLK, MEKK1, TAK1, and ASK1. In this review, we focus on recent progress in the SAPK/JNK group of MAPK-signaling pathways in mouse embryonic stem (ES) cells, embryos, and adult mice (Fig. 1). It is becoming clear that SAPK/JNK activation regulates many cellular processes such as gene expression, cell survival and apoptosis (1-4).

Molecular mechanism of SAPK/JNK activation in ES cells

SAPK/JNK is activated not only by many types of cellular stress including changes in osmolarity, UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin, and tunicamycin, but also by serum, lysophosphatidic acid (LPA), and inflammatory cytokines, interleukin-1ß (IL-1β) and tumor necrosis factor α (TNF-α). The activated SAPK/JNK phosphorylates a number of substrates including transcription factors, c-Jun, Jun D, and ATF-2, to regulate gene expression for stress responses. Activation of SAPK/JNK requires the dual phosphorylation of Tyr and Thr residues located in a Thr-Pro-Tyr motif in the activation loop between VII and VIII of the kinase domain (5). The phosphorylation is catalyzed by the dual specificity kinases, SEK1 and MKK7, which are capable of catalyzing the phosphorylation of both Thr and Tyr residues.

Recent studies have shown that SEK1 has a preference for the phosphorylation of Tyr residue, while MKK7 for Thr residue of SAPK/JNK *in vitro* and that both phosphorylation results in the synergistic activation of SAPK/JNK (Fig. 2A) (6-8). Strong support for this activation mechanism has been obtained from studies of SEK1- and MKK7-gene disruption in ES cells. The severe impairment of SAPK/JNK activation observed in $mkk7^{-/-}$ ES cells was accompanied with a loss of the Thr-phosphorylation of SAPK/JNK, without marked reduction in its Tyr-phosphorylated level. On the other hand, Thr-phosphorylation of SAPK/JNK in $sek1^{-/-}$ ES cells was also attenuated, in addition to a decreased level of its Tyr-phosphorylation. These results indicate that the Tyr and Thr residues of SAPK/JNK are sequentially phosphorylated by SEK1 and MKK7, respectively, in stress-stimulated ES cells (Fig. 2B) (9, 10).

Involvement of "scaffold proteins" has also been reported for the correct proceeding of SAPK/JNK-

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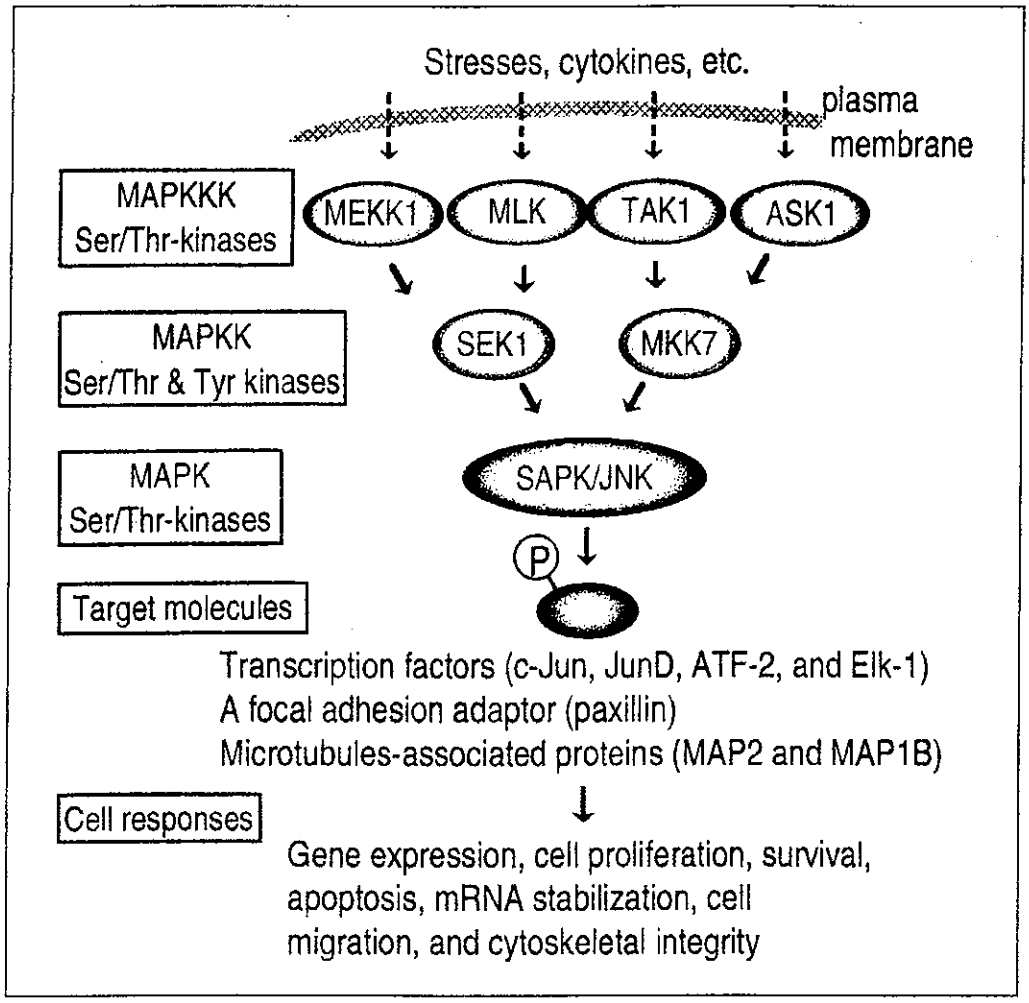

Fig. 1 - SAPK/JNK-signaling pathways involved in a variety of cell responses. SAPK/JNK is activated by extracellular stimuli including stresses and cytokines through kinase cascades. Activated MAPKKKs such as MEKK1, MLK, TAK1, and ASK1 transmit the signal to two MAPKK, SEK1 and MKK7. SAPK/JNK activated by SEK1 and/or MKK7 phosphorylates target molecules such as c-Jun, paxillin, and MAP2 to regulate a wide range of cell functions.

signaling pathway. The scaffold proteins, JIP-1, JIP-2, JIP-3, JSAP1, and JLP, organize the components of SAPK/JNK cascade to secure SAPK/JNK-signaling specificity in mammalian cells (11-15). JIP-1, JIP-2, and JIP-3 bind to SAPK/JNK, MKK7, and mixed-lineage protein kinases (MLKs). JSAP1 is an alternatively spliced variant of JIP3, however, associates with SAPK/JNK, SEK1, and MEKK1. JLP acts as a scaffold protein to bring together Max and c-Myc along with SAPK/JNK and p38, as well as their upstream SEK1 and MEKK3. MEKK1 itself plays a role as a scaffold protein that regulates SEK1 (Fig. 2C) (16). A theoretical consideration indicates that a single set of the scaffold-supported complex, which contains either SEK1 or MKK7, can not induce the sequential phosphorylation, since the synergistic activation of SAPK/JNK requires both SEK1 and MKK7 as observed in ES cells. Therefore, two sets of scaffold complexes, one containing SEK1 and the other MKK7, must exist closely in ES cells. Indeed, we have screened and found a predicted "binder"-like protein, which connects the two MAPKK (MKK7 and SEK1) cascades (Fig. 2D). Characterization of the protein in SAPK/JNK-signaling pathway is currently under investigation in our laboratory.

SAPK/JNK activation as a molecular switch in all-or-none manner

Recently, Ferrell et al have proposed an interesting concept that SAPK/JNK-signaling cascade could, in principle, function as a sensitivity amplifier, which converts graded inputs into more switch-like outputs, allowing the cascade to filter out noise and yet still respond decisively to supra-threshold stimuli (17, 18). They have shown in *Xenopus* oocytes, HeLa cells, HEK293 cells, and Jurkat T cells that SAPK/JNK responds to physiological and pathological stimuli, such as progesterone and sorbitol, in an all-or-none manner. The activation of SAPK/JNK by the stimuli was graded at the level of a population of oocytes, however, at the level of an individual oocyte, the stimulatory response appeared to be switch-like. Indeed, we have also observed a very steep concentration-dependent response in the activation of SAPK/JNK by hyper-osmolar stress, sorbitol, in murine ES cells (Fig. 3) (10). This suggests that the all-or-none type MAPK activation also occurs in mammalian cells at an individual cell level only when the two MAPKKs are simultaneously activated. Therefore, this MAPK signaling should

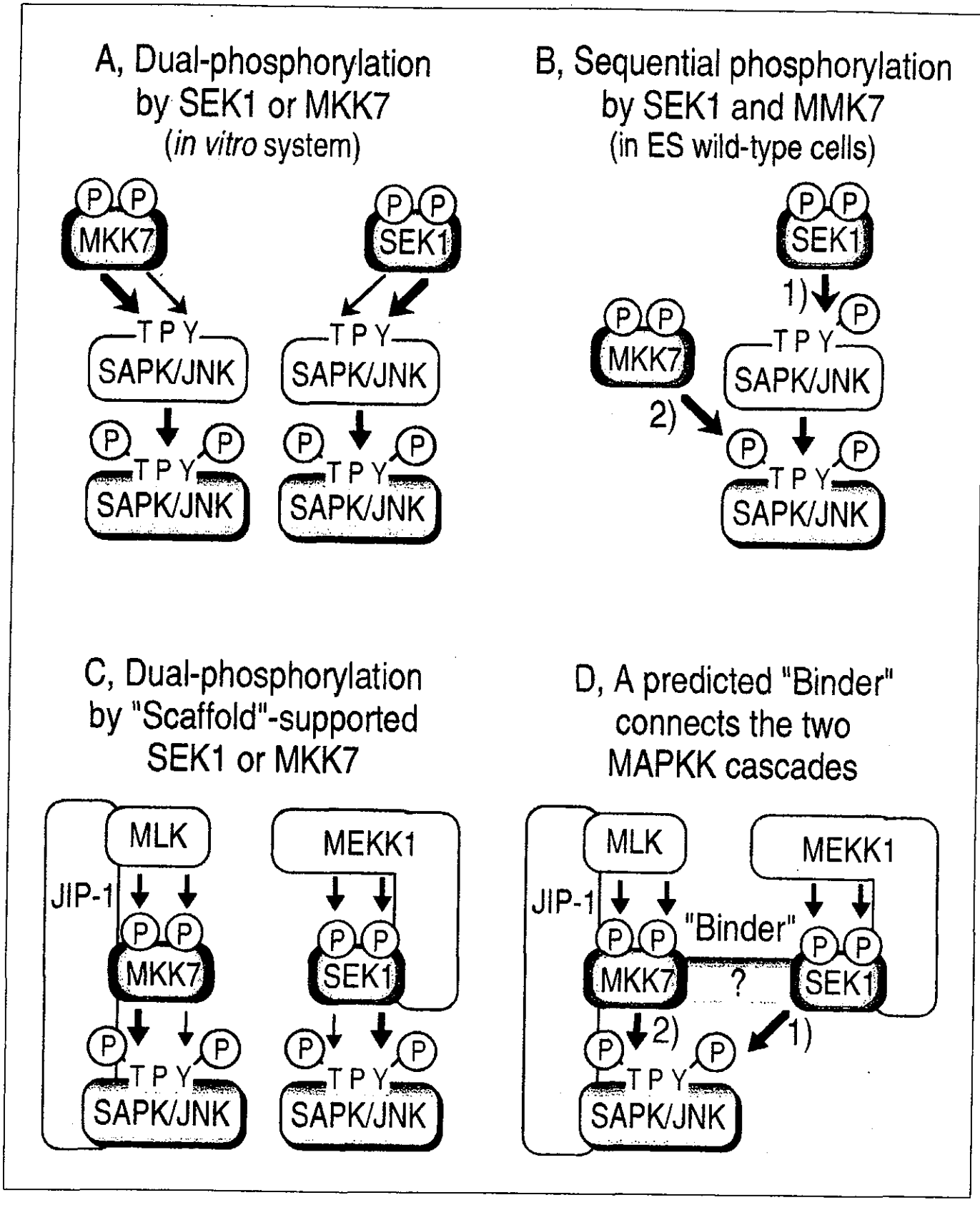

Fig. 2 - Schematic description of SAPK/JNK phosphorylation by SEK1 and MKK7 under various conditions. A) synergistic activation of SAPK/JNK by the dual-specificity kinase, SEK1 or MKK7, which has been reported in *in vitro* conditions (6-8). B) synergistic activation of SAPK/JNK through sequential phosphorylation by SEK1 and MKK7 in murine ES cells (9, 10). C) activation of SAPK/JNK by SEK1 or MKK7 associated with their scaffold proteins, JIP-1 and MEKK1 (11, 16). D) a predicted "binder" connects the two scaffold-supported MAPKK cascades (SEK1 and MKK7) for the sequential phosphorylation of SAPK/JNK as shown in panel B) TPY, Thr-Pro-Tyr motif.

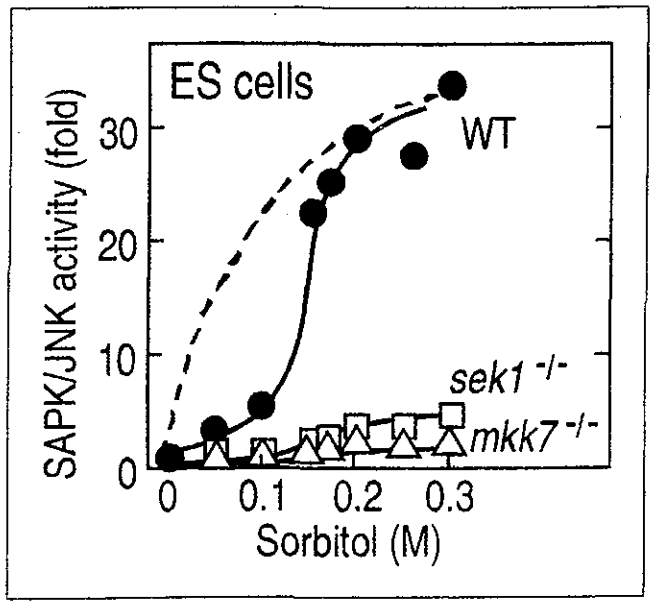

Fig. 3 - SAPK/JNK activation in response to hyper-osmolar stress (sorbitol) *requires both* SEK1 and MKK7 in ES cells. Wild-type, sek1$^{-/-}$, and mkk7$^{-/-}$ ES cells were stimulated with the indicated concentrations of sorbitol for 30 min.

strictly proceed without errors basically through the two separated signals, one activating SEK1 and another activating MKK7. Although the molecular mechanism whereby the two MAPKKs are simultaneously stimulated by various stress signals remains to be resolved, it is tempting to speculate that the two separated pathways leading to SAPK/JNK activation may exist and physiologically function as a fail-safe mechanism as proposed previously (9).

Role of SAPK/JNK in mouse development

Sek1, mkk7, and all three Jnk (Jnk1, 2, and 3) loci have been knocked out at present. Sek1$^{-/-}$ embryos die between embryonic day 10.5 (E10.5) and E12.5 with impaired liver formation and massive apoptosis (Fig. 4) (19-21). We have recently shown that SEK1-mediated SAPK/JNK pathway *downstream TNF-α* receptor 1 (TNFR1) participates in embryonic hepatoblast proliferation and survival via a pathway different from NF-κB-induced anti-apoptosis. On the other hand, mkk7$^{-/-}$ embryos die between E11.5–12.5 with similar defects in liver formation. These results indicate that SAPK/JNK activation mediated through SEK1 plus MKK7 plays indispensable roles in hepatoblast proliferation and survival during mouse embryogenesis (Fig. 5) (22).

JNK1 and JNK2 are widely expressed in many tissues, but JNK3 is expressed predominantly in nervous system. Mice deficient in the single gene of Jnk1, Jnk2, or Jnk3, and Jnk1/Jnk3- or Jnk2/Jnk3-double mutant mice all survived normally. Mice lacking both JNK1 and JNK2 die around E11 with severe dysregulation of apoptosis in brain. Specifically, there was a reduction of cell death in the lateral edges of hindbrain prior to neural tube closure. In contrast, increased apoptosis and caspase activation were found in the mutant forebrain. These results assign both pro-and anti-apoptotic functions to JNK1 and JNK2 in the development of the fetal brain (23, 24).

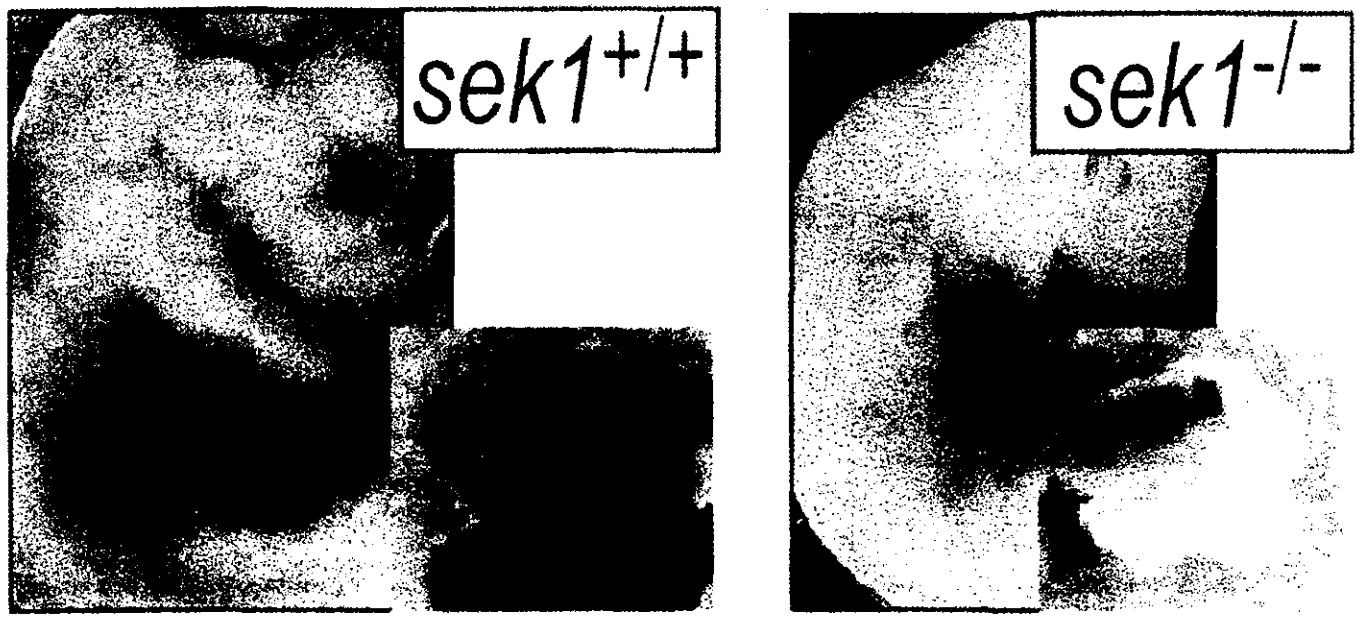

Fig. 4 - Defective liver formation in sek1$^{-/-}$ embryos. Appearance of wild-type and sek1$^{-/-}$ embryos and livers at embryonic day 12.5. Severe anemia is observed in sek1$^{-/-}$ embryos.

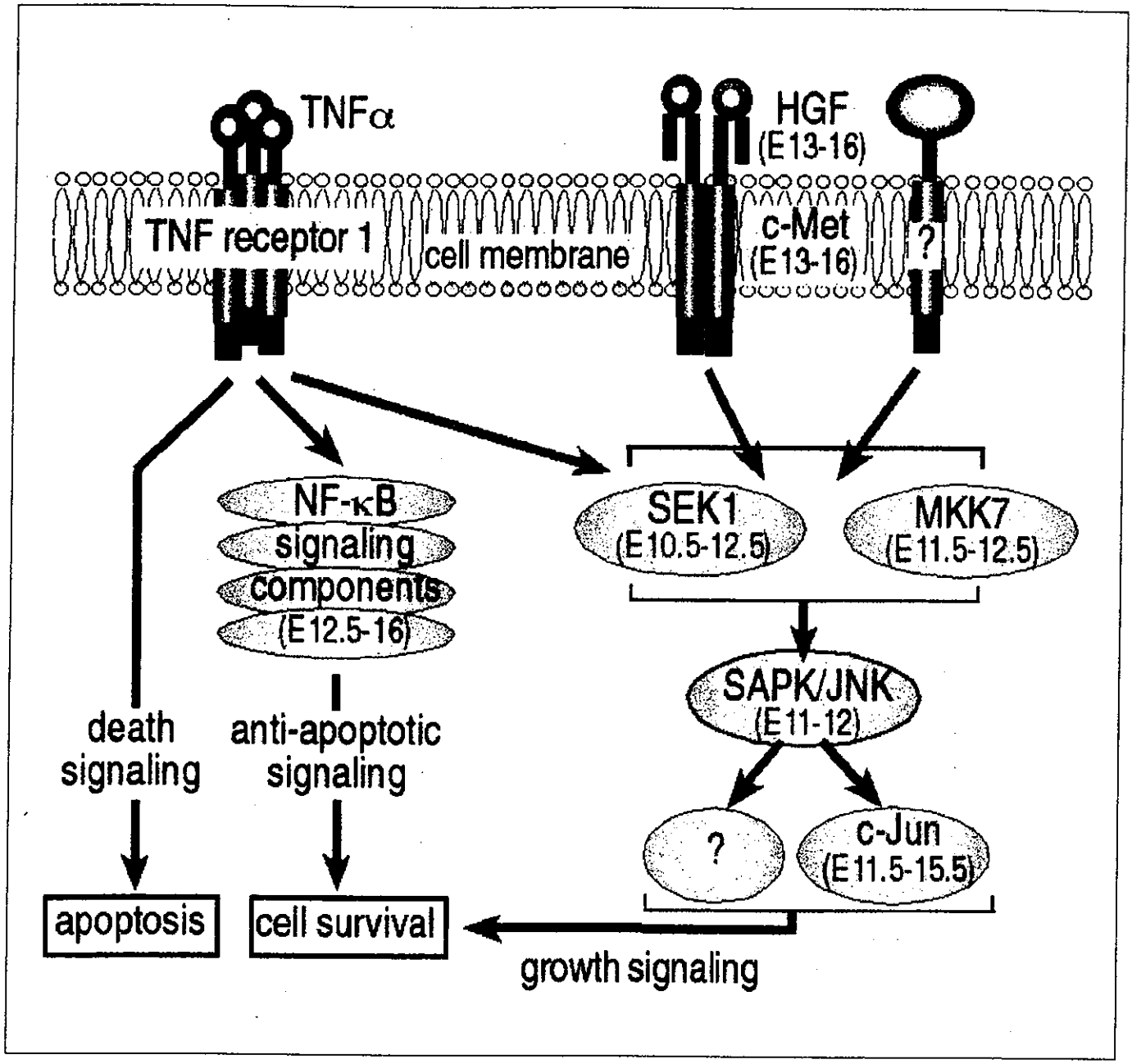

Fig. 5 - A proposed model for SAPK/JNK signaling pathway in hepatoblasts. The numbers in parentheses are dates of embryonic lethality reported in previous papers. TNFα elicits a wide range of biological responses, such as inflammation, tumor necrosis, differentiation, cell proliferation, and apoptosis, through the stimulation of its receptor, TNFR1. The induction of apoptosis, NF-κB activation, and SAPK/JNK activation are simultaneously mediated through TNFR1. SAPK/JNK activation is involved in cell growth, while activation of NF-κB protects against the apoptosis in hepatoblasts (22).

Role of SAPK/JNK in cell survival and apoptosis

In mammalian cells, apoptotic signaling cascades can be divided into two broad categories: the intrinsic (mitochondria-dependent) and the extrinsic (death receptor-mediated) pathways. The initiation of mitochondria-dependent pathway requires a change in the organella membrane permeability that is prevented by anti-apoptotic molecules such as Bcl-2 and Bcl-X_L and promoted by pro-apoptotic molecules including Bax and Bak. The permeability change results in the release of mitochondrial proteins. One of the released proteins, cytochrome c, associates with Apaf1 and caspase 9 to activate the effector caspase 3 (25, 26). Cellular stresses such as UV irradiation and heat shock mediate apoptosis through the mitochondria-dependent pathway (27). However, upstream signaling that regulates the pro-apoptotic molecules remains to be elucidated. Recently, the involvement of SAPK/JNK activation in pro-apoptotic function has been suggested in the study with $Jnk1^{-/-}$ $Jnk2^{-/-}$ and $mkk4^{-/-}$ $mkk7^{-/-}$ mouse

embryonic fibroblasts (MEFs). Both Jnk1$^{-/-}$ Jnk2$^{-/-}$ and mkk4$^{-/-}$mkk7$^{-/-}$ MEFs exhibited profound defects in stress-induced apoptosis (28, 29). Furthermore, it has been reported that JNK activation causes the release of apoptogenic factors such as cytochrome c and Smac from isolated mitochondria in a cell-free system (30, 31). These results strongly suggest that the SAPK/JNK activation directly regulates mitochondria-dependent apoptosis in pro-apoptotic direction.

Recently, we generated ES cells lacking both MKK4 and MKK7 to reevaluate the role of SAPK/JNK activation in the stress-induced and mitochondria-dependent apoptosis. We utilize mouse ES cells in terms of the following advantages: 1) ES cells are a prototype of all cell lineages and can be differentiated into MEF-like cells with retinoic acid, 2) ES cells do not express death receptors including Fas and TNFR1, but have stress-induced, mitochondria-dependent apoptotic pathway, and 3) the molecular mechanism of SAPK/JNK activation is well characterized in ES cells. Interestingly, SAPK/JNK activation is not required for stress-induced and mitochondria-dependent apoptosis in ES and MEF-like cells (submitted). Thus, the physiological role of SAPK/JNK activation in cell survival and apoptosis is still controversial, suggesting an anti-apoptotic, a pro-apoptotic, or no function in these processes dependent on the types of cells and stimuli (32).

Role of SAPK/JNK in mouse immune responses

CD4$^+$ and C8$^+$ T cells are two subsets of peripheral T cells that play important roles during an immune response. After antigen stimulation, CD4$^+$ T cells differentiate into effector Th1 or Th2 cells that secret cytokines to help modulate the type of immune response that is generated. Th1 cell promote cell-mediated immunity against intracellular microbial pathogens by expressing interferon-γ, interleukin (IL)-2, and lymphotoxin, whereas Th2 cells promote humoral immunity against parasites and extracellular pathgens by expressing IL-4, IL-5, IL-9, IL-10, and IL-13. CD8$^+$ T cells differentiate into cytotoxic T cells to help defend the host during the cell-mediated immune response. Thus, defective T cell development to Th1 and Th2 cells results in impaired immune responses. Furthermore, inappropriate activation of T cells initiates and perpetuates many autoimmune diseases including rheumatoid, asthma, inflammatory bowel disease, and multiple sclerosis. It has been reported that T cells from mice deficient in the Jnk1 or Jnk2 gene have a defect in functional differentiation into Th1 or Th2 subsets (33, 34). Sabapathy et al have reported that JNK1 positively regulates mature T cell activation and that JNK1 and JNK2 have similar and overlapping roles in T cell function (35). Conze et al have also reported that JNK1 is required for CD8$^+$ T cell activation, however, that JNK1 and JNK2 have distinct functions in CD4$^+$ T cell differentiation and CD8$^+$ T cell activation (36). Furthermore, we found defective T cell activation, whereas Swat et al. found normal activation of T cells lacking SEK1 (37, 38). These results indicate that the magnitude of the stimuli, the expression of JNK isoforms, and the activation level of SAPK/JNK are important factors determining the direction and efficiency of T cell activation (35). Thus, SAPK/JNK appears to be involved in the differentiation and activation of T cells, though its precise molecular mechanisms are still controversial.

Other physiological roles and target molecules of SAPK/JNK

As described above, SAPK/JNK regulates embryonic development including cell proliferation, survival, and apoptosis, and immune responses including T cell differentiation and activation. Furthermore, it has been reported that SAPK/JNK regulates mRNA stabilization, cell migration, and cytoskeletal integrity (Fig. 1). Turnover of mRNA is an important mechanism for the regulation of gene expression in organisms from bacteria to mammals. Regulation of mRNA half-life can influence normal cell proliferation, differentiation and oncogenesis. PB-3c mast cells produce IL-3 upon stimulation with extracellular signals, and its short-lived (half-life is about 30 min) mRNA is stabilized by Ca^{2+} ionophores. Using an active MEKK1 and a dominant-negative mutant of JNK, it has been indicated that SAPK/JNK is involved in the regulation of IL-3 mRNA turnover in mast cells (39). SAPK/JNK is required for *Drosophila* dorsal closure and is also essential for cell migration in mammalian cells. Rat bladder tumor epithelial cells (NBT-II) exhibit rapid keratinocyte-like movement. Interestingly, SP600125, a specific inhibitor of JNK, suppresses the movement. Several experiments indicate that JNK1 phosphorylates serine 178 on paxillin, a focal adhesion adaptor, in NBT-II cells. Expression of a paxillin mutant (Ser178 to Ala) inhibited the migration of the cells. Thus, phosphorylation of paxillin by SAPK/JNK seems to be essential for maintaining the labile adhesions required for rapid cell migration (40). Dynamic assembly and disassembly of microtubules is essential for a variety of cellular functions, such as maintenance of cell morphology and polarity, cell division, cell locomotion, and intracellular trafficking. JNK1-deficient mice exhibit progressive degeneration of long nerve fibers and loss of microtubule integrity in dendrites. Dendritic degeneration of neuronal microtubules is associated with hypo-phosphorylation of microtubule assembly-promoting protein (MAP) 2 and its reduced ability to promote tubulin polymerization. Thus, JNK1 is required for maintaining the cytoskeletal integrity of neuronal cells and is a critical regulator of MAP activity and microtubule assembly (41).

Conclusions

Data continues to emerge implicating the SAPK/JNK-signaling pathway in a number of physiological functions that may be involved in human disease including autoimmune, anti-inflammatory, neurogenerative diseases, and cancers. In fact, mutations in Jnk3 gene were identified in human brain tumors (42). Similarly, mutations in sek1 gene have been identified in human cancers of pancreas, lung, breast, colon, and prostate as tumor suppressor genes (43-45). Under these circumstances, several pharmaceutical companies have been working on the discovery of SAPK/JNK-related drugs such as an anthrapyrazolone and SP600125. Efforts by many researchers in this field may help to find effective drugs in the near future.

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References

1. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000; 103: 239-52.
2. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001; 410: 37-40.
3. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opini Gene Dev 2002; 12: 14-21.
4. Manning AM, Davis RJ. Targeting JNK for therapeutic benefit: from junk to gold? Nature Rev Mol Drug Disc 2003; 2: 554-65.
5. Derijard B, Hibi M, Wu IH, et al. JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994; 76: 1025-37.
6. Lawler S, Fleming Y, Goedert M, Cohen P. Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases *in vitro*. Curr Biol 1998; 8: 1387-90.
7. Lisnock J, Griffin P, Calaycay J, et al. Activation of JNK3α1 requires both MKK4 and MKK7: Kinetic characterization of *in vitro* phosphorylated JNK3α1. Biochemistry 2000; 39: 3141-8.
8. Fleming Y, Armstrong CG, Morrice N, Paterson A, Goedert M, Cohen P. Synergistic activation of stress-activated protein kinase 1/c-Jun N-terminal kinase (SAPK1/JNK) isoforms by mitogen-activated protein kinase 4 (MKK4) and MKK7. Biochem J 2000; 352: 145-54.
9. Wada T, Nagakawa K, Watanabe T, et al. Impaired synergistic activation of stress activated protein kinase SAPK/JNK in mouse embryonic stem cells lacking SEK1/MKK4. J Biol Chem 2001; 276: 30892-7.
10. Kishimoto H, Nagakawa K, Watanabe T, et al. Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. J Biol Chem 2003; 278: 16595-601.
11. Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ. A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 1998; 281: 1671-4.
12. Whitmarh AJ, Davis RJ. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem Sci 1998; 23: 481-5.
13. Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ. The JIP group of mitogen-activated protein kinase scaffold proteins. Mol Cell Biol 1999; 19: 7245-54.
14. Ito M, Yoshioka K, Akechi M, et al. JSAP1, a novel Jun N-terminal protein kinase (JNK)-binding protein that functions as a scaffold factor in the JNK signaling pathway. Mol Cell Biol 1999; 19: 7539-48.
15. Lee CM, Onesime D, Reddy CD, Dhanasekaran N, Reddy EP. JLP: A scaffolding protein that tethers JNK/p38MAPK signaling modules and transcription factors. Proc Natl Acad Sci USA 2002; 99: 14189-94.
16. Xia Y, Wu Z, Su B, Murray B, Karin M. JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. Genes Dev 1998; 12: 3369-81.
17. Bagowski CP, Ferrell JE. Bistability in the JNK cascade. Curr Biol 2001; 11: 1176-82.
18. Bagowski CP, Besser J, Frey CR, Ferrell JE. The JNK cascade as a biochemical switch in mammalian cells: Ultrasensitive and all-or-none responses. Curr Biol 2003; 13: 315-20.
19. Yang D, Tournier C, Wysk M, et al. Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH_2-terminal kinase activation, and defects in AP-1 transcriptional activity. Proc Natl Acad Sci USA 1997; 94: 3004-9.
20. Ganiatsas S, Kwee L, Fujiwara Y, et al. SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proc Natl Acad Sci USA 1998; 95: 6881-6.
21. Nishina H, Vaz C, Billia P, et al. Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. Development 1999; 126: 505-16.
22. Watanabe T, Nakagawa K, Ohata S, et al. SEK1/MKK4-mediated SAPK/JNK signaling participates in embryonic hepatoblast proliferation via a pathway different from NF-κB-induced anti-apoptosis. Dev Biol 2002; 250: 332-47.

23. Kuan CY, Yang DD, Roy DRS, et al. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 1999; 22: 667-76.
24. Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF. Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. Mech Dev 1999; 89: 115-24.
25. Peng L, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91: 479-89.
26. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nature Rev Mol Cell Bio 2003; 4: 552-65.
27. Yoshida H, Kong YY, Yoshida R, et al. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 1998; 94: 739-50.
28. Tournier C, Hess P, Yang DD, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000; 288: 870-4.
29. Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. Genes Dev 2001; 15: 1419-26.
30. Aoki H, Kang PM, Hampe J, et al. Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. J Biol Chem 2002; 277: 10244-50.
31. Chauhan D, Li G, Hideshima T, et al. JNK-dependent release of mitochondrial protein, Smac, during apoptosis in multiple myeloma (MM) cells. J Biol Chem 2003; 278: 17593-6.
32. Lin A. Activation of the JNK signaling pathway: breaking the brake on apoptosis. BioEssays 2002; 25: 17-24.
33. Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ, Flavell RA. Defective T cell differentiation in the absence of Jnk1. Science 1998; 282: 2092-5.
34. Yang DD, Conze D, Whitmarsh AJ, et al. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity 1998; 9: 575-85.
35. Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF. c-Jun NH_2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. J Exp Med 2001; 193: 317-28.
36. Conze D, Krahl T, Kennedy N, et al. c-Jun NH_2-terminal kinase (JNK)1 and JNK2 have distinct roles in $CD8^+$ T cell activation. J Exp Med 2002; 195, 811-23.
37. Nishina H, Bachmann M, Oliveira AJ, et al. Impaired CD28-mediated interleukin 2 production and proliferation in stress kinase SAPK/ERK1 kinase (SEK1)/mitogen-activated protein kinase kinase 4 (MKK4)-deficient T lymphocytes. J Exp Med 1997; 186: 941-53.
38. Swat W, Fujikawa K, Ganiatsas S, et al. SEK1/MKK4 is required for maintenance of a normal peripheral lymphoid compartment but not for lymphocyte development. Immunity 1998; 8: 625-34.
39. Ming XF, Kaiser M, Moroni C. c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells. EMBO J 1998; 17: 6039-48.
40. Huang C, Rjfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. Nature 2003; 424: 219-23.
41. Chang L, Jones Y, Ellisman MH, Goldstein LSB, Karin M. JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtuble-associated proteins. Dev Cell 2003; 4: 521-33.
42. Yoshida S, Fukino K, Harada H, et al. The c-Jun NH_2-terminal kinase 3 (JNK3) gene: Genomic structure, chromosomal assignment, and loss of expression in brain tumors. J Hum Genet 2001;46: 182-7.
43. Teng DH, Perry WL, Hogan JK, et al. Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res 1997; 57: 4177-82.
44. Su GH, Hilgers W, Shekher MC, et al. Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res 1998; 58: 2339-42.
45. Yoshida BA, Dubauskas Z, Chekmareva MA, et al. Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Res 1999; 59: 5483-7.

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水晶体の形成遺伝子とその変異

Genetics on the Lens Morphogenesis and Its Malformation

東　範行*

要　約

発生は転写因子遺伝子群がカスケードをなして働く一連のシステムである．眼においては*Pax6*を頂点とする遺伝子カスケードが存在する．*Pax6*はまずヒトの先天無虹彩の原因遺伝子として見つかり，下等動物の初期胚に導入すると眼が異所性にできることから，眼形成のmaster control遺伝子であると考えられるようになった．ヒト発生眼でも広範に発現し，無虹彩のほかに，前眼部形成不全，先天白内障，黄斑低形成，視神経形成異常で変異が見つかったことから，発生における働きはヒトでも眼のほぼ全体を網羅していることが判明した．さらに*Pax6*の下流にあって，前眼部を形成する遺伝子*Pitx*，*Eya1*が見つかり，先天白内障と前眼部形成異常でその変異が見出された．また，鶏やアフリカツメガエルでは*Pax6*の下流で水晶体を形成するmaster control遺伝子*L-Maf*が発見された．水晶体の発生において，これらの形態形成遺伝子はクリスタリンをはじめとする水晶体特異蛋白の発現を制御していることも明らかになった．そして，これらの構造蛋白の変異も先天白内障で次々と見つかっている．これら水晶体形成遺伝子に変異が起これば同様な表現型が生ずるので，疾患概念も変化しつつある．これら発生に関する遺伝子システムの解明は，疾患の成因への理解や，遺伝相談に有用であるが，さらには将来の遺伝子治療や再生医学に結びつくことが期待される．

はじめに

眼は複雑な構造をもつ器官であり，遺伝性疾患も多いことから，これまで眼科領域では多くの疾患で原因遺伝子が発見されてきた．代表的なものとしては，Leber視神経症（ミトコンドリア遺伝子の変異による），網膜色素変性症（rhodopsin，peripherin，cGMPなど），小口病（arreston，rhodopsin kinase），色覚異常（red-green opsin），角膜ジストロフィ（kerato-epithelin）などがある．Leber視神経症におけるミトコンドリア遺伝子は特殊であるが，その他多くの遺伝性眼疾患は各組織に特異的に発現する構造遺伝子や酵素をコードする遺伝子の変異が原因である．一方，形態形成（発生）においては組織構築に関わる蛋白をコードする遺伝子も重要であるが，転写因子やシグナル伝達物質がきわめて重要な役割を果たしている．Leber黒内障（guanylate cyclaseの変異による）やNorrie病（Norrie disease，*ND*遺伝子，ムチンや成長因子と類似），網膜分離症（*XLRS1*遺伝子，網膜間の接着に関与）などは前者であり，先天無虹彩（*PAX6*遺伝子）などは後者である．

水晶体は表面外胚葉から形成される特異な組織であり，最近，その発生に関わる多くの遺伝子が発見された．一方，先天白内障は発生期に起こる水晶体の形成異常であるが，これらの発生遺伝子や水晶体に特異的に発現する蛋白の変異によって起こることが明らかになってきた．これら基礎研究と遺伝学研究から，水晶体の発生や疾患に関する概念は大きく変化してきている．ここでは，眼球およびその中の水晶体の形成に関わる遺伝子と，先天白内障の遺伝子変異について最近の知見を述べる．

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I 器官・組織の発生に関わる遺伝子

細胞の核の中に存在する遺伝子は，その翻訳する蛋白の性格によって，構造蛋白，酵素，シグナル伝達物質，転写因子などに大別されるが，発生においては転写因子が重要な役割を果たしている．転写因子は遺伝子調節蛋白ともよばれ，他のDNAのおもにpromoter領域に結合して，その遺伝子の発現スイッチをon offする司令塔の役割をする蛋白である．働きの場は細胞核内で，微量にしか存在しないが，細胞が特異的機能を果たすために適切な遺伝子を適切量発現させるための司令塔の役割をもち，構造蛋白，酵素，シグナル伝達物質などはいずれもその支配下にある．ことに発生においては，複雑な組織・器官が分化するために各時期に適切な遺伝子が発現するネットワークが必要であり，そのプログラムに従って整然と形成過程は進まなければならない．たとえば，手が形成される場合には，ある時期に中胚葉細胞が増殖して手根骨，指骨，筋肉になり，外胚葉細胞から皮膚ができ，さらにある時期に指間の細胞が一斉にアポトーシスを起こすことによって各指が離れる．動物によっては，アポトーシスが完全に起こらなければ指間にヒレが残るわけである．したがって，発生（形態形成）においては転写因子遺伝子ネットワークを解明することは，一連の工程の設計図を明らかにすることを意味する．

発生に関わる遺伝子は，その中にhomeoboxという構造をもつことが特徴である．Homeoboxは生物種を越えて発生に関与する遺伝子のきわめて多くに共通して含まれる180の塩基対であり，これは翻訳されて60アミノ酸homeodomainとなる．Homeodomainは転写因子における標的DNA結合部位として働くが，その普遍性から，homeoboxをもつ遺伝子は発生における転写因子遺伝子，位置や空間を決定する遺伝子の指標であると考えられるようになった．

さらに，遺伝子が意外な場所に転用されるのも特徴である．眼を作る遺伝子は，同時に中枢の発生にも関与していることが多い．眼と中枢は神経系として共通点も多いが，まったく関係のない腎臓や膵臓などで同じ遺伝子が働いていることがある．これは，眼と腎臓が同起源ということではなく，遺伝子（あるいは翻訳される蛋白）が1つの固定した機能に縛られず，融通性に富んでいて，少し条件を換えれば他の働きもできることを意味している．そして，生物は進化して複雑化する際に，しばしばこの転用を利用してきた．新しい遺伝子を作ることもあったが，これは大変なことであり，使えるものはそのままあるいは少し違えて使うほうが，はるかに効率的である．激烈な進化の競争のなかで，生物はゆっくり新しい遺伝子を創作している余裕はなかったのである．そして，遺伝子がこのように多用されているため，同じ遺伝子障害によって多彩な複数臓器障害の症候群が起こることになる．

眼は最も複雑な構造をした器官であり，角膜，水晶体，網膜などその一部の組織をとっても，発生には膨大な転写因子ネットワークがあるはずである．そして，現在までにさまざまな転写因子遺伝子が見つかり，複数臓器に発現しているものも多かった．そして，これらに変異が起こればヒト疾患の原因となることも明らかになってきた．このなかで，後に述べる*PAX6*遺伝子の発見が最も重要である．これが眼全体を作る遺伝子ネットワークの頂点に存在するmaster control遺伝子であるからである．

II 眼形成のmaster control遺伝子*PAX6*の発見と動物間の共通性

*PAX*遺伝子群はpaired boxとhomeoboxを共通モチーフとしてもつ遺伝子familyで，PAX蛋白ではpaired boxとhomeoboxから翻訳される部位paired domainとhomeodomainの2つが標的遺伝子への結合部位となる（図1）．この遺伝子群は最初にショウジョウバエで発見されたが，脊椎動物では9種見つかっており，*PAX6*は6番目に発見された．ヒトでは最初に先天無虹彩の候補遺伝子として染色体11p13領域の欠失部位からpositional cloningによって発見され[1]，多くの変異が発見されて無虹彩の原因遺伝子であることが確定した（図2，3）．その後，マウスやラットで変異があると小眼球になる*small eye (Sey)*遺伝子や，ショウジョウバエで複眼が形成されない*eyeless*遺伝子と同じものであることが判明し，*Pax6*に統一された（遺伝子の表記はすべてが大文字ならヒト，頭だけが大文字ならばマウスなど他のほ乳類，全部小文字ならばショウジョウバエなどの下等動物

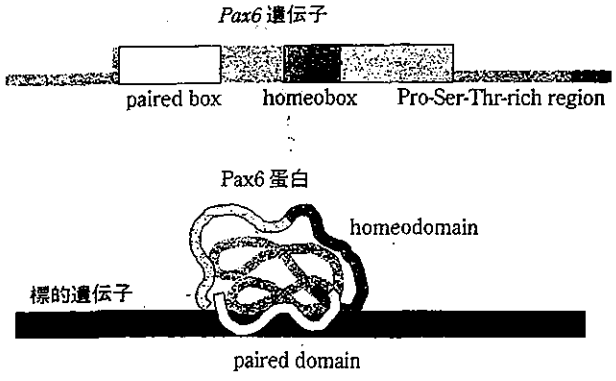


図1 *PAX6*遺伝子と，これから翻訳されるPAX6蛋白
PAX6蛋白はおもにpaired domainが標的DNAに接着して，これを制御する．

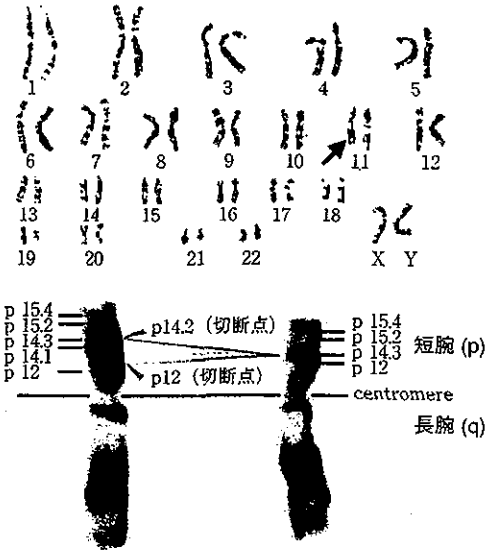
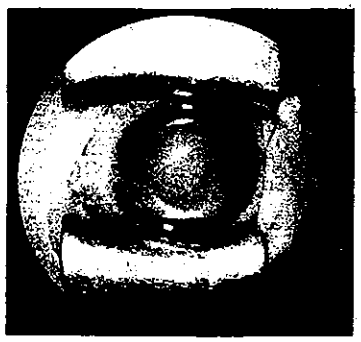


図2 先天無虹彩の染色体異常
（11番短腕欠損）
正常　　11番短腕欠損



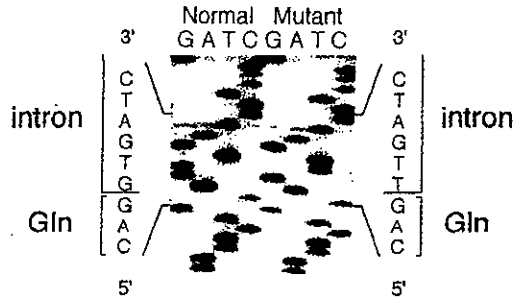


図3 黄斑と視神経の低形成を伴う先天無虹彩の*PAX6*遺伝子変異（スプライシング変異）

のものを表す．また遺伝子は斜体文字，翻訳された蛋白は標準体文字で書かれる）．

さらに，ショウジョウバエで初期胚のさまざまな部位にこの遺伝子を発現させたところ（target expression），触覚や翅，肢などで異所性に複眼が発生し，しかもこれらが光を感じたため，眼という器官全体を1つ作ってしまうような強力な遺伝子であることが明らかになった[2]．器官が形成される場合，その過程が整然と行われるためには，全体を支配する遺伝子（master control遺伝子）があるだろうと予測されていたが，昆虫のような下等動物とはいえ，眼という最も複雑な器官でその遺伝子が見つかったのである（図4）．

その後，さまざまな動物で*Pax6*遺伝子が見つかり，

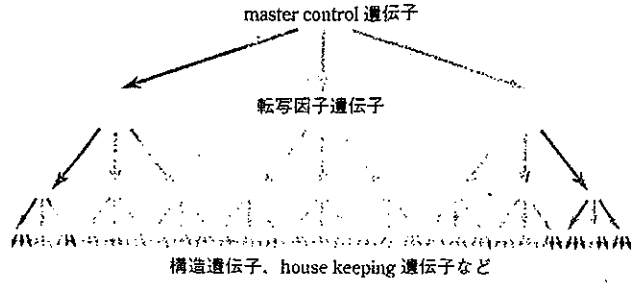


図4 形態形成遺伝子カスケード

脊椎動物，軟体動物の眼や昆虫の複眼だけではなく，プラナリアの原始的な眼や線虫の光感受性細胞にも存在していることが明らかになった．ほとんどすべての動物の