# Results Regarding Self Renewal and Differentiation of Cells Composing Inner Ear Derived Spheres

**Roxana Vintila[1,2], Oana Gavriliuc[1,3]**

*[1]University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania*
*[2]University of Medicine"Vasile Goldis", Arad, Romania*
*[3]Banat's University of Agricultural Sciences and Veterinary Medicine from Timişoara, 300645-Timisoara, Calea Aradului, 119, Romania*

**Abstract**
The purpose of the ongoing research is to improve our current skills and knowledge in stem cell isolation, cultivation and differentiation from the vestibular epithelia of young mice.
We harvested utricles and sacculi from 7 days old mice. Obtained cells were cultivated at 37ºC and 5% CO2 in DMEM with F12 Nutrient mixture, B27, N2 supplement, IGF-1 and EGF. Sphere pluripotency was established with Nanog and Oct-4 stem cell markers. We mechanically dissociated primary spheres and cultivated them. Cells were characterized by immunofluorescence and immunohistochemistry for myosinVIIA (hair cell marker), nestin (intermediate filament VI marker) and beta III tubulin.
We proved that vestibular epithelia contains pluripotent stem cells which formed spheres. Sphere-derived cells' pluripotency was demonstrated by the expression of nanog, oct 4 and nestin markers. Also, after sphere dissociation we obtained a higher number of spheres beeing pluripotent and capable of self-renewal. We obtained through differentiation different cell types including neuron like-cells which were positive for myosin VIIA, nestin and beta III tubulin.
Utricular epithelia of seven days old mice contains sufficient pluripotent stem cells which generate spheres.
Cells obtained from utricular epithelia are pluripotent because they express nanog, oct 4, nestin, characteristic for cell progenitors.

**Keywords**: inner ear, pluripotency, stem cells

## 1. Introduction

Some drugs like the group of aminoglycosides, strong noise, chemotherapeutic agents, aging, determine 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, 8] 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 [4-7]. 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.

One way of identifying cell pluripotency is by testing their capacity of self renewal and not last their capacity of differentiation into specialized cells. In order to evidence the presence of these characteristics in cultivated cells we used 2 ways of analysis. Those were the following:

1. Individualization and dissociation of cells composing a sphere and their separate cultivation

* Corresponding author: Roxana Vintila,
Tel: 0721934535, 0256-432306,
Email: Roxty@yahoo.com

in the culture media in order to obtain information about their power of replication and self renewal.
2. Adding to the culture media growth factors like EGF and IGF-1 in order to intensify cell multiplying and evidencing the capacity of sphere cells to differentiate into specialized cells

## 2. Materials and methods

We obtained organotopypic cultures of utricles (n=150) 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.

### Self-renewal capacity analysis in cells composing the spheres

From ears harvested from ≈ 15 mice/experiment we harvested selected cells supposed to be stem cells, which were cultivated after in the incubator following the method mentioned above. After 7 days the incubated cells formed around 130 spheres (Figure 1).
In order to detect if utricular cells which form the spheres are capable of self- renewal, we mechanically dissociated the spheres into individual cells which we cultivated in a fresh nutrient media. After their individual cultivation in the same conditions as the ones they belonged, in the presence of IGF-1 and EGF, at 3 days we detected a number of around 230 secondary spheres. Continuing their cultivation another 7 days, their number reached 350 secondary generation spheres.
All this demonstrates that sphere dissociated individual cells are capable to replicate and to keep their capacity to stay nearby their ascendants forming spheres.
Adding EGF and IGF-1 to the culture media double intensified the sphere derived cell replication after their mechanically dissociation. The number of spheres obtained from the second generation doubled after adding growth factors to the culture media compared to the cultivation in their absence.

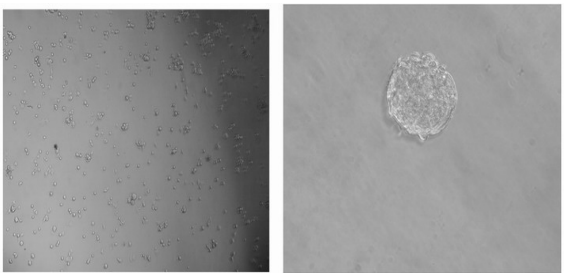


**Figure 1.** Sphere in culture media

From one mouse we could harvest an average of $13x10^4$ cells. Their cultivation in culture media in presence of EGF and IGF-1 lead to the formation of spheres. Their mechanical dissociation into individual cells and their reculture lead to formation of 230 secondary spheres after 3 days. After another 3 days the number of spheres doubled.

### Analyzing the differentiation capacity of cell composing the spheres

In order to obtain certain data regarding the pluripotence of cells obtained by us from the inner ear of young mice (7 days), which form spheres "in vitro", we tried to provoce their differentiation. For this we transferred the spheres, one by one, with the help of the pipette, on a culture media containing fibronectin on chamber slides, and FCS 100% in other nuncs, with the purpose of determining their attachment to the bottom of the plate. The used culture media was DMEM high glucose and FCS 10%. The media was replaced after 16 hours of cultivation with serum free high

glucose media DMEM, F12 Nutrient mixture, B27 supplement and N2, without growth factors.
This media was changed daily with fresh media. After 1 day we could observe both complete adhered spheres beginning to differentiate as well as spheres in suspension (Figures 2, 3). Followed under the microscope, the cells took different shapes and morphologies, some with elongations, others poliedric, or neuron like (Figure 4). During cultivation they formed an entire net, in which cells interconnected with their elongations. For us it was clear that these cells formed by in vitro differentiation of pluripotent stem cells harvested from the inner ear. The results we expose in the following pictures.

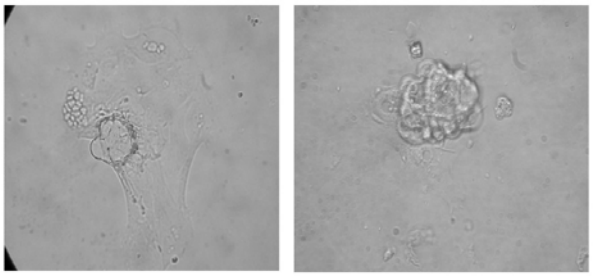


**Figure 2.** Adhered spheres which tend to occupy the bottom of the plate by circular expansion

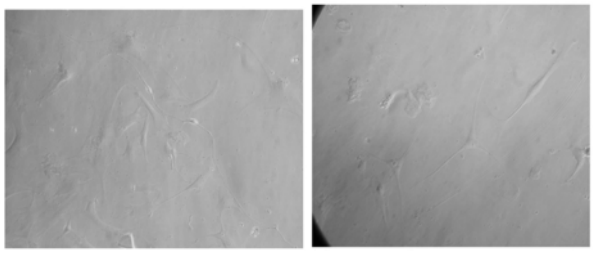


**Figure 3.** Microscopic image in phase contrast: neuron-like cells which interconnect together with cells of different morphologies resulting following sphere derived cell differentiation

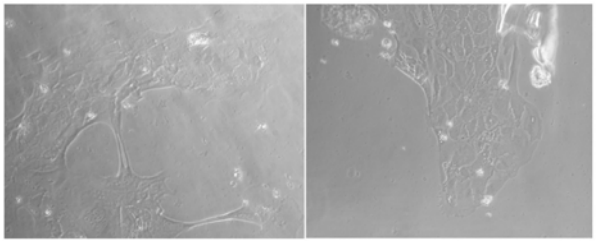


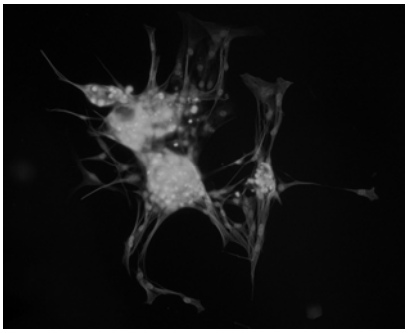
**Figure 4.** Microscopic image in phase contrast: cells of different morphologies resulting following sphere derived cell differentiation

**Describing the nature of cells originating from spheres**

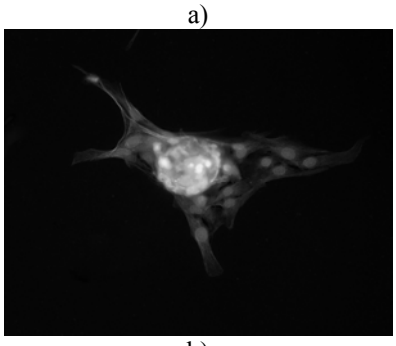
In order to convince ourselves that cells composing the spheres obtained in culture are the result of differentiation into neuronal cells, neuron- like or sensorial cells, we stained them by immunoflorescence or immunohistochemistry. For this we used the following techniques.

*1) Immnuoflorescent assay of sphere derived cells*

In order to be able to perform sphere staining we placed the spheres on adherent tissue slides for three hours in culture media without IgF- 1, EGF. We performed afterwards their staining with help of monoclonal antibodies against nestin, nanog (pluripotency markers), alfa tubulin (cytoskeleton marker), and beta III tubulin (neural progenitors).
Obtained results suggest that spheres express pluripotency markers like nestin, nanog (Figure 5). We could visualize with help of alfa tubulin cells which began to differentiate from spheres after 2 hours of cultivation. We evidenced sphere derived cells positive for beta III tubulin, marker for neural progenitors.



a)



b)

**Figure 5.** Imunoflorescence
a) Nestin (red), Falloidin (blue), Alfa tubulin (green), Propidium Iodide stained nuclei (orange)
b) Nanog (green), Nestin (red)

*2) Regarding the capacity of sphere derived stem cell differentiation*

In order to obtain differentiated cells in culture we used fresh cultivated cells harvested from vestibular organs, sphere derived cells after mechanical dissociation and individual spheres. Thez have all been placed on fibernectin in the absence of growth factors. In order to obtain a clear image upon differentiated cells we used a cytoskeleton marker called alfa tubulin (Figure 6). We obtained cells of different morphologies (star shaped, pavimentous) and we observed also neuron like cells which interconnected by their elongations (Figure 7).

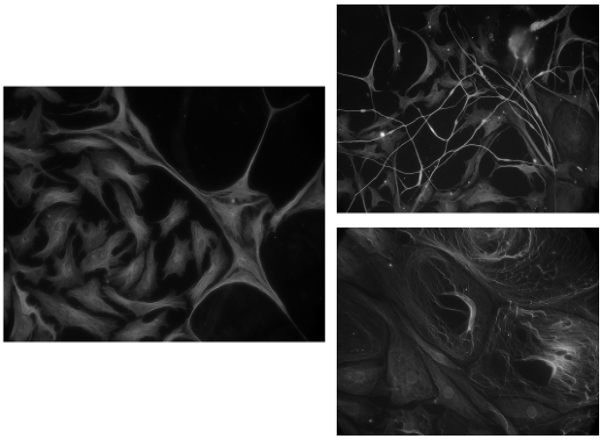


**Figure 6.** Alfa tubulin staining of differentiated sphere derived cells

*3) Sphere derived cells staining by immunoflorescence*

In order to evidence the characteristics of cells composing the first and second generation spheres (which resulted after mechanical dissociation), we performed the staining with primary monoclonal mouse anti mouse antinestin (Santa Cruz Biotehnologies) and monoclonal goat anti mouse antibody (Santa Cruz Biotechnologies). Secondary antibodies were Alexa 546 goat anti mouse anti nestin (Invitrogen Molecular Probes) and FITC donkey anti goat (Santa Cruz).

After in vitro differentiation we evidenced the presence of cells with neuron like extensions which were immunopositive for nestin. Cell morphology was different and we observed neuron like cells which were interconnecting as well as elongated or pavement epithelia. We didn't detect the presence of myosin VIIA marker. We also stained neuronal like cells for beta III tubulin, a neuronal marker, which showed to be present in these cells (Figure 8).

We expose the results in the following pictures:

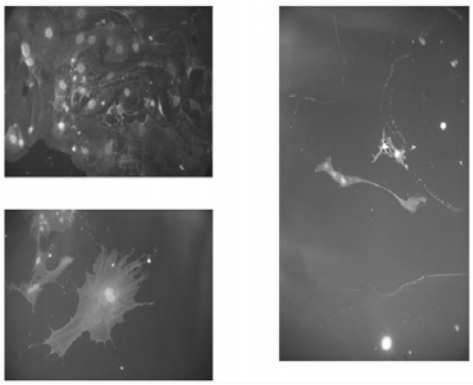


**Figure 7.** Immunoflorescence: positive nestin (red) cells, starshaped, along with poliedric cells, resulted after sphere derived cell differentiation; positive Dapi nuclei (blue)

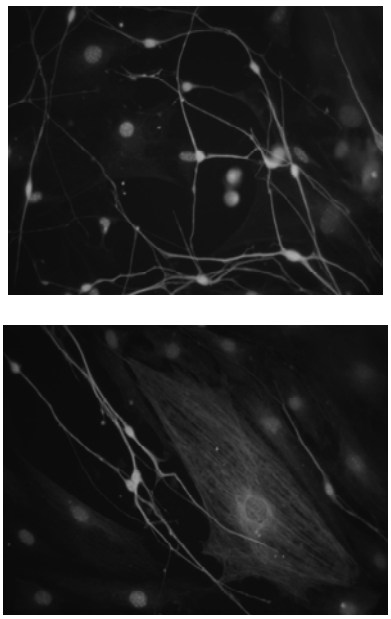


**Figure 8.** Beta III tubulin expression in neurons differentiated from spheres placed on fibronectin coated tissue culture slide chambers

## *Immunohystochemical analysis*

In order to characterize in detail differentiated cells we used specific antibodies for myosin VIIA (hair cell marker), nestin (marker for intermediary filament type VI), synaptophysine (glycoprotein from synatic vesicles).

For immunohystochemistry we used the following antibodies policlonal primary antibody antihuman antisynaptophysin (Dako), antinestin rabbit anti

mouse policlonal antibody (Abcam), anti myosin VIIA goat anti mouse (Santa Cruz). As secondary antibody we used the LSAB+ Dako kit, which stains in red all the cells disregarding the primary antibody. Nuclei were stained blue with the help of hematoxilin eosin. Stained cells were positive for nestin, myosin VIIA, but negative for synaptophysin (probably because of the antihuman sinaptophysin antibody). (Figures 9, 10, 11).

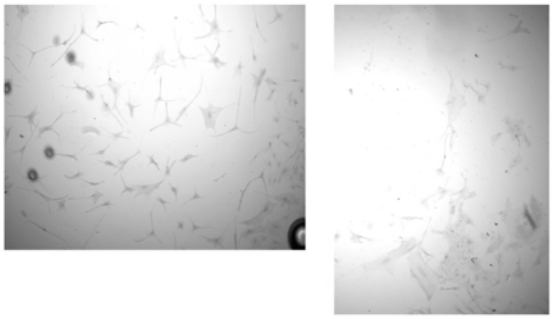


**Figure 9.** Immunohystochemistry: neuron like nestin positive cells along with positive nestin (red) cells, starshaped, along with poliedric cells, resulted after sphere derived cell differentiation; positive hematoxilin-eosin nuclei (blue)

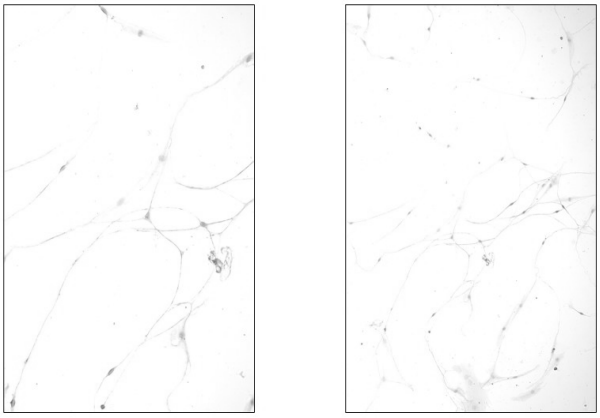


**Figure 10.** Positive neuronal like net (red) resulted after sphere derived cell differentiation; positive hematoxilin- eosin nuclei (blue)

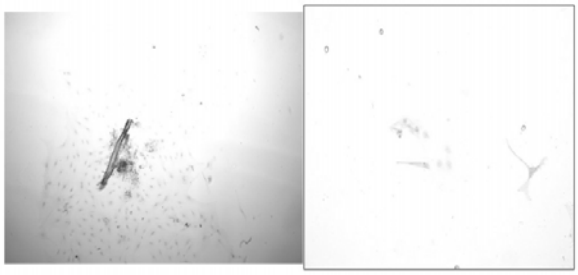


**Figure 11.** Immunohystochemistry: positive myosin VII A cells (hair cell marker) (red) resulted after sphere derived cell differentiation

## 3. Results and discussion

In addition to the vestibular system, the mammalian cochlea in neonates contains cells that have properties of stem cells based on sphere formation and selfrenewal [19-21]. These cells differ from the normal post mitotic cells present in the organ of Corti and vestibular system. The cells form clonal spheres that can be propagated in culture and have from 1-3 stem cells per sphere. The capacity of sphere formation decreased with age. Studies have shown that adult vestibular stem cells are pluripotent: they differentiate into cell types corresponding to all three germ layers, endoderm, mesoderm, ectoderm and this includes neurons and hair cells in vitro [1-4] indicates that these endogenous cells are true stem cells [21]. Kazuo Oshima et al [22] presented a stepwise guidance protocol starting with mouse embryonic stem and induced pluripotent stem cells, which were directed toward becoming ectoderm capable of responding to otic-inducing growth factors. The resulting otic progenitor cells were subjected to varying differentiation conditions, one of which promoted the organization of the cells into epithelial clusters displaying hair cell-like cells with stereociliary bundles. Bundle-bearing cells in these clusters responded to mechanical stimulation with currents that were reminiscent of immature hair cell transduction currents. Diensthuber et al noticed that the sphere population derived from mouse cochlear sensory epithelium cells was heterogeneous, consisting of morphologically distinct sphere types, hereby classified as solid, transitional, and hollow. Cochlear sensory epithelium-derived stem/progenitor cells initially give rise to small solid spheres, which subsequently transition into hollow spheres, a change that is accompanied by epithelial differentiation of the majority of sphere cells. Only solid spheres, and to a lesser extent, transitional spheres, appeared to harbor self-renewing stem cells, whereas hollow spheres could not be consistently propagated. Solid spheres contained significantly more rapidly cycling Pax-2-expressing presumptive otic progenitor cells than hollow spheres [17, 18]. They conclude that cochlear sensory epithelium cell populations initially give rise to small solid spheres that have self-renewing capacity before they subsequently convert into hollow spheres, a process that is accompanied by loss of stemness

and reduced ability to spontaneously give rise to hair cell-like cells. Solid spheres might, therefore, represent the most suitable sphere type for cell-based assays or animal model transplantation studies aimed at development of cell replacement therapies[17, 18]. Inner ear stem cells can be isolated by neurosphere formation from the vestibular organs and the cochlea. The cells are pluripotent, with the potential to become hair cells and neurons, the cochlear cell types whose loss causes deafness. The authors describe the control of cell fate decisions that determine the phenotype adopted by these progenitors, and we determine whether differentiation to sensory neurons is preferred over other types of neurons. Differentiation of progenitor cells recapitulated developmental pathways of embryonic sensory neurons. Based on marker expression, retinoic acid increased the yield of neurons and the percentage of sensory neurons obtained and caused a sharp increase in Pax2, a key transcription factor of cranial placodes. Markers of embryonic auditory and other sensory neurons, GATA3, Brn3a, and islet1, could be detected after 3 days of differentiation of the cells, and markers of the sensory phenotype, peripherin, calretinin, TrkC, and TrkB were expressed after 10 days[20]. The basic helix-loop-helix transcription factor Atoh1 (also known as Math1) has emerged as a candidate for gene-based treatment of auditory and vestibular disorders. Atoh1 is expressed in the prosensory patches that give rise to the auditory and vestibular sensory epithelia. The *Atoh1* gene is essential for hair cell development as its targeted disruption results in the absence of auditory and vestibular hair cells [13, 14, 23]. In birds and many nonmammalian vertebrates, supporting cells are able to produce new hair cells through two mechanisms: transdifferentiation and mitotic regeneration. In the latter process, normally postmitotic supporting cells reenter the cell cycle, divide and give rise to both hair cells and supporting cells. An essential question is whether the mammalian supporting cell is terminally postmitotic, having permanently lost the capacity to reenter the cell cycle, or whether manipulation of genes that regulate cell cycle progression could induce the supporting cell to reenter mitosis. The answer may lie with the cyclin-dependent kinase inhibitor p27Kip1 (also known as Cdkn1b), which occurs in the developing organ of Corti from embryonic days 12–14 and correlates with cessation of cell division of the otic epithelial progenitors that give rise to hair cells and supporting cells. In the maturing inner ear, p27Kip1 is downregulated in hair cells but its expression persists in all classes of supporting cells [13, 14, 15, 23]. In the inner ear, the Brn-3c protein is found only in auditory and vestibular hair cells, and the Brn-3a and Brn-3b proteins are found only in subsets of spiral and vestibular ganglion neurons. Mice carrying a targeted deletion of the Brn-3c gene are deaf and have impaired balance. These defects reflect a complete loss of auditory and vestibular hair cells during the late embryonic and early postnatal period and a secondary loss of spiral and vestibular ganglion neurons[7]. Despite the inability of the mammalian inner ear to regenerate lost hair cells, there is evidence for cells with regenerative capacity because stem cells can be isolated from vestibular sensory epithelia and from the neonatal cochlea. Challenges and recent progress toward identification of the intrinsic and extrinsic signaling pathways that could be used to re-establish stemness in the mammalian organ of Corti are discussed [5-10]. Beisel K. propose a two-step approach using a first set of transcription factors to enhance the generation of inducible pluripotent stem (iPS) cells and a second set of factors to initiate the differentiation of hair cells. Recent evidence regarding ear development and stem cell research strongly suggest that microRNAs will be an important new regulatory factor in both iPS cell formation and differentiation to reprogram cells into hair cells. In addition, we highlight currently insurmountable obstacles to the successful transformation of stem cells into hair cell precursors and their injection into the cochlear canal to replace lost hair cells [16].

## Conclusions

From our research we conclude the following:

1. Utricular epithelia of seven days old mice contains sufficient pluripotent stem cells which, under special conditions, can generate spheres
2. Cells harvested from utricular epithelia are pluripotent stem cells which, under special conditions, can give rise to spheres or precursors of the 3 embryonar germ layers: endoderm, mesoderm, ectoderm

3. Utricular epithelia of seven days old mice contains sufficient pluripotent stem cells which, under special conditions, can generate spheres

4. Cells harvested from utricular epithelia are pluripotent stem cells which, under special conditions, can give rise to spheres or precursors of the 3 embryonar germ layers: endoderm, mesoderm, ectoderm

5. Cells obtained from utricular epithelia are pluripotent due to the fact that after sphere aggregation they manifest markers fro nanog, oct 4 as well as markers for nestin gene, characteristic for cell progenitors

6. Individualization and dissociation of cells composing spheres and their separate cultivation in culture media lead to formation of a larger number of spheres than the number rom which we initially started, proving the fact that cells composing spheres are pluripotent and capable of self renewal

7. Adding of growth factors like EGF and IGF-1 to the culture media intensified the replication of cells and evidenced the capacity of differentiating into specialized cells which compose the spheres

8. Spheres cultivated on fibernectin gave rise through differentiation to cells of different sizes: star- like cells, neuron- like cells. These cells were positive for myosin VII A (hair cell marker), nestin (intermediate filament marker type VI), but negative for synaptophysin, a glycoprotein present in synaptic vesicles. This fact can be due to the used antibody, which has antihuman specificity.

## Aknowledgement

This work was cofinanced from the European Social Fund through Sectoral Operational Programm Human Resources Development 2007-2013, project number POSDRU/89/1.5/S/63258 ”Postdoctoral school for zootechnical biodiversity and food biotehnology based on the eco-economy and the bio-economy required by eco-san-genesys”

## References

1. Li, H., Roblin, G., Liu, H., Heller S. Generation of hair cells by stepwise differentiation of embryonic stem cells., PNAS, 2003, 11 100 (23), 13495–13500

2. Huawei Li, Hong Liu, Heller, S., Pluripotent stem cells from the adult mouse inner ear, Nature Medicine, 2003, 9 (10), 1293-1299

3. Lin, J., Feng, L., Fukudomem, S., Hamajima, Y., Huang, T., Levine, S., Cochlear stem cells/progenitors and degenerative hearing disorders, Curr Med Chem., 2007,14(27), 2937-43

4. Matsui, J.I., Parker, M.A., Ryals B.M., Cotanche, D.A., Regeneration and replacement in the vertebrate inner ear. Drug Discov Today, 2005, 10(19), 1307-12, Review. PMID: 16214675

5. Parker, M.A., Cotanche, D.A., The potential use of stem cells for cochlear repair, Audiology and Neurotology, 2004, 9:72-80

6. Parker, M.A., Cotanche DA. The potential use of stem cells for cochlear repair. Audiol Neurootol., 2004, (2):72-80. Review. PMID: 14981355

7. Xiang, M., et al., Essentioal role of POU-domain factor Brn-3c in auditory and vestibular hair cell development. Proc. Natl. Acad. Sci. USA, 1997, 94, 9445-9450

8. Li. H., Corrales, C.E., Edge, A., Heller, S., Stem cells as therapy for hearing loss. Trends Mol Med., 2004 10 (7), 309-15. Review. PMID: 15242678

9. Kamiya, K., Ikeda, K., Inner ear cell therapy for hereditary deafness with multipotent stem cells, Nihon Rinsho, 2011, 69(12):2215-9

10. Ronaghi, M., Nasr, M., Heller, S., Concise review: Inner ear stem cells--an oxymoron, but why? Stem Cells, 2012 Jan, 30(1):69-74. doi: 10.1002/stem.785

11. Kopecky, B., Fritzsch, B., Regeneration of Hair Cells: Making Sense of All the Noise. Pharmaceuticals (Basel). 2011, 4(6), 848-879

12. de Felipe, M.M., Feijoo, Redondo A.F., García-Sancho, J., Schimmang, T., Alonso, M.B., Cell- and gene-therapy approaches to inner ear repair. Histol Histopathol. 2011, 26(7), 923-40. Review

13. Liu, X.W., Sun, J.W., Primary culture of neurospheres obtained from fetal mouse central nervous system and generation of inner ear hair cell immunophenotypes in vitro. J Laryngol Otol. 2011, 125(7), 686-91. Epub 2011 Apr 1214.

14. Fritzsch, B., Jahan, I., Pan, N., Kersigo, J., Duncan, J., Kopecky, B., Dissecting the molecular basis of organ of Corti development: Where are we now? Hear Res., 2011,276(1-2), 16-26. Epub 2011 Jan 21. Review.

15. El-Amraoui, A., Petit, C., Stem cell therapy in the inner ear: recent achievements and prospects]. Med Sci (Paris). 2010, 26(11), 981-5. Review. French

16. Beisel, K., Hansen, L., Soukup, G., Fritzsch, B., Regenerating cochlear hair cells: quo vadis stem cell. Cell Tissue Res. 2008, 333(3), 373-9. Epub 2008.

17. Diensthuber, M., Heller, S., Characterization of stem cells derived from the neonatal auditory sensory epithelium]. HNO. 2010, 58(11), 1056, 1058, 1060-6.

18. Diensthuber, M., Oshima, K., Heller, S., Stem/progenitor cells derived from the cochlear sensory epithelium give rise to spheres with distinct morphologies and features. J Assoc Res Otolaryngol. 2009, 10(2), 173-90. Epub 2009 Feb 27.

19. Oshima, K., Senn, P., Heller, S., Isolation of sphere-forming stem cells from the mouse inner ear. Methods Mol Biol. 2009, 493, 141-62.
20. Martinez-Monedero, R., Yi, E., Oshima, K., Glowatzki, E., Edge, A.S., Differentiation of inner ear stem cells to functional sensory neurons. Dev Neurobiol. 2008, 68(5), 669-84.
21. Martinez-Monedero, R., Oshima, K., Heller, S., Edge, A., The potential role of endogenous stem cells in regeneration of the inner ear, Hearing Research 2007, 227 (1-2), 48-52
22. Oshima, K., Shin, K., Diensthuber, M., Peng, A.W., Ricci, A.J., Heller, S., Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell. 2010, 14, 141(4), 704-16.
23. Brigande, J.V., Heller, S., Quo vadis, hair cell regeneration? Nat. Neurosci. 2009, 12 (6), 679- 685