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Abstract

To test transdifferentiation of retinal pigment epithelial (RPE) cells in suspension culture, chick embryonic RPE sheets and dissociated RPE cells were cultured for two months in a non-adherent dish for suspension culture. RPE cells, isolated as a sheet, aggregated immediately and remained the same size with their differentiated characteristics for two months. The presence of basic fibroblast growth factor (bFGF) at concentrations of 10 ng/ml or higher induced the formation of a spherical lentoid structure which was positive for crystallin and bFGF receptor. In contrast, dissociated RPE cells did reaggregate but did not develop the lentoid structure even in the presence of bFGF. The transdifferentiation of RPE cells to the lentoid structure in this study was in contrast to their transdifferentiation to the retina, as reported in a previous study.

KEYWORDS: basic fibroblast growth factor, multicellular spheroids, retinal pigment epithelium, transdifferentiation, lens, lentoid

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Transdifferentiation of Chick Embryonic Retinal Pigment Epithelial Cells to Lentoid Structure in Suspension Culture

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To test transdifferentiation of retinal pigment epithelial (RPE) cells in suspension culture, chick embryonic RPE sheets and dissociated RPE cells were cultured for two months in a non-adherent dish for suspension culture. RPE cells, isolated as a sheet, aggregated immediately and remained the same size with their differentiated characteristics for two months. The presence of basic fibroblast growth factor (bFGF) at concentrations of 10 ng/ml or higher induced the formation of a spherical lentoid structure which was positive for crystallin and bFGF receptor. In contrast, dissociated RPE cells did reaggregate but did not develop the lentoid structure even in the presence of bFGF. The transdifferentiation of RPE cells to the lentoid structure in this study was in contrast to their transdifferentiation to the retina, as reported in a previous study.

Key words: basic fibroblast growth factor, multicellular spheroids, retinal pigment epithelium, transdifferentiation, lens, lentoid

Pigment epithelial cells of the eye including those of the retina and iris have a potential for transdifferentiating into other types of cells (1-3). The lens can be regenerated from iris pigment epithelium *in vivo* after its surgical removal in such urodeles as newt and salamanders (4, 5). The retina can also be regenerated from retinal pigment epithelium (RPE) *in vivo* after extirpation of the retina or transection of the optic nerve in urodeles (6-8) and in frogs (9). More recently, the retina has been shown to regenerate from RPE *in vivo* after its surgical removal in chick embryos (10-13). Basic fibroblast growth factor (bFGF) is known to play a crucial role in such transdifferentiation (12-15).

Cells, cultured in a non-adherent environment, form cellular aggregates called multicellular spheroids (16-18). Cells in a three-dimensional structure of multicellular spheroids show a greater tendency toward differentiation than proliferation, compared with cells in the usual two-dimensional adherent culture. The RPE of chick embryos have, indeed, been shown to transdifferentiate to the retina *in vitro* when cultured with bFGF under agitation of the media to avoid RPE sheets from attaching to the bottom of dishes (19). In this study, RPE was cultured with bFGF in a non-adherent dish for suspension culture (20-22) in expectation of its transdifferentiation to the retina. However, the transdifferentiation to the lentoid structure instead was observed. Thus, we examined the conditions underlying this transdifferentiation to the lentoid structure.

Materials and Methods

Egg shells were disinfected with 70 % alcohol, and 7.5-day-old chick embryos were taken out from a small opening. Eyes were enucleated and cut at the midperiphery of the globe, and anterior halves were removed together with the vitreous gel. The retina at this embryonic stage was pealed off easily from the eye cup (22). After the removal of the retina, the area of the optic fissure was excised, and the remaining eye cup was divided into 4 pieces. The tissues were then incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) in Dulbecco's modified Eagle's medium (DMEM) for 40 min at 37 °C, which allowed RPE to separate as a sheet from the eye cup. The RPE was placed in wells of a 24-multidish for suspension culture (Sumilon, Osaka, Japan), as a sheet or as dissociated cells after treatment with 0.25 % trypsin and 1 mM EDTA in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (Gibco BRL, Gaithersburg, MD,

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USA) for 15 min at 37°C. bFGF (Human FGF basic [157aa], R & D Systems, Minneapolis, MN, USA) was added to the media of RPE cells at a concentration of 0.1, 1, 10, or 200 ng/ml.

For light microscopy, multicellular spheroids were fixed with 3.7 % formaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, dehydrated with a graded series of ethanol and xylene, and then embedded in paraffin. Paraffin sections were cut and stained with hematoxylin-eosin. For electron microscopy, multicellular spheroids were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, fixed further with 1 % osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 3 hours at room temperature, dehydrated with a graded series of ethanol and propylene oxide, and embedded in epoxy resin (Epon 812 Resin, Taab Laboratories Equipments, Berk, UK). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with an electron microscope (Hitachi, HS-9, Katsuta, Japan).

For immunohistochemistry, paraffin sections were deparaffinized with xylene and washed with phosphate-buffered saline (PBS, pH 7.4) after being processed by a graded series of ethanol. Sections were incubated with goat normal serum for 30 min to block the non-specific binding, and incubated with anti-rhodopsin antibody (Rabbit Anti-bovine Rhodopsin: LSL, Tokyo, × 2000 dilution), anti-crystallin antibody (Rabbit Antibody to Alpha B Crystallin: SeroTec, Oxford, England, × 2000 dilution), or anti-bFGF receptor antibody (Anti-Chicken-FGF Receptor, Rabbit Antiserum: Upstate Biotechnology, Lake Placid, NY, USA, × 1000 dilution) for 90 min at 37°C. After being washed 3 times each for 10 min in PBS containing 0.05 % Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA), sections were incubated with biotinylated goat antibody against rabbit IgG for 60 min at 37°C and washed again. Sections were then incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC: Vector Stain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature and washed. Sections were color-developed with 0.1 % diaminobenzidine tetrahydrochloride and 0.02 % hydrogen peroxide in 0.1 M Tris buffer (pH 7.4) for 30 min, and counter-stained with 1 % methyl green for 30 min.

Results

RPE cells, isolated as a sheet, rolled up into cellular aggregates during the following day of culture (Fig. 1a). The absence of other types of cells, especially of retinal cells in cellular aggregates, was confirmed by light microscopic observation (Fig. 2a). The presence of bFGF at a concentration of 10 ng/ml or 200 ng/ml induced the growth of a nonpigmented mass from pigmented cellular aggregates at around 2 weeks (Fig. 1b), and resulted in the formation of a large nonpigmented mass at 4 weeks (Fig. 1c). RPE aggregates in the absence of bFGF remained stationary for the 4 weeks of culture (Fig. 1d).

Histologically, the nonpigmented mass was composed of spindle-shaped, layered cells with eosinophilic cytoplasms (Fig. 2b, 2c), which were markedly positive for crystallin and also for bFGF receptor, while negative for rhodopsin (Fig. 2d, 2e, 2f) at 4 weeks. This lentoid structure stopped growing in size at around 4 weeks and remained stationary afterwards until 8 weeks of culture.

The lentoid structure was formed from aggregated RPE sheets within 4 weeks in all 24 wells of a microplate at the concentration of 200 ng/ml of bFGF, while the lentoid structure developed only in 9 wells out of 24 wells at the concentration of 10 ng/ml. The presence of bFGF at a concentration of 1 ng/ml or 0.1 ng/ml, in contrast, did not induce the formation of the lentoid structure. Furthermore, dispersed RPE cells did aggregate to form multicellular spheroids, but did not develop a lentoid structure even in the presence of bFGF at the higher concentrations.

Ultrastructurally, lens-epithelial-like cells appeared initially along the outside of RPE clumps (Fig. 3a). Cells in the lentoid structure showed continuous intercellular junctions with neighboring cells, appearing as dense and closely apposed cellular membranes, characteristic of the lens *in vivo* (Fig. 3b). As a control, RPE cells, cultured for 4 weeks in the absence of bFGF, maintained their differentiated state with such polarity as apical microvilli and basal infoldings with basal lamina (Fig. 3c, 3d). The polarity of cells in cellular aggregates was in the direction of their apical sides out.

Discussion

The present study demonstrated that the presence of bFGF induced transdifferentiation of RPE cells to the lentoid structure, but not to the retina as described previously by Pittack *et al.* (19). We used chick embryos at 7.5 days of age, corresponding to the Hamburger-Hamilton stage 32 (23), which were older than embryos

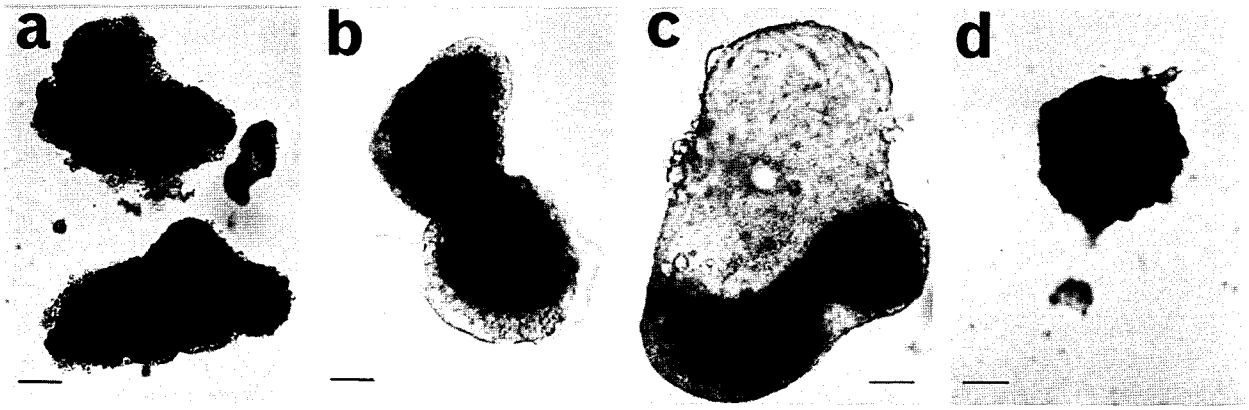

Fig. 1 Process of the formation of multicellular spheroids from chick embryonic retinal pigment epithelial sheets at 1 day (**a**), 2 weeks (**b**), and 4 weeks (**c**) in the presence of basic fibroblast growth factor (bFGF) at a concentration of 200 ng/ml, in contrast with a spheroid at 4 weeks in the absence of bFGF (**d**). A transparent mass can be seen growing out of a pigmented aggregate (**b**, **c**). Bar = 100 μm.

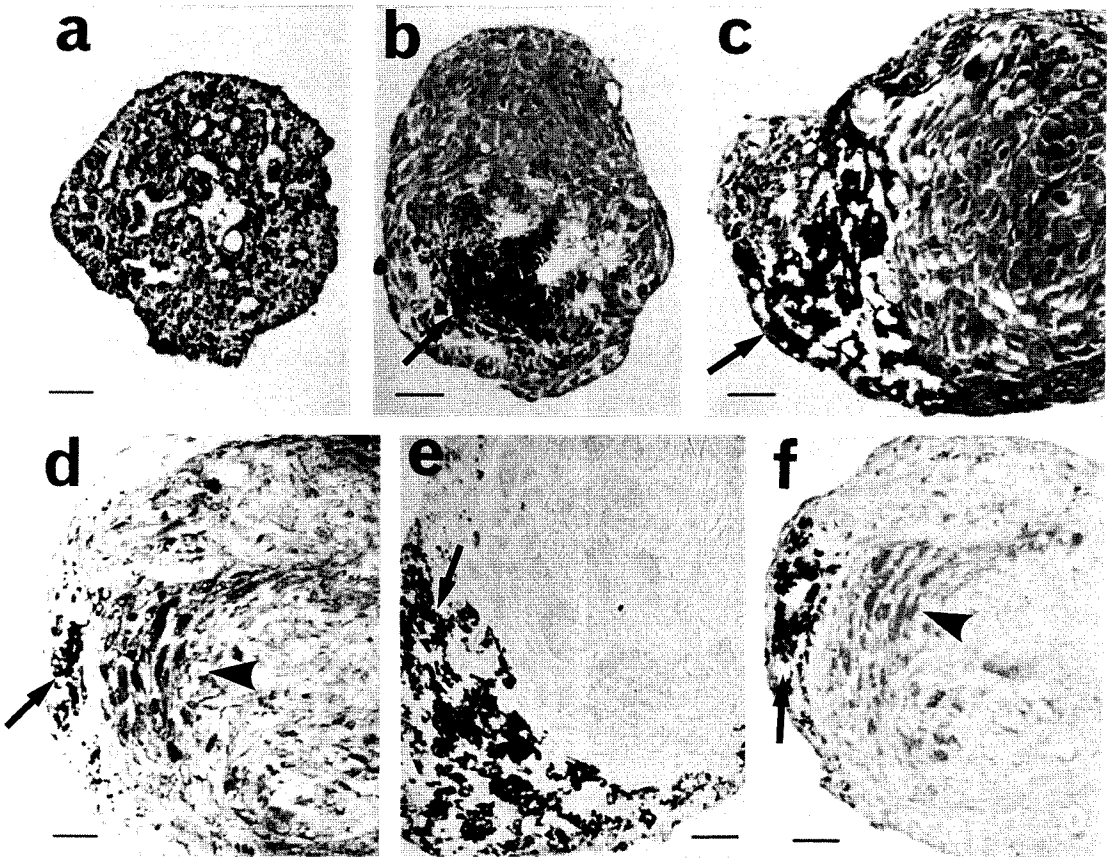

Fig. 2 Histological structure of multicellular spheroids formed by chick embryonic retinal pigment epithelial sheets and their immunohistochemistry. Spheroids with well-differentiated retinal pigment epithelial cells at 1 day (**a**) give rise to a mass with eosinophilic cells growing in size at 2 weeks (**b**) and at 4 weeks (**c**) in the presence of basic fibroblast growth factor (bFGF) at a concentration of 200 ng/ml. These cells in multicellular spheroids at 4 weeks are positive for crystallin (arrowhead in **d**) and bFGF receptor (arrowhead in **f**), but negative for rhodopsin (**e**). Arrows indicate collection of pigmented cells. Hematoxylin-eosin for **a**-**c**. Bar = 50 μm.

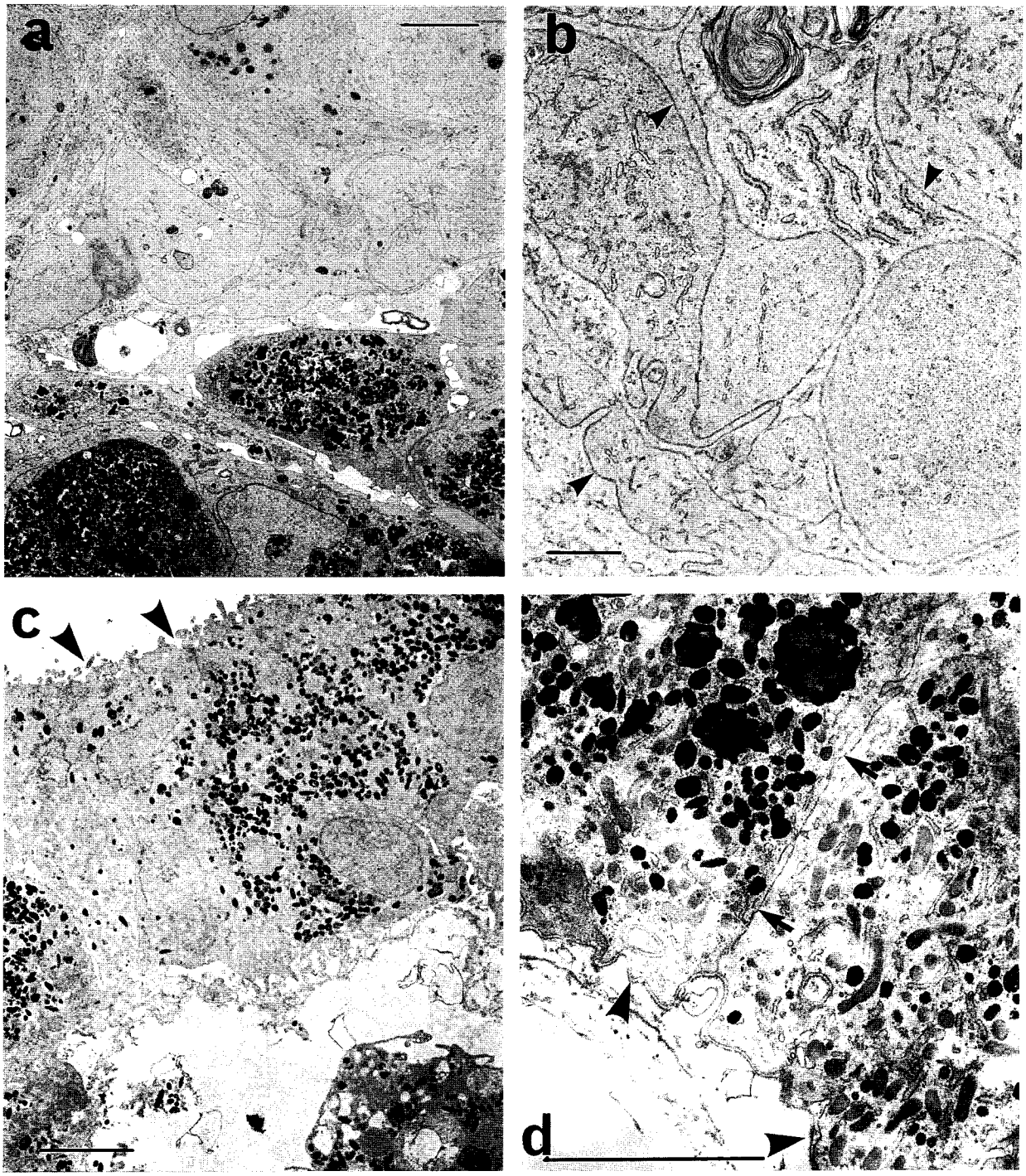

Fig. 3 Electron micrographs of multicellular spheroids at 4 weeks formed by chick embryonic retinal pigment epithelial sheets in the presence of 200 ng/ml basic fibroblast growth factor (bFGF) (**a**, **b**) and in its absence (**c**, **d**). Nonpigmented cells appear outside of a core of retinal pigment epithelial cells (**a**). Note the marked intercellular junctions among lens-like cells (arrowheads in **b**). Retinal pigment epithelial cells maintain their polarity with such features as apical microvilli (arrowheads in **c**) and basal infoldings with basal lamina (arrowheads in **d**). Note continuous intercellular junctions (arrows in **d**). Uranyl acetate and lead citrate stain. Bar = 5 μm in **a**, **c** and **d**. Bar = 1 μm in **b**.

at the stage 24–34 (3.5–8.0 days) which they used. We also did not use RPE cells around the optic fissure where it was difficult to isolate RPE from the retina completely, in contrast to the study performed by Pittack *et al.*, which made no mention of such care (19). We used embryos at stage 32 since this was the earliest stage at which the RPE could be separated completely from the retina outside the optic fissure. In this sense, transdifferentiation of RPE to the retina in the study by Pittack *et al.* (19) could be explained also by the presence of multipotent cells at earlier stages of development or by contamination with retinal precursors present in the optic fissure.

The reasons why the transdifferentiation to the retina was not observed in the present study could be: a) stage 32 was too late for RPE to transdifferentiate to the retina, b) RPE used in our study was completely separate from the optic fissure where the retina and RPE were continuous, and c) the media in our floating culture was not shaken as was that in the culture by Pittack *et al.* (19), which might influence the way the RPE sheets rolled up into aggregate forms.

The concentration of bFGF was found to be critical for the transdifferentiation of RPE to the lentoid structure in our system. The transdifferentiation occurred at concentrations of 10 ng/ml or higher, and higher concentrations resulted in a higher rate of the transdifferentiation. Dispersion of cells by treatment of an RPE sheet with trypsin did result in reaggregation of these cells, but did not lead to their transdifferentiation to the lentoid structure, meaning that the loss of proper orientation of each cell in a group of cells would abolish the induction of transdifferentiation. These two factors underlying the transdifferentiation to the lentoid structure in this study, the concentration of bFGF and proper cell-cell contact, were the same as those found for transdifferentiation to the retina in the previous study by Pittack *et al.* (19). In addition, the co-expression of crystallin with bFGF receptor in transdifferentiated lens cells, found in the present study, suggests that a closed loop of bFGF, as a signal, enhances the transdifferentiation.

Our floating culture system allowed embryonic RPE cells to survive as multicellular spheroids for two months. Multicellular spheroids, formed in a dish for suspension culture, would be a convenient *in vitro* system for studying the transdifferentiation which occurs *in vivo* in urodeles, and also be suitable for examining the effect of various factors upon the transdifferentiation. In a non-adherent dish for culture, lentoid structures were indeed formed earlier after the beginning of incubation from RPE cells cultured as multicellular spheroids than RPE cells in the usual adherent culture (1, 15, 24). In addition, differentiated or transdifferentiated multicellular spheroids might be used as transplants for RPE or lenses in the future.

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