

# The loss of gap junctional cell-to-cell communication is coupled with dedifferentiation of retinal pigmented epithelial cells in the course of transdifferentiation into the lens

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**ABSTRACT** Retinal pigmented epithelial cells (PECs) of the chick embryo can be cultured as a monolayer of melanized hexagonal cells. Modifications of the culture condition make the cells lose most of the phenotypes and further transdifferentiate into lentoid bodies within a few weeks. Ultrastructural observations showed that PECs and the lentoids have gap junctions with distinct morphology. Diffusion of a fluorescent dye confirmed the presence of gap junctions in both phenotypes. However, cells in the intermediate stage of transdifferentiation, which show neither the phenotype of the PEC nor that of the lentoid and are called dedifferentiated PECs here, have almost no gap junction structure. We propose the possibility that the dedifferentiation of PECs and the loss of cell-to-cell communication are tightly coupled events. This cell culture system is a suitable material for further studying this relationship by cellular and molecular approaches.

**KEY WORDS:** *transdifferentiation, retinal pigmented epithelium, lens, gap junction, ultrastructure*

## Introduction

Retinal pigmented epithelial cells (PECs) of the chick embryo can be purely isolated and cultured *in vitro*. When the PECs become confluent, cells reconstruct a simple epithelium made up of melanized hexagonal cells in the culture dish. Long-term culture for two to three months of the primary cells with repeated transfers yields aggregates of transparent cells which have many of the ultrastructural and biochemical characteristics of the lens; thus these aggregates are called lentoids (Eguchi and Okada, 1973). This phenotypic change of the progeny of the pigmented cells to lens cells was confirmed by clonal cell culture and called transdifferentiation. Many other examples of such changes of phenotype have been described thereafter (see review, Okada, 1991; Eguchi and Kodama, 1993).

Modifications of culture conditions by Itoh and Eguchi (1986) have made the process of the transdifferentiation of PECs rapid and uniform, thus permitting the analysis of this process by biochemical and molecular biological methods. When PECs are cultured in the presence of phenylthiourea and testicular hyaluronidase, cells lose their melanosomes and an ordered structure as a simple epithelium and proliferate without the contact inhibition of growth. This "dedifferentiated" state without either of the major phenotypes of the PEC and the lens can be maintained up to two months by transferring before reaching confluence. Redifferentiation into the PECs is elicited by the removal of phenylthiourea and hyaluronidase and the addition of ascorbic

acid. Differentiation into the lens cells is induced by culturing in the presence of phenylthiourea, hyaluronidase and ascorbic acid at high cell density. It was later shown that the hyaluronidase activity was not necessary to elicit this change but the basic fibroblast growth factor which was contained in the hyaluronidase specimen in a small amount played a critical role to enhance the phenotypic changes (Hyuga *et al.*, 1993). We investigated the ultrastructure of the cells during each step of transdifferentiation and found that there is a characteristic and reproducible change in the status of the gap junction.

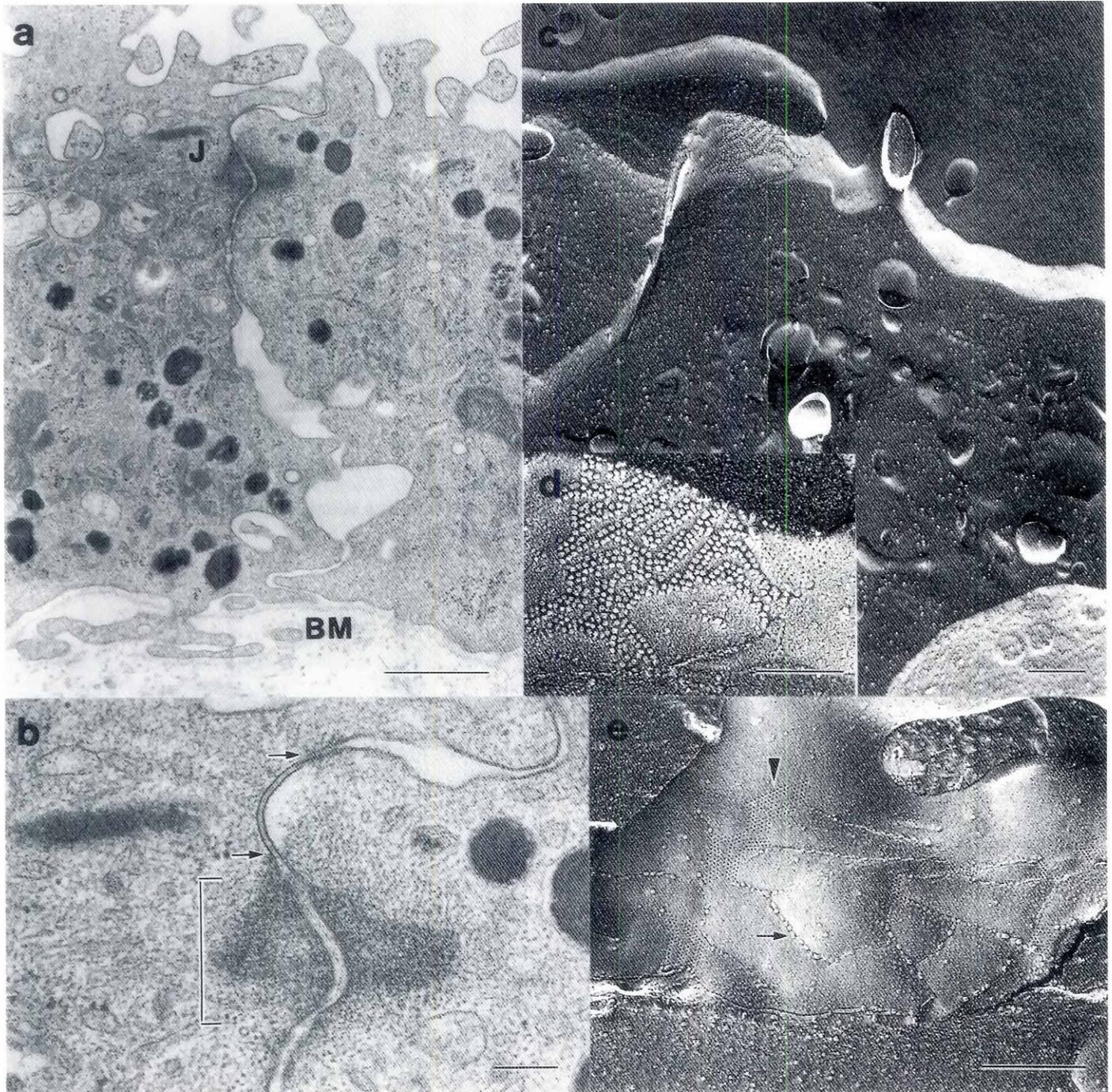
The gap junction was first characterized as a form of cell-cell junction where two cells meet with a narrow and constant gap between the outer lamina of the cell membrane and it is now generally agreed that the gap junction serves as a pathway of ions and small molecules between the cytoplasm of neighboring cells (reviews, Musil and Goodenough, 1990; Bennett *et al.*, 1991; Holder *et al.*, 1993). The recent finding that the gap junction is found in one of the most primitive multicellular organisms, a mesozoan

*Abbreviations used in this paper:* PEC, pigmented epithelial cell; EF, Eagle's minimum essential medium-8% fetal calf serum; EdFPH, Eagle's minimum essential medium-8% dialyzed fetal calf serum-0.5 mM phenylthiourea-250 U/ml ovine testicular hyaluronidase; EdFPHA, EdFPH-0.15 mM ascorbic acid; dePEC, dedifferentiated pigmented epithelial cell; CB, 0.1 M sodium cacodylate buffer (pH 7.4); RITC-dextran, rhodamine B isothiocyanate conjugated dextran; IMP, intramembranous particle.

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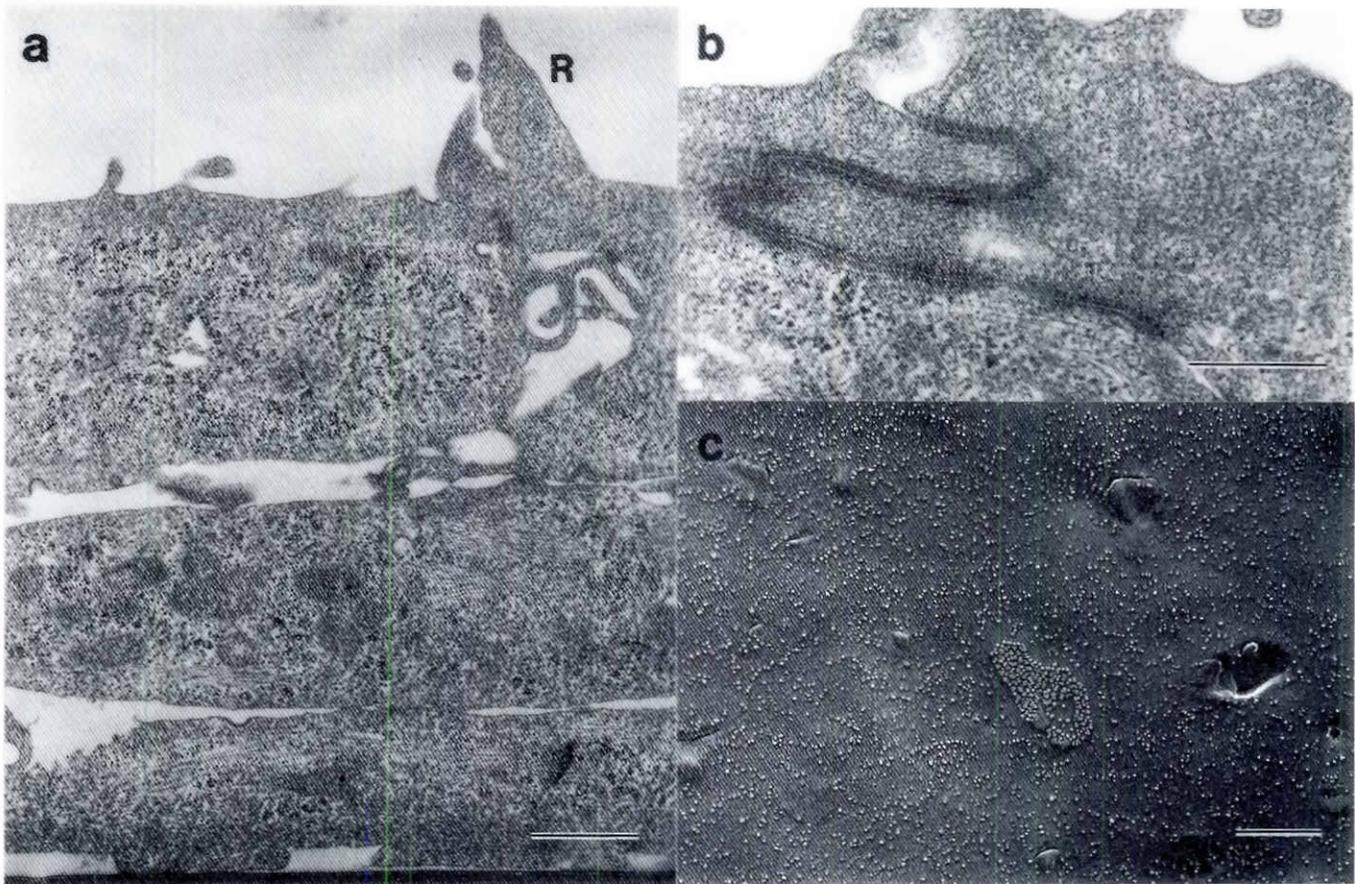
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**Fig. 1. Electron microscopic observation of gap junctions in cultured pigmented epithelial cells (PECs) of the retina.** (a) Low-power view of an ultrathin section cut vertically to the plastic dish shows apico-basal polarity of PECs. J: junctional complex, BM: basement membrane (lamina densa). (b) Higher magnification of junctional complex shows the gap junction (between arrows) and adherens junction (bracket). (c-e) Freeze-fracture images of PEC. Higher magnification of the gap junction (d) reveals the packed hexagonal array of IMPs on the P-face. E-face of a similar area (e) shows corresponding array of pits (arrowhead) and a linear array representing the tight junction (arrow). Bars, a, 1  $\mu$ m, b,c,e, 200 nm, d, 100 nm.

*Dyccemid* (Revel, 1988), suggests that the gap junction has its origin in the very beginning of multicellularity. Inhibition of gap junction by specific antibodies induced defects in the organogenic process in the early embryo (Warner *et al.*, 1984), suggesting the

crucial importance of the gap junction in cell differentiation and morphogenesis. However a whole early embryo is too complicated a system to ask precisely what roles gap junctions play in the process of cell differentiation.



**Fig. 2. Electron microscopic observation of dedifferentiated PECs (dePECs).** (a) Low-power view of dePECs sectioned vertically to the plastic dish. R: ruffled membrane. (b) Junctional structures are observed just beneath the surface exposed to culture medium. They include the adherens junction, but not the gap junction. (c) Freeze-fracture images show the very rare presence of clustered IMPs. Bars, a, 1  $\mu$ m, b, 500 nm, c, 200 nm.

We will describe in this paper how gap junctions change in the course of the phenotypic changes of PECs and how important this system may be in studying the role of gap junctions in cell differentiation.

## Results

### Ultrastructural observations of PECs in the course of transdifferentiation into lentoids

#### PEC

When retinal PECs of the chick embryo became confluent in a culture dish after vigorous proliferation in EF medium, they started to accumulate melanosomes and reconstituted a simple epithelium with a regular hexagonal cellular pattern, which is very similar to the pigmented epithelium *in vivo*. Observations with a transmission electron microscope showed that the junctional complex at the subapical level and the basement membrane on the basal surface were also similar to the *in situ* tissue, thus the apico-basal polarity of the morphology was very conspicuous (Fig. 1a). As reported earlier (Crawford, 1980), the junctional complex of the PECs *in vitro* consisted of the tight junction, the adherens junction and the gap junction, but lacked desmosomes. This composition was the same as *in vivo*. The gap junction was usually located at the most apical portion of the junctional complex (Fig. 1b). Freeze-fracture images

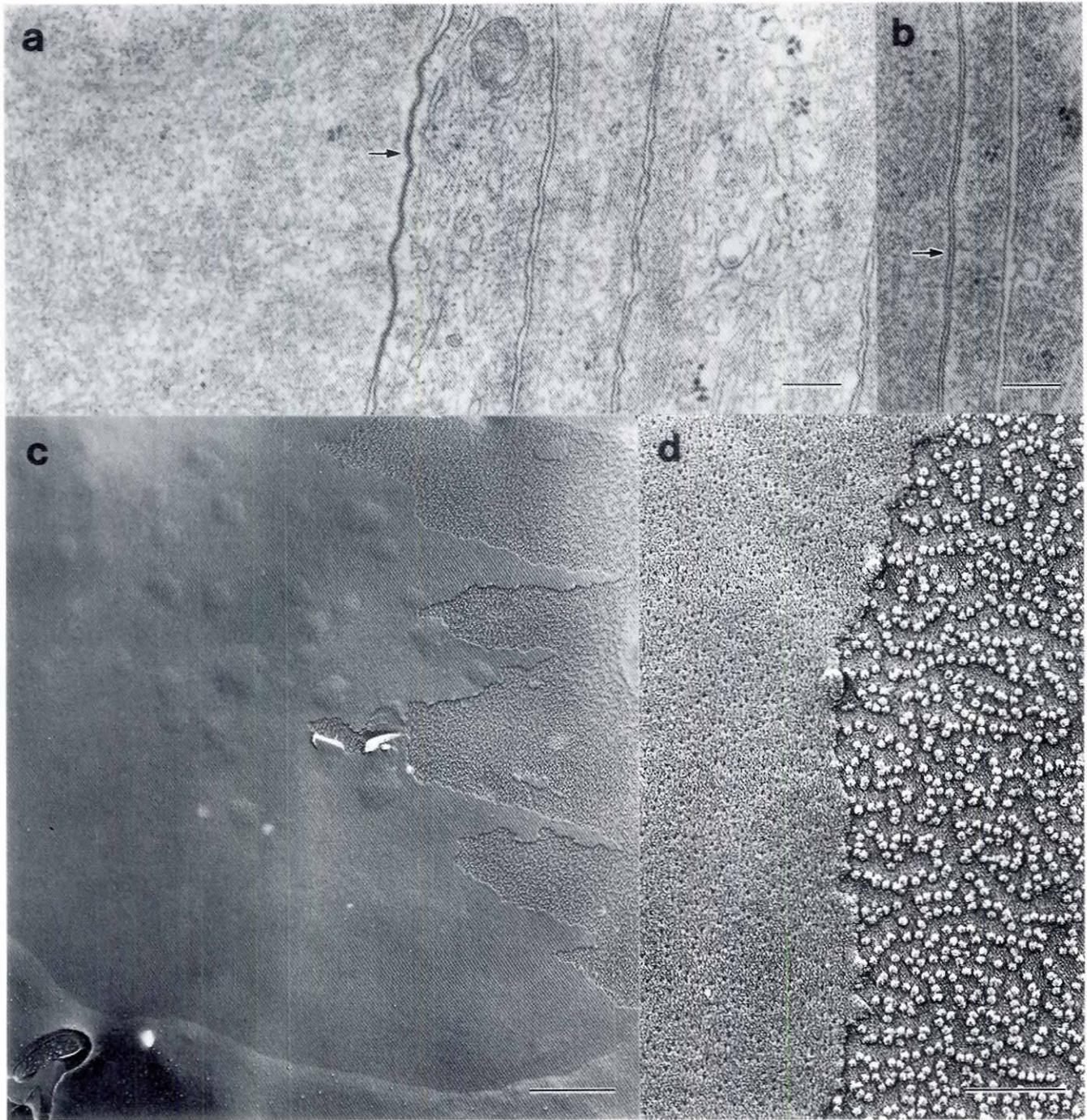
confirmed the presence of the gap junction and further showed that the intramembranous particles (IMPs) on the P-face were arranged in a regular hexagonal array, usually in double or triple rows (Fig. 1c,d). Pits on the E-face showed a very similar arrangement (Fig. 1e). Gap junction particles of PECs were always observed adjacent to the tight junction which appeared as a continuous linear arrangement of IMPs (Fig. 1e).

#### DePEC

In the dedifferentiation medium (EdFPH), PECs became unpigmented and lost the contact inhibition of growth. Ultrastructural observations showed that they had lost the polarized morphology characteristic to the PECs, as exemplified by the loss of the basement membrane (Fig. 2a). We could not observe any gap junctions in the transmission images, although small clusters of IMPs were occasionally observed in the freeze-fracture images (Fig. 2c). In no case was the tight junction observed. The adherens junction was the only junctional structure in dePECs (Fig. 2b), and the presence of the adherens junction at the sub-apical level was the only recognizable polarity of morphology in the dePECs.

#### Lentoids

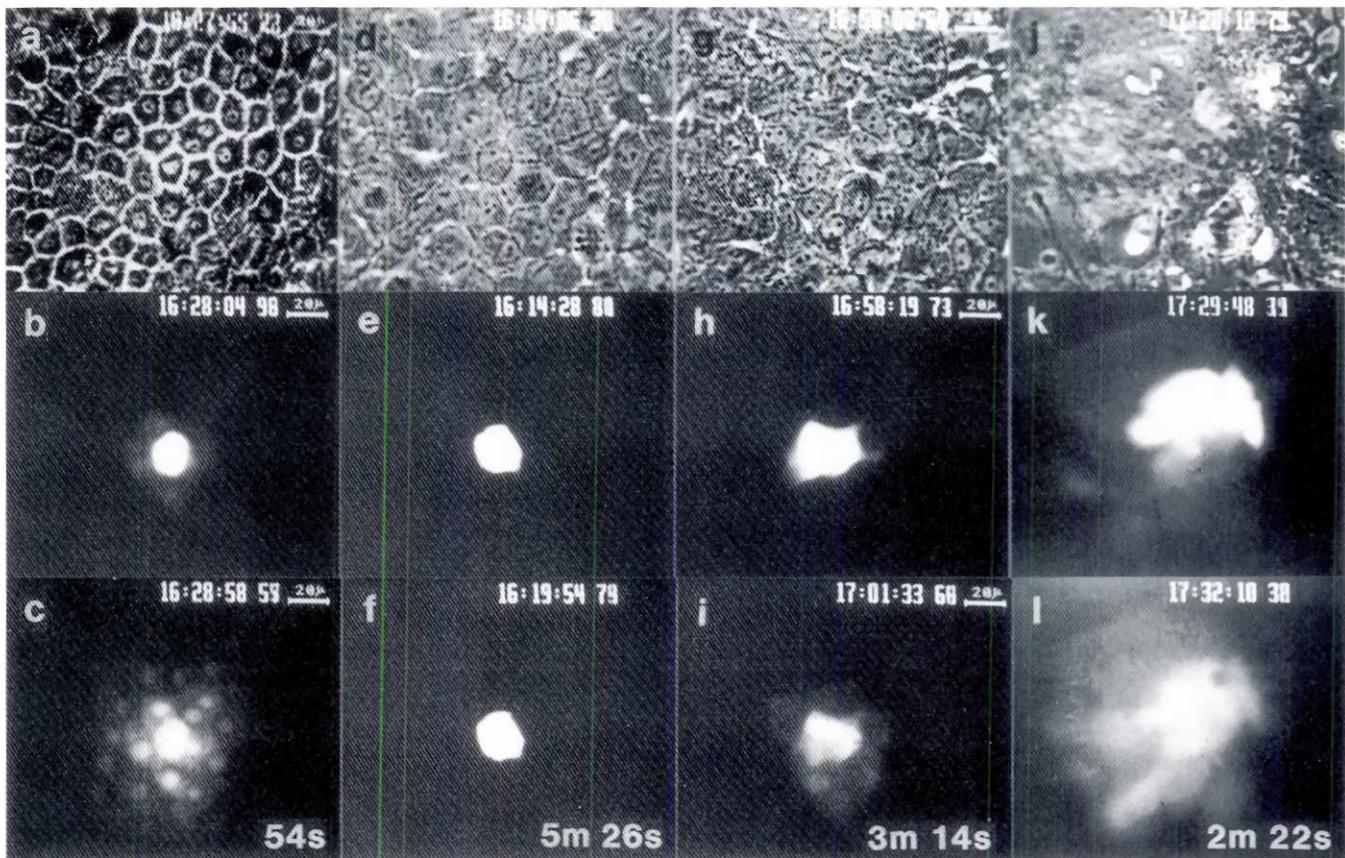
As reported earlier (Eguchi and Okada, 1973), cells in lentoid bodies lacked most of the organelles, and the most conspicuous



**Fig. 3. Electron microscopic observation of lentoids formed by transdifferentiation of PECs.** (a) Cytoplasm of the lentoid shows few organelles. Junctions with no gap between the outer leaflets of plasma membrane (arrow) resemble gap junctions of the lens fiber. Another type of junction with a wide gap is also present (b, arrow). (c) Freeze-fracture images show large area of clustered IMPs (whole area above the concave line at the bottom). To the right of the zig-zag line, P-face with particles is seen, and to the left, E-face with pits. (d) Higher magnification of a similar replica shows particles and pits arranged randomly dispersed. Bars, a, b, 200 nm, c, 500 nm, d, 100 nm.

content of the cytoplasm were the free ribosomes (Fig. 3a). These characteristics coincided with those of the lens fiber cells of *in vivo* lenses. Transmission electron microscopy revealed the presence of membrane region which seemed to represent the gap junction (Fig. 3a). We could also recognize a region of cell membrane with

high electron density and a wide gap between the outer lamina of the cell membrane (Fig. 3b). These two kinds of membrane specialization had two characteristics in common: a) the distance between two opposing cell membranes was constant, and b) there was an electron-lucent space over the cytoplasmic surface of the



**Fig. 4.** Video-recording of the diffusion of fluorescent dye through PECs, dePECs and lentoid. (Upper row) Phase-contrast images of PECs (a), dePECs (d, g) and lentoid (j). (Middle row) Fluorescent images just after the injection of Lucifer Yellow. (Lower row) Fluorescent images after incubation for a period indicated on the lower right corner. Numbers on the upper right corner of each figure show the time of recording (hour: minute: second). Diffusion of the dye occurred in PECs and lentoids but very little if any, and slow diffusion was seen in dePECs. All figures are of the same magnification. Bars, 20  $\mu$ m.

cell membrane on which short fibrous or granular projections were observed. Although the former structure had a morphology similar to that observed in the *in vivo* lens (Goodenough, 1979), no comparable structure to the latter one has yet been reported. Freeze-fracture images confirmed the presence of a wide area of the gap junction (Fig. 3c) where IMPs were arranged randomly on the P-face and the corresponding pits on the E-face showed a similar arrangement (Fig. 3d). This random arrangement of IMPs was characteristic to the gap junctions between the lens fiber cells (Benedetti *et al.*, 1976; Goodenough, 1979), and showed a sharp contrast to the gap junctions between PECs (cf. Fig. 1d).

#### Dye diffusion assay of the gap junction

The diffusion of small molecules between adjacent cells, an indication of the presence of the gap junction, was assayed by the injection of fluorescent dyes. When a single cell in the monolayer of the PECs *in vitro* was injected with Lucifer Yellow (molecular weight 457), the dye rapidly diffused in a uniform manner to all neighboring cells (Fig. 4a-c). When rhodamine B isothiocyanate conjugated dextran (RITC-dextran) of average molecular weight 17,200 was injected together with Lucifer Yellow, RITC-dextran remained in the injected cell (Fig. 5a) while Lucifer Yellow diffused to neighboring cells (Fig. 5b) just as it did when injected alone. This result showed that the Lucifer Yellow diffused through a passage

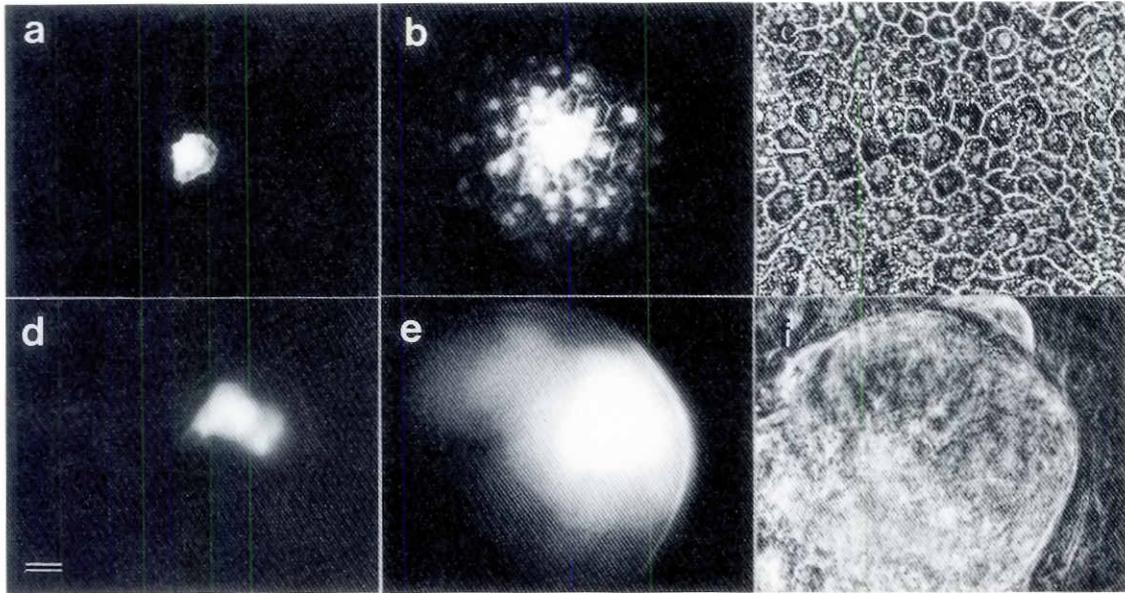
which only allows small molecules to get through.

In contrast, when a single cell of dePECs was injected with the Lucifer Yellow, the dye stayed in the same cell for at least several minutes (Fig. 4d-f). In a few cases, diffusion of the dye into a limited number of neighboring cells was observed (Fig. 4g-i). This coincides with the ultrastructural observation that a small number of gap junctions were observed among dePECs (cf. Fig. 2c). The overall tendency of the difference of dye diffusion between PECs and dePECs was obtained by a semi-quantitative analysis. The histogram of the number of the cells which were recognizable under the fluorescent microscope 5 min after injection of Lucifer Yellow showed a clear decrease in the degree of dye-coupling among the dePECs (Fig. 6).

Diffusion of the Lucifer Yellow was observed among cells in the lentoid body (Fig. 4j-l). Co-injection with the RITC-dextran showed that the RITC-dextran could not diffuse in the lentoid (Fig. 5d), while the Lucifer Yellow did (Fig. 5e), showing that the gap junction observed by morphological methods is a functional one.

#### Discussion

The present study clearly showed that gap junctional cell-to-cell communication is almost completely lost in the dePECs. Ultrastructural observation showed that the loss is through the



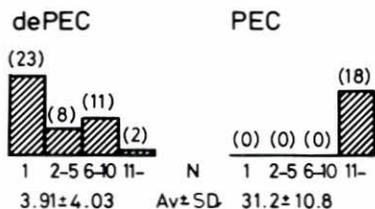
**Fig. 5. Co-injection of RITC-dextran and Lucifer Yellow into PECs and lentoids.** (a,d) Fluorescence of RITC. (b,e) Fluorescence of Lucifer Yellow. (c,f) Phase-contrast images. Figures in the upper row and the lower row show the same microscopic field from the culture of PECs and lentoids, respectively. In both cases, extensive diffusion of Lucifer Yellow was observed 5 min after the injection but no diffusion of RITC-dextran occurred. All figures are of the same magnification. Bar, 20  $\mu$ m.

disappearance of the gap junctional structure itself, rather than through the closure of the existing junctional channels. The growth characteristics of the dePECs are very different from those of the PECs in that dePECs grow without contact inhibition of growth and form a multilayer in the culture dish (Itoh and Eguchi, 1986). Analyses of gene expression in the dePECs recently showed that all the genes specifically expressed in the PECs are not expressed in the dePECs, while the expression of one of the protooncogenes, *c-myc*, is dramatically enhanced in the dePECs (Agata *et al.*, 1993). We can find a very similar combination of events in hepatocytes after partial hepatectomy *in vivo*. Yee and Revel (1978) showed that from 28 h to 40 h after partial hepatectomy very little gap junctional structure can be observed between hepatocytes. This period coincides with a mitotic peak. In addition, the expression of *c-myc* is found to be enhanced in the regenerating liver (Makino *et al.*, 1984). While the mechanisms linking these events are still unknown, the loss of gap junctions, the stimulation of cell growth and the expression of *c-myc* should be closely linked

phenomena and we suppose that the dedifferentiation of PECs can be a good model to study the relationship between them. The analysis of the expression of connexins, a major component of the gap junction, in the dePECs should be interesting because in the case of hepatocytes the downregulation of gap junction structure was recently shown to be controlled post-transcriptionally, i.e. the amount of the connexin transcripts remained unchanged after the hepatectomy (Kren *et al.*, 1993).

The PECs of the retina were repeatedly shown to have a characteristic junctional complex made up of tight junction, gap junction, and adherens junction and a very similar junctional complex is observed also in *in vitro* culture (Hudspeth and Yee, 1973; Middleton and Pegrum, 1976; Crawford, 1980; Kniesel and Wolburg, 1993). Our culture of retinal PECs from 9-day-old chick embryos also showed the presence of the gap junction at the apical-most level which consists of tightly packed array of connexins. Preliminary study showed that chicken connexin 43 is expressed in the PECs (Kodama, unpublished).

The gap junction found in the lentoids formed in our culture has characteristics common with the lens fiber junctions. The arrangement of IMPs is always random, which shows a clear contrast to that seen in PECs. The diffusion of small molecules through the junction was confirmed with fluorescent dyes. Menko *et al.* (1987) precisely described the change of the pattern of IMPs in the course of lentoid formation *in vitro* from the lens epithelium of the chick embryo, and they showed that in the fully formed lentoids most of the junctional plaques present a random pattern of IMPs. Our observation is very similar to their results on fully formed lentoids and the arrangement of IMPs corresponding to the intermediate stages of lentoid formation was not observed. This should be because we picked up only morphologically apparent lentoids and removed most of the surrounding epithelial cells for freeze-fracture studies. Thus we can conclude that lentoid formation through the



**Fig. 6. Histograms showing the number of cells to which fluorescent dye was diffused 5 min after injection.** The number in parenthesis on each column shows the number of incidences. N: number of fluorescent cells. Av, SD: average and standard deviation of N, respectively.

transdifferentiation of the PECs is very similar to that from lens epithelial cells.

The presence of a small number of junctional plaques and infrequent slow diffusion of fluorescent dye may indicate that some of the dePECs partially maintained the gap junction of PECs at the time of observation. However, it is at the same time possible to interpret that a portion of the dePECs has acquired a phenotype of lens epithelial cells, because the small patches of IMPs in the dePECs are morphologically very similar to those observed at the beginning of lentoid formation from lens epithelial cells (Menko *et al.*, 1987). The analysis of expression of marker molecules of the lens epithelium is necessary to dissect this process. Chicken connexin 43 should be among them since it is expressed in the lens epithelium but not in the lens fiber (Musil *et al.*, 1990).

## Materials and Methods

### Materials

Fertilized eggs of white leghorn chickens were purchased from a local hatchery (Matsumoto Yokeien, Okazaki). Ovine testicular hyaluronidase (product number H-6254, lot number 79F-0098), Lucifer Yellow CH and RITC-dextran were purchased from Sigma Chemical Company. Fetal calf serum (lot number SF90223) was purchased from Bocknek (Canada). Other reagents were purchased from Wako Pure Chemical Industries, Ltd.

### Cell culture

All the cell culture methods were as described previously (Itoh and Eguchi, 1986). Retinal PECs were isolated from 9-day-old chick embryos, dissociated and cultured in EF (Eagle's minimum essential medium-8% fetal calf serum) for a week, and further cultured with transferring about once a week in EF. Cells after the second or the third transfer were used as PECs. When cells after the first transfer were cultured in the dedifferentiation medium, EdFPH (Eagle's minimum essential medium-8% dialyzed fetal calf serum-0.5 mM phenylthiourea-250 U/ml ovine testicular hyaluronidase), they gradually lost phenotypes of PECs. After cells were further transferred in EdFPH twice or more, they had lost melanosomes and showed large surface membrane undulations. These cells were used as dePECs (dedifferentiated PECs). Spherical aggregates which appeared in the multilayered regions of dePEC culture, which had been cultured for one or two weeks after confluence in EdFPH supplemented with 0.15 mM ascorbic acid (EdFPHA), were used as lentoid bodies.

### Ultrastructural observations: ultrathin sections

Cells grown on plastic culture dishes were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) (CB) at 4°C for 1 h, postfixed with 1% OsO<sub>4</sub> in CB at 4°C for 1 h, block-stained with 2% uranyl acetate at room temperature for 1 h, dehydrated and embedded in Epok 812 (Oken Shoji, Tokyo). After the polymerization at 60°C for 3 days, resin was removed from the dish by dipping into liquid nitrogen, and the exposed surface was glued to a thin plate made of the same resin with epoxy adhesive to ensure the visualization of the cell substrate interface. Gold-silver sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed under a JEM1200EX transmission electron microscope (JEOL, Tokyo) at 80 kV.

### Ultrastructural observations: freeze-fracture

Cells grown on plastic dishes were fixed with 2.5% glutaraldehyde in CB at 4°C for 1 h, washed with CB, and infused with 30%(w/v) glycerol in CB at 4°C for 2 h. Cells were removed from the dish with scalpel blades. PECs and dePECs were cut out as a sheet and folded. Lentoids were marked under a phase-contrast microscope and cut out with least amount of surrounding epithelial cells. They were placed on 3 mm-diameter gold specimen stubs (Balzers Union) and frozen in freon-22 cooled with liquid nitrogen. Specimens were transferred to a JFD-7000S freeze-fracture device (JEOL, Tokyo), cut at -150°C, rotary-shadowed with platinum-carbon at an angle of 45° and with carbon at 90°, thawed and dissolved with

household bleach. The cleaned replica was put on specimen grids and observed under a JEM-1200EX transmission electron microscope at 80 kV. Photographs were reverse-processed to enhance contrast.

### Assay of dye coupling

Glass micropipettes were pulled from thin-walled glass capillary (1.0 mm o.d., 0.75 mm i.d., with filament; A-M Systems Inc., Everett). Cells were injected with 10%(w/v) Lucifer Yellow CH in 0.33 M LiCl by manually applying water-pressure with a glass syringe pushed with a fine-pitched screw (Narishige Scientific Instrument Lab.). The micropipette was set vertically downward beneath the center of the condenser lens of an inverted phase-contrast microscope (Injectoscope IMT2-SYF2; Olympus Optical Co. Ltd., Tokyo) so that the observation of the cells being injected was not disturbed. The micropipette was lowered until the tip penetrated the cell and touched the dish, and then raised a little to allow the injection of the dye. When the lentoid was injected, the micropipette was lowered until it penetrated the surface of the lentoid, which was judged from the wobbling of the lentoid. Sometimes RITC-dextran (average molecular weight 17,200) was dissolved at 20% (w/v) into the Lucifer Yellow solution and injected as above to confirm that a single cell was injected with the dye solution. Diffusion of the dye was visualized by the epifluorescence optics attached to the microscope and recorded with photographs (T-MAX 400, Kodak) or with a microscope image processor (C1966; Hamamatsu Photonics, Hamamatsu) equipped with an SIT video camera and a video tape recorder (BVH-2500; Sony, Tokyo). When the observation was through a video camera, a constant setting of the sensitivity of the camera was employed throughout observations to semiquantitize the observations. Photographic recording of the video images was done by directly photographing the CRT with a 35 mm camera. For the measurement of the intensity of the dye coupling, cells were shown on the CRT through video image processing and cells which exhibited enough fluorescence over the background were counted 5 min after the injection.

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