In Vitro Histogenesis of Human Embryonic Stem Cells into Retina Components

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We developed a protocol of *in vitro* differentiation of human embryonic stem cells into three-dimensional structures histologically and molecularly similar to the developing retina.

Key Words: *embryonic stem cells; differentiation; pigment epithelium; retina*

Cell therapy, in particular, transplantation of retinal neuroepithelium, can be a principally new approach to the therapy of retinal degeneration diseases. Transplantation of the retina is extremely rarely used because of the absence of standardized and renewable source of the tissue for transplantation. Human pluripotent SC can be a source of any human tissue and in case of creation of the technology of their targeted differentiation *in vitro* and selection can become a unique standardized source of cell and tissues for transplantation. Two types of human pluripotent cells are known: embryonic SC (ESC) and induced pluripotent SC [10]. There are reports on the development of ESC differentiation protocols into pigment retinal epithelial cells [1-5] and photoreceptor cells [6,9], but the possibility of controlled differentiation of this three-dimensional and multicomponent tissue remains an open question.

Here we analyze three-dimensional structures similar to developing eye retina, which were differentiated *in vitro* from human ESC.

MATERIALS AND METHODS

We used three strains of human ESC: hESM01, hESM03, and hESM04 [7,8]. The cells were cultured on gelatin-coated 35-mm Petri dishes (Costar) using mouse embryonic fibroblasts as the feeder in KODMEM (Invitrogen) containing 20% serum substitute (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 1 mM glutamine (Gibco), 1 mM amino acid mixture (Gibco), 4 ng/ml bFGF (Peprotech), and antibiotics. The cells were cultured at 37°C and 5% CO_2.

Alternatively, human ESC were cultured in defined serum-free medium mTESR1 (Stem Cell Technologies). Matrigel (BD Biosciences) was used as a substrate for culturing. The medium was replaced every day. The cells were passaged every 5-7 days using 1 mg/ml dispase (Invitrogen).

For induction of differentiation, ESC were cultured until subconfluence, treated with collagenase IV (for cells growth on the feeder) or dispase (for cells cultured without feeder), and transferred to a new Petri dish coated with gelatin, cultured in DMEM/F12 (HyClone) with N2 and B27 (Invitrogen), 50 ng/ml noggin, and 20 ng bFGF. The medium was replaced every other day. After 5-7 days, rosette-like structures, neuroepithelial precursors, appeared in the culture. After 2 weeks of culturing, the rosettes (up to 70% cells) were mechanically separated from other cells with a plastic tip, transferred to a new Perti dish, and the culturing was continued in DMEM/F12 containing 15% FBS with N2, 5 ng/ml BMP4, 20 ng/ml EGF, 20 ng/ml DKK1, and 5 ng/ml VEGF.

For preparing histological sections, the preparations were routinely embedded in araldite and then

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semithin (1-2 μ) and thin (60 nm) sections were sliced (Nova ultratome). Semithin sections were stained with methylene blue. Thin sections were contrasted with uranyl acetate and lead citrate.

Reverse transcription-PCR (RT-PCR) and histochemical analysis were carried out as described previously [7].

RESULTS

Culturing of ESC at a high density under conditions providing preferential development of neuroepithelium (addition of noggin, EGF, bFGF) and then (after 2 weeks) addition of BMP and serum to a concentration of 15% and culturing for 90 days led to the formation

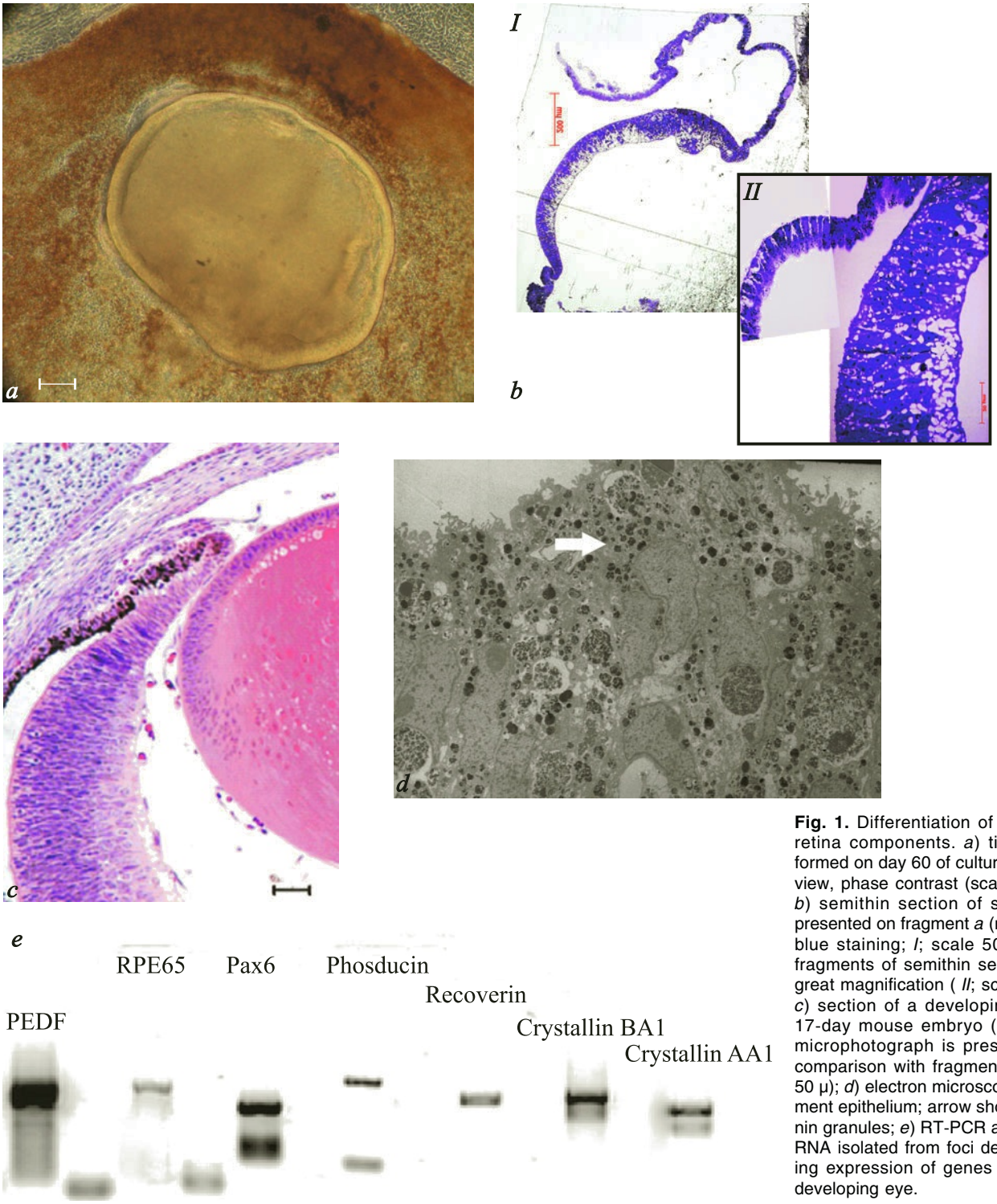

Fig. 1. Differentiation of ESC into retina components. *a*) tissue foci formed on day 60 of culturing: plane view, phase contrast (scale 250 μ); *b*) semithin section of structures presented on fragment *a* (methylene blue staining; *I*; scale 500 μ) and fragments of semithin section at a great magnification (*II*; scale 50 μ); *c*) section of a developing eye of 17-day mouse embryo (from [3]): microphotograph is presented for comparison with fragment *b* (scale 50 μ); *d*) electron microscopy of pigment epithelium; arrow shows melanin granules; *e*) RT-PCR analysis of RNA isolated from foci demonstrating expression of genes typical of developing eye.

of complex three-dimensional structures of neuroepithelial origin (Fig. 1, *a*).

Three-dimensional structures attained 3-4 mm in diameter. Individual histological foci lay on a sublayer consisting of neuronal and glial cells; they contained peripheral pigment cells of typical hexagonal shape with pigment granules. Several dense layers of pigment-free cells were seen on top of these structures. For more detailed histological and molecular analysis, these structures were mechanically separated from the sublayer of neuronal and glial cells.

Histological analysis of semithin sections confirmed complex histological structure of these foci (Fig. 1, *b*): the central zone consisted of several cell layers; to the periphery, the number of cell layers decreased and pigment content in them increased. The cross-section of the formed focus in general remained flattened mouse eye on day 17 of embryonic development [3] (Fig. 1, *b*, *c*). Electron microscopy of thin sections confirmed the presence of melanin granules in cells (Fig. 1, *d*). Immunohistochemical analysis of cryosections revealed specific localization of phosducin, recoverin, and connexin 43 (data not shown), which attested to the presence of photoreceptor cells in the studied structures. For more comprehensive molecular characteristics, RT-PCR analysis of gene expression specific for retinal cells was performed (Fig. 1, *d*; Table 1). Expression of genes typical for functional pigment retinal epithelium (PEDF, RPE65) was detected in the foci. Expression of phosducin and recoverin, as well as expression of transcription factor Pax6 confirmed immunohistochemical findings (Fig. 1, *e*). Moreover, in the material obtained from isolated foci, expression of crystallin (BA1 and AA) was found, which probably suggests that the material contains cells involved in lens formation. Thus, analysis of foci demonstrated the formation of structured tissues carrying markers of developing eye.

Similar structures were previously obtained from mouse ESC, but they were not completely characterized [4]. The formation of three-dimensional structures similar to the retina was previously observed during co-culturing of human ESC with stromal PA-6 cells [2]. Our finding suggest that targeted differentiation of human ESC into separate multicomponent tissue complexes similar by their histological and molecular characteristics to eye retina can be attained without co-coulturing of human ESC with other cells.

TABLE 1. Primers Used for RT-PCR Cells Differentiated from ESC

Pedf	acttcggctatgacctgtac
	accgagaaggagaatgctga
RPE65	gtgaccgattcaagccatct
	gtcactgcacagaattgcag
Recoverin	ggacttcaaggagtacgtca
	ttggcatcaggcgttcttca
Pax6	TCAGCACCAGTGTCTACCAACCAA
	ATCATAACTCCGCCCATTCACCGA
Phosducin	gcaggatatgcaccagaagc
	ggtatgctctaggacatgtac

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