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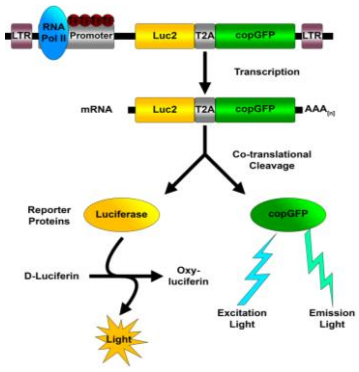
Towards Longitudinal Monitoring of Stem Cells after Transplantation in the Cochlea

Introduction

Stem cell therapy – in order to repair the cochlear nerve by supplementing lost spiral ganglion neurons – in conjunction with a cochlear implant (CI) could be highly beneficial in improving auditory performance of CI users. Hair-follicle-bulge-derived stem cells (HFBSCs) are promising candidates for cell-based inner ear therapy. To understand the interactions of these strategies, it is crucial to monitor *in vivo* the survival of the grafted stem cells as well as the functional status of the auditory nerve in CI-bearing, deafened animals. However, HFBSCs are new in the field of inner ear regeneration, while *in vivo* monitoring of stem cells transplanted in the cochlea of CI-bearing, ouabain-deafened animals has never been performed. Therefore, we have performed several feasibility studies.



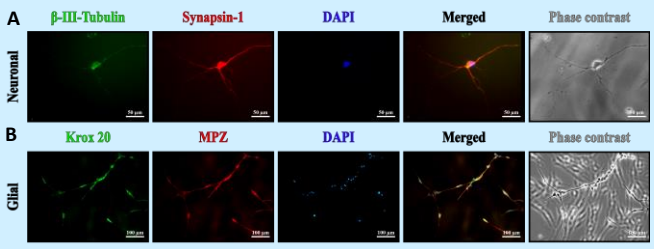
Schematic drawing explaining of basic principles of dual-reporter gene expression in genetically engineered cells. The lentiviral construct is composed of a promotor (either EF1α or DCX) and genes coding for copepod green fluorescent protein (copGFP) and codon-optimized firefly luciferase (Luc2). Both genes are coupled via a T2A-like sequence, which mediates co-translational cleavage and, hence, results in bicistronic expression. The inserts are flanked by long terminal repeats (LTR). TF: transcription factors; RNA Pol II: RNA polymerase II.

Hair Follicle Bulge Stem Cells (HFBSCs)

Advantages
- Multipotent
- Easy to harvest (Hu): plucked from the scalp
- Autologous source
- Neural progenitor
- Neural crest derived
- The hair follicle is an immune tolerant area

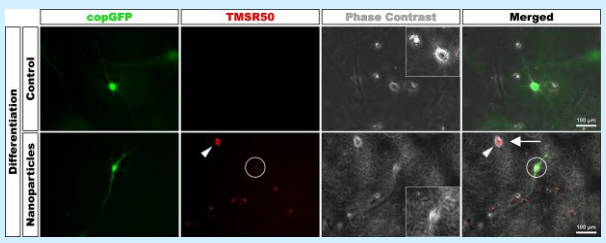
Disadvantage
- Mixed population

Generation of Neurons and Glial Cells from HFBSCs



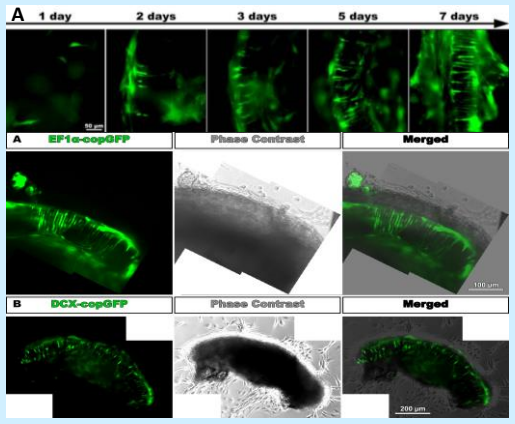
Human HFBSCs can differentiate into mature neurons and glia cells
A. HFBSCs 14 days after neuronal induction. Cells which preferred to grow underneath a cover glass, stained positive for class III β-tubulin (green) as well as synapsin-1 (red). The cell nucleus is stained with DAPI (blue). Merged image reveals co-localization of class III β-tubulin (green) and synapsin-1 (red). The phase-contrast image shows differences in long dendritic projections and thicker axon.
B. HFBSCs 15 days after glial induction. Glial cells underneath the cover glass are positive for the glial markers Krox20 (green) and myelin protein zero (MPZ; red). Nuclei are stained with DAPI (blue). Cells with yellow colour in the merged image are double-stained for MPZ (red) and Krox20 (green). The phase-contrast image depicts the spindle-shaped morphology of the glial cells

Genetic Modification AND Loading with Nanoparticles



***In vitro*: Luc2-copGFP-containing mouse HFBSCs with and without iron-containing TMSR50 nanoparticles differentiate alike**
Different neuron-like cells with elongated, branched projections developed over time (60 hours). In comparison, no differences were observed in the differentiation potential and morphology between loaded and non-loaded Luc2-copGFP-containing HFBSCs. While all cells contain nanoparticles after differentiation (arrowhead), not all cells are transduced (arrow). After differentiation, transduced cells retained the loaded TMSR50 nanoparticles, as indicated by the red fluorescence.

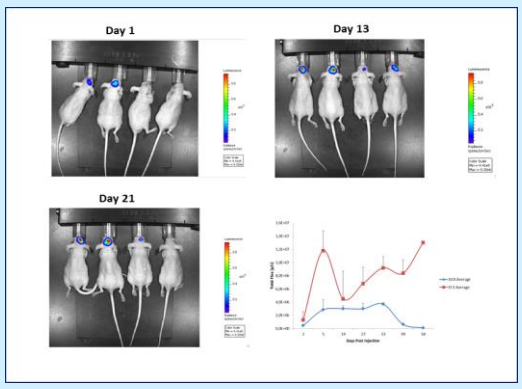
In Vitro: Integration of HFBSCs within Modiolar Explants



A. Distinct fascicular pattern visible within 5 days
One day after seeding, fluorescent mouse HFBSCs migrated under the quarter-turn mouse modiolar explant and began migrating into the explant within the next day (2 days). Over time, more EF1α-Luc2-copGFP HFBSCs settled in the explant (3 days) forming a distinct fascicular pattern after 5 days. Within the next 2 days the pattern enhanced as the number of HFBSCs within the explant increased.

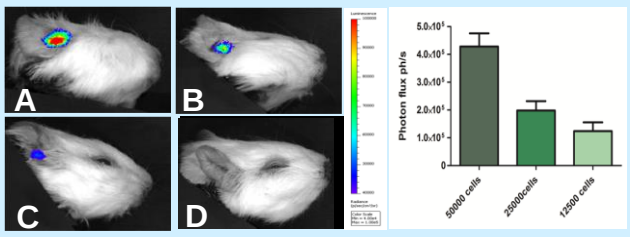
B. EF1α-Luc2-copGFP HFBSCs and DCX-Luc2-copGFP HFBSCs form a similar distinct fascicular pattern.
(A) Set of stitched fluorescence images showing the distinct fascicular pattern that is formed by the copGFP-expressing cells (green). The phase-contrast images show the morphology of the modiolus explant (grey) and the merged set of images reveals the localization of the green fluorescent EF1α-Luc2-copGFP HFBSCs within the explant. **(B)** DCX-Luc2-copGFP HFBSCs formed a similar fascicular pattern of fluorescent cells within the explant (green). The merged image of phase-contrast (grey) and fluorescence images depicts the location of DCX-Luc2-copGFP HFBSCs within the explant.

In Vivo: Neuronal Differentiation of Transduced Stem cells



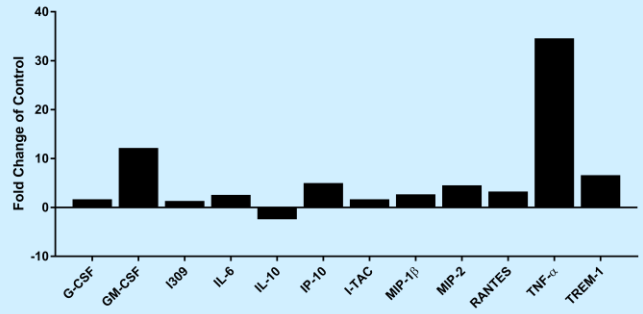
Spontaneous *in vivo* neuronal differentiation of engrafted mouse Luc2-copGFP HFBSCs
Bioluminescence imaging of HFBSCs transduced with the EF1α- Luc2-copGFP construct or DCX-Luc2-copGFP construct in mice with traumatic brain injury.
HFBSC transduced with the EF1α-Luc2-copGFP construct (control) express a highly bioluminescent signal after transplantation, which (after an initial drop) remains high for 58 days.
Bioluminescence imaging of DCX-Luc2-copGFP-HFBSCs revealed that these cells emit less light compared to the control. The signal from DCX-Luc2-copGFP HFBSCs remains low and the signal drops significantly between 33 days and 58 days. Bioluminescence is expressed as photon flux (ph/s: photons/second).

Feasibility of Visibility



HFBSCs can quantitatively be detected by means of bioluminescence imaging
Cell dilution series to determine the amount of transduced HFBSCs (containing the EF1α-Luc2-copGFP construct) needed to reach signal threshold for bioluminescence imaging.
Different amounts of cells were injected into the modiolus of the basal cochlear turn in the right ear. A bright bioluminescent signal was seen after injection of $5x10^4$ cells (*A*). Considerably lower signals were detected after injection of $2.5x10^4$ cells (*B*) and $1.25x10^4$ cells (*C*). Injection of $0.5x10^4$ cells (*D*) did not result in a detectable bioluminescent signal. ***(E)*** Quantitative measurement of the bioluminescent signal shows that the threshold for bioluminescence imaging lies between $0.5x10^4$ cells and $1.25x10^4$ cells. Bioluminescence is expressed as photon flux (ph/s: photons/second).

HFBSCs Immune Tolerance



HFBSC response to TNF-α:

- all factors used in this assay that recruit immune cells, such as GM-CSF or IP-10, increase 5-fold

- however, HFBSCs also produce more IL-6 which inhibits differentiation of dendritic cells and T-cell proliferation

- HFBSCs cells secrete high concentrations of TIMP-1, which was reported to promote oligodendrogenesis and myelination in general

- the results indicate that HFBSCs are more appropriate for SC therapy of brain injury than mesenchymal stem cells

Ouabain Does Not Induce Selective Degeneration of Type-I Spiral Ganglion Cells in Guinea Pigs

Group	Treatment	n	ABR Threshold	IHC	OHC	SGC
1	10 mM ouabain	2	elevated	normal	loss (n=2)	no loss (n=1) / loss (n=1)
2	1 mM ouabain	8	normal (n=2)	normal	no loss (n=2)	no loss (n=2)
			elevated (n=6)	normal	no loss (n=2)	no loss (n=2)
					loss (n=4)	no loss (n=2) / loss (n=2)
3	0.1 mM ouabain	4	normal	normal	normal	normal
4	0.01 mM ouabain	4	normal	normal	normal	normal
5	PBS	2	normal	normal	normal	normal

Conclusions

- **HFBSCs from human plucked hair can differentiate into neurons and glial cells**
- **HFBSCs tolerate transduction with lentiviral constructs containing Luc2 and copGFP and subsequent loading with iron-containing nano particles**
- **HFBSCs integrate within modiolar explants and differentiate into young neurons**
- **Neuronal differentiation of transduced HFBSCs can be monitored longitudinally *in vivo***
- **Live transduced stem cells can be visualized in the guinea pig cochlea**
- **HFBSCs show moderate immunoreactivity to TNF-α, but produce also immune modulatory IL-6**

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Acknowledgments

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