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PLURIPOTENT STEM CELLS FROM THE ADULT MOUSE UTRICLE

CELULE STEM PLURIPOTENTE DIN UTRICULA SOARECELUI ADULT

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Researchers discovered that cochlear epithelia in mice especially the vestibular one, contains stem cells that have the capacity to differentiate in sensorial auditory hair cell progenitors specific to the organ. They are reduced in number as the animal progresses in age. This process leads to a loss in the regenerative and proliferative potential of sensorial inner ear epithelia secondary to different injuries. Isolation, cultivation and than in vitro differentiation of vestibular stem cells could become a regenerative implant for acquired hearing loss. These were the motives that determined us to try to isolate, cultivate and finally differentiate vestibular stem cells from vestibular epithelia.Utricles from 7 days old mice NMRI were harvested, the otolites were removed, the utricles were trypsinized in order to isolate cells. Obtained cells were cultivated at 37ºC and 5% CO2 in DMEM with F12 Nutrient mixture, B27, N2 supplement. Pluripotency of obtained spheres was established with the help of stem cell markers Nanog and Oct-4. For identification of progenitor cells we used the marker, which reveals the gene which encodes the protein nestin. In all experiments we obtained floating colonies called spheres, formed by mitotic multiplying. For testing the pluripotency of spheres we used Nanog and Oct-4, two transcription factors that are expressed at high levels in stem cells and that we found to be expressed in our spheres. The presence of nestin mRNA in cells composing the spheres showed that these progressed to a progenitor cell stage.We concluded that utricular epithelia in 7 days old mice contains sufficient stem cells that can be cultivated and that can be later differentiated.

Key words: utricle, adult stem cells, spheres

Introduction

Some drugs like the group of aminoglycosides, strong noise, chemotheraputic agents, aging, provoke different degrees of hypoacusia or even

complete hearing loss. All these are due to irreversible auditory hair cell loss within the organ of Corti, especially through apoptosis (2, 3). Their regeneration is present only in some species of birds, by spontaneous differentiation induction of adult stem cells in the inner ear. Li et al (1, 2) revealed that both the utricle and the saccule and semicircular canals, contain pluripotent stem cells that could be induced to differentiate into auditory hair cells(4, 5, 6, 7). The purpose of the ongoing research is to improve our current skills and knowledge in stem cell isolation and cultivation from the utricular epithelia of the mouse and than to precise their pluripotency.

Material and Methods

Stem cell isolation

We obtained organotopypic cultures of utricles (n= 45) from postnatal day seven (p7) NMRI mouse pups. For each experiment we dissected 5-8 utricular maculae.The mice were sacrificed by decapitation. The temporal bones were dissected in PBS at pH 7,3 under sterile conditions. The utricles were dissected using sterile technique and were cultured free-floating (8 utricles per well) in 24-well tissue culture plates. We removed the overlying otoconia and the extramacular epithelial tissue together with the remains of the nerve fibers and separated the cells by an 15 min treatment with trypsin in PBS at 37°C. Afterwards the utricles were triturated in order to separate the cells. The enzymatic digest was stopped by addition of 5% FCS in DMEM\ high glucose medium (Invitrogen). Finally, the cells together with other cell aggregates and debris were passed through a 70 μm cell strainer. For sphere formation we plated the cells in plastic Petri dishes into serum free high glucose DMEM and F 12 Nutrient mixture, B27 supplement and N2 supplement. The utricles were incubated for 7 days hr at 37°C in a 5% CO2 and 95% air environment.

Testing the pluripotency of cells obtained from utricular epithelia

RNA purification: after sphere formation we extract them and use it for RNA purification and analysis. The RNA isolation from spheres was performed using a rapid method using Nucleo Spin RNA Kit (BD Biosciences) and Trizol reagent (Invitrogen). The total amount of isolated RNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies).

qRT-PCR: the total amount of RNA was diluted to3 ng/μl. For reverse transcription, we used total RNA, treated with RNase-free DNase (Qiagen). The DNase step (RNase-free DNase Set, Qiagen) was performed to remove genomic DNA. Following appropriate washings, RNA was eluted from the column in 20 μl nuclease-free water.

In order to see if our harvested cells from utricule are pluripotent, we use genetic markers like Oct 4, Nanog and GAPDH.

Expression of stem cell markers Nanog and Oct-4 and housekeeping genes GAPDH was assessed using gene specific primers (TaqMan from Applied Biosystems). First we accomplish the cDNA synthesis using High Capacity cDNA

Revers Transcripsion Kit from Applied Biosystem. After the cDNA synthesis we perform qRT-PCR. The reactions were assembled using cDNA, 2X PCR buffer, TaqMan probe for Nanog, Oct4 and GAPDH in a 15 µl reaction volume. The following protocol was performed: 10 min denature step at 95°C followed by 40 cycles of 95°C for 15 sec and step 2 for 60 sec at 60°C with a final melt from 45°C to 90°C using a Corbett Rotor Gene Real-Time PCR Machine. All samples were run using 2 parallels.

In vitro amplification – RT-PCR

For reverse transcription with One step RT-PCR kit (Qiagen), we used total RNA, treated with RNase-free DNase. Cycling parameters for different products were optimized to generate the highest amount of amplification product.

Specific parameters were: reverse transcription at 50°C for 31 min and inactivation at 95°C for 15 min followed by *x* cycles of denaturation step at 94°C for 1 min, annealing step at 66°C for 1 min for Nestin and 57°C for 1 min for Brn3.1 respectively, and extension at 72°C for 1 min. The number of cycles (*x*) was 30 for Nestin, and 35 for Brn 3.1. We used the following primer pairs:

Nestin primers: 5'-GCC GAG CTG GAG CGC GAG TTA GAG-3'
694 pb 5'-GCA AGG GGG AAG AGA AGG ATG TCG-3'

Brn3.1 primers 5'-GCC ATG CGC CGA GTT TGT C-3'
368 pb 5'-ATG GCG CCT AGA TGA TGC -3'

Results and Discussions

Results regarding the *in vitro* cultivation of epithelial cells harvested from the utricle

Adult stem cells have the capacity to generate many type of cells from the organism. Their capacity is due to their proliferation and differentiation potential. They are multiplying themselves by mytosis and form spheres and embryonic bodies. Our research demonstrated that isolated cells from the utricular epithelia, treated properly after 7 days of cultivation in DMEM media, F12, B27, are multiplying and form spheres.

Obtained spheres were variable both from size but also from their structure. There are small spheres formed by a smaller amount of cells and also bigger ones. In the first stages of development we can observe also hole spheres surrounded by one or 2 layers of cells. Later, the number of cells increases occupying the entire inner surface. In this way the spheres are becoming compact.

Spheres are formed from individual cells originating in the utricular macula sensory epithelium. In figure 2 *c, d, e* and *f* we show some aspects of spheres of different shapes and sizes.

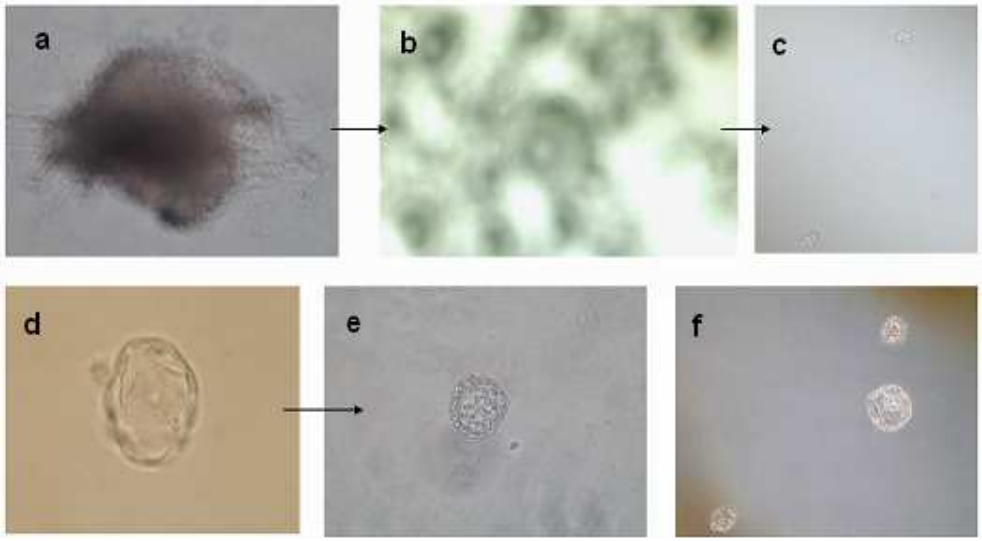

Fig. 1. a)Utricles after removal of otoconia. b) Utricular sensory epithelial cells. c) 2 days old spheres derived from utricular cells. d) Spheres formed after 4 days e) spheres at 7 days.

Results regarding pluripotency testing of utricular cells

a) Research done by Li et al with help of thyimidine analogue, 5-bromdeoxiuridine, demonstrated that obtained spheres harvested from utricular epithelia, represent clones of one and the same cell. Consequently, obtaining in culture of cell aggergates is the first prove that our cells isolated from utricular epithelia, are pluripotent adult stem cells, which possess high proliferative capacity.

b)*Total RNA analysis by RT- PCR*

In order to have a direct and objective argument that our isolated cells are pluripotent cells capable to form spheres and than embryoid bodies which give rise to sensorial hair cells we used RT- PCR analysis of cDNA obtained by reverstranscription from total RNA copied from Nanog and Oct 4 genes.

Nanog and Oct-4 are two transcription factors that are expressed at high levels in stem cells. These transcription factors regulate the expression of other genes during development and are found at high levels in the pluripotent cells. The downregulation of these three transcription factors correlates with the loss of pluripotency and self-renewal, and the beginning of subsequent differentiation steps. The role of Nanog and Oct-4 is important in embryonic development and maintenance of pluripotency.

Our quantitative gene expression data was normalized to the expression levels of control ("housekeeping") genes GAPDH - glyceraldehyde-3-phosphate dehydrogenase. GAPDH is one of the most commonly used housekeeping genes used in comparisons of gene expression data. An inherent assumption in the use of

housekeeping genes is that expression of the genes remains constant in the cells or tissues under investigation. Results are showed in figure 2.

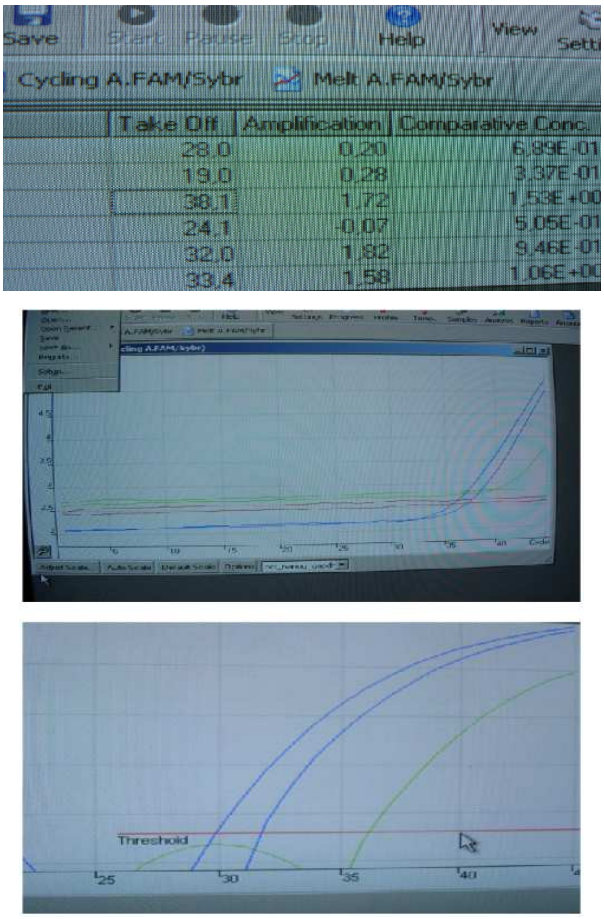

Fig. 2 Real time PCR analysis made with the help of this method revealed the presence of mRNA for Nanog and oct 4.

From fig. 2 we can observe that obtained spheres in culture contain sufficient mRNA copied from Nanog and oct 4 genes. This suggests that the 2 genes are functioning and synthesize the proteins that encodes them. The presence of these markers in cells composing the spheres, represents a solid argument which gives us the right to infer that in 7 days old mice exist enough pluripotent stem cells that can be artificially induced to enter proliferation and to differentiate into auditory hair cells.

c*) Presence of differentiated cell progenitors are proves for pluripotency*

Adult pluripotent stem cells, before they give rise to differentiated cells, are transforming themselves into precursor cells or progenitors and only afterwards

they reach the stage of specific cells for ecto-, endo-, mesoderm. As following, markers that define progenitor cells from a sphere, become objective probes of cellular pluripotency. In order to identify progenitor cells we use the marker for gene expression which encodes the protein nestin. In order to quantify the expression of nestin gene in cells contained by the spheres and to be able to establish if these cells are precursors of the 3 embryonic layers, we analyzed by RT-PCR the presence of mRNA encoding this gene. Obtained results are showed in fig. 3

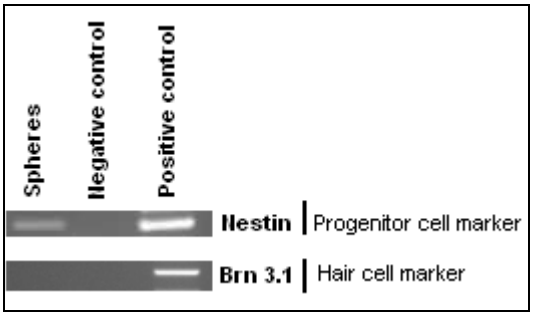

Fig. 3. RT-PCR analysis of expression of markers by spheres

In the figure above the RT- PCR reaction showed that the spheres we obtained were positive for nestin expression and negative for Brn 3.1, a marker of differentiated hair cells. Therefore, we conclude that the spheres are made out of early progenitor cells and not of differentiated cells. Expression of the transcription factor Brn3.1 serves as marker for the hair cell phenotype, next step of our experiments.

The image above reveals that the marker for nestin gene expression is present within cells composing the spheres obtained from utricular epithelia as well as in the cells of the controls. The presence of nestin marker in cells which compose the obtained spheres constitutes an argument that these are progenitor cells of utricular epithelial cells. They are a prove that utricular epithelia of 7 days old mice contains enough pluripotent stem cells which can differentiate into hair cell progenitors.

Conclusions

From our research we conclude the following conclusions:

1. Utricular apithelia in 7 days old mice contains sufficient pluripotent cells which, under special conditions, can generate spheres
2. Cells harvested from utricular epithelia are pluripotent and, under special conditions, they can give rise to spheres and precursors of the three layers: endoderm, ectoderm, mesoderm

3. Cells obtained from utricular epithelia are pluripotent because after their aggregation in spheres they manifest the markers nanog and oct 4, as well as the marker of nestin gene expression, characteristic for cell progenitors

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