

Role in Transdifferentiation of Retinal Pigment Epithelium of *silver* Mutant Quail: Localization of FGF and Related Molecules and Aberrant Migration Pattern of Neural Crest Cells during Eye Rudiment Formation

Masasuke Araki,^{*,1} Takako Takano,^{*} Tomoko Uemonsa,^{*}
Yoshifumi Nakane,^{†,2} Masaoki Tsudzuki,[‡] and Tomoko Kaneko[§]

^{*}Developmental Neurobiology Laboratory, Department of Biological Sciences, Nara Women's University, Nara 630-8506, Japan; [†]College of Agriculture, University of Osaka Prefecture, Osaka 599-8531, Japan; [‡]Laboratory of Animal Genetics, Faculty of Applied Biological Science, Hiroshima University, Hiroshima 739-8528, Japan; and [§]University of Shizuoka, Junior College, Shizuoka 422-8021, Japan

Homozygotes of the quail *silver* mutation, which have plumage color changes, also display a unique phenotype in the eye: during early embryonic development, the retinal pigment epithelium (RPE) spontaneously transdifferentiates into neural retinal tissue. *Mitf* is considered to be the responsible gene and to function similarly to the mouse microphthalmia mutation, and tissue interaction between RPE and surrounding mesenchymal tissue in organ culture has been shown to be essential for the initiation of the transdifferentiation process in which fibroblast growth factor (FGF) signaling is involved. The immunohistochemical results of the present study show that laminin and heparan sulfate proteoglycan, both acting as cofactors for FGF binding, are localized in the area of transdifferentiation of *silver* embryos much more abundantly than in wild-type embryos. More intense immunohistochemical staining with FGF-1 antibody, but not with FGF-2 antibody, is also found in the neural retina, RPE, and choroidal tissue of *silver* embryos than in wild-type embryos. HNK-1 immunohistochemistry revealed that clusters of HNK-1-positive cells (presumptive migrating neural crest cells) are frequently located around the developing eyes and in the posterior region of the *silver* embryonic eye. Finally, chick-quail chimerical eyes were made by grafting *silver* quail optic vesicles to chicken host embryos: in most cases, no transdifferentiation occurs in the *silver* RPE, but in a few cases, transdifferentiation occurs where *silver* quail cells predominate in the choroid tissue. These observations together with our previous *in vitro* study indicate that the *silver* mutation affects not only RPE cells but also cephalic neural crest cells, which migrate to the eye rudiment, and that these crest cells play an essential role in the transdifferentiation of RPE, possibly by modifying the FGF signaling pathway. The precise molecular mechanism involved in RPE-neural crest cell interaction is still unknown, and the quail *silver* mutation is considered to be a good experimental model for studying the role of neural crest cells in vertebrate eye development. © 2002 Elsevier Science (USA)

Key Words: retina; retinal pigment epithelium; neural crest cell; laminin; heparan sulfate; fibroblast growth factors; chimera embryo; transdifferentiation; *silver* mutation; *Mitf*.

INTRODUCTION

During development of the eye, the optic vesicle invaginates to form a double-walled optic cup. As invagination

continues, the two layers of the optic cup begin to differentiate in different directions; the outer layer becomes the retinal pigment epithelium (RPE) and the inner layer generates a variety of retinal cells constituting the neural retina (NR). While this developmental process proceeds, the proximal part of the optic vesicle faces the epidermis and the distal part makes contact with the mesenchymal tissue, a part of which is considered to be derived from neural crest

¹ To whom correspondence should be addressed. Fax: 81-742-20-3411. E-mail: masaaraki@cc.nara-wu.ac.jp.

² Present address: Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Yoshida-Konoe, Kyoto 606-8501.

cells (see review by Hall and Hörstadius, 1988). This indicates that the two parts of the optic vesicle encounter different environmental signals, which may activate or regulate the expression of several genes necessary for the development of either the RPE or the NR.

Earlier studies using classical transplantation techniques suggested that the surrounding tissues play an important role in the induction of the RPE and NR (Lopashov, 1963; Stone, 1950). Retinal regeneration or transdifferentiation has also been intensively studied to elucidate the mechanism involved in the tissue determination and maintenance of the NR and RPE properties (see reviews by Okada, 1991; Eguchi and Kodama, 1993). Several environmental factors have been shown to alter the stability of RPE cells; for example, intraocular administration of fibroblast growth factor (FGF-2) can induce NR generation from RPE in the early chick embryo (Park and Hollenberg, 1989, 1992). *In vitro* experiments also showed that retinal differentiation from the RPE of a chick embryo can be induced by FGF-1 or FGF-2 (Guillemot and Cepco, 1992; Zhao *et al.*, 1995; Pittack *et al.*, 1991). In addition to FGFs, extracellular matrix (ECM) components appear to be significant environmental cues for such switching. Laminin was reported to induce RPE cells of *Rana* tadpoles to transdifferentiate into neurons *in vitro* (Reh *et al.*, 1987). Because the biological activity of FGFs is dependent on their molecular interaction with heparan sulfate proteoglycans (HSPG) for high-affinity binding with FGF receptors (reviewed by Gospodarowicz, 1990), the distribution of HSPG in the ocular tissues seems to be crucial for FGF-induced RPE transdifferentiation.

An incompletely dominant autosomal gene *B* (Homma *et al.*, 1969) controls the silver plumage color mutation of the Japanese quail, and the mutation is believed to affect neural crest-derived cells, particularly the epidermal melanocytes. Homozygotes of this gene obtained from white-feathered quail show an interesting phenotype in the eyes, having a circular transparent area only around the posterior center of the eye (Fuji and Wakasugi, 1993). This area consists of double neural retinal layers due to aberrant differentiation of the RPE to form a new neural retinal layer. This observation suggests that the *silver* mutation gene plays a critical role in the RPE and that developmental analysis of the *silver* homozygote would promote understanding of the development of the eye. A candidate gene has been identified as *Mitf*, encoding a basic helix–loop–helix-leucine zipper (bHLH zip) transcriptional factor. The *Mitf* gene from the *silver* homozygote has an amino acid substitution in the basic region and is truncated in the C-terminal region (Mochii *et al.*, 1998).

A similar mutation has been reported in the eye development of the mouse microphthalmia (Packer, 1967; Scholtz and Chan, 1987), and it has been demonstrated that the *Mitf* gene controls the development of the RPE in addition to regulating the development of neural crest-derived melanocytes (Hodgkinson *et al.*, 1993; Hughes *et al.*, 1993). In the mouse, *Mitf* gene expression begins first in the entire optic vesicle, preceding the separation into presumptive NR and

RPE, and only later does its expression become restricted to the presumptive RPE, although it is still not known how the RPE hyperproliferates and displays a restricted area developing into a second retina when rendered nonfunctional in embryos with *microphthalmia* mutations (Nakayama *et al.*, 1998; Nguyen and Arnheiter, 2000).

In a previous study, we showed that the RPE of the early embryonic eyes of *silver* homozygotes (*B/B*), when cultured *in vitro* with the surrounding mesenchyme attached, change their fate and differentiate into NR in the same way as *silver* homozygotes develop *in vivo* (Araki *et al.*, 1998). We also showed that pure isolated RPEs do not transdifferentiate into NR when cultured alone. In the presence of either acidic FGF (FGF-1) or basic FGF (FGF-2), the RPE of the *B/B* mutant differentiates into NR cells in the absence of mesenchymal tissue, but the RPE of wild-type embryos only does so in the presence of 10–40 times as much FGF-1 or FGF-2. These observations suggest that FGFs may be key factors that induce the transdifferentiation of RPE in the *silver* mutant eyes. To answer the question of why only the restricted portion of the RPE differentiates into NR, determining spatial distributions of these candidate factors is of particular importance. Accordingly, in the present study, we investigated the localization of FGFs and related molecules in embryonic eyes.

We also attempted to determine whether any difference could be found in the distribution of neural crest cells between wild-type quail embryos and *silver* homozygotes to understand what role neural crest cell-derived mesenchymal cells play in transdifferentiation. To this end, migrating neural crest cells were examined by HNK-1 immunohistochemistry. Finally, quail–chick chimerical transplantation was carried out to produce altered genetic combinations of the RPE and adjacent mesenchymal cells. The results indicated that the emergence of the *silver* homozygotic phenotype (transdifferentiation of RPE into NR in only a limited region) depends on the genotype of the connective tissue cells adjoining the RPE. Thus, it was concluded that neural crest-derived mesenchymal cells migrating to the eye rudiments play a crucial role in the transdifferentiation of the *silver* mutation.

MATERIALS AND METHODS

Animals

Wild-type (*UOP-WT* line) and *silver* (*B*) mutant (Homma *et al.*, 1969) quail were used in this study. Eggs were collected daily and kept at 16°C until incubation at 38°C was started. Embryos of the *B/B* genotype were obtained by mating homozygotes among themselves. Embryo staging was done according to Hamburger and Hamilton (1951) or based on the somite number.

Immunohistochemistry

Immunohistochemistry was performed on developing embryos as described in a previous paper (Araki, 1992). Briefly, quail

embryos were fixed with ice-chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 6–10 h. After washing thoroughly with the same buffer containing 10% sucrose followed by a buffer containing 20% sucrose, embryos were embedded in Tissue-Tek, and 8- to 10- μ m sections were cut on a cryostat. Sections were processed for immunohistochemistry either by the indirect method or by the avidin-biotin-peroxidase complex (ABC) method. For the indirect method, the immunoreactive sites were detected by using a fluorescein-conjugated second antibody (Alexa-488; Molecular Probes), and for the ABC method, they were visualized by using hydrogen peroxide and diaminobenzidine.

The primary anti-laminin and anti-fibronectin antisera were purchased from E-Y Laboratory (San Mateo, CA) and diluted 2,000 times. Their specificities were confirmed as described in our previous paper (Ogawa et al., 1989). Monoclonal anti-FGF-1 and anti-FGF-2 were obtained from Upstate Biotechnology (NY) and BD Transduction (KY), and were diluted 800–1000 times. Their specificity was confirmed by immunoblot analysis and by staining sections with antibodies that had been incubated with an excess amount of FGF-1 or FGF-2. Monoclonal anti-heparan sulfate (10E4) was obtained from Seikagaku Corp. (Tokyo, Japan) and diluted 200 times. Its specificity was confirmed as described previously (David et al., 1992; Bai et al., 1994). Heparitinase treatment of sections prior to incubation with anti-heparan sulfate was also done according to the procedure described previously (Bai et al., 1994) to examine whether the reaction product was due to a reaction with a specific antibody binding to heparan sulfate.

Neural Crest Markers

Immunohistochemistry of HNK-1, a monoclonal antibody for a neural crest cell marker, was performed on sections of paraffin-embedded embryos and on whole embryonic preparations. Embryos between stages 10 and 32 were fixed in Bouin fixative and dehydrated with a graded series of ethanol. Whole embryo preparations were then rehydrated and processed for immunohistochemistry by the ABC method. The monoclonal antibody was purchased from Zymed Laboratories (San Francisco, CA).

Chimerical Transplantation of the Optic Vesicle

Isolated quail optic vesicles were transplanted into the corresponding portion of host chicken embryos. The host embryos used were between the 10- and 12-somite stages, and the stage was determined by injecting India ink beneath the embryos before the operation. Optic vesicles were removed from quail embryos between the 10- and 18-somite stages. For this purpose, quail embryos were placed in round-bottomed dishes filled with Hanks' solution supplemented with 2% fetal bovine serum (FBS). The right optic vesicle was carefully amputated with a fine knife at the proximal end of the vesicle together with the ectoderm and mesenchymal

tissue. The tissues were placed in ice-chilled Hanks' solution with FBS until grafting. The corresponding portion of a host chick embryo was cut *in ovo*, and the graft of the quail optic vesicle was placed in the corresponding cavity of the host. A few hours later, the amputated optic vesicle was examined to determine whether it had stuck to the brain vesicle of the host embryos. Eggs were incubated until embryonic day 6–9, when the embryos were fixed with Bouin fixative to prepare sections for ordinary histological staining with hematoxylin and eosin and for immunohistochemistry with quail cell-specific antibody (QCPN, obtained from DSHB of University of Iowa).

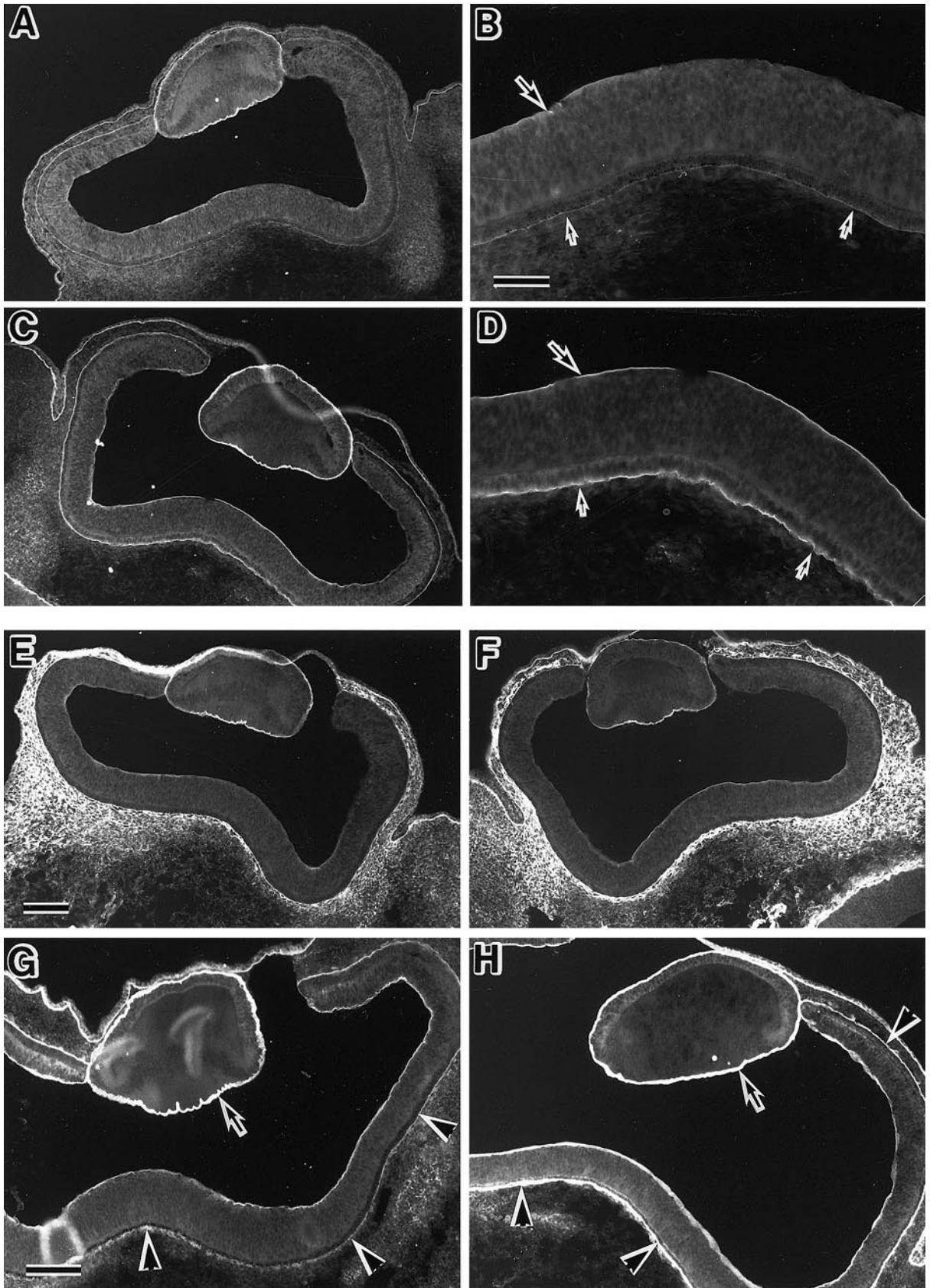
RESULTS

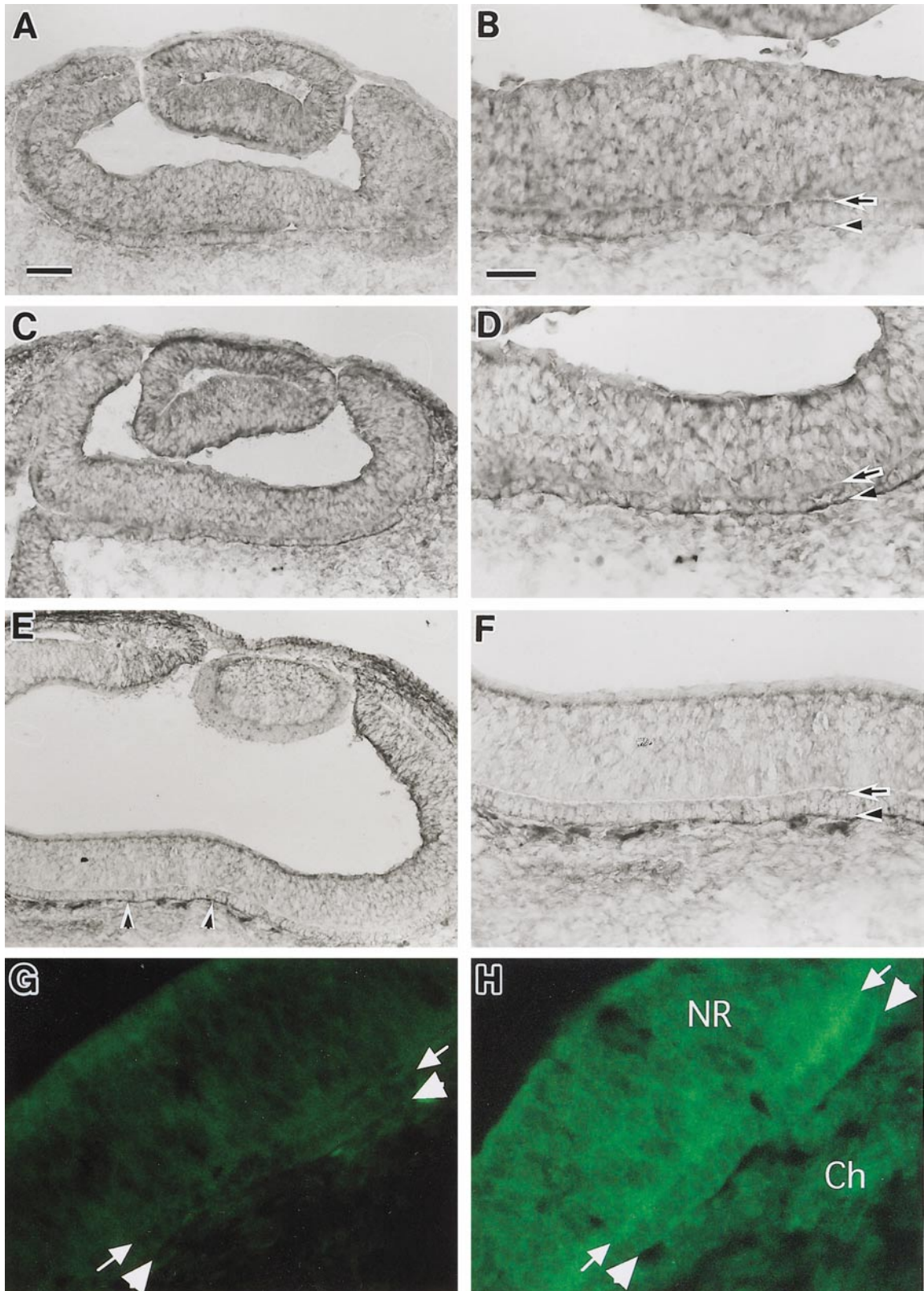
Localization of ECM Components and FGF-1

Laminin and fibronectin. The distribution of laminin-like immunoreactivity (laminin-LI) and fibronectin-like immunoreactivity (fibronectin-LI) in the head region was essentially the same as reported previously (Adler et al., 1985; Turksen et al., 1985; Cohen et al., 1987). No clear differences were observed in the overall distribution of laminin-LI and fibronectin-LI between the *silver* homozygotes (*B/B*) and wild-type embryos (Fig. 1). At embryonic stage 18, no difference was noticed in the distribution of laminin-LI between *B/B* and wild-type eyes. At embryonic stages 19–20, however, a significant difference in the intensity of laminin-LI was found in the RPE basal lamina (Figs. 1A and 1C): in the *B/B* eyes, a clearer and more intense band of laminin-LI was found in the RPE basal lamina both in the peripheral and posterior central portions than was observed in the wild-type eyes (Figs. 1C and 1D). In the eyes at stages 22–23, a prominent difference was found in the RPE basal lamina (Figs. 1G and 1H): the laminin-LI became more intense in the *B/B* eyes, whereas in the wild-type eyes, it remained faint or often showed a discontinuously stained band, although the peripheral portion (the future iris) of the RPE of wild-type embryos still showed intense laminin-LI. In contrast to laminin-LI, no apparent difference was noticed in fibronectin-LI between the *B/B* and wild-type embryos (Figs. 1E and 1F); intense staining of fibronectin-LI was seen in the RPE basal lamina and its surrounding connective tissues.

Heparan sulfate. HSPG occur in the extracellular matrix and are believed to play a significant role in the interaction between FGFs and their specific surface receptors (Rapraeger et al., 1991; Yayon et al., 1991). To detect HSPG-like immunoreactivity (HSPG-LI), we used an anti-

FIG. 1. Immunohistochemistry of laminin and fibronectin. (A–F) Embryonic eyes at stage 20 stained for laminin (A–D) or fibronectin (E, F). (G, H) Eyes at stage 23 stained for laminin. (A, B, E, G) Wild-type eyes. (C, D, F, H) *B/B* mutant eyes. Arrowheads in (B, D, G, H) and arrows in (B, D) indicate laminin-like immunostaining found at the Bruch's membrane of the RPE and at the inner limiting membrane of the NR, respectively. Note that more intense staining is seen in the Bruch's membrane of *B/B* mutant eyes than in wild-type eyes (compare B with D, and G with H), while no difference can be seen in the lens epithelium (arrows in G, H). Bar in (E) is 100 μ m and applies to (A, C, F). Bar in (B) is 50 μ m and applies to (D). Bar in (G) is 100 μ m and applies to (H).





body that was raised against HSPG and specifically recognizes N-sulfated glucosamine residues.

At stage 18, HSPG-LI was diffusely distributed in the lens, NR, RPE, and mesenchymal tissues around the RPE (Figs. 2A–2D). In the *B/B* eyes, the basement membranes of the lens epithelium, the inner surfaces of the NR and RPE, were lined with clear HSPG-LI materials (Figs. 2C and 2D), while those of wild-type eyes were not stained distinctly (Figs. 2A and 2B). At stage 20, the basement membranes of the NR and RPE were clearly stained in the *B/B* eyes (Figs. 2E and 2F), but not in the wild-type eyes. The mesenchymal tissue of the future iris portion became more intensely stained than in the previous stage (Fig. 2E). Essentially the same observations were made at stage 22. Heparitinase digestion of sections prior to immunohistochemical staining revealed an absence of HSPG-LI in the inner limiting membrane of the NR and the basement membrane of the RPE.

FGF-1 and FGF-2. During the embryonic stages examined (from stage 18 to 23), the intensity of FGF-1-like immunoreactivity (FGF-1-LI) in the ocular tissues tended to decrease. At embryonic stage 18, no difference could be discerned between *B/B* and wild-type embryos, and FGF-1-LI was diffusely distributed in the lens, NR, and RPE. At stage 20, the *B/B* RPE and NR were significantly more intensely stained than those of wild-type embryos. At stage 22, the difference in the staining intensity was more obvious (Figs. 2G and 2H); diffuse staining was found in the NR, RPE, and choroid tissue of *B/B* embryos, while little immunofluorescence could be detected in the wild-type RPE.

The localization of FGF-2-like immunoreactivity (FGF-2-LI) generally resembled that of FGF-1-LI (data not shown). However, no clear differences were noticed between *B/B* and wild-type eyes in the early stages. In contrast to the FGF-1-LI localization found at stages 20–22, the RPE and choroid tissue were mostly devoid of FGF-2-LI in both *B/B* and wild-type eyes.

Distribution of HNK-1-Positive Neural Crest Cells in the Optic Vesicle

Examination of whole-embryo preparations by HNK-1 immunohistochemistry showed temporal and spatial distributions of HNK-1-positive cells that were fundamentally the same as reported previously (Kuratani, 1997). During

early embryonic development (between the 10-somite and 18-somite stages), no significant differences were found between wild-type and *B/B* embryos, except that a few HNK-1-positive cells appeared to extend out toward the anterior procerebrum (Figs. 3A and 3B). Later in development, however, a conspicuous difference was observed from around the 28- to 36-somite stages (Figs. 3C–3F): numerous positively stained cells were found around the lens vesicle of *B/B* embryos, while only a few were found in the same region of the wild-type embryos. Since there were no clear differences in the earlier development, these observations suggest that more HNK-1-positive cells migrate to the developing eye region in *B/B* embryos. In regions other than the eyes, HNK-1-positive cells were distributed similarly in wild-type and *B/B* embryos.

In sections from *B/B* embryos at stage 18 (approximately 3 days of incubation), a few positively stained cells were often observed to be clustered in the mesenchymal layer (future choroid layer) adjoining the RPE layer (Fig. 3H), but in the wild-type embryos, only a few cells were observed in the same area (Fig. 3G), suggesting that neural crest cells of *B/B* embryos were still migrating to settle in the mesenchymal tissues at this time of development.

Transplantation of silver Optic Vesicle to Normal Chick Embryos

Chick embryos receiving a graft of quail optic vesicles were incubated until embryonic day 6–9 (stages 25–35). At these stages, the central part of the RPE has transdifferentiated into NR in *B/B* embryos, forming a multistratified layer. In a series of transplantations (3 successful cases) in which host chick embryos at the 10- to 11-somite stage received an optic vesicle graft from wild-type quail embryos at the same stage, the spliced optic vesicle developed normally. Both the neural retina and RPE developed normally, and the size of donor eyes was similar to the normal quail eye of this stage, and obviously smaller than the contralateral host chick eye. Close observation revealed that many of the fibroblastic cells in the choroid layer were chicken phenotype, and the sclera cartilage consisted of both chick and quail cells intermingled with each other. Some chick cells were found in the corneal epithelium, and numerous chick cells were also present in the cornea stroma.

FIG. 2. Immunohistochemistry of HSPG (A–F) and FGF-1 (G, H). (A–D) Eyes at stage 18. (E, F) Eyes at stage 20. (G, H) Eyes at stage 22. (A, B) Wild-type eyes. Diffuse immunostaining is found in the lens, NR, and RPE. In the RPE, immunostaining is not obvious at the basal side of the cells (lower arrowhead in B). (C, D) *Silver* mutant eyes at stage 20. (C) More intense staining is seen in the lens, NR, and RPE. In particular, intense immunoreactivity is found at the basal side of the RPE (lower arrowhead in D). (E, F) *Silver* eyes at stage 20. Immunostaining is found in the RPE basement membrane, particularly at the posterior portion (arrowheads). The basement membrane of the NR is rather uniformly stained. (G, H) FGF-1 fluorescent immunohistochemistry of wild-type eye (G) and *silver* mutant eye (H). The posterior region of eyes is shown. At this stage of development (stage 22), little fluorescence is detected in the retina (NR), RPE, and choroid tissue (Ch) of wild-type eyes (shown in G), whereas intense fluorescence is seen in *silver* homozygote eyes (in H). Arrows indicate the interface between the NR and RPE. Scale bar in (A) indicates 50 μ m, and applies to (C, E). Scale bar in (B) is 20 μ m, and applies to (D, F and G, H).

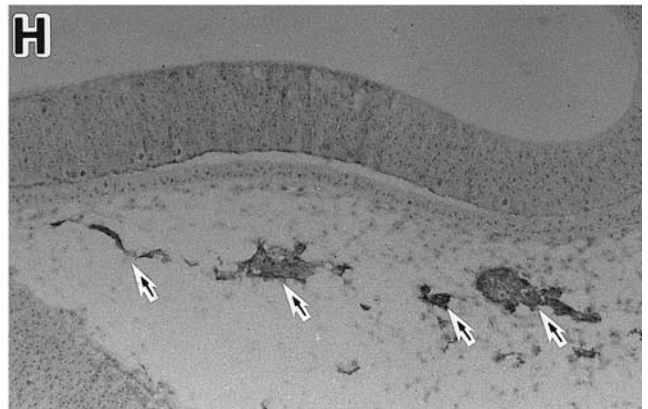
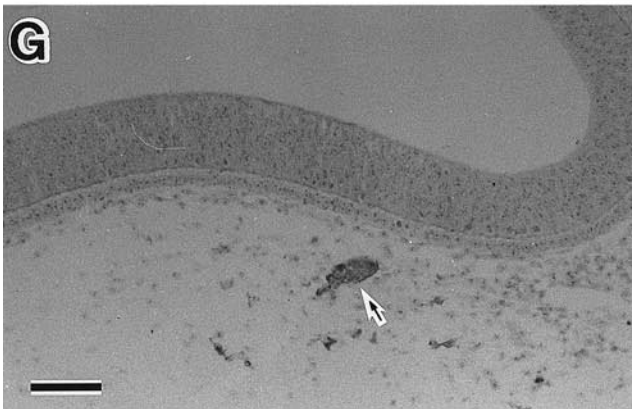
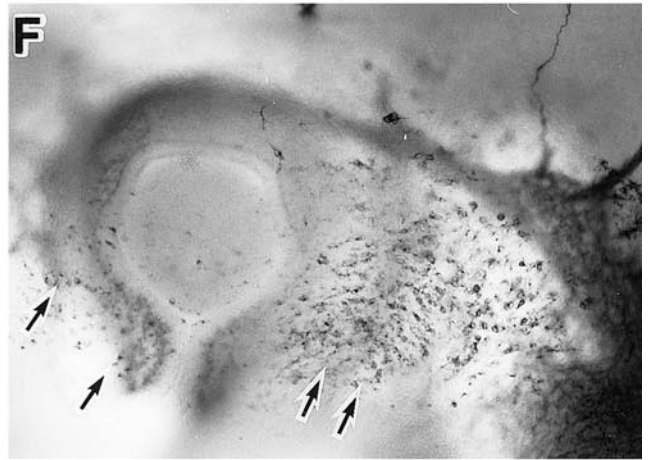
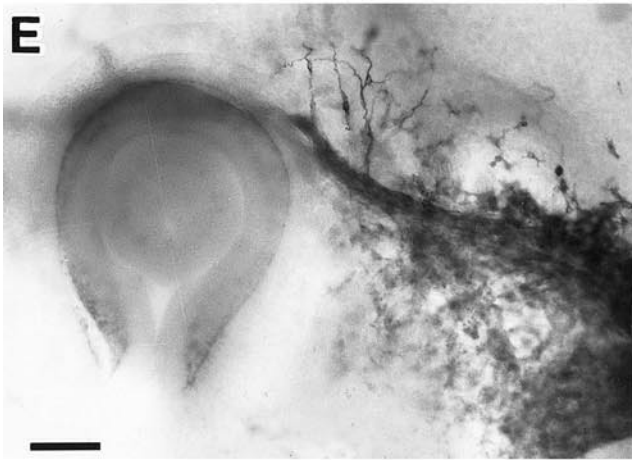
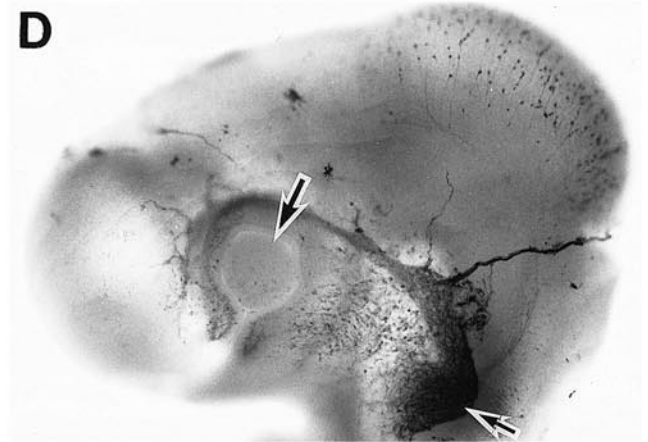
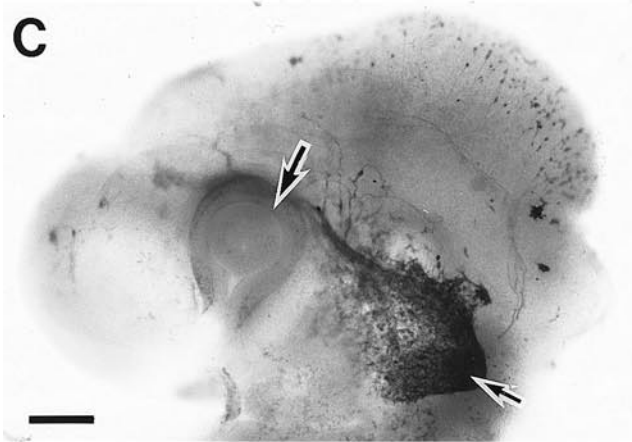
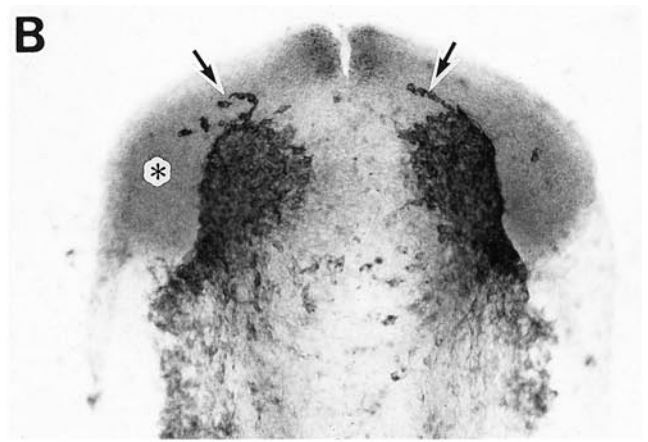
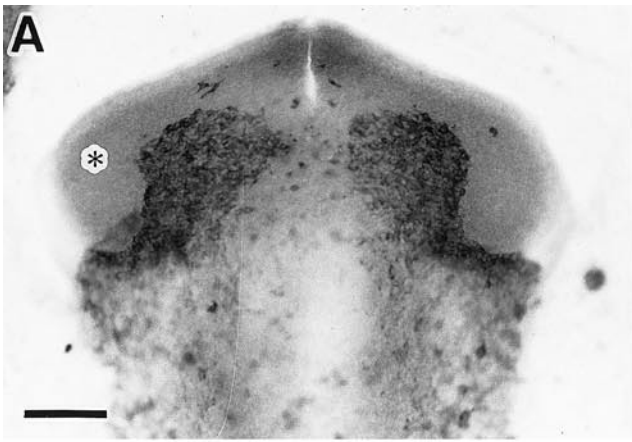


TABLE 1
Transplantation of *B/B* Optic Vesicle to Wild-Type Chick Embryo

Stage of donor embryos (somite number)	Total number of embryos	Number of embryos without transdifferentiation	Number of embryos with transdifferentiation
10-11	7	6	1
12-13	4	3	1
14-15	3	2	1
18	4	4	0
Total	18	15	3

Note. Grafts were removed from *B/B* quail embryos at different stages indicated and were spliced to host chicken embryos at the 10- to 12-somite stages. Eggs were incubated until embryonic day 6-9, and the development of RPE layer was examined whether RPE transdifferentiates into NR or not.

In a series of transplantations (18 successful cases) in which host chick embryos received an optic vesicle graft from *B/B* embryos, RPEs from the graft were always much less pigmented than host RPEs (Figs. 4A-4C), suggesting that the activity of melanin synthesis remained at a low level, as seen in unoperated *B/B* embryos. No abnormal features were observed in the facial development. Interestingly, RPEs from *B/B* optic vesicles formed a single epithelial layer and did not transform to NR structures in 15 of 18 successful cases (Table 1). Histological and immunocytochemical examinations revealed that numerous mesenchymal fibroblastic cells in the choroid layer were derived from the host chick embryos, though the proportion of chick cells varied from 60 to 85% when roughly counted in the sections (Figs. 4E and 5C-5F). In 3 cases, the posterior region of RPE transdifferentiated to NR, but less extensively than in unoperated *B/B* embryos (Figs. 4F, 4G, 5A, and 5B). Close examination revealed that the mesenchymal cells in the choroid layer facing the RPE were predominantly quail cells (Figs. 4G and 5B). It appeared that optic vesicle grafts from younger embryos (before the 14-somite stage) resulted in more frequent transdifferentiation, but this needs to be reexamined in future studies.

As a control experiment, *B/B* embryos received an optic vesicle graft from other *B/B* embryos (homotypic transplantation) to examine whether the loss of transdifferentiation in most of the heterotypic grafts might simply be due to the procedure of truncation of optic vesicle rudiments. In 2 of 10 cases, the embryo developed to stage 25 (6 days of incubation), and the results showed that the posterior part of the RPE from the graft transdifferentiated to a multilayered NR structure. All other host embryos died 2 or 3 days after the operation, probably due to the mutant embryos being too feeble to survive the graft operation.

DISCUSSION

During embryonic development of the *silver* homozygote, an ectopic NR is formed by the transdifferentiation of the RPE. This occurs only in the posterior portion of the eye and is initiated at the embryonic stage between days 4 and 5 when pigmentation is already found in wild-type quail embryos. Mochii *et al.* (1998) reported that the *Mitf* gene, encoding a bHLH zip transcription factor, has an amino acid substitution in the basic region and is truncated in the C-terminal region in the *silver* homozygote. Many mutations in mouse *Mitf* have been reported to induce a variety of abnormalities in melanocytes, osteoclasts, and mast cells in addition to the anomaly in the eye (Hodgkinson *et al.*, 1993; Nakayama *et al.*, 1998). It has been speculated that *silver* phenotypes are also caused by the mutation of *Mitf*, and *Mitf* appears to play a crucial role in RPE transdifferentiation (Mochii *et al.*, 1998). However, our previous study indicated that the RPE of *silver* homozygotes undergoes transdifferentiation only when cultured in combination with periocular mesenchyme and does not transdifferentiate to NR when cultured alone (Araki *et al.*, 1998). We also showed that the isolated RPE transdifferentiates into NR upon the addition of FGF and that the RPE of *silver* homozygotes is 10-40 times as susceptible to FGFs (FGF-1 and FGF-2) as RPE of the wild type (Araki *et al.*, 1998). These results strongly suggest that RPE-mesenchymal interaction is essential for RPE transdifferentiation and that mesenchymal cells are also affected by the *silver* mutation, in addition to RPE cells. Accordingly, we decided to investigate what roles mesenchymal cells play in RPE transdifferentiation and what molecules are involved in the RPE-

FIG. 3. Distribution of neural crest cells during embryonic development. Whole mount-prepared embryos and paraffin-embedded sections were stained with HNK-1 antibody. (A, C, E, G) Wild-type embryos. (B, D, F, H) *silver* homozygotes. (A, B) Embryos at the 10-somite stage. Numerous positive cells are migrating toward the optic vesicle (asterisk), and a few stained cells appear extending out toward the anterior proencephalon in the *silver* embryos (arrows in B). (C-F) Embryos at the 31- to 32-somite stage. Nerve fibers of intensely stained trigeminal ganglia (small arrows) extend to the developing eyes (large arrows), which are shown at a higher magnification in (E) and (F). In wild-type embryos (E), only a few cells are found surrounding the eye, whereas in *silver* mutant eyes (F), numerous stained cells (arrows) are observed around the eye. (G, H) Embryos at stage 18 (3 days of incubation). In the mutant eye (H), clusters of stained cells are found in the connective tissue (future choroid tissue) in the posterior region (arrows). Scale bars in (A, C, E, G) indicate 100, 200, 100, and 50 μm and apply to (B, D, F, H), respectively.

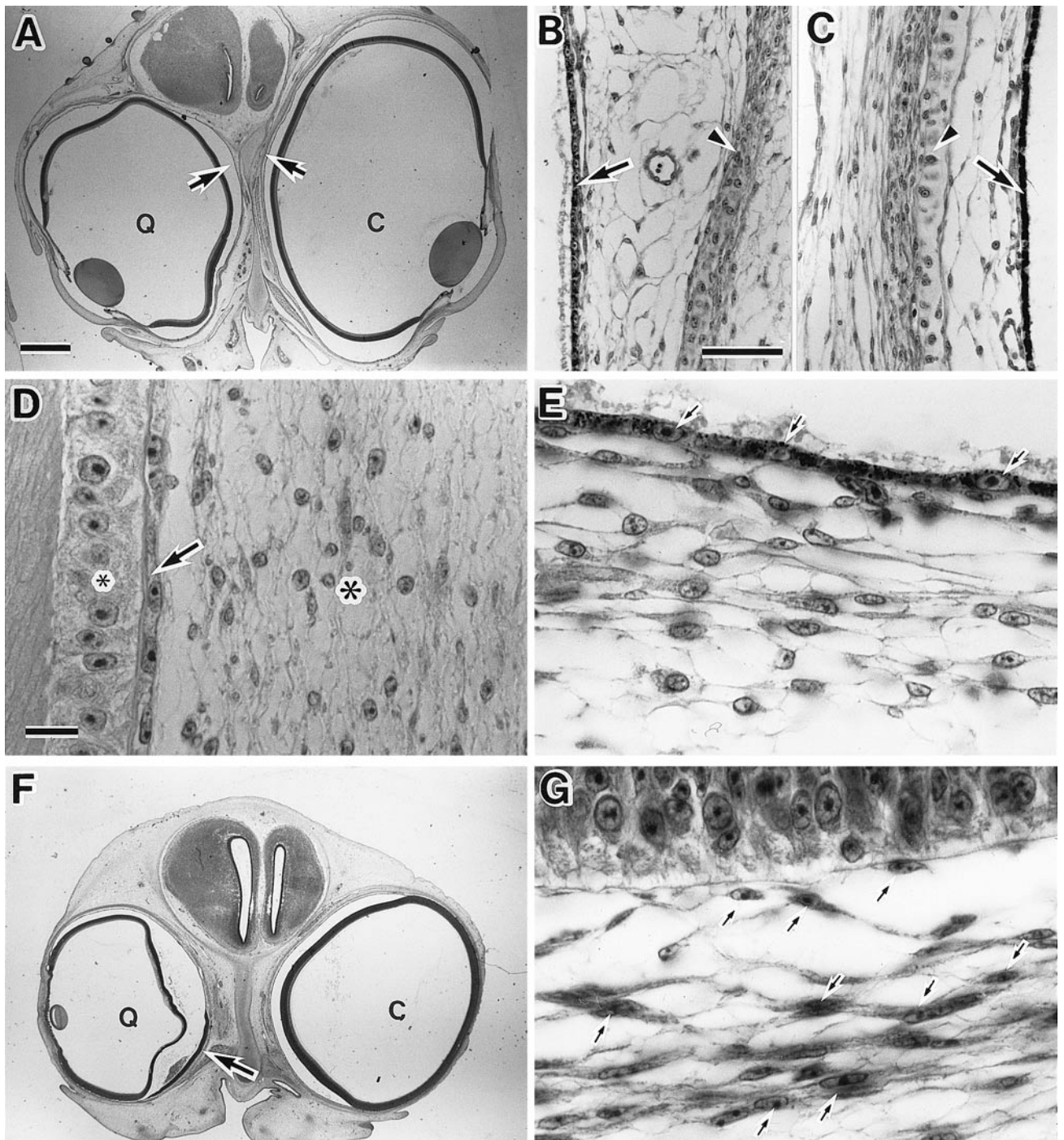


FIG. 4. Embryonic transplantation of quail optic vesicles to chick host embryos. Chick–quail chimerical embryos were prepared to analyze the effect of migrating mesenchymal cells. Transplanted embryos were fixed on embryonic day 9–10, and frontal sections were stained with hematoxylin and eosin. (A, F) Eyes derived from grafted quail optic vesicle (Q) are significantly smaller than the host eyes (C). In (A), the RPE is indicated by arrows and shown at a high magnification in (B, C). (B) The donor quail eye; no sign of transdifferentiation can be found. The RPE (indicated by arrows) is significantly less pigmented in the quail RPE than in the host chick eyes (C). The scleral cartilage is also differentiated in both eyes (arrowheads). In (E), a part of the RPE in (B) is shown at a higher magnification. The arrowheads indicate RPE cell nuclei, which display condensed nuclear staining characteristic of quail cells. In the adjoining connective tissue, most of the mesenchymal cells are chick cells. In (D), the lens and cornea regions in (A) are shown at a higher magnification. The small asterisk, arrow, and large asterisk indicate the lens epithelium, corneal epithelium, and corneal stroma, respectively. Cells in the lens and corneal epithelia are donor quail cells, whereas many chick cells can be seen in the corneal stroma. (F) Another case of embryonic transplantation, in which the RPE has transdifferentiated into NR in the posterior region, as indicated by an arrow in (F) and at a higher magnification in (G). In (G), cells in the newly formed second retina are quail type, and most cells in the adjoining connective tissue are also quail type (arrows). Roughly estimated, 70% of the mesenchymal cells in that area are quail cells. Scale bars in (A, B) indicate 1000 and 50 μm , and apply to (F, C). The scale bar in (D) indicates 10 μm and applies to (E, G).

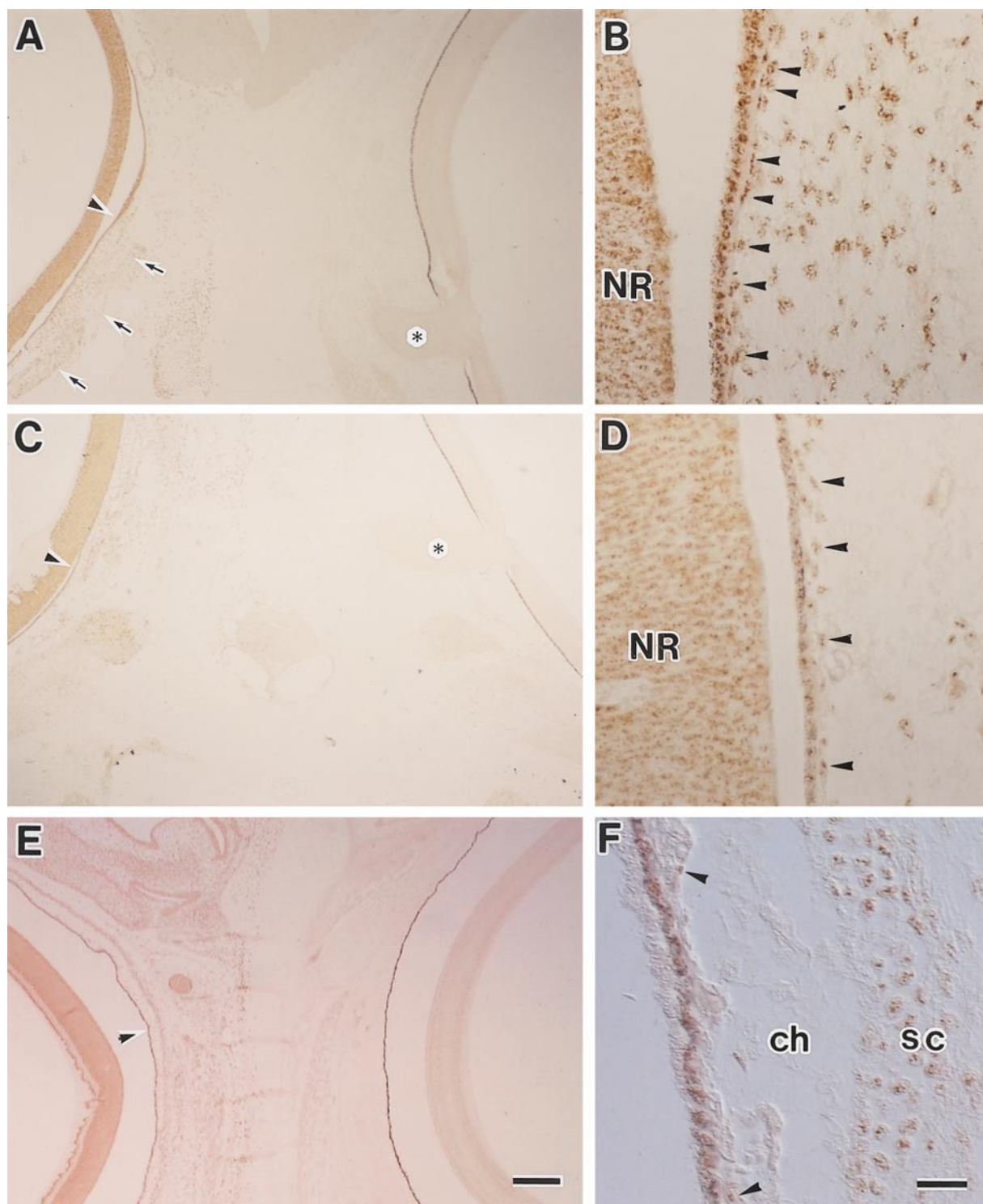


FIG. 5. Immunohistochemistry of quail cell nuclear marker (QCPN) in embryonic transplantation of quail optic vesicles to chick host embryos. Embryos were fixed on day 6 (A, B and C, D) and on day 10 (E, F). The parts indicated by arrowheads in (A, C, E) are shown at a higher magnification in (B, D, F), respectively. The asterisks show an optic nerve. In (A), the posterior part of the RPE shows thickening of the layer, indicating an area of transdifferentiation of the RPE. Arrowheads indicate QCPN-positive cell clusters observed in the mesenchymal tissue. In (C, E), no transdifferentiation occurs in the RPE. At a higher magnification, many positive quail cells are found in the mesenchymal tissue adjoining transdifferentiating RPE (arrowheads in B), whereas fewer quail cells are observed in the corresponding region of eyes without transdifferentiation (D, F). ch, choroidal tissue, sc, scleral cartilage. The scale bar in (E) indicates 200 μm and applies to (A, C), and the scale bar in (F) is 20 μm and applies to (B, D).

mesenchyme tissue interaction. We found that RPE transdifferentiation does not depend merely on the RPE genotype, but also on the genetic type of the mesenchymal cells adjacent to the RPE. Our findings also suggest that FGFs and related receptor molecules may play crucial roles in the processes.

FGF-1 Is a Potential Signal for RPE Transdifferentiation in silver Homozygote Embryos

Mature NR contains relatively high levels of FGF-1 and FGF-2, but in the early stage of development, only trace amounts of FGFs are present (Kalcheim and Neufeld, 1990; Schnürch and Risau, 1991). Although a previous immunohistochemical study demonstrated that a low level of FGF-2-like immunoreactivity (FGF-2-LI) is found in the RPE of day 6 chick embryos (Consigli et al., 1993), little is known about avian embryos at much earlier stages. Our present observations showed that the NR, RPE, and choroid tissue of the *silver* homozygote eye was immunoreactive for FGF-1 as early as embryonic day 3, when the RPE of the wild-type eye appeared to be negative. In contrast to FGF-1 distribution, the FGF-2 level remained low between stages 18 and 24 and did not differ substantially between *silver* homozygote and wild-type embryos.

In the cultures of isolated RPE from *silver* homozygote embryos, FGF-1 was more effective than FGF-2 for RPE transdifferentiation (Araki et al., 1998). The present immunohistochemical observations on FGF localization suggest that FGF-1 is a possible candidate as a signal molecule that induces RPE transdifferentiation in the *silver* eye. Correspondingly, two types of FGF receptors (types 1 and 2) are expressed in the ocular tissues of early chicken embryos (Ohuchi et al., 1994; Tchong et al., 1994), and FGFR1 is expressed in the prospective lens, NR, and RPE, while FGFR2 is expressed predominantly in the periocular mesenchymal tissues (Ohuchi et al., 1994). FGFR2 expression is also found in the epithelium during mouse embryonic organogenesis (Jackson et al., 1997; Celli et al., 1998), and a recent study conducted by targeted disruption of the mouse FGFR2 (IIIb) gene suggested a key role for the receptor in mesenchymal-epithelial signaling during early organogenesis (De Moerloose et al., 2000). It has yet to be determined which type of FGFR functions in the RPE-mesenchyme interaction during RPE transdifferentiation.

Enhanced Accumulation of HSPG and Laminin in the Basement Membrane of silver Mutant RPE

The activity of FGFs is modulated through their interaction with the ECM components (for reviews, see Gospodarowicz, 1990; Mason, 1994). Several studies have indicated that cell surface heparan sulfate is directly involved in FGF cell signaling (Yayon et al., 1991; Flaumenhaft et al., 1990). Heparan sulfate is essential for high-affinity binding of FGFs to FGFRs, and without heparan sulfate, FGFs cannot carry out their biological functions. The present observa-

tions demonstrated that the RPE basement membrane of the *silver* homozygote embryo was stained for both heparan sulfate and laminin more intensely than that of the wild-type embryo. Since this difference in ECM distribution was found at the embryonic stage when the RPE of the *silver* homozygote had not yet been committed to differentiate to NR, it can be speculated that the intense distribution of ECM components in the *silver* homozygote RPE may direct the fate of RPE cells to transdifferentiation through the action of anchoring trophic factors (FGF-1 is a possible candidate). A study of light-microscopic autoradiography with labeled FGF-2 revealed binding sites in early embryonic chicken eyes (Cirillo et al., 1990); these sites are localized mainly in basement membranes, including Bruch's membrane, and are highly sensitive to heparitinase digestion and to competition with heparin.

Neural Crest Cell Migration May Be Affected in silver Homozygote Embryos

In the early embryos of stages 10–12 (10- to 17-somite stages), few differences were found in the spatial distribution of HNK-1-positive neural crest cells between the *silver* homozygotes and wild-type embryos. At around stage 17 (30-somite stage), however, the differences were conspicuous, i.e., in the *silver* homozygote embryos, numerous HNK-1 cells were distributed around the optic cup surrounding the lens vesicle, but very few, if any, were found in the optic cup of the wild-type embryos. This indicates that, in the later stage of development, more numerous neural crest cells migrate toward the optic cup in the *silver* homozygote than in the wild type. On paraffin-embedded sections at around stage 18, numerous HNK-1 cells gathered in the mesenchymal tissues beneath the RPE in *silver* homozygotes, but very few were found there in the wild-type embryos. In other regions, such as the dorsal surface of the brain vesicle, trigeminal ganglion, and otic vesicle, little or no obvious differences were discerned between *silver* and wild-type embryos. These observations clearly suggest that the spatial distribution pattern and/or the migration pathway were affected by the *silver* mutation, particularly in the region of the eye. The distribution does not seem to be affected in the early stages of development (somite formation) but only during early organogenesis of the eye (differentiation of the lens, retina, and RPE).

The present tissue combination experiment suggests an essential role of neural crest cells for RPE transdifferentiation. The melanin content of the RPE of the grafted *silver* donor still remained at a level as low as that of ordinary *silver* homozygote RPE, but transdifferentiation of the RPE did not usually occur. The mesenchymal cells juxtaposed with RPE were mostly derived from host chick embryos, which are considered to have migrated to the grafted optic vesicle after it is spliced. These migrating cells, though not all of them, have a neural crest origin. In rare cases, transdifferentiation was observed, but much less extensively than in unoperated *silver* embryos, and there, the

quail mesenchymal cells were predominant. Although there is a slight tendency that more frequent transdifferentiation occurs in donor optic vesicles removed from earlier embryonic stages, there appears to be no substantial difference. It is speculated that transdifferentiation might have occurred when the host cell migration to the graft happened to be disturbed due to grafting procedure. The results suggest that, for RPE transdifferentiation in *silver* embryos, neural crest cells (and other migrating mesenchymal cells) need to migrate into the optic vesicle continually for a certain period of development.

Cranial neural crest cells are involved in the formation of the face, including the eye and nose. The neural crest cells from the anterior midbrain migrate ventrolaterally to the presumptive eye and nose in rats (Tan and Morriss-Kay, 1986) and in mice (Serbedzija *et al.*, 1992). The migration takes place before the induction of lens placodes, suggesting that these neural crest cells contribute to the formation of the eyes and nose. The neural crest cells migrate from the edge of the neuroepithelium at 10 days postcoitum in rat embryos before closure of the neural tube, and migrate toward specific destinations in a highly segmented manner. Matsuo *et al.* (1993) reported an anomaly in the neural crest cell migration of the homozygotes of rat *small eye* (*rSey*) mutation, where neural crest cells accumulate in the area around the optic vesicle but do not migrate any further to the frontonasal area. These studies indicate that neural crest cells play a crucial role in eye morphogenesis.

RPE-Mesenchymal Cell Interaction Is Essential for Transdifferentiation of RPE in Silver Embryos: A Hypothetical Mechanism of Transfating in silver RPE

Although the molecular basis of phenotypic switching from RPE to NR is currently unclear, some components in the ECM, including laminin and fibronectin, have been proposed as important signals for phenotypic expression. Reh and Nagy (1987) observed that, during the initial stages of retinal regeneration in *Rana pipiens* tadpoles, RPE cells come into contact with vitreal vascular ECM, which contains some materials that are different from those in Bruch's membrane, and that the first new neuronal progenitors arise in association with this vitreal vascular membrane. It has also been found that larval Rana RPE cells undergo transdifferentiation to neural cells when cultured on a laminin-coated culture dish (Reh *et al.*, 1987). These findings suggest that retinal regeneration is initiated by changes in the composition of the ECM, which the RPE cells may contact early in this process (Pittack *et al.*, 1991). Since HSPG has been shown to form complexes with both laminin and FGFs (Baird and Ling, 1987), changes in the distribution of ECM components may direct the fate of RPE cells through the action of FGFs or other related substances. Intraocularly administered FGFs have been shown to induce transdifferentiation of RPE to NR in early chicken embryos (Park and Hollenberg, 1989). Our observations that

the distribution of laminin and heparan sulfate is much more intense in the RPE-mesenchyme interface of *silver* homozygotes than in that of the wild type, and that FGF-1 also shows intense localization in the similar region of *silver* homozygotes, indicate that FGF-1 is probably involved in the transdifferentiation of RPE in *silver* homozygote embryos. Our previous *in vitro* experiments suggested that the source of FGF-1 is the mesenchymal tissue (Araki *et al.*, 1998). The present graft experiments also provide support for the essential role of mesenchymal cells for RPE transdifferentiation in the *silver* embryos. Periocular mesenchymal cells are derived from the neural crest, although other mesodermal cells also contribute to the formation of periocular tissue. It is plausible that the mutation also occurs in these periocular mesenchymal cells (probably neural crest cells).

Evidence from salamander development indicates that the direction of neural crest cell migration may be instructed by the ECM over which they migrate (Löfberg *et al.*, 1989; Perris *et al.*, 1990). In chick embryos, the mesoderm appears to control where and when the neural crest cells can migrate (Bronner-Fraser and Stern, 1991), and the migration of chick cranial neural crest cells can be severely altered when antibodies to fibronectin, fibronectin receptors, tenascin, or laminin-HSPG are injected into the developing embryo, although these antibodies do not severely alter the migration of chick trunk neural crest cells (Poole and Thiery, 1986; Perris and Bronner-Fraser, 1989). For the moment, we conjecture that alterations in the distribution of ECM components, particularly laminin-HSPG, may subsequently affect the distribution of migrating neural crest cells and FGF distribution. In further studies, it will be of particular importance to study how *mitf* mutations affect the migration of neural crest and which cells are responsible for the up-regulated expression of ECM components and FGFs.

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