<http://www.diva-portal.org>

Preprint

This is the submitted version of a paper published in *Acta Oto-Laryngologica*.

Citation for the original published paper (version of record):

Edin, F., Liu, W., Boström, M., Magnusson, P., Rask-Andersen, H. (2014)

Differentiation of human neural progenitor cell-derived spiral ganglion-like neurons: a time-lapse video study.

Acta Oto-Laryngologica, 134(5): 441-447

<http://dx.doi.org/10.3109/00016489.2013.875220>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-224707>

Differentiation of human neural progenitor cell-derived spiral ganglion-like neurons

A Time-Lapse Video Study

Fredrik Edin^{1,*}, Wei Liu¹, Marja Boström¹, Peetra U. Magnusson² and Helge Rask-Andersen¹

¹Department of Otolaryngology, Uppsala University Hospital, 75185 Uppsala, Sweden

²Department of Immunology, Genetics and Pathology, Clinical Immunology,
Dag Hammarskölds väg 20, 752 37 Uppsala

**Corresponding author:*

Fredrik Edin

Department of Surgical Sciences, Section of Otolaryngology

Uppsala University Hospital

SE-751 85 Uppsala

Sweden

Phone: +46-18-6115458

Fax: +46-18-500979

E-mail: fredrik.edin@surgsci.uu.se

Abstract

Conclusions: Human neural progenitor cells can differentiate into spiral ganglion-like cells when exposed to inner ear-associated growth factors. Phenotype bears resemblance to human sphere-derived neurons.

Objective: To establish an *in vitro* model for the human auditory nerve to replace and complement *in vivo* animal experiments and ultimately human *in vivo* transplantation.

Methods: Human neural progenitors were differentiated under conditions developed for *in vitro* survival of human primary spiral ganglion culture with media containing growth factors associated with inner ear development. Differentiation was documented using time-lapse video microscopy. Time-dependent marker expression was evaluated using immunocytochemistry with fluorescence- and laser confocal microscopy.

Results: Within 14 days of differentiation, neural progenitors adopted neural phenotype and expressed spiral ganglion-associated markers.

Abbreviations:

SG	Spiral ganglion
SGN	Spiral ganglion neuron
IHC	Inner hair cell
HC	Hair cell
ABI	Auditory Brain stem Implant
CI	Cochlear Implant
hNPC	Human Neural Progenitor Cell
BDNF	Brain-Derived Neurotrophic Factor
NT-3	Neurotrophin-3
GDNF	Glial cell line-derived neurotrophic factor
TLVM	Time-lapse video microscopy
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2
Nf-m	Neurofilament 160

Introduction

Human organ of Corti constitutes a cell mosaic of polarized sensory receptors with surrounding supporting cells. The number of hair cell receptors are remarkably low, approximately 15 000. About 3 400 of these are so-called inner hair cells (IHC) (1) which convey neural signals to the CNS through 35 000 primary afferents (2); the bipolar type I spiral ganglion neurons (SGN).

Sensory-neural deafness is most frequently caused by loss of inner ear hair cell (HC) function and can be alleviated by cochlear implantation (CI). In conditions disrupting cochlear nerve function or in genetic diseases causing severe loss of primary afferents with preserved hair cells patients can be treated with auditory brain stem implantation (ABI) to restore some hearing. Potentially these individuals could be treated with nerve cell replacement (3).

Stem cells have been verified in the adult inner ear (4) suggesting some regenerative potential (5). Neural stem cell marker nestin is expressed in the developing and mature mammalian inner ear (4, 6). Despite ethical and immunological concerns transplanted stem cells hold considerable interest for regenerating auditory neurons and even hair cells (7).

In previous studies, spiral ganglion-like (SG) cells have been produced from mammalian stem cells, including human (8-10). Here we differentiated and expanded human neural progenitor cells (hNPC). Morphology and protein expression was analyzed using time-lapse video, immunofluorescence and confocal microscopy. The hNPCs were differentiated in culture conditions developed for primary cultured human and guinea pig SG cells (5). Cells were compared with primary human sphere derived neurons.

Materials and Methods

Culturing neural progenitor cells

The ENStem-A cell line is an adherent non-immortalized commercially available (Millipore) hNPC derived from the WA09 (H9) human embryonic stem cell line (WiCell).

hNPC were cultured in Neurobasal medium (Gibco) supplemented with 2% B27 supplement (Gibco), 1 % L-glutamine (Gibco) and 0.04 % gentamicin during both expansion and differentiation. Medium was replaced every second or third day. Proliferating cells were passaged using Accutase (Millipore).

To stimulate proliferation 20 ng/mL of fibroblast growth factor-2 (FGF-2, Millipore) and 10 ng/mL of leukemia inhibitory factor (LIF, Millipore) was added to the media.

During differentiation FGF-2 and LIF were withdrawn and replaced with 20 ng/mL glial cell-derived neurotrophic factor (GDNF, Millipore), brain-derived neurotrophic factor (BDNF, Millipore) and neurotrophin-3 (NT-3, Millipore) to promote spiral ganglion-like differentiation. Cells were normally differentiated for 14 – 18 days.

During both expansion and differentiation tissue culture plastic was double coated with poly-L-ornithine (Sigma-Aldrich) and laminin (mouse, Millipore).

Time-lapse video microscopy (TLVM)

TLVM recordings were made using an inverted microscope (Nikon TE2000) fitted with an incubator and Nikon Perfect Focus System controlled via NIS Elements software (version 3.10, SP3, Hotfix 1). Pictures were taken every 1 – 2 minutes for 12 – 48 hours and movies were saved in a compacted avi-format at an increased frame rate with one frame every 70 ms.

Videos were also recorded using a second inverted, incubator fitted, microscope (Zeiss Axiovert 135, Germany) with a video camera connected to a digital surveillance recorder (Sony, Japan) taking one picture every third min.

Immunohistochemistry

Cells were fixed for 20 min at room temperature in 4 % paraformaldehyde solution. The paraformaldehyde solution was removed and cells washed 3 x 5 min with phosphate buffered saline (PBS, Gibco). Fixated specimen were treated with 0.4 % Triton X-100 detergent for 30 min at room temperature, followed by application of primary antibody (see Table 1 for antibodies) dissolved in 2 % BSA blocking solution and overnight incubation at 4 °C. After washing 3 x 5 min with PBS the secondary antibody diluted 1:300 in a 2 % BSA solution was applied, secondary antibodies used were Alexa Flour 488 (goat anti rabbit: A11008, donkey anti rabbit: A21206 or rabbit anti mouse A11059; Life Technologies) or Alexa 555 (goat anti mouse: A21422, donkey anti goat: A21432 or rabbit anti mouse: A21431; Life Technologies), and incubated for 2 hours at room temperature. Specimens were washed 3 x 5 min with PBS and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories).

Stained samples were studied in an inverted fluorescent microscope (Nikon TE2000, Japan) equipped with bright field, fluorescence unit (filters for emission spectra at 358, 461 and 555 nm) and confocal laser (Nikon D-Eclipse C1, three lasers) processed with image merging software (NIS-elements and EZ-C1, Nikon).

Image processing

Measurements and image analysis was performed using ImageJ (1.43u).

Results

Differentiating hNPC morphology

TLVM demonstrated in great detail the temporary events during the maturation process of the hNPC. Video microscopy display cell metamorphosis from undifferentiated to mature stage. In addition, axonal sprouting and growth cone formation were documented. After an initial stage of proliferation, cell apoptosis increased. Rosette formations were common with hNPC with peripherally directed cell processes in colonies (11). Cell nuclei changed structure and were birefringent having a diameter around 15x20 µm. Neurons were bi-, tri- or multipolar. Nuclear translocation could be observed (Figure 1).

The growth cones (GC) frequently fasciculated with adjacent cells and formed networks interacting with both neural- and non-neural cells. One cell was traced for 16 hours between day 18 and 19 of differentiation. Seventeen images were chosen, showing the hourly progress (Figure 2). The hNPC-derived neurons were morphologically similar to primary stem cell-derived neurons observed earlier (Figure 2, B) (5).

Differentiation markers

Immunofluorescence imaging was performed at day 0, 7 and 14 on hNPC cells with markers for neural differentiation (Table 1). Neural phenotype coincided with expression of markers found in mature SG, such as the synaptic glutamate receptor GlutR1 and BDNF-receptor neurotrophic tyrosine kinase receptor type 2 (NTRK2) (Figure 3 A, B) (12). Glutamate is one of the most common signaling molecules of the nervous system whilst BDNF has been shown to increase survival in cultured SG neurons. Neuron specific structural filaments Neurofilament 160 (Nf-m) and class III beta-tubulin (Tuj 1) (Figure 3 D, F) were also found.

During differentiation Neurogenin 1 (Neurog1) (Figure 3 C) was strongly expressed and cells also stained positive for Brn3a, both highly important transcription factors in stem cells differentiating towards SG lineage (13). No expression of GATA3, a transcription factor of vital importance during inner ear embryogenesis (14) was detected.

Cells also expressed calcium binding protein S-100 (Figure 3 F) which is expressed in SG neurons (15). DAPI staining of cell nuclei showed that many neurons displayed more than one cell nucleus (Figure 3 A, C, E). Olig1 was strongly expressed showing that cells followed an oligodendrocyte lineage as shown by strong Olig1 expression that also to some extent overlapped with Tuj 1 expression.

Confocal microscopy (Figure 4) revealed additional details regarding the differentiation process. Some cells co-expressed Nf-m and S100 during the entire maturation process and many cells co-expressed Nf-m and NTRK2 (Figure 4 B). Nf-m was more expressed in the perinuclear area and hillock region while NTRK2 was more evenly distributed in the cell including axons. Staining for inner ear associated gap junction protein Connexin 30 (Cx30) and Nf-m (Figure 4 C) showed that neural and non-neural-like cells interacted physically.

Discussion

Here, we could explore and monitor the growth and maturation of hNPC:s, using time-lapse video microscopy and immunofluorescent staining.

The hNPC were readily differentiated into SG-like cells under the influence of culture conditions previously developed for primary cultured guinea pig and human SG neurons. Morphologically, we saw consistent similarities between the differentiated hNPC and human sphere-derived neurons. Cells had an ovoid cell soma and smaller nuclear size compared to primary type 1 SG neurons which has a round cell soma and prominent nucleus (16).

TLVM revealed a rapid axonal outgrowth as early as day 7. Axonal sprouting was preceded by physical cell interaction and rosette formations; a phenomenon previously linked to neural progenitor cells. Cells also underwent extensive cell division and apoptosis. Differentiation gave rise to neurons whose axonal processes that fasciculate forming bundles, which stain positive for Tuj 1. The differentiation process (Figure 2) continued even after 18 days suggesting on-going maturation process. TLVM capturing showed that cell behavior was influenced by light. The microscope equipped with an active shutter illuminating cells for only 1 second every time a picture was taken had little effect on cells, but continuously illuminated cells preferred growing on apoptotic cells and migrating out of the lit field. This illumination effect could also be temperature-dependent.

Sprouting was predominantly bi-directed, but tri- and multipolar cells also formed. If transplanted, cell polarity may be influenced by signal cues from remaining scaffold of the auditory nerve with responsiveness relying on level of differentiation in transplanted cells and state of remaining nerve. The intriguing activity in network formations with nuclear translocation and cell migration suggested that network formations could potentially be architected through application of e.g. electric fields or gradients of neurotrophic factors. At which stage these cells can generate action potentials is currently under investigation.

During the maturation process, cells expressed SG-associated markers NTRK2, Tuj 1, Nf-m and GlutR1. Developmental markers Neurog1 and Brn3a both expressed during SG development were also found. GATA3 however, was not verified, suggesting that cells do not strictly follow a SG-specific differentiation route, but although GATA3 has an important function during embryonic development, it may not be necessary for functional integration into a mature auditory nerve. It is also possible that since these cells are selected as neural progenitors their differentiation path is more restricted, also within the neural lineage. Co-expression of Olig1 and Tuj 1 was surprising and suggested that cells had not fully matured into neurons. Cells also expressed Cx30 and to some extent also S-100 and GFAP, suggesting a differentiation along glial cell lineage.

When considering transplantation an important issue is the functional integration of hNPCs connecting to native type 1 hair cells. Previous studies have shown that stem cells implanted into animal cochlea remain in the tissue and differentiate into mature neurons with axonal outgrowth towards organ of Corti, IHC and brain stem (17, 18). This process has even been suggested to result in improved hearing threshold in chemically deafened animals (19). Since stem cells capable of neural differentiation reside in the adult human cochleae (5), it is feasible that humans also express cues necessary for stem cell differentiation and integration. Hair cell-like cells have also been produced *in vitro* (20). However, due to immunological concerns and the potentially tumorigenic nature of stem cells, cell replacement as a clinical tool to treat hearing impairment is still in the future.

Conflict of interest

No conflict of interest.

Acknowledgements

This paper was produced thanks to funding from private donations, the EU, ALF grants, Stiftelsen Tysta Skolan, Hörselskadades Riksförbund and the Research Council (A0290401, A0290402).

References

1. Spoendlin H, Schrott A. The Block Surface Method for Evaluation of Human Inner Ears. *Acta oto-laryngologica*. 1987;104(s436):25-36.
2. Glueckert R, Pfaller K, Kinnefors A, Rask-Andersen H, Schrott-Fischer A. The human spiral ganglion: new insights into ultrastructure, survival rate and implications for cochlear implants. *Audiology & neuro-otology*. 2005 Sep-Oct;10(5):258-73. PubMed PMID: 15925863.
3. Altschuler RA, O'Shea KS, Miller JM. Stem cell transplantation for auditory nerve replacement. *Hearing research*. 2008 8//;242(1-2):110-6.
4. Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. *Nat Med*. 2003 10//print;9(10):1293-9.
5. Rask-Andersen H, Bostrom M, Gerdin B, Kinnefors A, Nyberg G, Engstrand T, et al. Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. *Hearing research*. 2005 May;203(1-2):180-91. PubMed PMID: 15855043.
6. Oshima K, Grimm C, Corrales CE, Senn P, Martinez Monedero R, Géléoc GG, et al. Differential Distribution of Stem Cells in the Auditory and Vestibular Organs of the Inner Ear. *JARO*. 2007 2007/03/01;8(1):18-31. English.
7. Martinez-Monedero R, Edge AS. Stem cells for the replacement of inner ear neurons and hair cells. *The International journal of developmental biology*. 2007;51(6-7):655-61. PubMed PMID: 17891724.
8. Shi F, Corrales CE, Liberman MC, Edge AS. BMP4 induction of sensory neurons from human embryonic stem cells and reinnervation of sensory epithelium. *The European journal of neuroscience*. 2007 Dec;26(11):3016-23. PubMed PMID: 18005071.
9. Pfingst BE, Bowling SA, Colesa DJ, Garadat SN, Raphael Y, Shibata SB, et al. Cochlear infrastructure for electrical hearing. *Hearing research*. 2011 Nov;281(1-2):65-73. PubMed PMID: 21605648. Pubmed Central PMCID: 3208788.
10. Wei D, Jin Z, Järlebark L, Scarfone E, Ulfendahl M. Survival, synaptogenesis, and regeneration of adult mouse spiral ganglion neurons in vitro. *Developmental neurobiology*. 2007;67(1):108-22.
11. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nature biotechnology*. 2001 Dec;19(12):1129-33. PubMed PMID: 11731781.
12. Liu W, Kinnefors A, Boström M, Rask-Andersen H. Expression of TrkB and BDNF in human cochlea—an immunohistochemical study. *Cell and tissue research*. 2011 2011/08/01;345(2):213-21. English.
13. Huang EJ, Liu W, Fritzsche B, Bianchi LM, Reichardt LF, Xiang M. Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons. *Development*. 2001 July 1, 2001;128(13):2421-32.
14. Duncan JS, Lim K-C, Engel JD, Fritzsche B. Limited inner ear morphogenesis and neurosensory development are possible in the absence of GATA3. *International Journal of Developmental Biology*. 2011;55(3):297.
15. Kubo T, Anniko M, Hsu W-J. Image Analysis of the Human Inner Ear. *Acta oto-laryngologica*. 1998;118(1):56-63.
16. Wei liu MB, Helge Rask-Andersen. Expression of peripherin in the pig spiral ganglion – aspects of nerve injury and regeneration. *Acta oto-laryngologica*. 2009;129(6):608-14.
17. Palmgren B, Jiao Y, Novozhilova E, Stupp SI, Olivius P. Survival, migration and differentiation of mouse tau-GFP embryonic stem cells transplanted into the rat auditory nerve. *Experimental Neurology*. 2012 6//;235(2):599-609.
18. Nishimura K, Nakagawa T, Ono K, Ogita H, Sakamoto T, Yamamoto N, et al. Transplantation of mouse induced pluripotent stem cells into the cochlea. *Neuroreport*. 2009 Sep 23;20(14):1250-4. PubMed PMID: 19625987.
19. Chen W, Jongkamonwiwat N, Abbas L, Eshtan SJ, Johnson SL, Kuhn S, et al. Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. *Nature*. 2012 10/11/print;490(7419):278-82.
20. Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S. Mechanosensitive Hair Cell-like Cells from Embryonic and Induced Pluripotent Stem Cells. *Cell*. 2010 5/14;141(4):704-16.

Figure legends:

Figure 1 Phase contrast imaging of hNPC cell differentiated for 18 days making a nerve-associated transport of its nucleus called “nuclear translocation”. Frames are from time-lapse video recording and are taken at 6 time points 10 minutes apart. The nucleus moves in total 43 μm (0.86 $\mu\text{m}/\text{min}$) from first to last picture.

Figure 2 A) A single hNPC-cell video recorded for 1 – 16 hours with phase contrast microscopy between its 18th and 19th day in differentiation media. Red circle indicates cell of interest. B) A phase contrast image of a sphere-derived neuron derived from human cochlea as previously shown (6). In the time-lapse video it is also possible to see how surrounding cells pull and tug on the differentiating cell and appear to guide it into shape.

Figure 3 Fluorescence imaging of hNPC-cells differentiated for 14 days immunostained for A) glutamate receptor GlutR1 (green), B) the BDNF growth factor receptor NTRK2 (green), C) neural differentiation factor Neurogenin 1 (green), D) Neurofilament 160 (Nf-m, green), E) Neuron-specific class III beta-tubulin (Tuj 1), F) calcium binding protein S-100 (green). G) Shows cells in F) in brightfield. Nuclei in A – F are detected by DAPI (blue).

Figure 4 hNPCs differentiated for 14 days imaged with laser confocal microscopy after double staining for neurofilament 160 (Nfm) (red) and A) GFAP (green), B) the BDNF growth factor receptor NTRK2 (green), C) inner ear associated gap junction protein Cx30 (green) and D) NTRK2 (green). Nuclei in A – D are detected by DAPI (blue).

Figure 1

Figure 2

Figure 3

Figure 4