Structure and Cell Composition of Spheres Cultured from Human Fetal Retina

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The structure and cell composition of spheres obtained by culturing human fetal retinal cells after 15, 18, 22-23, and 24 weeks of gestation were studied. The cells were cultured as neurospheres: in serum-free medium with growth factors, in which they formed floating spheres. Immunocytochemical analysis showed that cell proliferation in the spheres decreased with increasing fetal age. Stem/progenitor cells, neuroblasts, and photoreceptors were detected in the spheres. Glial cells were detected only in spheres originating from 22- and 24-week fetuses. All spheres, irrespective of age and duration of culturing, consisted of numerous cell rosettes, each histotypically similar to the neuroblastic layer of the developing retina.

Key Words: *human retina; stem and progenitor cells; tissue culture; immunocytochemistry*

Introduction of methods for culturing of stem and progenitor cells in experimental cell biology promoted creation of technologies of substitution and stimulatory cell therapy. Neural stem and progenitor cells actively investigated in recent years are a potential source for cell transplantation in neurodegenerative diseases of the central nervous system and eye retina [4,14,20].

Multiplication and maintenance of cells from mammalian and human retina in tissue culture are important for basic and applied research [3,11]. It was shown that neural progenitor cells from human retina can be isolated at the stage of embryonic development (when the retina is not yet differentiated) and grown in tissue culture [12,13,15,18, 23]. The capacity to neurosphere formation from neural retinal cells decreases with age and disappeared during the postnatal period, which is attributed to starting differentiation of stem and progenitor cells from the neuroblastic layer [6,7]. On the other hand, it was shown that progenitor cells are retained in adult human retina and can form spheres during culturing [16]. The main source of stem cells in adult eye is the ciliary pigmented epithelium [7,21].

Selection of adequate conditions for culturing is extremely important for effective production of progenitor cells. It was shown on rodent and human cultures that energy substrates, hormones, fibroblast growth factor, and epidermal growth factor, regulating the growth of photoreceptors, neurons, and glial cells are essential for the maintenance of retinal progenitor cells [5,8,10,12,18,22]. Experimental data on the production of cloned neurospheres from rodent and human fetal retina are contradictory. The development of human neurospheres ceased during the second passage [9,23]. However, it seems possible to obtain them in experiments on mice [11,15].

We studied the structure and cell composition of spheres obtained by culturing of human fetal retinal cells at different terms of gestation.

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MATERIALS AND METHODS

Our method for culturing of neurospheres from the brain, which proved to be effective [17], was used for culturing human retinal cells.

The eyes of nonviable aborted human fetuses of 15-, 18-, 22-23-, and 24-week gestation were obtained from licensed institutions of Ministry of Health of the Russian Federation within the framework of legislation of the Russian Federation on public health protection and in accordance with approved list of medical indications. Fetal age corresponded to terms determined by the obstetrician.

The retina was isolated under a binocular microscope, put into medium, crushed, and pipetted to prepare a suspension. The cells were cultured in serum-free DMEM/F12 with N2-supplement (1:100), main fibroblast growth factor (20 ng/ml), epidermal growth factor (20 ng/ml), and leukemia-inhibiting factor (10 ng/ml), heparin (8 μg/ml) and penicillin/streptomycin (100 μg/ml). The initial viability of cells in the suspension was at least 70%. The cells were cultured in suspension at a density of 2×10^6/ml; the medium (25-75%) was replaced every 4-5 days. If large cell aggregations formed, the culture was repeatedly pipetted. Culturing was carried out at 37°C and 5% CO_2 for 20 days (30 days for the retina from a 24-week fetus).

For immunocytochemical analysis spheroids were fixed in 4% paraform in 0.01 M phosphate buffer (pH 7.4). The cultures were then washed and incubated overnight on the cold with the first antibodies. The following antibodies were used: to human nestin (Chemicon, 1:200; Abcam, 1:100), glial fibrillary acidic protein (GFAP; Chemicon, 1:200), β-III tubulin (Abcam, 1:200), vimentin (Chemicon, undiluted), neurofilament protein 200 (ICN, 1:10), Ki67 protein (Abcam, 1:50), recoverin (Moscow State University, 1:20), fibronectin (Santa Cruz, 1:50), and connexin-43 (Sigma, 1:200). The cells were then stained with second antibodies with fluorescent stains Cy-2 or Texas Red (Jackson). The nuclei were stained with Hoechst 33342. After staining the spheres were placed onto glass in a glycerol drop and covered with a slide, using small pedicles, so that the three-dimensional spheres were not damaged. The preparations were examined under an Opton-III fluorescent microscope with Nikon Coolpix 4500 digital camera.

RESULTS

Analysis of cultures derived from human fetal retinas at weeks 15, 18, 22, and 24 of gestation showed that cells formed floating spherical aggregations (spheres) in all cases. Some of them resembled neurospheres described previously [17] by shape and small size, others were larger aggregations consisting of several spheres stuck to each other.

Retinospheres produced from the retina of 15-week fetuses were formed from cells distributed chaotically or forming concentrical structures, which was detected by Hoechst 33342 (nuclear fluorescent stain) staining (Fig. 1, *a*). Immunohistochemical staining with antibodies to Ki67 protein (proliferation marker) showed many positive cells, located in small retinospheres and large aggregations in their entire volume (Fig. 1, *b*). Along with chaotic distribution, in some spheres the distribution of stained cells indicated structural orderliness of proliferating cells. In these cases labeled cells formed concentrical rosette-like structures, similar to those detected by Hoechst 33342 staining. Double staining with antibodies to Ki67 and nestin showed that these nestin-positive cells were abundant in all spheres, their processes formed a dense network and some fibers penetrated into cell rosettes (Fig. 1, *c*). Antibodies to recoverin (a protein of photoreceptor cell and cone bipolar cells) clearly distinguished the groups and solitary cells in the spheres (Fig. 2, *a*). Cell groups consisting of several tens or hundreds cells were located in spheres without apparent order. The groups of cells presented as round formations, with cells positioned radially, centrally symmetrically spherically, or circularly, which coincided with structures detected by Hoechst 33342 and Ki67 staining. This attests to generation of photoreceptors in the cultures. Recoverin-positive cells did not express Ki67, because they were in a state of differentiation. Double staining for nestin and recoverin showed that fibers of nestin-positive cells passed between recoverin-positive cells (Fig. 2, *b*), similarly as during interactions in the native fetal retina. A characteristic feature of nestin-positive cells in developing human retina is staining of the processes alone, and hence, no cell bodies are seen in the outer layer, where they are located. It was hypothesized that the bodies of nestin-positive cell in cultures lied among recoverin-positive cells similarly to their distribution during normal development. Staining with antibodies to β-III tubulin (early neuroblasts) showed that labeled cells formed a dense network in spheres. They had small round bodies with pronounced thin processes and by their morphology were similar to poorly differentiated ganglial cells (Fig. 2, *c*). No cells positively stained for GFAP (marker of differentiated Muller cells and astrocytes) were found in these cultures.

Similar spheroids with numerous cells expressing Ki67 formed in the culture of retina from an 18-week fetuses (Fig. 3, *a*). Dividing cells were

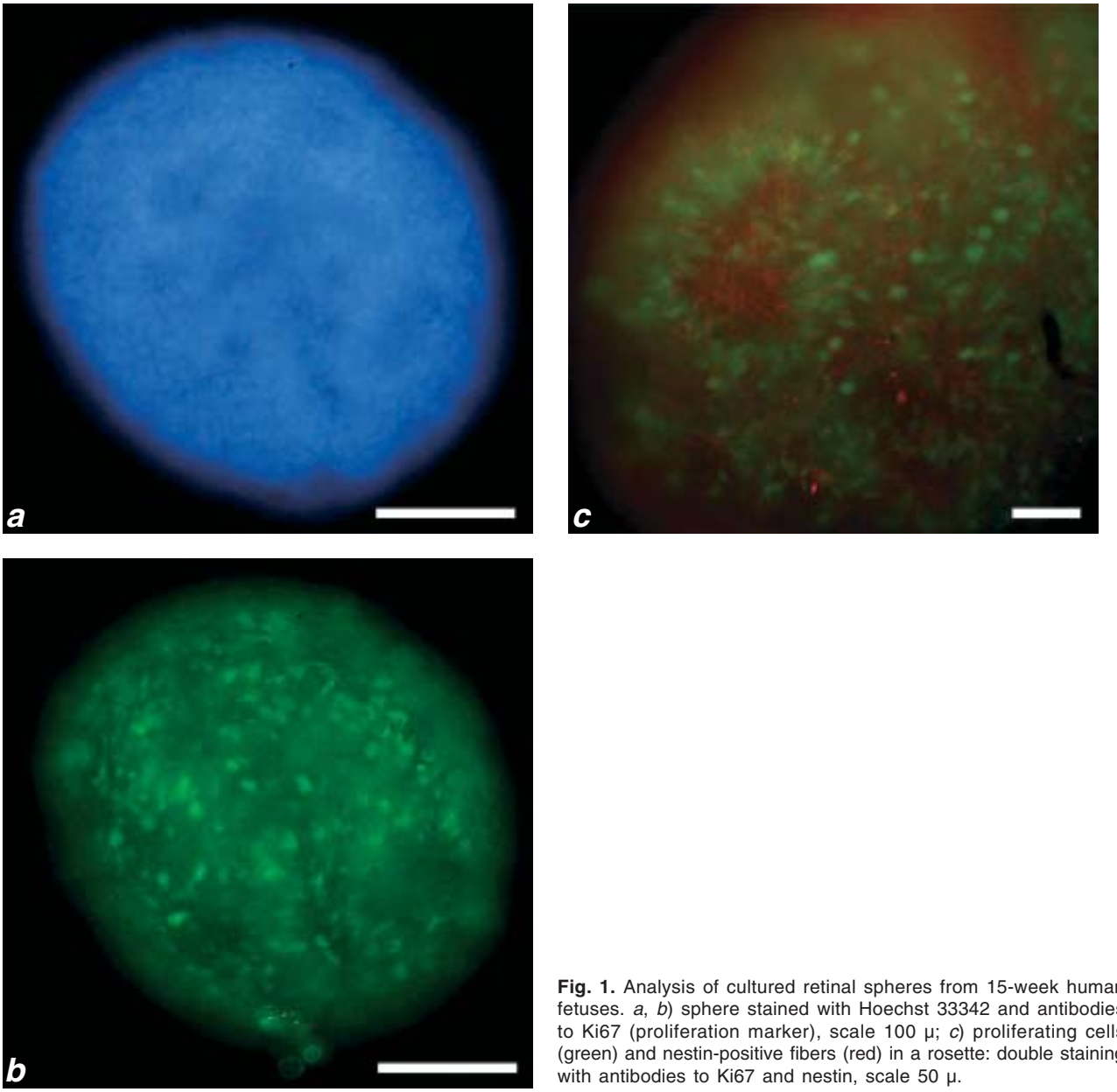

Fig. 1. Analysis of cultured retinal spheres from 15-week human fetuses. *a*, *b*) sphere stained with Hoechst 33342 and antibodies to Ki67 (proliferation marker), scale 100 μ; *c*) proliferating cells (green) and nestin-positive fibers (red) in a rosette: double staining with antibodies to Ki67 and nestin, scale 50 μ.

scattered in the entire volume of the sphere and formed concentrical accumulations. The fibers of nestin-positive cells were typically arranged in compact cords (Fig. 3, *b*). The fibers of cells stained for vimentin (marker of poorly differentiated cells) were distributed similarly. This suggests that nestin and vimentin were expressed in the same cells. The cords of nestin-positive fibers surrounded the rosettes consisting of recoverin-positive photoreceptor cells, presenting as concentrical accumulations of radially oriented cells with a cavity in the center. Photoreceptors (cones at this stage of development) were oriented by their future outer segments inside the rosette. Double staining for nestin and recoverin showed that nestin-positive fibers were positioned radially, passing through accumulations of cells expressing recoverin. β-III Tubulin-positive cells, shaped as photoreceptors (this presumably indicating their continuing differentiation), were distributed also radially between recoverin-positive cells (Fig. 3, *c*, *d*). The bulk of neuroblasts stained for β-III tubulin were located at the outer surface of recoverin-positive cell accumulations filling the entire space between them. Neuroblasts were bipolar cells with long poorly branched processes spreading to the periphery and encircling the accumulations of recoverin-positive cells. Staining for neurofilaments showed individual unipolar cells with small processes. No cells expressing GFAP, connexin-43, and fibronectin were detected in spheres.

Small and larger retinospheres formed in retinal culture from 22-23-week fetuses. The number of Ki67-positive cells decreased significantly in the spheres; these cells formed small chaotic groups mainly between the rosettes of recoverin-positive cells and were rarely seen in their inner part (Fig. 4, *a*). This means that there were virtually no proliferating cells in the photoreceptor layer. β-III Tubulin-positive radially oriented cells were clearly seen in the rosettes. Their apical processes were short and thick, basal ones (axon-type) were very thin and long, forming bundles at the outer surface of rosettes. In some cases symmetrically positioned cells expressing β-III tubulin were seen in these rosettes. Many neuroblasts stained for β-III tubulin were located outside of recoverin-positive rosettes (Fig. 4, *b*). The distribution of nestin- and vimentin-expressing cells coincided with that in cultures derived from 18-week fetuses. Staining with antibodies to recoverin and vimentin showed that vimentin-positive fibers lay between recoverin-positive cells and formed a radial backbone (Fig. 4, *c*, *d*). All these cell populations formed histotypical structures resembling the outer neuroblast layer of the developing retina.

Glial cells expressing GFAP were detected in this culture. Their distribution in spheres was uncommon: they formed pronounced convex clones of different size on the surface of spheres (Fig. 4, *d*). Long fibers originated from some clones, the processes on other cells did not spread beyond the clone.

Sufficiently uniform small spheres formed in cell culture derived from the retina of 24-week human fetuses after 30-day culturing; these spheres consisted of numerous cell rosettes, detected by Hoechst 33342 staining (Fig. 5, *a*). Staining with antibodies to recoverin and Ki67 confirmed that these rosettes were bulky clusters of radially oriented recoverin-positive cells with nuclei lying at the periphery. No dividing cells were detected in rosettes. However, one sphere contained many proliferating cells, which attested to different cell composition in different spheres. Staining of spheres with antibodies to recoverin and nestin showed that the major part of nestin-positive fibers were located outside the rosettes and were rarely seen inside (Fig. 5, *b*). This distribution of fibers was detected by staining for vimentin. Many neuroblasts expres-

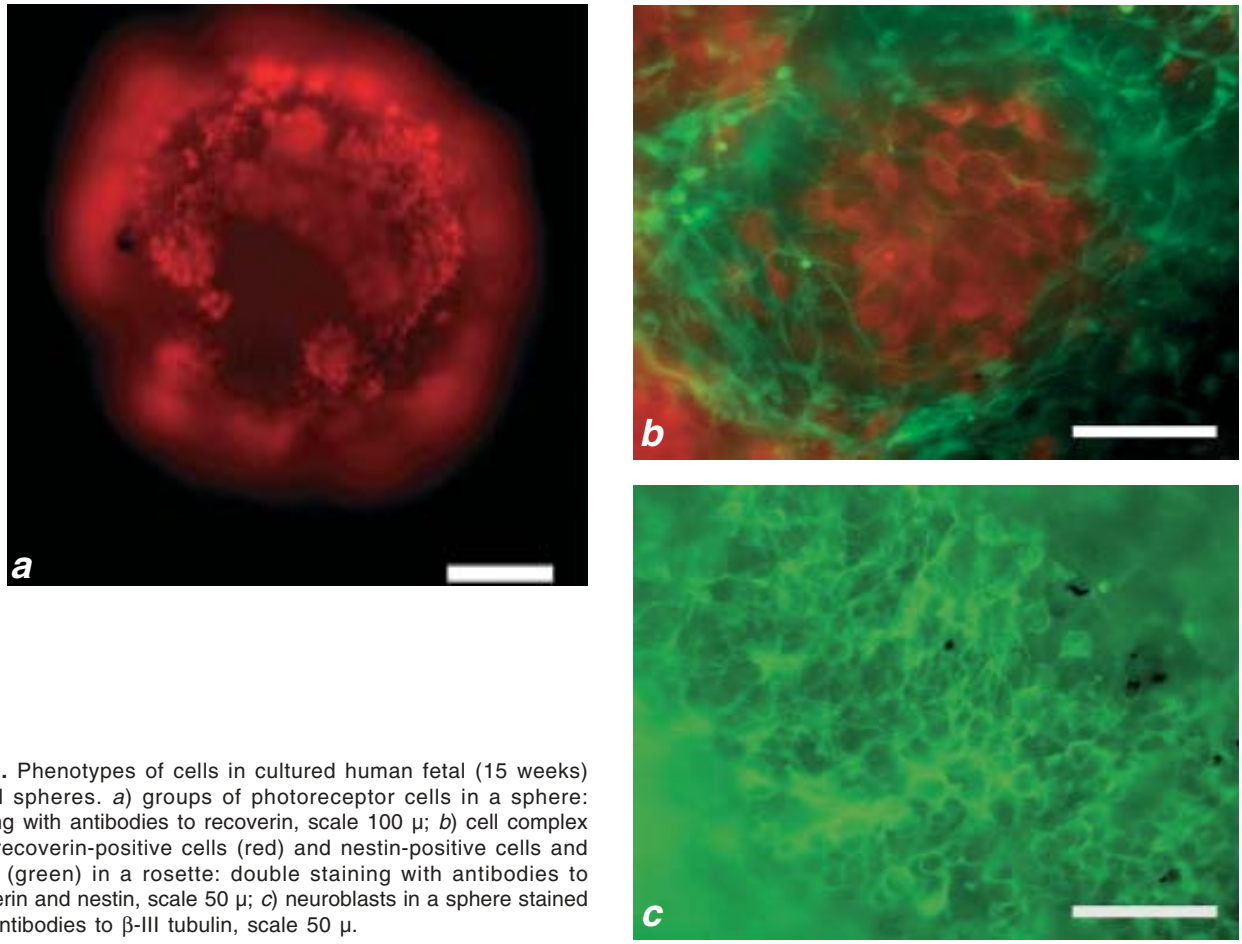

Fig. 2. Phenotypes of cells in cultured human fetal (15 weeks) retinal spheres. *a*) groups of photoreceptor cells in a sphere: staining with antibodies to recoverin, scale 100 μ; *b*) cell complex from recoverin-positive cells (red) and nestin-positive cells and fibers (green) in a rosette: double staining with antibodies to recoverin and nestin, scale 50 μ; *c*) neuroblasts in a sphere stained with antibodies to β-III tubulin, scale 50 μ.

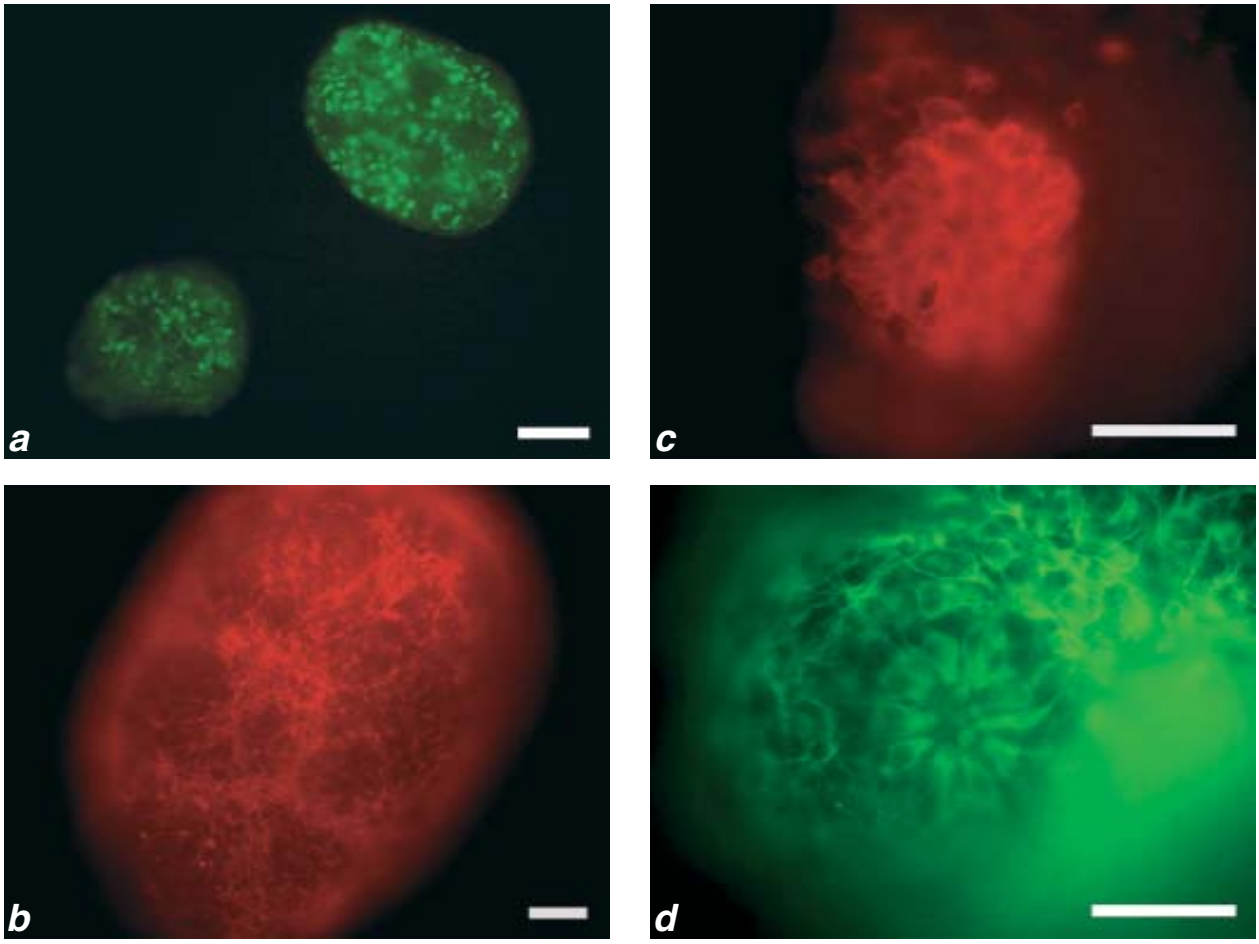

Fig. 3. Immunohistochemical analysis of cultured spheres of 18-week human fetal retina. *a*) cell proliferation: staining with antibodies to Ki67, scale 100 μ; *b*) distribution of nestin-positive fibers: staining with antibodies to nestin, scale 50 μ; *c*, *d*) cell rosette: double staining for recoverin (red) and β-III tubulin (green), scale 50 μ.

sing β-III tubulin were found in zones with nestin-positive fibers. These neuroblasts were uni- and bipolar with long processes, often surrounding the rosettes (Fig. 5, *c*). Some rare β-III tubulin-positive cells resembled horizontal cells not only by shape, but also by spatial location. Neuroblasts detected in rosettes were located radially, some of them migrating from the center to the periphery beyond the photoreceptor cells. Staining with antibodies to GFAP showed that astrocytes formed structures resembling clones on the surface of spheres. The spheres sometimes contain few these formations connected to each other with fibrous processes. Double staining for GFAP and Ki67 showed active cell proliferation inside these structures (Fig. 5, *d*).

Small bundles of fibers were detected in spheres with antibodies to neurofilaments. Staining with antibodies to connexin-43 and fibronectin showed no labeled cells.

Morphological studies of cultures derived from retinal cells of 15-, 18-, 22-23-, and 24-week human fetuses cultured without feeder in a serum-free medium supplemented with basic fibroblast growth factor, epidermal growth factor, and leukemia-inhibiting factor showed the formation of free floating spheres and their aggregations in all cultures, irrespective of the term of fetal retina development. This is in line with the data obtained in culturing of human retinal cells [6,12,13,23]. In our cultures the spheres were pipetted during each replacement of the medium, and by the end of culturing the number of spheres increased. These were effectively growing secondary spheres, which disagrees with our previous data [23]. The structure of retinal spheres differs significantly from neurospheres derived from the fetal brain. All retinal spheres studied (irrespective of their age and duration of culturing) consisted of numerous cell rosettes and cells scattered among them, while neurospheres sometimes contained solitary rosettes [2].

According to the data of immunocytochemical analysis of retinal spheres, their cells still prolife-

rated after 20 and 30 days in culture. However, the cell capacity to division decreased with fetal age. We found numerous dividing cells in all studied spheres from 15- and 18-week retinas, which contradicts the data on drastically decreasing proliferation during culturing of cells from 10-13-week human retina [9]. Virtually no proliferating cells were seen in spheres derived from 22-24-week retina, except solitary spheres with large numbers of dividing cells, indicating the differences in the cell composition of spheres. It seems that some spheres were formed from retinal fragments with great number of progenitor cells (ciliary area). However, the summary cell proliferation in these cultures was low, which is in line with previous results obtained on a culture of 25-week retina [13].

Phenotypical markers detected various cell types in the studied cultures. Expression of nestin (neural stem and progenitor cell protein) was detected in all cultures. The density of these cells did not de-

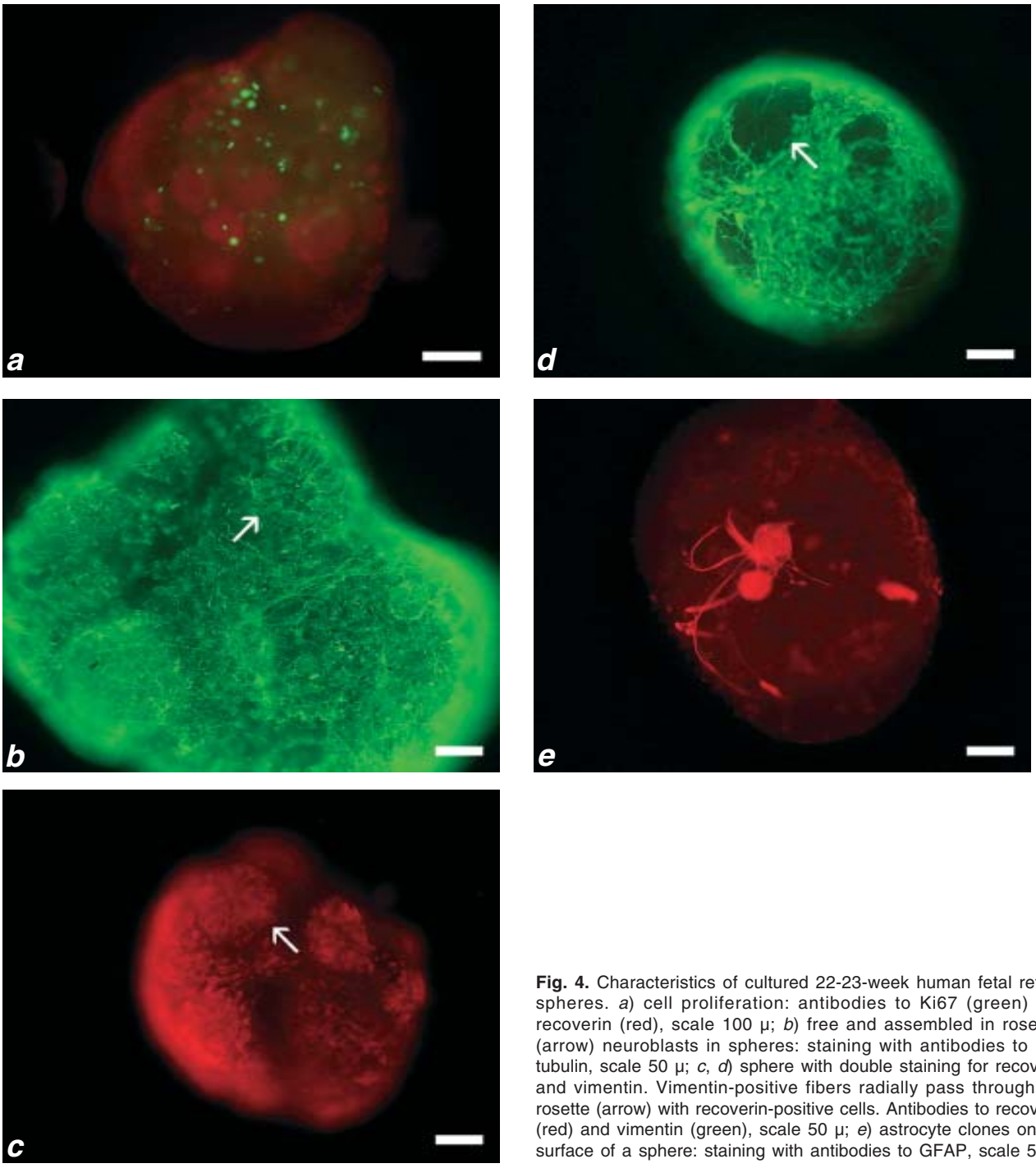

Fig. 4. Characteristics of cultured 22-23-week human fetal retinal spheres. *a*) cell proliferation: antibodies to Ki67 (green) and recoverin (red), scale 100 μ; *b*) free and assembled in rosettes (arrow) neuroblasts in spheres: staining with antibodies to β-III tubulin, scale 50 μ; *c*, *d*) sphere with double staining for recoverin and vimentin. Vimentin-positive fibers radially pass through the rosette (arrow) with recoverin-positive cells. Antibodies to recoverin (red) and vimentin (green), scale 50 μ; *e*) astrocyte clones on the surface of a sphere: staining with antibodies to GFAP, scale 50 μ.

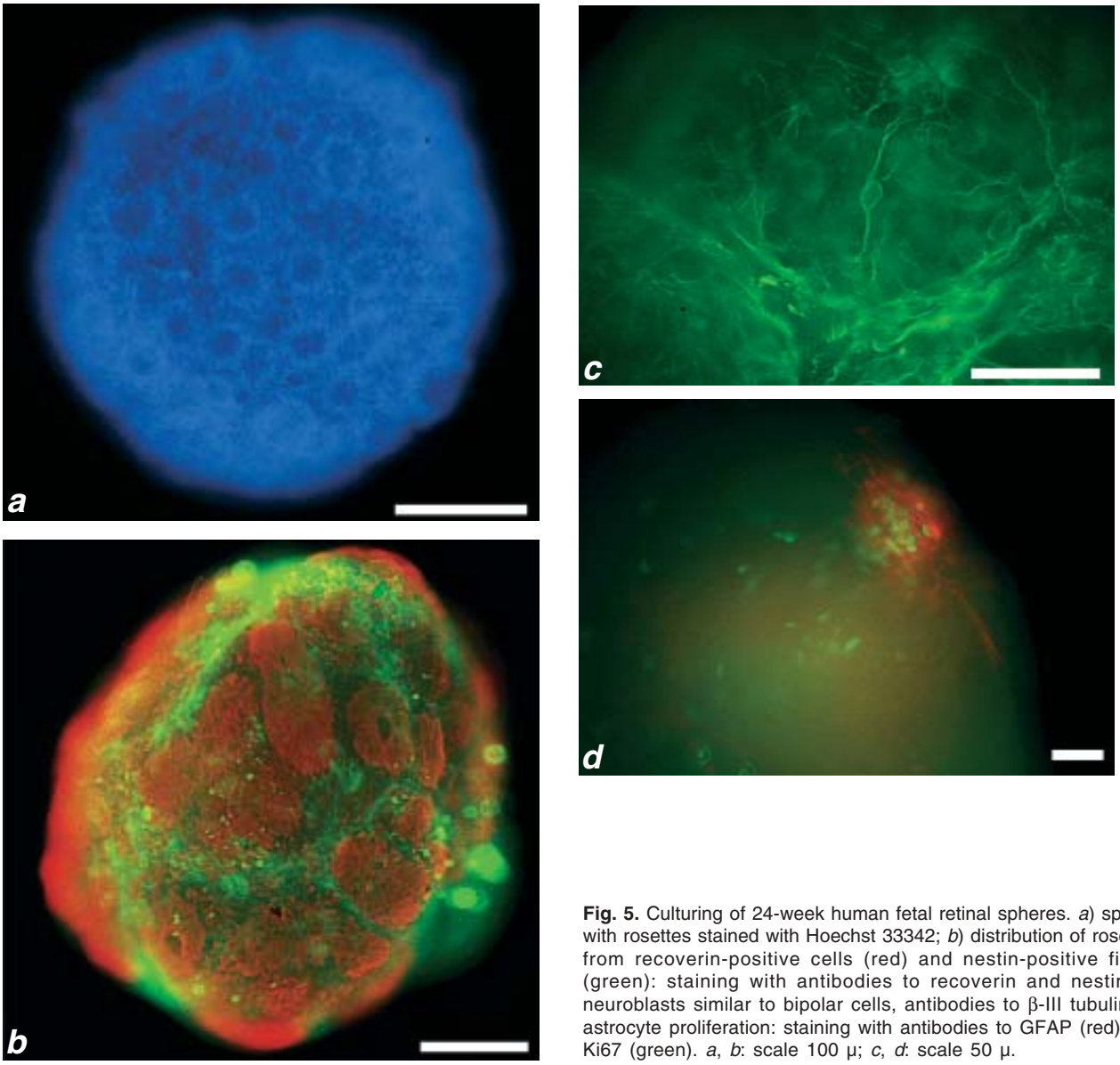

Fig. 5. Culturing of 24-week human fetal retinal spheres. *a*) sphere with rosettes stained with Hoechst 33342; *b*) distribution of rosettes from recoverin-positive cells (red) and nestin-positive fibers (green): staining with antibodies to recoverin and nestin; *c*) neuroblasts similar to bipolar cells, antibodies to β-III tubulin; *d*) astrocyte proliferation: staining with antibodies to GFAP (red) and Ki67 (green). *a*, *b*: scale 100 μ; *c*, *d*: scale 50 μ.

crease in different cultures. The distribution of nestin coincided with that of vimentin (progenitor cell marker) and did not coincide with that of astrocyte GFAP, similarly to that observed in developing human retina. Many neuroblasts expressing β-III tubulin were detected in all cultures; these cells were phenotypically similar to ganglioblasts, bipolars, and even horizontal cells detected in cultures from late retina.

Since photoreceptor differentiation (recoverin expression) in native human retina starts during week 10 of gestation, these cells were present in our cultures [1]. Numerous photoreceptor (recoverin-positive) cells were detected in retinal spheres of all ages, which were well preserved during culturing without signs of degradation, though previous studies on adherent cultures detected a negligible number of recoverin-positive cells [12,13,23]. It seems that the generation of photoreceptors continued in cultures from 15- and 18-week retinas, because proliferating cells were detected in rosettes, but this was not observed in cultures originating from late retinas. In our experiments glial cells were detected only in spheres cultured from 22- and 24-week retina. Cells expressing GFAP proliferated and formed clone-like structures on the surface of spheres, but were not located inside them. These were not Muller cells, but astrocytes migrating into the retina via the optic nerve starting from week 20 of fetal development [19]. Cultured Muller cells did not express GFAP. This indicates that culturing conditions were adequate and did not provoke reactive gliosis in them, which was presumably observed in our previous experiments [9,23].

The cells in the spheres were not chaotically scattered, but formed well-organized histotypical structures, including rosettes consisting of radially positioned nestin-vimentin-positive cells, photoreceptors, and neuroblasts, and nestin-vimentin-positive cells and neuroblasts surrounding them. Thus organized cells were obviously similar to the structure of the outer layer of human fetal retina. Moreover, the location of astrocytes on spherical surface was spatially similar to their distribution in the layer of ganglionic cells and their fibers in the native retina. The mechanisms of formation of rosette-like structures in spheres remain unknown. Presumably, small cell aggregations are retained during preparation of suspension or specific cell adhesion is possible.

Hence, the main result of our study is a proven fact that viable specific complexes of progenitor cells with unique contacts and microenvironment essential for the maintenance of long proliferation and normal differentiation of retinal cells are retained for a long time in the system of cultured spheres used in our study. Further experimental studies of cultured spheres will presumably clear out some basic problems in the developmental biology and cell therapy for degeneration of the retina in humans.

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