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Research Article

Expression of FGF2 and Nucleostemin in Models of Retinal Regeneration in the Newt Under Conditions of 3D Organotypic Culture *In vitro*

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Abstract

The models of retinal regeneration in the adult newt *Pleurodeles waltl* (Urodela) were adapted for studies of the effects of regulatory factors in 3D organotypic culture *in vitro*. We found that these conditions of *in vitro* culturing of retinal pigmented epithelium and neural retina whole amount are appropriate and give an opportunity to reproduce the processes taking place *in vivo*. We studied the newt retinal cell behavior and regenerative responses to exogenous fibroblast growth factor 2. We found that Fgf2 added to cultivation medium could increse proliferation of retinal pigment epithelial - derived dedifferentiating cells and subsequent formation of the first cell row of retinal rudiment. Cultivated newt neural retina isolated from pigment epithelium demonstrated good viability and potential to self-regeneration by its own low differentiated cell precursors at the retinal periphery. In samples of neural retina we revealed the up-regulation of *Fgf2* and nucleostemin *(Ns)* genes. Meanwhile in RPE with underlying choroid and scleral coat *Fgf2* gene expression was at low, hardly detectable level but expression of *Ns* gene markedly increased. Results suggest the role of endogenous (neural retina) and exogenous (retinal pigmented epithelium) Fgf2 in the processes of retinal regeneration in two different models of newt eye tissue regeneration. *Ns* gene expression increased in parallel to *Fgf2* is probably included in Fgf2 - mediated regulatory network.

Keywords: *Newt; Retinal Pigment Epithelium; Neural Retina; FGF2; Nucleostemin; Culture In Vitro*

Abbreviations

RPEs: Retinal Pigment Epithelium Cells; 3D: Three-Dimensional; Fgf: Fibroblast Growth Factor; ESCs : Embryonic Stem Cells; H-E: Hematoxylin Eosine; Brdu: Bromodeoxyuridine; PCR:Polymerase Chain Reaction

Introduction

The newt eye tissues are widely used in studies of different aspects of eye tissue regeneration and remodeling. The differentiation of all types of neurons and glial cells during retinal regeneration in the adult newt *in vivo* is based on the retinal pigment epithelium cells (RPECs) reprogramming [1-3]. As a result of RPECs phenotype conversion the population of actively proliferating multipotent neuroblasts – cell sources for retina regeneration are formed. Fgf2 (fibroblast growth factor2) - mediated signaling pathway is proposed as the key one in the network controlling the multistage processes of retinal development and regeneration [4-7]. Some works are devoted also to the study of the role of nucleolar proteins that take a part in the epigenetic control of gene expression in different cellular processes. One from the most interesting nucleolar proteins is nucleostemin (Ns, GNL3/Gnl3) that is identified in the pluripotent embryonic stem cells (ESCs), in undifferentiated precursors of the neural cells, and some lines of cancer cells [8-10]. The role of Gnl3 in the maintenance of cell viability, in controlling of cell cycle progression in stem cells and cancer cells is shown [9-11]. The important role of Gnl3 was also determined for various cellular processes in mice embryogenesis [12]. It was suggested that GTP-binding protein Gnl3 is a participant of mitogenic signal transmission in the cells [9]. So, it is reasonable to make attempt to clarify the role of *Fgf2* and *Gnl3* genes in retina regeneration on the

models *in vitro* and *in vivo*. For that we have recently identified *Fgf2* and nucleostemin *NS* (Gnl3) genes in the genome of the adult newt *P. waltl* and have given structural characteristics of these genes nucleotide sequences [13,14].

RPE conversion to the retina in the newt is a multistage process that includes RPE destabilization, dedifferentiation, a formation of retinal neuroblasts *de novo*, their active proliferation and subsequent differentiation of RPE descendant cells to the retinal neurons and glial cells [1,15,16]. Neural retina itself has an additional also well established cell source for restoration/regeneration at the periphery presented by low differentiated slowly amplifying cells. The share of their participation in retinal restoration depends on the type of retinal trauma (type of surgery) used in the experiment [17].

The aim of this work is to study the effect of exogenous Fgf2 upon the adult newt RPECs behavior in 3D organotypic culture *in vitro* and investigate the expression pattern of *Fgf2* and *Ns* genes in the neural retina and RPECs at the time window of cell regenerative responses in these eye tissues in 3D organotypic culture.

Materials and Methods

Animal Model

The adult newts *Pleurodeles waltl* (6 months) were used in experiments. Animals were bred in the aquarium of the Koltzov Institute of Developmental Biology, Russian Academy of Sciences (RAS). All experiments were performed in accordance with protocols approved by the Commissions on Bioethics RAS and the Koltzov Institute of Developmental Biology RAS. After anesthesia by trikain methanesulfonate MS 222 (Sigma, USA) dissolved in 0.65% NaCl physiological solution (1:1000), neural retina and RPE with underlying choroidal and scleral coats were isolated from newt eyes by microsurgery methods described in [18,19].

3D Organotypic Culture *In vitro*

In experiments we used a method of long-term rotary 3D organotypic culture developed earlier [18,19]. The 3D organotypic cultivation of eye tissues was conducted in the miniroller RM-1 (Elmi, Latvia). Isolated neural retina and RPE with underlying tissues of the eye back wall were cultivated under conditions of permanent rotation of the flasks (20 ml, Wheadon). The duration of eye tissue culturing was 14 and 21 days, rotation speed was 40 rev/min at 20°C. Culture medium was changed to fresh one once a week. The medium consisted of medium 199 (70%), bidistilled water (30%), 1M HEPES buffer (30 ml/100 ml of medium), gentamicin sulfate (200 ul /100 ml of medium) and 10% fetal bovine serum (Hyclon, USA). Routine histology and immunochemistry were applied for the analysis of eye tissue morphology, cell behavior and proliferation just after culturing (14 and 21 days). Commercial FGF2 (Sigma, USA) ($1x10^{-8}$ g / ml) and DNA precursor (BrdU) (Zymed, USA) (0.5 ml of solution per 5 ml of the medium) were added at the time of medium change. The eye tissues samples cultivated in the absence of exogenous FGF2 in the cultivation medium served as the control of Fgf2 effect. Experiments with Fgf2 and BrdU addition were performed for RPE samples only.

Preparation of Histological-and Cryosections. Analysis of Cell Survival and Proliferation. Ocular tissue samples were obtained on the 14^{th} and 21^{st} days of culturing. Just after the miniroller was stopped they were fixed. Changes of tissue and cell morphology were analyzed on thin and semi-thin cross-sections. The sections were processed and stained with H-E and toluidine blue, as described earlier [18].

The proliferative activity of RPECs and RPECs - derived retinal cell precurcors were studied after sustained delivery of BrdU. After cultivation these samples were fixed in 4% PFA prepared on 0.1 M PBS (pH 7.4), for 4 hours at room temperature. Then they were washed (3 times in 0.1 M PBS), incubated in 5% and 20% sucrose, and frozen at 70°C in Tissue Tec OCT (Leica, Germany). The serial sections (7μ) were prepared using M1900 cryostat (Leica, Germany). Cell survival was evaluated by identification of pyknotic nuclei on H-E stained sections and by TUNEL assay detecting apoptotic cells. For TUNEL assay (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling) cryosections of cultured eye tissues were processed in accordance with TUNEL manufacturer's protocol (Promega Corporation, USA). Immunochemistry with antibody against BrdU was performed as described earlier [19]. Positive (labeling of small intestinal epithelium cells) and negative (only secondary antibodies) controls were used in BrdU labeling immunohistochemical detection. After staining by DNA marker Hoechst 33342 (Leica, Germany), the sections were embedded in Vectashield (Vector Laboratories, USA). Images were analysed with microscope Leica DM RXA2. Digital images were processed using PC equipped with Lite software package.

PCR analysis

The mRNA of genes in the eye tissues was detected by a semi-quantitative polymerase chain reaction (PCR). RNA was isolated using the TRI reagent (MRC, United States), treated with the DNase Turbo enzyme (Ambion, USA) to eliminate the contamination with the genomic DNA. The first strand of cDNA was synthesized using the Omniscript RT Kit (Quiagen, Germany). cDNA from the tissues (isolated neural retina and RPE (with underlying choroid and sclera) were normalized for the *Gapdh* gene of the *P. waltl.*

The specific primers were constructed using Beacon Designer 7.2 software with optimization for self-dimerization, cross-dimerization and hairpin ΔG close to 0, GC% between 40 and 60, amplicon length below 200 bp and similar T_m for different primer pairs. Primers were tested for specificity using NCBI Primer-Blast search in the nucleotide collection (nr/nt) database for amphibia; none elicited any products on unintended targets. PCR performed with the cDNA templates derived from the eye tissues using the amplification kit (Silex, Russia) in a Mastercycler Personal instrument (Eppendorf, Germany). qPCR was performed on Applied Biosystems Step One Plus system. The total reaction volume was 25 µl and contained 5 µl 5× HS Sybr+High ROX 5x PCR mix (Evrogen), 0.2 µl cDNA from RT reaction (substituted by water in negative controls), 1 µl of each forward and reverse primer working solutions and molecular-grade water for PCR applications. After initial optimization, we chose to use 3-step PCR program consisting of an initial denaturation stage at 95°C for 1 min, followed by 38 cycles (95°C for 10 s, T_a for 20 s, elongation at 72°C for 30 s, detection during the elongation step) and a melt curve (temperature increasing from 60°C to 95°C with 0.5°C increments) to ensure amplification of only one product with expected T_m. Each reaction was replicated 4 times.

The structures of the primers for qPCR were: *gapdh* 5'-CGGAATCAACGGATTTGG-3' (forward) and 5'-TACTGAGATGGGTACCTGCG-3' (reverse), the size of the PCR fragment was 148 bp.; *Fgf2* 5'-GACCCAAGAGGCTGTACTGCA-3' (forward) and 5'-GTTGTTGGACTCCAGTCGCTC-3' (reverse), 252 bp.; *Ns*: 5'-GTCAGCCTCACTAGTATCAAAG-3' (forward) and 5'-CAGTCGGAGTGATCATAGTTTC-3' (reverse), 369 bp.; *rpe65* 5'-CTGACGGGGGAATACCTCAAC-3' (forward) and 5'-CTGTGGAAACTCAAAGGCTAGGC-3' (reverse), 400 bp. The Gene Ruler (Fermentas, Lithuania) was used as markers of DNA fragment lengths. Statistical analysis was performed after exporting the data in Microsoft Excel. ΔΔCt method was used to calculate relative quantities (RQs) for each gene of interest in each sample within a run, normalized by reference gene. Relative quantities from all runs were then transported to STATISTICA to calculate mean RQs and standard deviation for each gene of interest in each sample and to determine the statistical significance of the differences between observed values and control value (1.0) by single sample *t*-test.

Results and Discussion

Retinal Pigmented Epithelium *in vitro*

In recent years the number of works that use organotypic culturing of isolated eye tissues of the adult lower and higher vertebrates for solving different issues has increased (see review [20]). This approach allows get and observe populations of proliferating and dedifferentiated cells, monitor the processes of retinal regeneration and reconstruction [18 -21]. The advantage of the approach is the similarity of cell processes in eye tissue models to those *in vivo* ("*in vitro* – like – *in vivo*"), a possibility to study of cell responses to the influence of different exogenous factors added to the culture medium in controlled concentrations.

In our study we focused on RPE - the main cell source for retina regeneration in adult newts [1,17]. In the way of culturing the newt posterior eye sector (RPE+choroid+scleral coat) does not change its shape and RPE layer keeps initial topology. In samples (all together) RPECs death was somewhat different after 14 days *in vitro* but did not exceed 15% as it was determined by approximate count of cell pyknosis. TUNEL assay demonstrated comparable results – from 14-17% to 20 - 25% in the way of long culturing (14 and 21 days). The majority of RPECs maintained phenotype closed to that of the native one, but some others left the layer, came out and pretended to form the second row – the row of partially depigmented cells. In addition we could observe another type of RPECs behavior – minor part of RPECs went beyond the layer and demonstrated phenotype close to melanophage one described earlier by Keefe [17] under conditions *in vivo*. The formation of the layer of partially depigmented RPE - derived cells, as well as the acquisition of melanophage morphology was also revealed in the cases of retinal regeneration *in vivo* in other newt species [17, 22]. This means that the behavior of RPE cells in 3D *in vitro* system is similar to that at the early steps of retinal regeneration after its surgical removal *in vivo*. Cases of RPECs transformation into macrophage-like cells were also mentioned for rabbit RPECs *in vivo* [23].

On 14 and 21 days *in vitro* BrdU labeling showed rare individual RPECs synthesizing DNA, the figures of mitoses in those samples were not observed. This confirms previously obtained findings reflecting the peculiarities in the behavior of newt RPE *in vitro*, where cells have much longer S-phase period than that *in vivo* and, as a result, demonstrated reduced proliferative activity [24]. However, we stress, that the newt RPECs are able to enter S-phase and synthesize DNA. Probably such peculiarity of newt RPE cell cycle *in vitro* is associated with the deficiency of cell division stimulating factors under *in vitro* conditions. Comparing our results with the data of literature [1,7] it can be assumed that in the newt retina regeneration *in vivo* the additional signals outgoing from surrounding tissues of the eye allow RPECs not only dedifferentiate but also produce a number of neural progenitor neuroblast cells and, as a result of their high proliferative activity, to form developing retinal anlage. Studies on the other models both *in vivo* and *in vitro* suggest RPE cell type conversion (transdifferentiation) and proliferation can be stimulated by exogenously administered Fgf2 [4-7].

In our study it is appeared that exogenous Fgf2 can change the phenotype of RPE cells and also modify the behavior of RPECs in the system of 3D-organotypic culturing (Figure1a,c). As we determined on the 14th day of culturing Fgf2 helps to maintain proliferative activity and viability of DNA synthesizing cells of RPE. At that time RPECs dedifferentiation was often observed in the epithelial layer in the presence and absence (control) of Fgf2 in the medium. In both cases it manifested in a partial loss of pigment granules but RPECs morphology was somewhat different: it was more or less regular one when FGF2 was added (experiment) and melanophage-like in the control. In the case of Fgf2 addition BrdU staining showed number of cells in S-phase, more often at the periphery of the layer (Figure1b, d). Later on the 21st day *in vitro* the processes of RPECs reprogramming and proliferation influenced by the presence of FGF2 in culture medium cover the majority of RPE cell population and could be observed in the peripheral and central part of RPE layer. This finding correlates with the data on the delayed effect of Fgf2 as a result of *Fgf2* genes down regulation at the beginning of RPE dependent retinal regeneration in the newt *in vivo* [13]. At 21st day *in vitro* the process of RPECs transformation to melanophores was slowed down: only a few melanophage-like cells were detected. In sum our findings are in an agreement with data of other previously reported studies gotten on other models of RPECs conversion in relation to FGF2 [25,26].

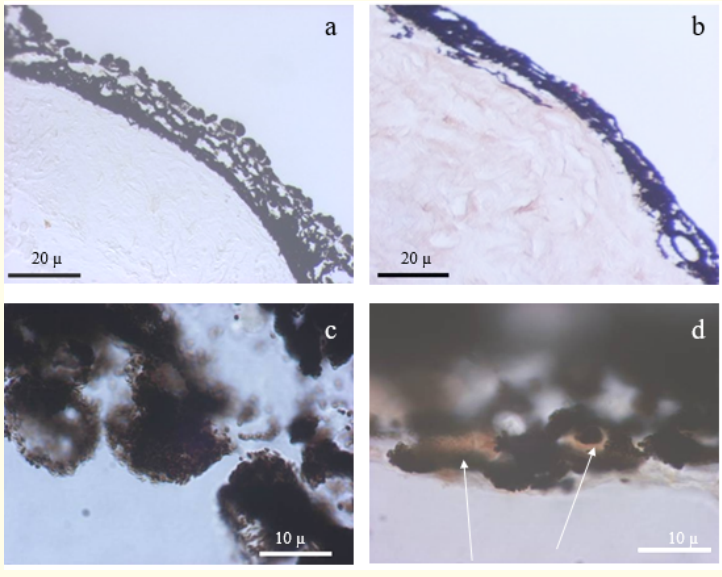


Figure 1: *Retinal pigmented epithelium of the newt on 14 day of organotypic culturing. a, c – in culture medium deprived of Fgf2; b, d – in the presence of Fgf2 in culture medium. Arrows show BrdU positive cells.*

Using semi-quantitative PCR we observed very low level of *Ns* gene expression in the native RPE, however at the 14th day of culturing it was detectable well enough (Table). This finding can be attributed to the process of RPECs dedifferentiation in which *Ns* could be a cofactor of cell type conversion [27]. Nevertheless *Ns* gene expression in RPE (native and *in vitro*) was less pronounced than that in neural

retina *in vivo* and *in vitro* (Table). In parallel the differentiation of RPE and neural retina was confirmed by an analysis of gene expression specific for retinal pigment epithelium (RPE65) and for neural retina (Tubulin b2, Rhodopsin) (Table).

Using PCR we did not reveal *Fgf2* expression in RPE tissue samples (Table). Possibly it is due to this method sensitivity when working on pigmented tissues. On the other hand, this result may reflect RPECs behavior – low level of proliferative activity in newt RPE native [28] and cultivated on 14 day *in vitro*. Real-time PCR and immunohistochemistry we carried out earlier showed that signal transmission through Fgf2 is suppressed *in vivo* before initiation of RPE cell proliferation soon (7-14 days) but not immediately after retina removal in the newt *P. waltl* [13]. Using microarray technology other researchers noted a decrease of Fgf2 ligand and Fgf2 receptors' expression in different regenerating systems of the newt at the early stages of regeneration [29]. Thus, in accordance with literature and our recent data Fgf2 is not a primary factor initially triggering a process of cell type reprogramming. Obviously the mitogenic effect of the Fgf2 is manifested later when RPECs have already entered cell cycle and cell proliferation initiated.

Despite of this, qPCR results analysis showed the insignificant up-regulation of *Fgf2* gene expression as well as *Ns* gene in RPECs on 14 day of culturing when exogenous Fgf2 was added to the medium when comparing with the control (absence of the factor in the medium) (Figure 2). One can propose that exogenously added FGF2 can still activate transcription of corresponding genes but this influence is not strong. Down regulation of rpe65 genes specific for RPE tissue correlates most likely with dedifferentiation of RPECs on 14 day *in vitro*, the phenomenon we observed on morphological pictures of the tissue described above.

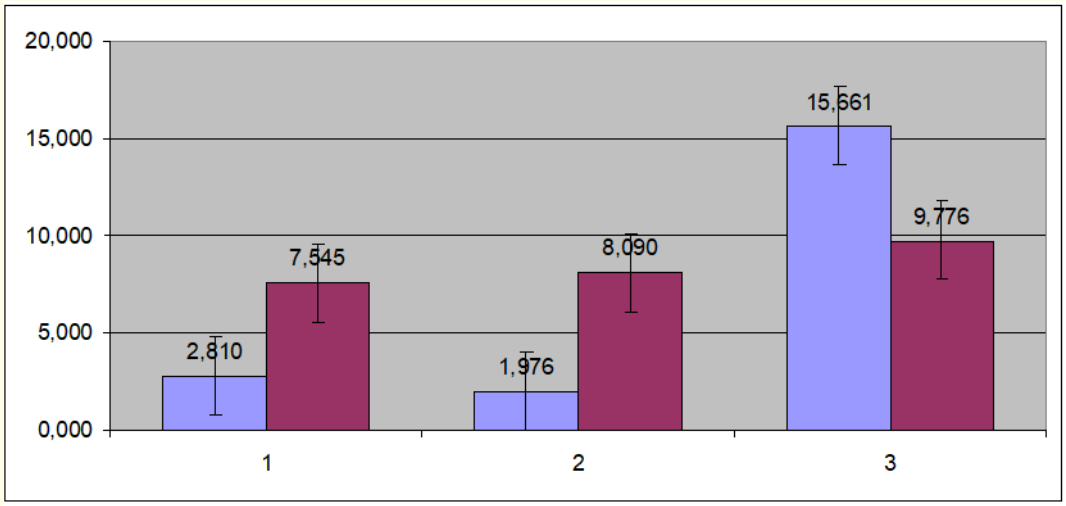


Figure 2: *QPCR on 14 day of RPE culturing in rotary 3D organotypic culture in vitro in the presence of Fgf2 in the medium (red colums) and absence of the factor (blue color). 1- endogenic fgf2; 2 – Ns; 3 - rpe65.*

In the case of *Ns* gene expression detected both by RCR and qPCR (Table, Figure 2) we obtained match results that can be interpreted also as correlated with RPE cell reprogramming taking place on 14 day *in vitro*.

Neural Retina *in vitro*

3D organotypic culturing *in vitro* of the neural retina gave us results comparable with those obtained in other our studies [13,21]. In roller culture, retinal explants formed closed "spheroids" (Figure 3a,b). On 14 day *in vitro* some cells in the outer and inner portions of retinal "spheroids" underwent a death. We observed pictures of pyknoses as well as TUNEL positive cells, however their number did not exceed 10-15%. At the area of spheroid closure (corresponding to the retinal periphery) cells in M phase were easily detected at the 14th and 21st days of culturing (Figure 3c,d). Pictures of mitoses were quite often (3-4 per tissue 7µ section). Cells at the retinal periphery in the

newt represent minor addional to RPE cell source for retinal regeneration [17]. Analysis of semi-thin sections revealed the hypertrophy of Müller glial cells, some of which were in M-phase also. These observations suggest the internal cell reserve for retina regeneration in the newt is involved in the process of retinal restoration under 3D organotypic culturing as well it is *in vivo*.

Isolated newt retinas cultured for 3 weeks demonstrated more frequent mitotic cells that could be found in each section of retinal samples. Many of cells had characteristic features of neuroblasts easily determined on semi-thin sections. Retinal layered organization was disturbed due to these, dedifferentiated cells' migration to the sites of local restoration of retinal structure.

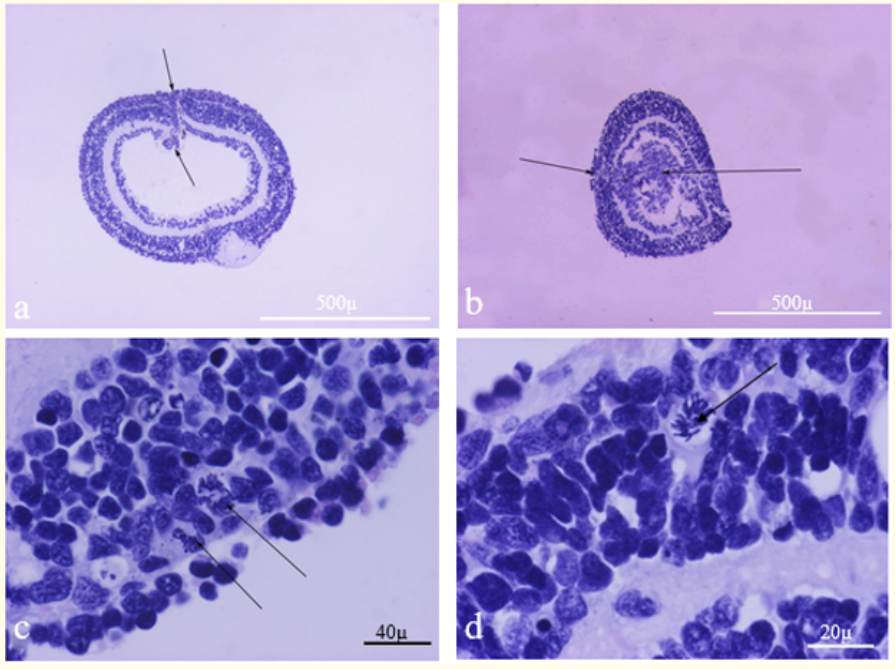


Figure 3: *Neural retina of the adult newt under conditions of organotypic culturing in vitro. a,b – general view of retinal spheroids on 14 (a) and 21 (b) days in vitro. Arrows show the area of retinal closing at the periphery and formation of cell mass as a result of cell divisions and migration to the cavity of spheroid.*

Usage of PCR analysis showed the up-regulation of the *Fgf2* and *Ns* gene expression in newt neural retina after 14 days of culturing *in vitro* (Table). Results obtained in morphological and PCR studies allow us to make some assumptions. First, as we have found earlier and now, newt neural retina cultivated whole mount under conditions of rotary culture accumulated low differentiated cells. This is in correlation with the increase of *Fgf2* and *Ns* gene expression level. Secondly, *Fgf2* gene up-regulation confirms that the neural retina is one of the tissue source of Fgf2 under conditions of eye experimental damage. Similar results were obtained also in cases of retinal detachment, optic nerve crosscut or partial removal – experimental surgery approaches resulted in RPECs reprogramming *in vivo* (7,17). Third, up-regulation of *Ns* gene may be down stream up-regulated due to *Fgf2* gene expression increase. In the study of Kafienah and colleagues [27] it was shown a dose-dependent increase of nucleostemin expression in proliferating human bone marrow stem cells under the influence of the Fgf2 signaling. Furthermore, the authors have shown that the usage of specific knockdown of nucleostemin by small interfering RNA (siRNA) abolished the proliferative effect of Fgf2 indicating the functional relationship between these genes.

Tubulin beta2 (Tub b2) is known as the cytoskeleton protein responsible for neurite outgrowth in neural tissue development and regeneration while Rhodopsin (Rho) is specific for retina and its gene regulation conserved in vertebrates [31]. The increase of *Tub b2* and the decrease of *Rho* gene expression are in consistent with the state of neural is retina explant on 14 day *in vitro* when its function is inhibited and regeneration takes place.

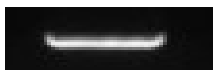
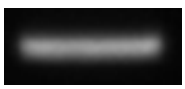
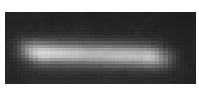
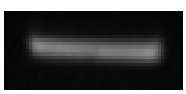
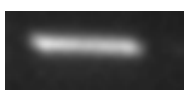
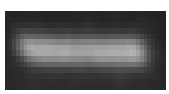
	Native neural retina	Neural retina, 14 day *in vitro*	Native RPE	RPE, 14 day *in vitro*
GABDH				
RPE65				
Fgf2				
Tub b2				
Rho				
Ns				

Table 1: *PCR analysis of gene expression in RPECs and neural retina of the adult newt P.waltl, in freshly isolated (conditionally native) samples and in the samples obtained on 14 day in vitro.*

Conclusion

Our study showed results useful for investigation of RPECs dependent and independent retinal regeneration *in vivo* and *in vitro*. As we established 3D organotypic culturing of the newt RPE and isolated neural retina whole amount is appropriate approach since it allows reproduce cell processes that take place *in vivo*. Under these conditions *in vitro* we studied of the role of Fgf2 and Ns (nucleostemin) in regulation of early stages of retinal regeneration by RPECs and isolated neural retina by its own cell sources. We found that DNA-synthesizing activity of RPECs increased in the presence of Fgf2 exogeneously added to the medium. In parallel to that slight increase of *Fgf2* gene expression in RPECs on 14 day of *in vitro* culturing took place also. Isolated newt neural retina regenerated *in vitro* by its low differentiated cells located at the periphery. These cells and ones of Müller glia demonstrated migration, mitotic activity, and production of neuroblasts for restoration. These processes in the neural retina correlated with up-regulation of *Fgf2* and *Ns* gene expression suggesting an important role of these two factors in RPE independent retinal regeneration. Our findings confirmed other data on the key role of FGF2 in cell proliferation in eye regeneration and development. Results showed also that nucleostemin can be one more participant in the control of retinal and RPE cell behavior, probably in FGF2-mediated molecular network. Our results are among other data giving key information to develop methods and approaches for stimulating eye tissue regeneration due to endogenic cell reserve.

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Conflict of Interest

The authors state that the manuscript has not been published previously.

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