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Original scientific paper

Development of the Fetal Neural Retina *in Vitro* and in Ectopic Transplants *in Vivo*

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ABSTRACT

Investigation of the developmental potential of immature tissues is important for novel approaches to human regenerative medicine. Development of the fetal neural retina has therefore been investigated in two experimental systems. Retinas were microsurgically isolated from 20-days-old rat fetuses and cultivated in vitro for 12 days or transplanted in vivo under the kidney capsule of adult males for as long as 6 months. Shedding of the photoreceptor outer segment which is a process occurring at the terminal stage of photoreceptor differentiation was observed in culture by transmission electron microscopy (TEM). In transplants, no photoreceptors were found although markers of terminal neural and glial differentiation (e.g. synaptophysin, chromogranin and glial fibrilary acidic protein – GFAP) along with the molecules involved in the process of differentiation (guidance molecule semaphorin IIIA and chondroitin sulfate proteoglycan) were expressed. Semaphorin was differentially expressed being absent from older transplants. Proliferating cell nuclear antigen and nestin (marker of undifferentiated neural cells) were still weakly expressed even in six-months-old transplants. We could conclude that in both our experimental systems fetal neural retina proceeded to differentiate further on. However, even in long-term ectopic transplants a small population of cells still retained the potential for proliferation and has not yet reached the stage of terminal differentiation.

Key words: *rats, retina, fetal tissue transplantation, in vitro*

Introduction

During the past decade attempts have been done to restore visual function in humans suffering from different retinal diseases by transplantation of the fetal retina or retinal cells. These attempts were largely unsuccessful although in some cases a slight improvement was reported[1,2]. It seems that main problem is the lack of proper functional integration of transplanted cells within the diseased retina[3].

According to some animal studies, the solution to this problem can be found in usage of different kinds of multipotent or pluripotent undifferentiated cells. The existence of stem cells in adult mammalian retina was discovered[4] similarly as in the adult brain[5,6]. Stem cells from the CNS, embryonic stem cells (ES cells), retinal progenitor cells (RPCs) and even marrow stromal cells (MSCs) seem to possess plasticity necessary for differentiation of photoreceptors and integration to the retina under the influence of a specific microenvironment[3,7]. However, the maintenance of »stemness« without tumorigenic potential most certainly requires a strict control in adult tissues because there is increasing evidence that a variety of human neoplasms may result from transformation of normal stem and progenitor cells[8]. In a recent study, subretinal transplantation of *in vitro* neurally selected mouse embryonic stem cells gave rise to teratomas which affected almost all layers of the eye[9]. To avoid such unwanted side-effects, further *in vitro* and *in vivo* animal research especially considering the developmental potential of various types of immature cells has to be done.

It was shown for retina that some extrinsic cues could change competence of tissue and developmental outcome[10]. Therefore in this study developmental potential for growth and differentiation of fetal rat retina was investigated outside of its normal microenvironment in an original organ-culture model *in vitro* and in long-term *in vivo* subcapsular kidney transplants[11,12].

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Material and Methods

Isolation of the neural retina

Fisher strain rats were bred at the Department of Biology, School of Medicine, Zagreb under conventional conditions with a standard diet. Animals were mated overnight and the finding of the vaginal plug designated the day 0 of pregnancy. Animals were killed in ether and 20-day-old fetuses were isolated. Eyeballs were enucleated with watchmaker's forceps and rinsed in Phosphate Buffered Saline (PBS). Under the dissecting microscope, a deep incision was done by Graeffe's knife on sclera near the optic nerve area. After spontaneous ablation of the neural retina, it was easy to isolate it and care was taken to avoid contamination with cells from other eye structures.

Cultivation in vitro

Retinas were gently rinsed in PBS and two of the explants were placed separately on a lens paper supported by a stainless steel grid in an organ tissue culture disposable dish (Falcon No. 3037). Medium was added to the dish well to wet the lens paper and retinas were grown at the gas-liquid interface. Eagle's Minimal Essential Medium with Hank's balanced salt solution (MEM) and fifty per cent rat serum was used as cultivation medium. The rat blood from aortas of adult male Fisher rats was recovered, immediately centrifuged and serum was inactivated at 56 °C for 30 min. Serum was sterilized through a 0.22 μm Milipore filter. Retinas were grown in an incubator for 12 days in 5% CO_2 and 95% air at 37 °C. The culture medium was usually changed every other day during the culture period.

Transplantation in vivo

Retinas were transplanted to an ectopic site in adult male Fisher rats. Rats were anaesthetized with ether and a paravertebral incision through skin and muscle was done to approach the kidney. An incision on the kidney capsule was done and a small pocket was formed under the capsule to place the explant. The skin and musculature wound was closed by 16 mm Michel's clumps. Transplants were grown *in vivo* for maximum of 180 days (50, 120 or 180 days).

Immunohistochemistry

For immunohistochemical analysis mild fixation during 24 hours with St Marie's solution (1% acetic acid in 96% ethanol, +4 °C) was used. Explants were dehydrated and embedded in paraffin at 56 °C. Serial sections (5 μm) were put on silanized slides (S3003; DAKO, Glostrup, Denmark) and air-dried for 24 hours at room temperature. Sections were deparaffinized in xylene (2 × 5 min), treated with absolute ethanol and 96% ethanol (2 × 3 min) and H_2O (30 seconds). Sections were placed in retrieval solution (S2031, DAKO) in a plastic covered jar, heated in a microwave oven for 5 minutes at 700W and cooled in the buffer for 1 minute at room temperature. This procedure was repeated three times. Finally, sections were cooled for 30 minutes and transferred to PBS for 5 minutes. Monoclonal mouse anti-Proliferating cell nuclear antigen (PCNA), Clone PC 10, (M0879, DAKO), was diluted to 1:100; monoclonal mouse anti-rat nestin, (Rat 401, Hybridoma Bank, Iowa) to 1:5; monoclonal mouse anti-Opsin (O4886, Sigma, St.Louis, MO) to 1:50; while polyclonal rabbit anti-cow Glial fibrillary acidic protein (GFAP; N1506, DAKO), polyclonal rabbit anti-bovine S-100a (N1519, DAKO), polyclonal rabbit anti-human Chromogranin A (N1535, DAKO) and polyclonal rabbit anti-human Synaptophysin (N1566, DAKO) were ready to use. DakoCytomation En Vision Doublestain Kit (K1395, DAKO) was divided and for detection of GFAP APAAP method (Alkaline Phosphatase, Fast Red) was used while for detection of synaptophysin, chromogranin A and S-100 PAP method (horseradish peroxidase, diaminobenzidine-DAB) was used. Negative controls were treated with an unspecific antibody (Rabbit Ig – 1506 or 1566 or 1535 and 1617 mouse IgG_1, DAKO). Labeled streptavidin-biotin kit (DAKO LSAB® 2 Kit, HRP) for usage on rat tissue was used for detection of PCNA according to manufacturer's instructions. Sections were briefly counterstained with hematoxylin, washed first with distilled water, then for 20 min in tap water and again for 3 min in distilled water, and covered with 50% glycerol in PBS. For antibodies listed below sections were deparaffinized and heated for antigen retrieval as described above. They were washed three times in phosphate-buffered saline (0,1M PBS; pH 7.4), blocked in a solution of 3% bovine serum albumin (BSA) with 2% normal horse or normal rabbit serum (depending upon the secondary antibody used) and 0.05% Triton X-100 (v/v; Sigma) in PBS for 2 hours. After blocking, sections were incubated in the primary antibodies: goat polyclonal anti-SemaIIIA (Santa Cruz Biotechnology, Santa Cruz, USA) (1:100); Chondroitin sulfate proteoglycan (CS-56), mouse monoclonal IgM (SIGMA) (1:200); anti-human Fibronectin (SIGMA) (1:1000); SMI312 monoclonal mouse anti-Neurofilament H Cocktail, Mouse IgG_1 and IgM (1:500) as well as synaptosomal associated protein SNAP-25 (25 kDA) (SMI81) mouse monoclonal IgG1 (1:1000) which were from Sternberger Monoclonals Inc. Lutherville, MD, USA, in a humid chamber overnight at 4 °C. On the following day, for immunoperoxidase staining sections were washed three times in PBS and then, depending upon the used primary antibody, sections were incubated in biotinylated: rabbit anti-goat or or horse anti-mouse antibody (dilution 1:200) for 1 hour. After further three washes in PBS, sections were incubated in avidin/biotin solution (1:500, Vectastain ABC kit, Vector, Burlingame, CA) for 1 hour, followed by three times washing. Sections were then immersed in the nickel-DAB chromogen solution (2.5% nickel sulphate and 0.02% DAB in 0.175 M sodium acetate activated with 0.01% (v/v) H_2O_2) until a dark black precipitates were formed. Sections were washed in PBS, dehydrated through increasing concentrations of ethanol, immersed in two changes of 100% xylene and coverslipped in DePeX mounting medium (Sigma Chemicals, St.Louis, MO).

Transmission electron microscopy

For analysis by transmission electron microscopy (TEM), explants were immediately transferred from culture to 4% glutaraldehyde fixative where they were kept for two hours and then postfixed in 1% OsO_4 during two hours. They were washed three times for 10 min in 0,1M phosphate buffer, dehydrated in ascending concentrations of ethanol and finally transferred to ethanol and acetone (1:1) for 30 min and 100% acetone for 30 minutes. They were kept in Durcopan (Balzers, Lichtenstein) diluted in acetone (1:1) for two hours, embedded in Durcopan and kept at 56 °C for 3 days. Serial semi-thin sections (0.9 μm) were cut and stained with 1% toluidine blue. Light microscopy analysis on semi-thin sections was used to localize retinal tissue in transplants. Ultrathin sections (70 nm) of areas containing retinal tissue were made, mounted on copper grids and contrasted with lead citrate and uranyl acetate. A Zeiss 902A transmission microscope was used for ultrastructural analysis (Centre for Electron Microscopy, School of Medicine University of Zagreb).

Results

Fetal rat retina survived in *in vitro* explants although the normal tissue architecture was lost and rosettes were formed (not shown). Various types of neural and glial cells were recognized in explants by electron microscopy and even a photoreceptor in the process of shedding its outer segment was found (Figure 1).

20-days-old fetal neuroretina transplants survived well and differentiated under the rat kidney capsule for the period of six months (Figure 2). It formed rosettes and abundant neuropil (Figure 3a). By using a panel of appropriate antibodies, neuronal and glial differentiation was assessed in transplants. Neurofilaments in axons were detected through expression of SMI312 (Figure 3b). The expression of glial markers Glial Fibrilary Acidic Protein (Figure 3c) and S-100 (not shown) was also abundant in all transplants. Expression of synaptophysin (Figure 3d) and of synaptosomal associated protein 25kD – SMI 81 (Figure 3e) as well as of chromogranin A was also detected (Figure 3f) but immunoreactivity to opsin was not found.

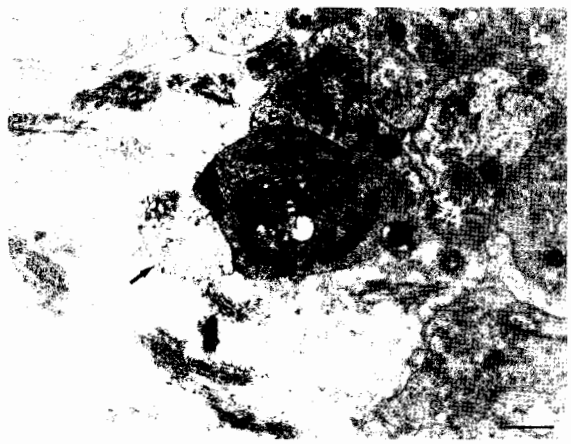

Fig. 1. Differentiation of the fetal neural rat retina in vitro. A degenerating outer segment of a photoreceptor in an explant cultivated with rat serum (arrow). Transmission Electron Microscopy. Scale bar = 2 μm.

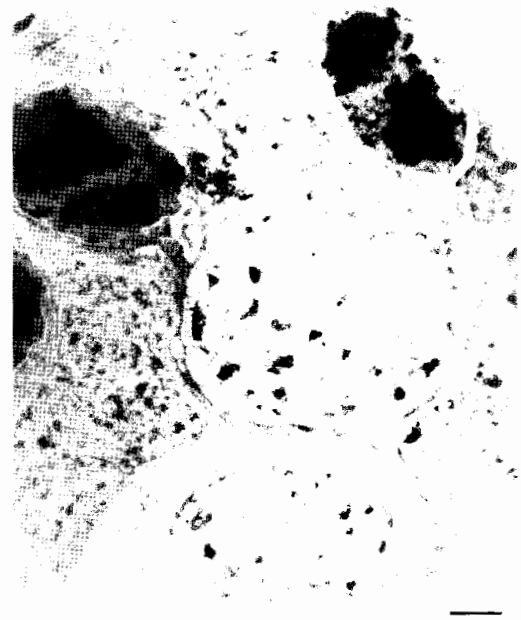

Fig. 2. Differentiatio of the fetal neural rat retina in transplants under the kidney capsule. Note the variety of neural elements characteristic for plexiform layers. Transmission Electron Microscopy. Scale bar = 2 μm.

Axon guidance molecule semaphorin IIIA (Sema III A) was scattered throughout the 50-days-old transplanted retinal tissue in a punctiforme manner (Figure 4b). Sometimes it was found to characteristically decorate cells. It was not expressed in transplants that spent 120 days under the kidney capsule. Chondroitin sulfate proteoglycan (CS-56) was expressed in 50-days-old transplants (Figure 4c). Fibronectin was absent from retinal transplants but was found in the kidney capsule (Figure 4d). Immunoreactivity for proliferation marker (Proliferating Cell Nuclear Antigen – PCNA) was found in all transplants (Figure 4e).

In six-months-old transplants we have also found low expression of nestin (Figure 4f).

Discussion

In concordance with our previous results[12], fetal neural retina survived *in vitro* in our original organ-culture system for 12 days. In this experiment a photoreceptor in the process of shedding its outer segment was found, meaning that in our organ culture system photoreceptors not only differentiated further to the second stage of differentiation but were also able to execute the whole differentiation program. The outer segment of the photo-

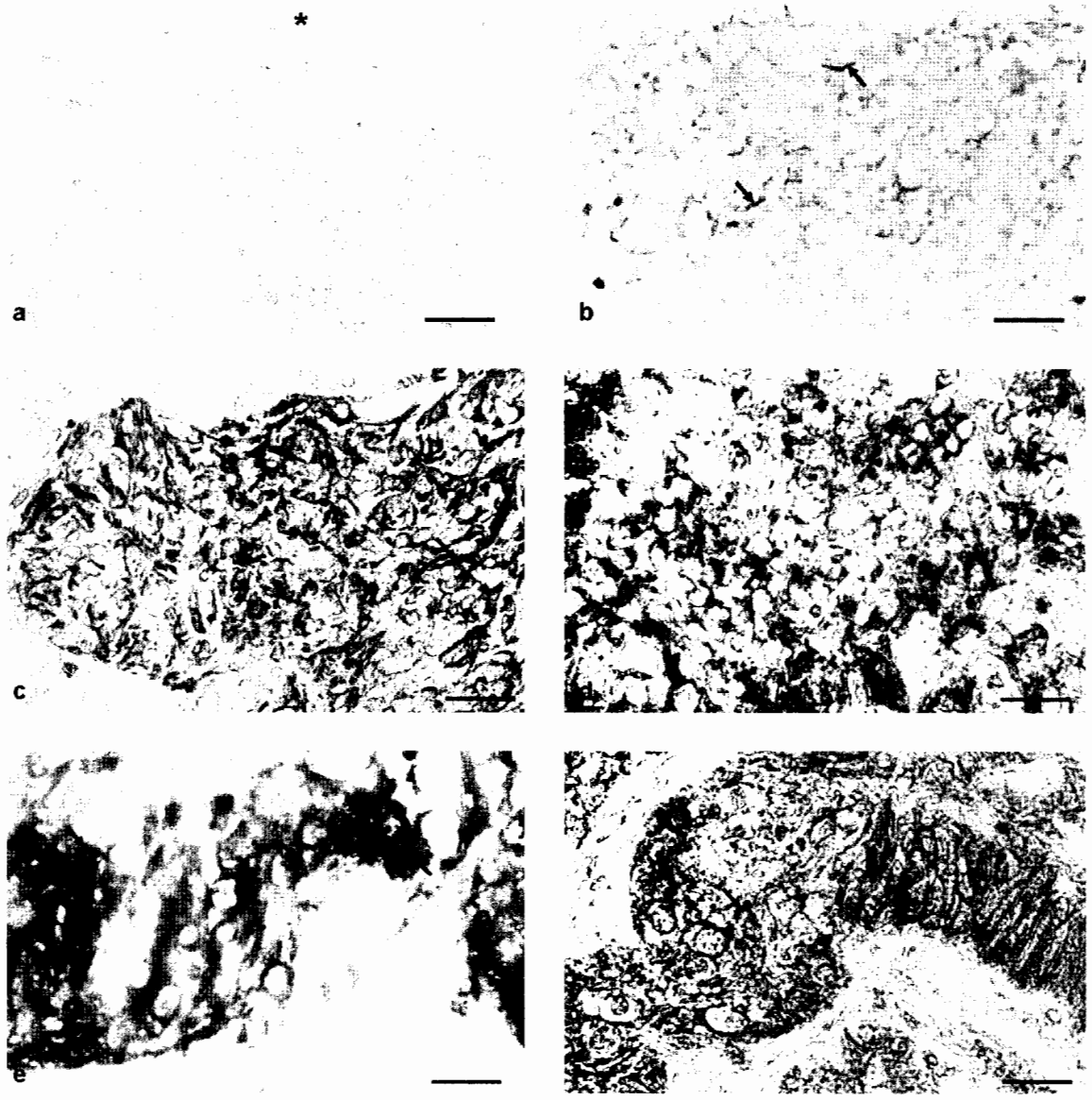

Fig. 3. Differentiation of the fetal neural rat retina in transplants under the kidney capsule. a) Negative control. Note typical rosettes within the transplants and the kidney capsule (asterix). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 100 μm; b) Anti-Neurofilament H (SMI312) expression in a 180-days-old transplant (arrows). Diaminobenzidine, metal enhancer. Scale bar = 100 μm; c) Glial Fibrilary Acidic Protein (GFAP) expression in a 50-days-old transplant (arrows). Fast Red, counterstained by hematoxylin. Scale bar = 100 μm; d) Synaptophysin expression in a 180-days-old transplant (arrow). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 50 μm. e) Synaptosomal associated protein SNAP-25 (SMI81) expression in a 180-days-old transplant (arrow). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 50 μm; f) Chromogranin A expression in a 180-days-old transplant (arrow). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 50 μm.

receptor consists of flattened discs which are constantly shed from their apical portion, while at the same time new vesicles and flattened discs are forming at the basal cell region and then gradually migrate to the apex to be eventually aborted. The whole process of migration, from assembly at the basal cell region to apical shedding takes from 9 to 13 days in humans[13]. In the rat, neural retina is fully mature at around 12 days after the birth[14,15] which period was approximately spanned by our *in vitro* system.

In the second experiment fetal retina exerted capacity for a long-term six-months survival[11] under the kidney capsule. In contrast to this, adult rat retina did not survive at all in ectopic subcapsular kidney transplants[16]. Fetal retina, because of its immaturity, should be more adaptable to the ectopic environment than mature retina which seems to need a specific microenvironment for its maintenance[17,18]. However, isolated human embryonic neuroretina could not survive upon transplantation to the mesenterium of the nude mice[19] which points to the

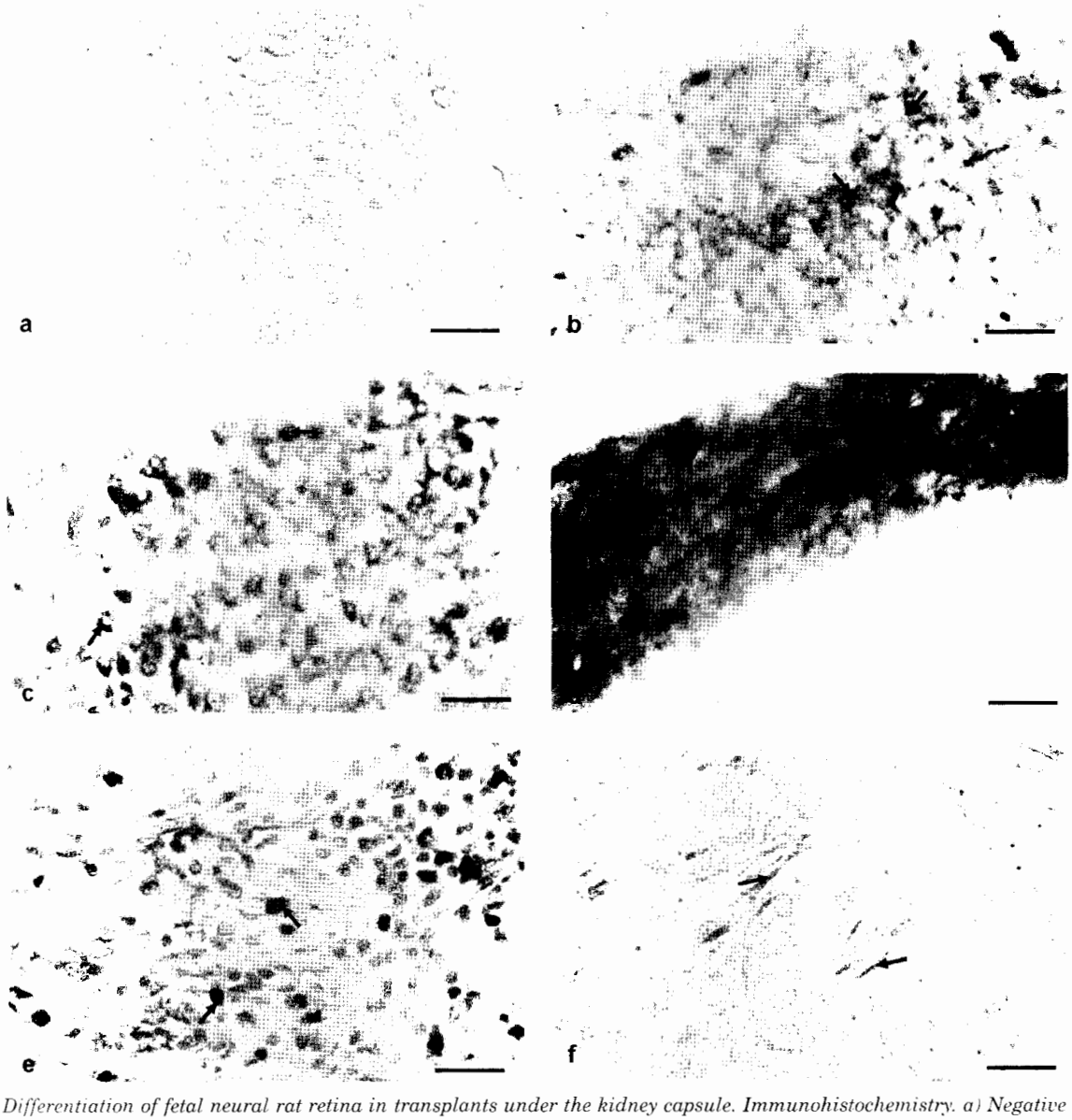

Fig. 4. Differentiation of fetal neural rat retina in transplants under the kidney capsule. Immunohistochemistry. a) Negative control. Diaminobenzidine counterstained by hematoxylin. Scale bar = 50 μm; b) Semaphorin IIIA expression in 50-days-old transplant (arrow). Diaminobenzidine, metal enhancer. Scale bar = 100 μm; c) Chondroitin sulfate proteoglycan expression in a 50-days-old transplant (arrow). Diaminobenzidine, metal enhancer. Scale bar = 100 μm; d) Fibronectin expression in the kidney capsule (arrow). Diaminobenzidine, metal enhancer. Scale bar = 50 μm; e) PCNA expression in a 120-days-old transplant (arrow). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 100 μm; f) Nestin expression in a 180-days-old transplant (arrow). Diaminobenzidine, counterstained by hematoxylin. Scale bar = 50 μm.

superiority of the subcapsular kidney space for ectopic transplantation experiments that has been also noted in experiments with postimplantation rat embryos[20,21,22].

Fetal retina in transplants formed rosettes which showed that the normal tissue architecture was lost[12]. In spite of this disorganization, neural and glial cells with abundant neuropil could be discerned. By using a panel of appropriate antibodies[23,24,25], neuronal differentiation together with the presence of synapses and glial differentiation was assessed in transplants. Chromogranin A, which in human fetal development was found to be typical for more mature retina and not for the early retinal anlage[26], was also detected showing that the maturation of the retina was proceeding well at this ectopic site.

However, we could not recognize typical photoreceptors. It is possible that photoreceptors could not fully differentiate and/or survive in our transplants without retinal pigmented epithelium (RPE) which was found to be necessary for *in vivo* morphogenesis of the mouse retina[27]. Photoreceptor differentiation was preserved in long-term transplants of human retina in nude rats also only when they were combined with RPE[24]. The second

reason for the necessity of RPE could be the function of its cells to phagocytize the shed photoreceptor outer segment tips. In Royal College of Surgeons mutant rats, inability of RPE for such a function leads to photoreceptor degeneration[28]. Because our 12-day *in vitro* system allowed differentiation of receptors even in the absence of RPE it is possible that the main problem in our long-term transplants was connected to the compromised survival of photoreceptors.

Semaphorin IIIA (Sema III A), an axon guidance molecule[29,30], was characteristically expressed in 50-days-old transplanted retinal tissue. It was not expressed in transplants that spent 120 days under the kidney capsule and therefore Sema IIIA exerted differential expression during development. This is consistent with the finding that during normal embryogenesis its expression is found in the retina but after birth becomes progressively restricted to specific sets of CNS neurons[29].

Chondroitin sulfate proteoglycan (CS-56) known to be a regulator of neuronal patterning in the retina[31] was expressed in 50-days-old transplants. Fibronectin, which is known to be involved in *in vitro* migration of neural crest cells[32] and retinal ganglion cells[33] was absent from our retinal transplants but was found in the kidney capsule. That at least indicated that the transplant contained only neural retina without contamination of other eye tissues containing fibronectin in their basement membranes[34].

Shown by the expression of the Proliferating Cell Nuclear Antigen (PCNA), even in our 6-months-old transplants some cells were retained in the cycling compartment meaning that those cells have not yet been terminally differentiated[35]. In a recent experiment, rat bone-marrow cells transplanted into subretinal space differentiated to retinal cells which did not express PCNA[7]. It was shown that PCNA expression is absent from the human adult differentiated retina but is present in the retinal tumor retinoblastoma[36].

In six-months-old transplants we have also found low expression of nestin known to be present in variety of progenitor cells[37] although it seems that its expression, at least in the brain, is not indicative of mitotic activity[38].

Expression of nestin and of proliferation markers was induced after laser injury in Müller cells of the adult rat retina[39]. In our experiments normal tissue architecture was not preserved and it is possible that expression of proliferation and progenitor cell markers for a long period of 6 months was due to the loss of the proper microenvironment necessary for terminal differentiation[3,17]. Moreover, we were dealing with ectopic transplants. Although transplanted to the subretinal space, mouse neural cells derived *in vitro* from pluripotent ES cells gave rise to teratoma containing various tissues such as bone, muscle etc. which invaded the whole eye. This means that their wide developmental potential could not be entirely directed to the wanted developmental outcome in spite of the more specific environment. As pointed out by authors, this effect was not discovered at first but only after a relatively long period of two months[9]. In subcapsular kidney transplants normal gastrulating embryos can produce even more malignant teratocarcinoma and yolk sac carcinoma[40].

By this investigation we have shown first that our simple *in vitro* organ Culture system seems to be favorable for full maturation of photoreceptors even in the absence of RPE and normal tissue architecture. Secondly, although ectopic retinal transplants did not behave as malignant tumors, we found that they were still harboring a small number of proliferating and immature cells. Expression of nestin and a proliferation marker in the functional engraftment of human ES cell-derived dopaminergic neurons to the parkinsonian rats was discussed as basis for »phenotypic instability and undifferentiated expansion«[41].

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RAZVOJ FETALNE NEURALNE MREŽNICE *IN VITRO* I U EKTOPIČNIM TRANSPLANTATIMA *IN VIVO*

SAŽETAK

Istraživanje razvojnog potencijala nezrelih tkiva važno je za nove pristupe humanoj regenerativnoj medicini, pa smo u ovom radu istraživali razvoj fetalne neuralne mrežnice u dva eksperimentalna sustava. Retine su mikrokirurški izolirane iz 20-dnevnih štakorskih zametaka te kultivirane *in vitro* tijekom 12 dana ili su transplantirane pod bubrežnu čahuru odraslih mužjaka gdje su provele do 6 mjeseci. U kulturi smo transmisijskom elektronskom mikroskopijom (TEM) pronašli odbacivanje vanjskog segmenta fotoreceptora što predstavlja proces koji se javlja u završnoj fazi diferencijacije. U transplantatima nismo pronašli fotoreceptore iako su bili prisutni biljezi završne neuralne i glijalne diferencijacije (npr. sinaptofizin, kromogranin, glijalni fibrilarni kiseli protein-GFAP) zajedno s molekulama uključenim u proces diferencijacije (vodič neurona semaforin IIIA i hondroitin sulfat proteoglikan). Semaforin je iskazao diferencijalnu ekspresiju budući se nije eksprimirao u najstarijim transplantatima. Jezgrin antigen proliferirajućih stanica (PCNA) i nestin (biljeg nediferenciranih živčanih stanica) bili su eksprimirani čak i u šestomjesečnim transplantatima. Može se zaključiti da se u oba naša eksperimentalna sustava fetalna neuralna mrežnica nastavila diferencirati. Ipak, čak i u najstarijim ektopičnim transplantatima mala populacija stanica zadržala je potencijal za proliferaciju i nije dostigla stadij terminalne diferencijacije.