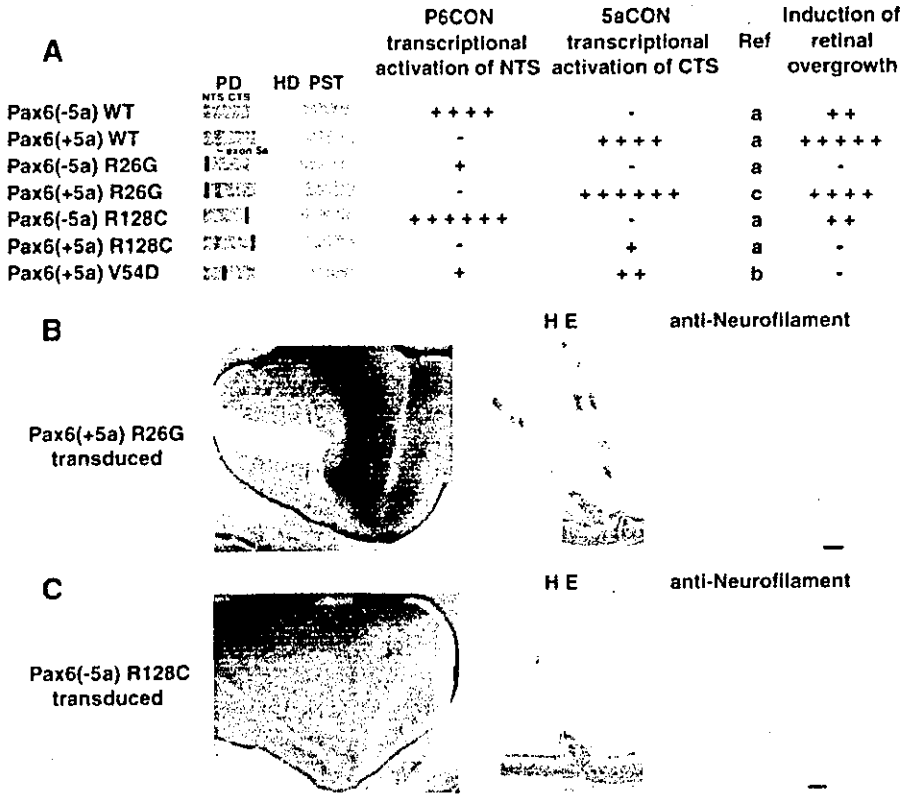

Figure 6. Effect of missense mutations of the *Pax6* gene on retinal overgrowth. (**A**) Schematic structure of the Pax6 wild-type and mutant (R26G, R128C and V54D) proteins with or without exon 5a that were used in this study. Our *in vitro* functional assays using P6CON- and 5aCON-CAT reporters in P19 cells have been reported previously [a, Yamaguchi *et al.* (22); b, Azuma *et al.* (5)] or are reported for the first time in this study (c). The effects of the mutants on overgrowth of the retina are also summarized. PD, paired domain (red, NTS; purple, CTS; blue, exon 5a; black bar, missense mutation); HD, homeodomain; PST, proline–serine–threonine rich transactivating domain. Each of the Pax6 mutants was electroporated into the right eye of HH stage 16 chick embryos and the changes around HH stage 35 were observed. (**B**) An eye that misexpresses Pax6(+5a) that carries the R26G mutation. The split eyeball shows the string- or stick-like structure of the overgrowing retina (left panel) (Pe, the pecten). Sections stained with HE and anti-neurofilament antibody suggest that the overgrowing tissues are thick bundles of nerve fibre and immature retina tissues (right panels, bar scale 100 μm). (**C**) An eye that misexpresses Pax6(−5a) that carries the R128C mutation. The split eyeball shows areas of swelling on the retina with fine fibres (left panel). Sections stained with HE and anti-neurofilament antibody reveal excessive differentiation of ganglion cells and their nerve fibres (right panels, bar scale 100 μm).

each mutant at each developmental stage is available in Supplementary Material.

DISCUSSION

We have shown here that when Pax6 is overexpressed in the developing chick eye, it induces ectopic differentiation of the retina. Compared with the effect of Pax6(−5a), Pax6(+5a) induces a remarkable artificial retina-like structure. Intriguingly, the ectopic retina-like structure induced by Pax6(+5a) is highly differentiated and contains well-formed retinal layers that express cone-specific colour opsins. We believe that the retinal overgrowth reported here is not an artifact but rather an exaggeration of the natural role of Pax6(+5a) in retinal development, namely, in the formation of the retinal area where visual cells highly accumulate. The assumption is based on two lines of evidence, as described subsequently.

First, Pax6(+5a) is expressed in a region of the developing retina where visual cells are densely packed (Figs 1 and 2). Previous studies have revealed that Pax6(+5a) is abundantly expressed in the lens and iris (37,38), but the expression pattern of Pax6(+5a) in the retina has not been clarified. As shown in previous studies and in the study reported here, the expression of the two Pax6 isoforms in the developing eye seems highly regulated at the levels of transcription and mRNA splicing (39,40).

Secondly, there is a clear correlation between the mutations in Pax6(+5a) that are associated with abnormal foveal formation in humans and that affect ectopic retinal formation in chick embryos. The V54D and R128C mutations disturbed the ectopic retinal structures induced by Pax6(+5a) as shown in Figure 6, while previous genetic analyses showed that these mutations are associated with foveal hypoplasia in human patients (4,5,26). As the V54D mutation in exon 5a should not affect the structure of Pax6(−5a), these observations suggest that Pax6(+5a) probably plays an important role in the formation of the fovea. Curiously, the V54D

mutation had only a modest effect on the transactivation activity of Pax6(+5a) in our reporter assay using P19 cells. It may be that a putative retina-specific cofactor that is not expressed in P19 cells may regulate the Pax6(+5a) activity in a V54D mutation-sensitive manner, thereby causing the apparent discrepancy. Alternatively, the V54D mutation may show a more potent effect when *cis* elements that diverge from the consensus sequences are used.

The two Pax6 isoforms seem to function differently in a qualitative rather than quantitative fashion. Pax6(−5a) overexpression does induce ectopic retina-like tissues. However, the incidence is far lower and the structures induced are far more immature when compared with those induced by Pax6(+5a) overexpression. As shown in Figure 6, the R26G mutation in the NTS and the R128C mutation in the CTS selectively impaired the induction of aberrant retinal structures by Pax6(−5a) and Pax6(+5a), respectively. Previous *in vitro* assays showed that Pax6(−5a) and Pax(+5a) bind to the distinct consensus sequences P6CON and 5aCON via different DNA-binding domains, namely, the NTS and the CTS, respectively. Thus, it is very likely that Pax6(−5a) and Pax6(+5a) have a different structural requirement for retinal development independently of each other and via different mechanisms. As these experiments were done in the retina that has endogenous Pax6 proteins, however, there is also a possibility that Pax6(+5a) exerts its effect on retinal development through modulation of Pax6(−5a) activity.

A different mechanism for Pax6-mediated gene regulation has been identified in *D. melanogaster* (41). There are four Pax6-related genes in *Drosophila*, namely *eyeless, twin of eyeless, eyegone* and *twin of eyegone*. Among them, *eyegone* has strong structural similarity with Pax6(+5a) and has been linked to growth control in the *Drosophila* eye. Overexpression of human Pax6(+5a) but not of Pax6(−5a) in *Drosophila* larvae induces strong overgrowth. Similarity of eyegone and Pax6(+5a) at a functional level is indicated by our data showing that overexpression of human Pax6(+5a) induces strong overgrowth of retina in the vertebrate eye.

Recently, mice lacking the Pax6(+5a) isoform were shown to have iris hypoplasia (38). Thus, the iris may be another part of the eye that is controlled by the Pax6(+5a) isoform. However, the knock-out mice showed no apparent abnormality in the retina. This does not conflict with our data, however, because mice intrinsically lack areas of high dense visual cells, including the fovea.

The regional expression of Pax6(+5a) may also be related to eyeball structure. It has been reported that a strictly controlled level of Pax6 expression is critical for the normal development of eyes. Transgenic mice carrying multiple copies of the Pax6 gene manifest severe eye anomalies and microphthalmos (42), while the same abnormalities are observed in mice with haploinsufficiency of this gene (43). However, microphthalmos is often associated with eye anomalies in which numerous eye tissues are affected (44,45). As Pax6 is expressed in numerous eye tissues throughout development (15–17), it may be that in the transgenic mice, the eye tissues, each of which expresses an abnormal dose of the gene (either loss-of-function or gain-of-function), affect neighbouring tissues and disturb their mutual relationship in eyeball growth, resulting in microphthalmos. In contrast, *in ovo* electroporation is able to transfer genes to a selected tissue. In our experiment, overexpression of Pax6 in the chick retina primordium caused enlarged eyes. The outer coat of the eyeball corresponding to areas of Pax6(+5a) misexpression was prominently enlarged. It is thought that retinal growth influences eyeball growth (1,45), and that the accumulation of retinal cells in the temporal posterior area may cause a larger growth in the temporal side of the eyeball than in the nasal side. Regional expression of the Pax6(+5a) isoform in the temporal posterior retina may lead to eyeball asymmetry.

Our observations also have implications regarding phylogenic development. The retinal layer structures are much more complex in vertebrates than in invertebrates. Structures that caused the visual cells to congregate at high density, such as the fovea, area centralis and visual streak, and eyeball asymmetry first appeared in fishes (1–3). The splice variant of Pax6 with exon 5a is present in vertebrates but not in invertebrates (20,21,38) except for *Drosophila*, which has *eyegone*, a putative homologue of Pax6(+5a) (42). Therefore, the acquisition of the Pax6 splice variant during evolution may have contributed to the formation of highly organized eye architectures that yield better vision. Thereafter, vertebrates may have preserved exon 5a so that they could form a restricted retinal domain that has high visual acuity.

The mechanism that regulates Pax6 alternative splicing has not yet been elucidated. Areas where retinal cells accumulate, including the visual streak, area centralis, and fovea, are positioned to promote visual acuity among animal species. Thus, further studies should focus on the signalling molecules that regulate the expression of Pax6 isoforms. In reproductive medicine research, studies have focused on transferring transcriptional factors into stem cells (46). As Pax6 induces the ectopic formation of eyes in flies (13) and frogs (14), this gene may be useful for regenerating regional eye tissue in vertebrates as well. Our results indicate that the use of Pax6(+5a) may be more suitable than Pax6(−5a) for reproducing highly differentiated retinal structures.

MATERIALS AND METHODS

Immunohistochemistry and *in situ* hybridization

A monoclonal antibody against chicken Pax6 that reacts to both Pax6(−5a) and Pax6(+5a) in chicken, monkey and human tissues has been described previously (16,17). A polyclonal antibody against the 14 amino acid residues encoded by exon 5a (THADAKVQVLDNQN) was raised by immunizing New Zealand white rabbits with a synthetic peptide. After purification, the immunoreactivity of the antibody was confirmed by ELISA and its specificity was further assayed by western blotting (data not shown). Antibodies against GFP (Clontech), 5-bromo-2′-deoxyuridine (BrdU; DAKO), Islet-1 protein (DSHB), Chx10 protein (Exalpha Biologicals), neurofilament H (DAKO) and peanut agglutinin (Vector) were purchased. Specimens were fixed in 4% paraformaldehyde, embedded in a Tissue-Tek OCT compound (Sankyo, Tokyo), and cryo-sliced into 8 μm sections. The sections were stained with haematoxylin and eosin (HE), or with a specific antibody followed by visualization with peroxydase

and diaminobenzidine. Section *in situ* hybridization was performed as described (47). Probes were prepared from plasmids that contain chick *Musashi* (*Eco*RI, *T7* polymerase), *Six3* (*Hin*dIII, *T3*) and *Rx* (*Hin*dIII, *T3*).

RNA isolation and RT–PCR

Total RNA was isolated from tissues excised from one to five chick embryos using an RNeasy Mini Kit (Qiagen) and converted to cDNA by a standard procedure using SuperScript II reverse transcriptase and adapter primers (GibcoBRL). cDNA was amplified under nonsaturating PCR conditions using the following primer sets: chicken *Pax6*, 5′-CGGCAG AAGATCGTGGAACTCG and 5′-GCACTCTCGTTTATA CTGCGCTAT [this yields a 207 bp band for *Pax6(−5a)* and a 249 bp band for *Pax6(+5a)*]; chicken *blue opsin*, 5′-GGCCTTTATGTTCCTCCTCATCG and 5′-CAGATGA CGAGGAAGCGCTCGA (297 bp); *green opsin*, 5′-TCCCT GGTGGTCTTGGCCATAG and 5′-TGCCTCTCGGACTTT GCAGATGA (320 bp); *violet opsin*, 5′-CTACCTACAG ACGGCCTTCATG and 5′-GCAGATAACGATGTAACG CTCGA (310 bp); and *rhodopsin*, 5′-GGCTGCCTACAT GTTCATGCTGA and 5′-ACGGCCAGGACGACGAGT GAC (281 bp). The PCR products were separated by gel electrophoresis. To standardize the RNA amounts, *β-actin* was also amplified by PCR with its specific primers: 5′-GT GGGTCGCCCCAGACATCA and 5′-CTCCTTGATGTCAC GCACAATTTC (540 bp). The PCR amplication involved 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. It should be noted that the alternative splicing exon of the human and mouse *Pax6* genes is situated between exon 5 and 6 and is known as 5a. However, the *Pax6* gene structure of the chick strain we used has not yet been fully determined. It may be that the alternative splicing exon of the chick may later be designated differently. For example, it has been suggested that this exon in the quail *Pax6* gene should be denoted as exon 4a. Nevertheless, in this report, we employ the term 5a to indicate the alternative splicing exon in the chick *Pax6* gene.

In ovo electroporation

Expression plasmids [pCAGGS-PAX6(−5a) and pCAGGS-PAX6(+5a)] carry the entire human *PAX6* coding region with or without exon 5a under the control of a cytomegalovirus enhancer and chicken β-actin promoter (5,22). The mutant forms of PAX6 expression plasmid were generated by PCR-based *in vitro* mutagenesis (5,22,27). Fertilized eggs of a domestic chick strain were purchased from Nisseizai (Tokyo). A small window was opened for access, and phosphate buffered saline was poured over the embryo to obtain appropriate resistance. The eggs were injected with ~0.1 μl of the DNA solution that contains an expression construct for GFP (pCAGGS–GFP) and one of the Pax6 expression plasmids (5 mg/ml) together with a fastgreen dye. The dye confirms that the injection was correctly targeted. Eggs, in which early changes are examined, were also injected with BrdU (0.3 mg/ml). The DNA solution was either injected into a region that is close to the primitive retina in the right optic cup or directly into the retina of the right eye of the embryos with a sharp glass pipette. The head of the embryo was then placed between platinum electrodes and electric pulses were applied (25–40 V, 90 ms, one to six times) with a CUY 21 electroporator (BEX Co., Tokyo). The egg-shells were sealed and the embryos were allowed to develop in humidified incubators at 38°C.

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

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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

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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 larvae* (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

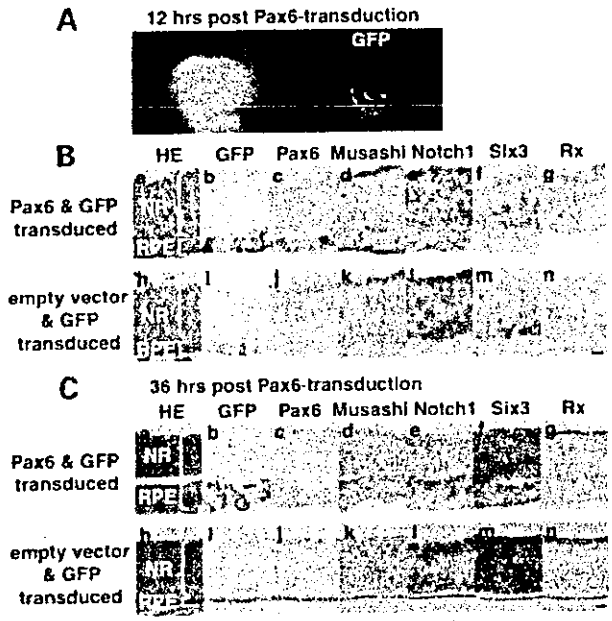

Figure 1. Early phases of NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene. The *Pax6* and *GFP* genes were misexpressed into the outer layer of the optic cup of stage 18 chick embryos. Eyes were examined 12 h (A and B) and 36 h (C) after electroporation. (A) Expression of 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 μm.

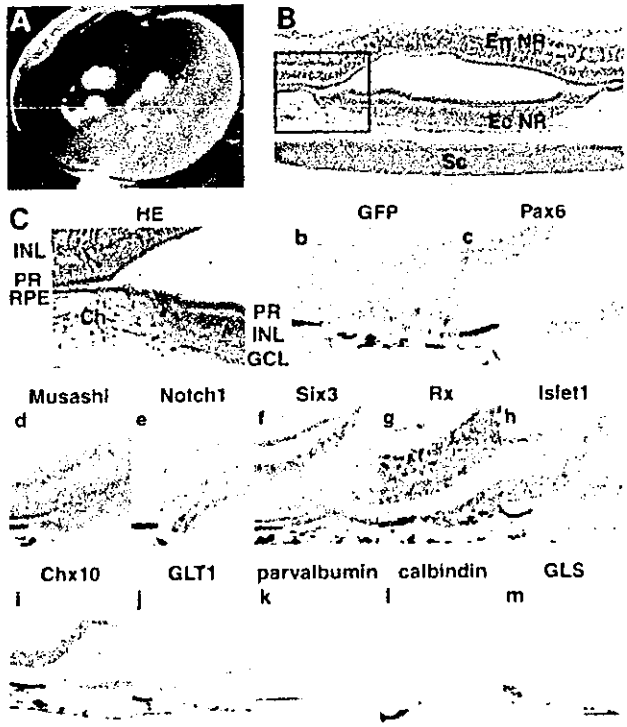

Figure 2. Fully structured NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene. The *Pax6* gene was misexpressed into the RPE layer of stage 24 chick embryos, and eyes were examined at stage 40. (A) In a half of the eyeball, patched areas of white swelling tissue are scattered. 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). EcNR, the ectopic NR transdiffcrentiated 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 μm. The results shown are representative of more than 200 independent experiments.

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 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).

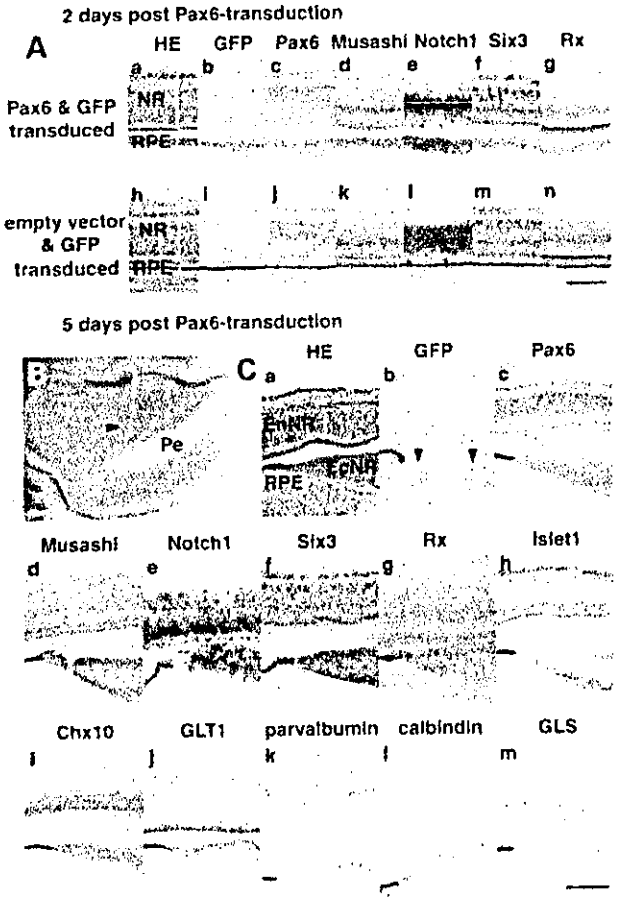

Figure 3. NR transdifferentiation from RPE cells by electroporation of the *Pax6* gene at a late stage. The *Pax6* and *GFP* genes were misexpressed into the RPE layer at stage 35. Eyes were examined 2 days (at stage 37) (A) and 5 days (at stage 40) (B and C) post electroporation. (A) Light microscopy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

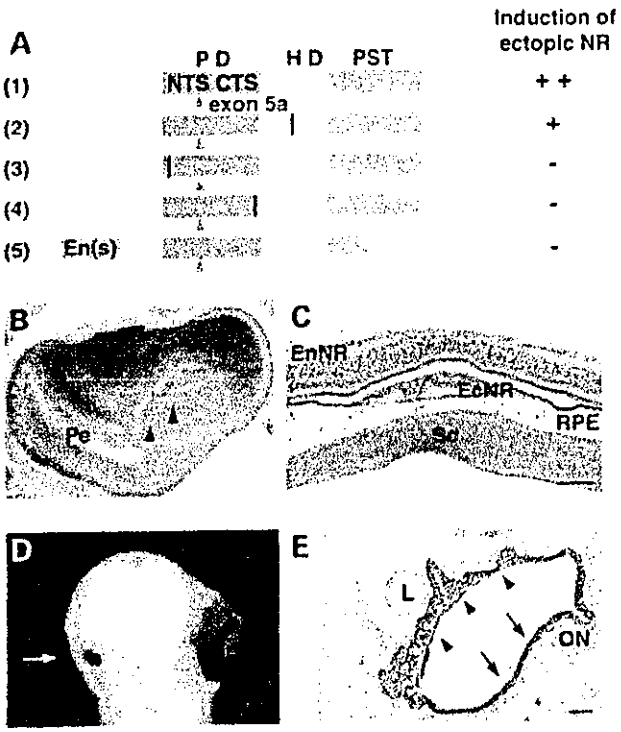

Figure 4. Effect of missense mutations or repression of the Pax6 gene in NR transdifferentiation. (**A**) Structure of the *Pax6* cDNA (1), Pax6 mutants [F258S (2), R26G (3) and R128C (4)] and En(s)–Pax6delC+ (5) used in these studies. The effects of the mutants and repression on NR transdifferentiation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

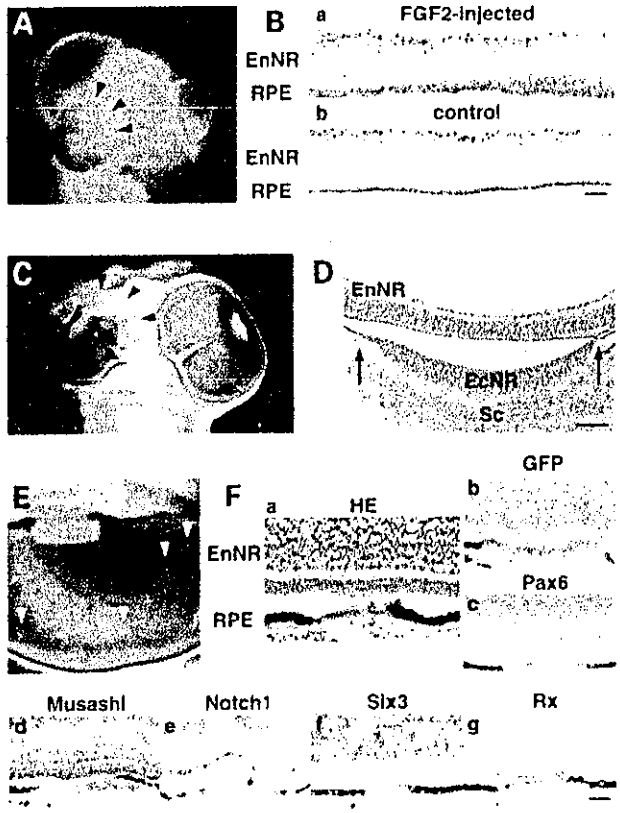

Figure 5. Expression of Pax6 in the ectopic NR transdifferentiated from RPE by FGFs treatment. (**A** and **B**) A stage 20 embryo, in which FGF2 was injected 12 h before (stage 18). (A) In the anterior half of the eye, the RPE layers lose pigments (arrowheads). (B) Immunohistochemistry shows expression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

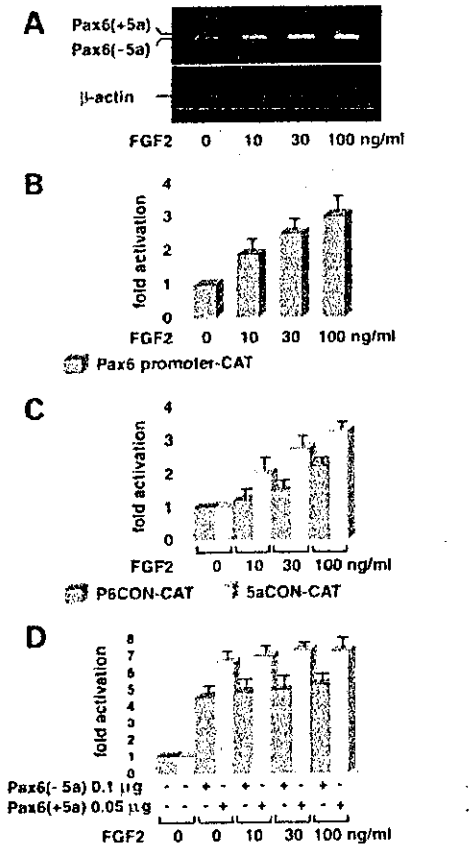

Figure 6. Effect of FGFs on Pax6 expression by functional assay. (**A**) Semi-quantitative analyses for expression levels of endogenous *Pax6* by RT–PCR in P19 cells treated with FGF2. The results shown are representative of three independent experiments. (**B** and **C**) CAT activities in P19 cells 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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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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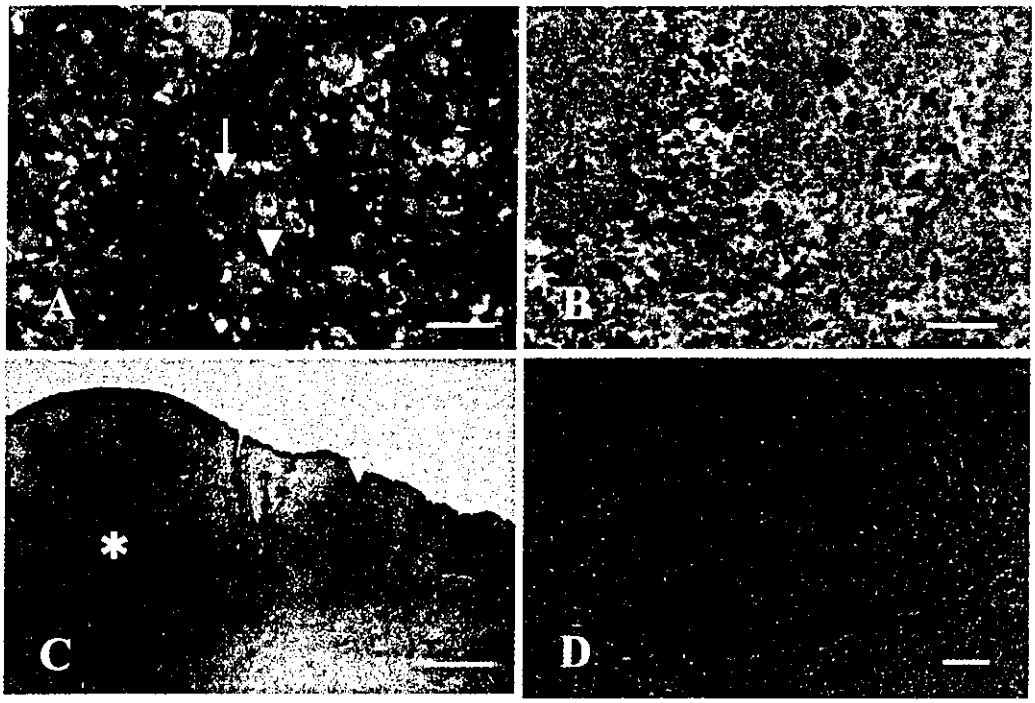

Figure 1. **A** Photomicrograph of the first eyelid excision shows lesion with foamy histiocytes (*arrowhead*) and characteristic Touton giant cells (*arrow*). H&E, bar = 50μm. **B** Immunolabeling for CD68 is positive in all sections. Bar = 100μm. **C** Low-power photomicrograph of the fourth eyelid 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.

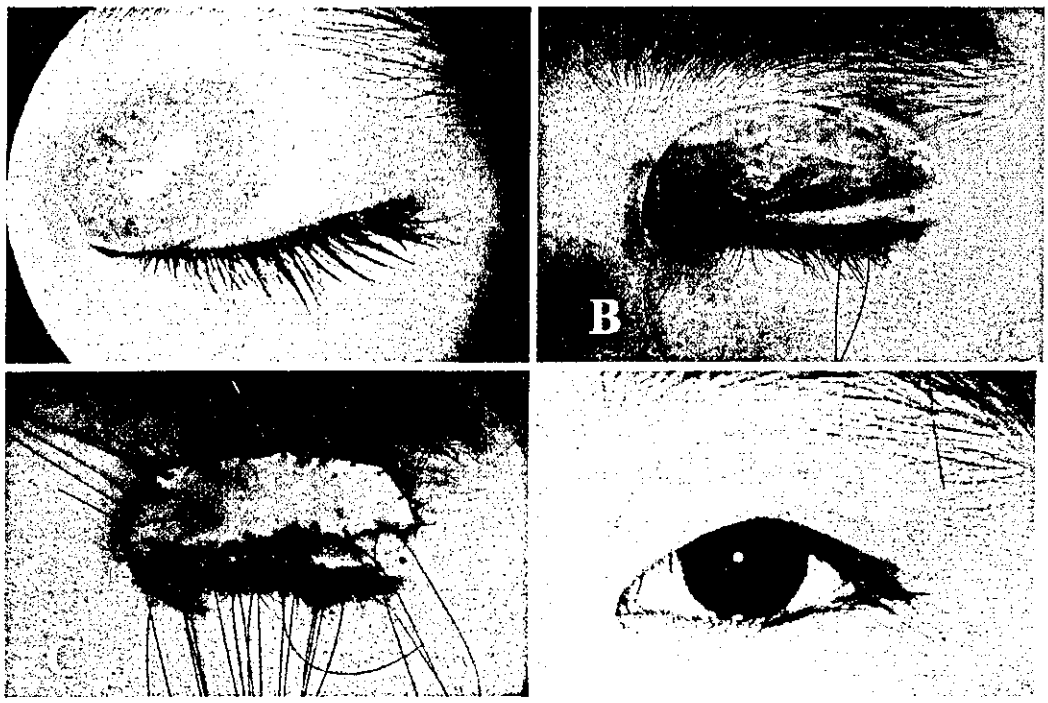

Figure 2. **A** The recurrent lesion has grown to a reddish, 14 × 19-mm mass. **B** The fourth operation: after complete excision of the mass with surrounding cutaneous tissue, orbital fat tissue is placed on the levator muscle to form a recipient bed. **C** The skin graft from the preauricular area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

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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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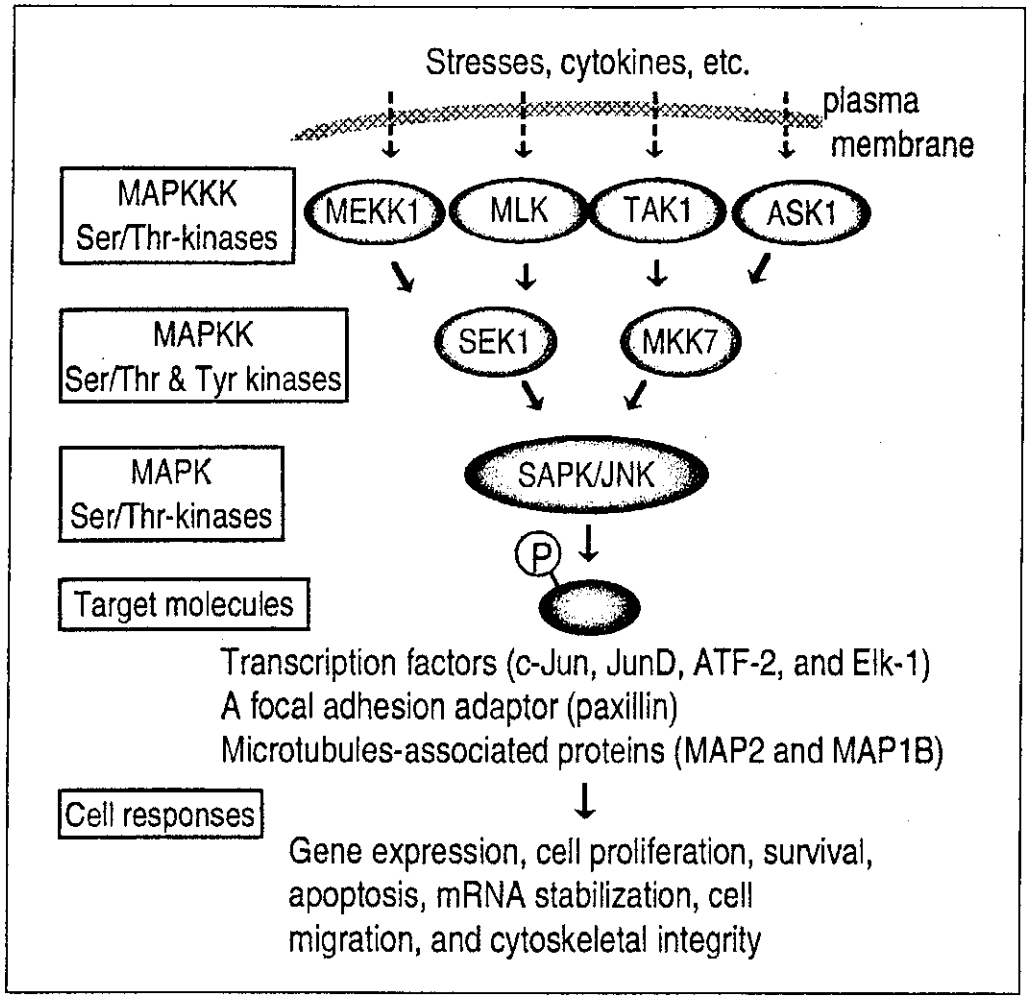

Fig. 1 - SAPK/JNK-signaling pathways involved in a variety of cell responses. SAPK/JNK is activated by extracellular stimuli including stresses and cytokines through kinase cascades. Activated MAPKKKs such as MEKK1, MLK, TAK1, and ASK1 transmit the signal to two MAPKK, SEK1 and MKK7. SAPK/JNK activated 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

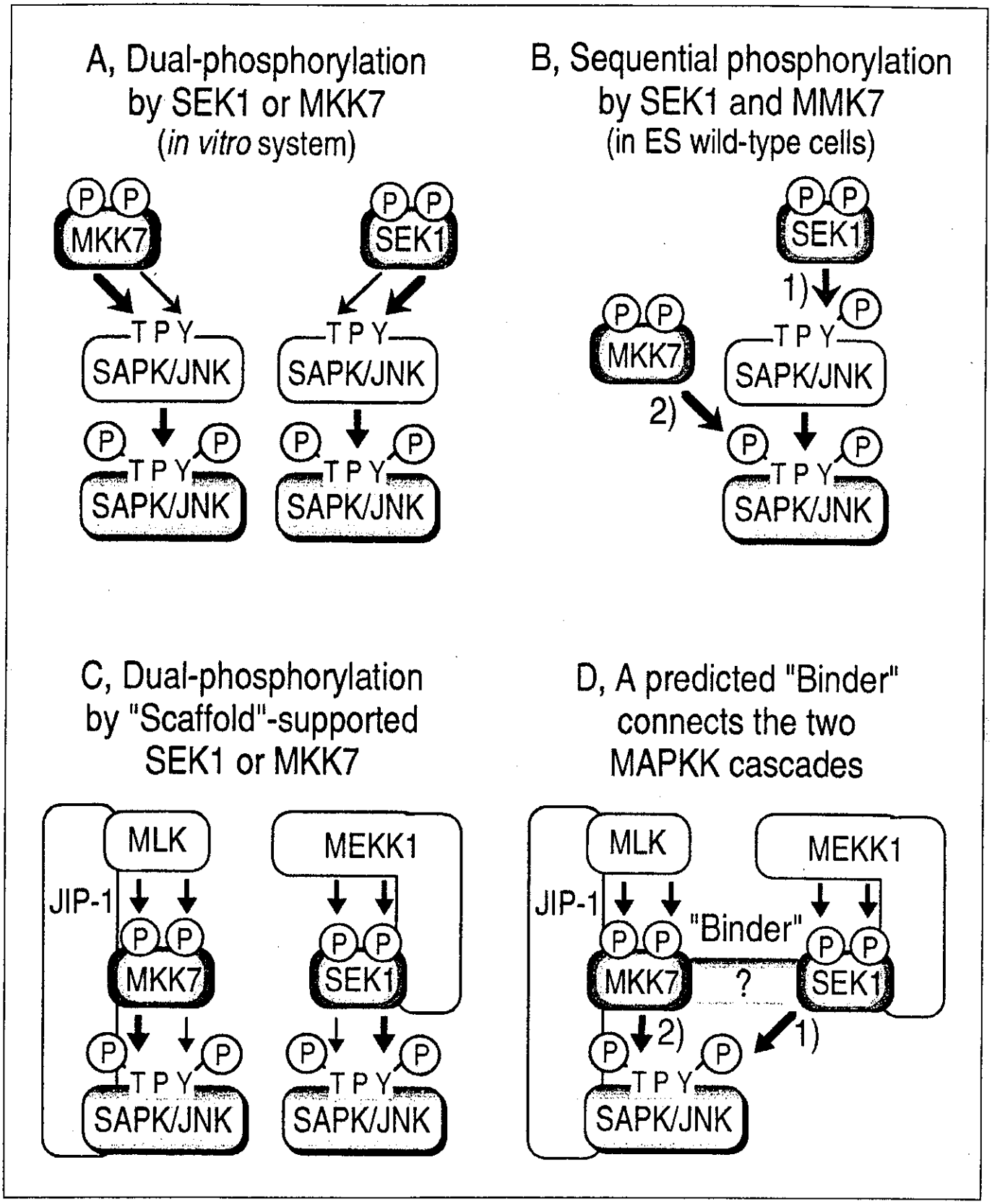

Fig. 2 - Schematic description of SAPK/JNK phosphorylation by SEK1 and MKK7 under various conditions. A) synergistic activation of SAPK/JNK by the dual-specificity kinase, SEK1 or MKK7, which has been reported in *in vitro* conditions (6-8). B) synergistic activation of SAPK/JNK through sequential phosphorylation 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.

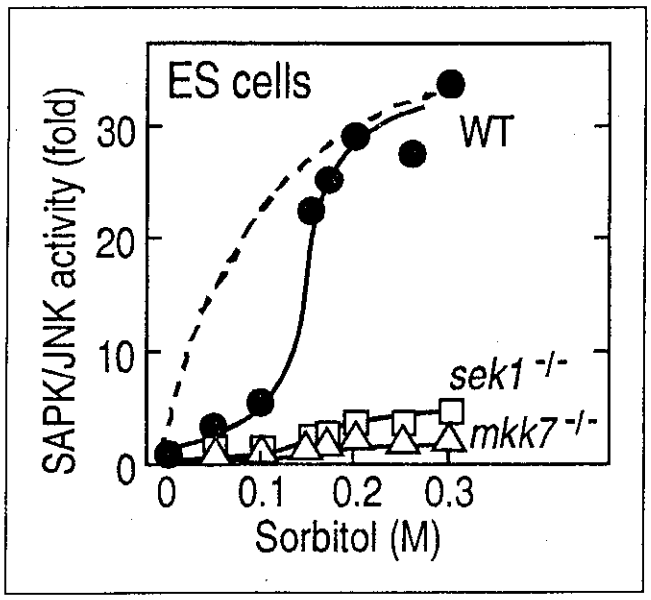

Fig. 3 - SAPK/JNK activation in response to hyper-osmolar stress (sorbitol) requires both SEK1 and MKK7 in ES cells. Wild-type, sek1$^{-/-}$, and mkk7$^{-/-}$ ES cells were stimulated with the indicated concentrations of sorbitol for 30 min.

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).

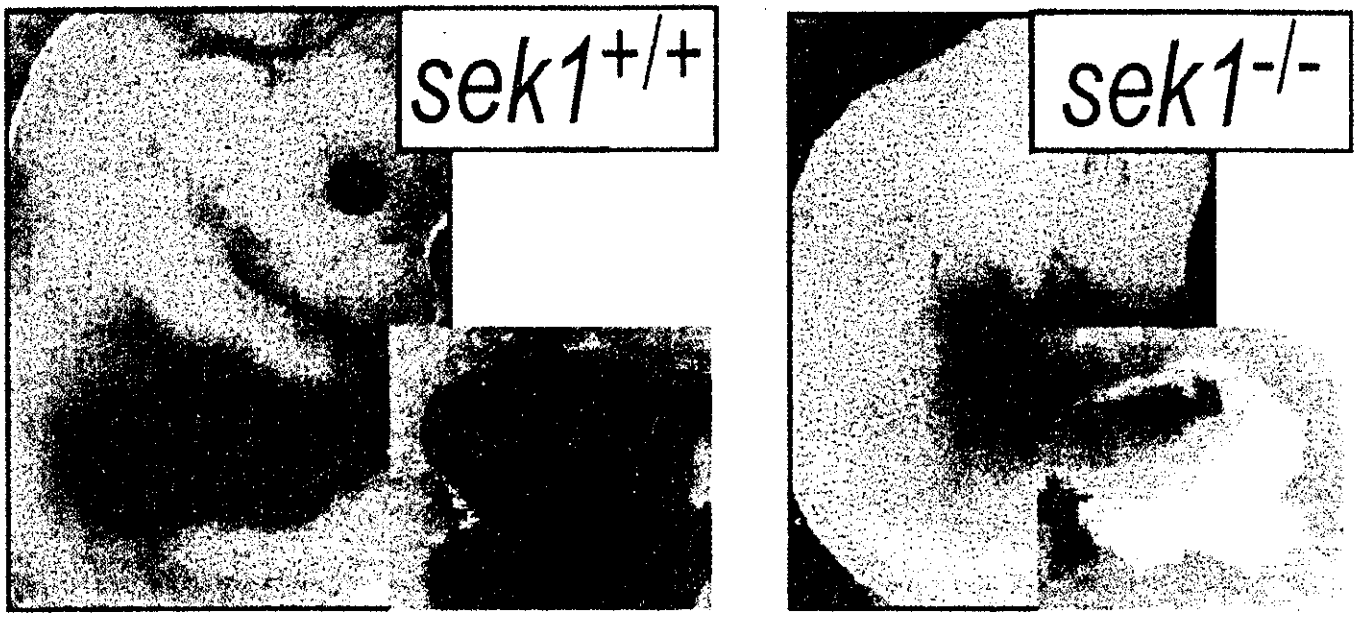

Fig. 4 - Defective liver formation in sek1$^{-/-}$ embryos. Appearance of wild-type and sek1$^{-/-}$ embryos and livers at embryonic day 12.5. Severe anemia is observed in sek1$^{-/-}$ embryos.