Multimodal visualization of adult stem cells in inner ear and brain pathology

Schomann, T.

Chapter 8

General discussion

Summary

The aim of this thesis was to investigate the feasibility of multimodal visualization techniques to observe adult stem cells, in particular HFBSCs, in the living animal. Due to the novelty of HFBSCs in the field of inner ear research, a series of proof-of-principle experiments have been undertaken to investigate if these cells can undergo neuronal differentiation, tolerate genetic modification with lentiviral constructs containing the genes coding for reporter proteins, and tolerate subsequent loading with nanoparticles *in vitro* (Chapters 2 and 3). In addition, it was of importance to examine if HFBSCs do integrate into modiolar tissue and if they can be visualized in the cochlea of the guinea pig (Chapters 4 and 5). Lastly, we performed *in vivo* studies to investigate the ototoxic effect of ouabain in guinea pigs and the behavior of HFBSCs in mice with traumatic brain injury (Chapters 6 and 7). The main outcomes of this research are summarized in the first part of this chapter to give a general overview. In addition, major issues that might have influenced those outcomes will be discussed. The second part will solely focus on future research.

Main outcomes and respective implications

Objective 1: The isolation, expansion and neural differentiation of stem cells from human plucked hair.

In the study described in Chapter 2, the human hair follicle was investigated as a source for autologous SCs [1]. We were able to obtain hair follicles with an easy, minimally invasive technique that is almost painless for the patient. From the harvested hair follicles HFBSCs were isolated, which still possess the immunophenotype of NCSCs, e.g., nestin-positive, SOX9-positive, but SOX10-negative [2], and neural and glial differentiation potential after isolation, expansion and cryopreservation.

However, the isolated and cultured HFBSCs remain a heterogenous population consisting of various cell types. The exact composition of the HFBSC cultures and the various cell types contaminating the cultures, such as fibroblasts, are still not exactly defined. Thus, to obtain a (more) purified stem cell culture, unwanted cells need to be removed. In our studies, this was achieved by “selection at the gate”, during which cultures with obvious unwanted cell outgrowth from the hair follicle,

such as keratinocytes and fibroblasts, were immediately discarded.

A second and next step for purification could be the cultivation in serum-free medium under sphere-forming conditions to eliminate fibroblasts, which require a combination of growth factors and an adhering matrix to survive [3, 4]. Nevertheless, it has to be taken into consideration that the process of purification and multiplication of stem cells derived from adult tissues each have their own specific problems. It could be, for instance, that transdifferentiation is required to achieve the right dermal lineage or that spontaneous differentiation into an unwanted phenotype may play a role.

Regarding induced pluripotent stem cells (iPSCs) as a source for homologous stem cells, the genetic manipulation to reprogram somatic cells into pluripotent stem cells is still not safe and is currently under debate with respect to epigenetic changes that may occur during the process of reprogramming [5, 6].

Therefore, HFBSCs which can be harvested almost painlessly from the patient and which are multipotent in the ectodermal lineage may be a good type of stem cell to be used parallel to iPSCs in neural regeneration studies. In this way, the most favorable source for cell-based regenerative medicine can be evaluated.

Objective 2: Rule out the possible cytotoxic effects of lentiviral transduction and subsequent loading with nanoparticles on cell viability and proliferation of HFBSCs *in vitro*.

We isolated HFBSCs from hair follicles of mouse whisker pads and investigated the possible cytotoxic effects of different labeling techniques in Chapter 3 [7]. HFBSCs were stably transduced with a lentiviral construct containing genes coding for Luc2 and copGFP, enabling visualization by means of FLI and BLI, respectively. After loading HFBSCs with SPIO nanoparticles, we were able to image them by means of MRI. Finally, we could rule out that the applied techniques had any adverse effect upon proliferation and differentiation capacity of HFBSCs.

During this experiment, silica-coated NEO-STEM™ TMSR50 nanoparticles, which contain a magnetic core and a fluorescent dye, were used. Thus, TMSR50 could enable visualization of loaded cells by means of MRI *in vivo* and immunofluorescent

analysis of histological sections. This quite detailed study was necessary to prove that multimodal (in-depth) visualization of HFBSCs is possible, which opens the way to use this technique in animals larger than mice, e.g., guinea pigs.

However, in a later study (Chapter 6) we also used the FDA-approved SPIO suspension ferumoxytol, which is not cytotoxic [8]. We were able to show retention of ferumoxytol in HFBSCs for at least 48 days *in vivo*. Ferumoxytol does not allow FLI and, thus, it cannot be visualized together with specific (cell) markers after immunofluorescent staining. Therefore, after successful immunohistochemistry and FLI, we applied Perls' Prussian blue staining, followed by DAB intensification, on the same tissue sections to visualize ferumoxytol within the cells. Finally, we merged the images obtained from the immunofluorescent staining with the images showing staining for ferumoxytol (and other iron oxide) within the tissue sections revealing retention of ferumoxytol by HFBSCs.

This elaborate procedure would not have been necessary in case of SPIO nanoparticles with a silica coating that contains a fluorescent dye, such as NEO-STEM™ TMSR50. In addition, such a dye would enable *in vivo* visualization when using one of the near-infrared fluorescence imaging windows, in which light absorption and autofluorescence by living tissue is minimal, thus, resulting in an improved penetration depth. However, NEO-STEM™ TMSR50 and other NEO-STEM™ variants as well as NEO-LIVE™, which contains a NIRF dye, are not commercially available anymore. This led to the use of ferumoxytol in Chapters 5 and 7.

Objective 3: Investigate migration, integration, and neural differentiation potential of HFBSCs in co-culture with modiolus explants from adult mice.

HFBSCs were cultured together with modiolus explants to investigate if they would integrate into the tissue and subsequently differentiate into neural (progenitor) cells [9]. In Chapter 4, we showed that HFBSCs migrate towards and into explants of modiolus tissue. The cells incorporated into the explant and formed a distinct fascicular pattern. Depending on this micro-environment, cells adapted a neuronal phenotype as shown with DCX-copGFP-transduced HFBSCs and immunofluorescence.

An improved and/or more mature pattern might have been obtained after a prolonged period, but during co-culture the modiolus tissue tended to be overgrown by cells. In addition, the explant deteriorated with time. Furthermore, image quality could be improved by employing a confocal laser scanning microscope instead of regular fluorescence microscopy. Nevertheless, we showed that HFBSCs are able to differentiate towards a neuronal phenotype when the cells are in contact with modiolar tissue, which makes them advantageous for future cell-based auditory nerve therapy.

Objective 4: The feasibility of BLI for the visualization of transduced cells after engraftment in the intact guinea pig cochlea.

The guinea pig is a commonly used animal model in the field of inner ear research. However, it is rarely used in the field of molecular imaging. In order to investigate the therapeutic potential of SCs in the inner ear it is necessary to combine the field of hearing research with the field of molecular imaging. Therefore, we investigated if it is possible to image transduced cells by means of BLI after transplantation in the inner ear of guinea pigs. This is particularly challenging since the cochlea is embedded within the auditory bulla consisting of compact bone with a high mineral density, which could block signal detection during molecular optical imaging. Our results in Chapter 5 show that we obtained a distinct, quantifiable bioluminescent signal, which emanates from the external auditory meatus of the guinea pig cadavers.

In light of the 3Rs guiding principles, which specify the replacement, reduction, and refinement of the use of animals in research, we decided to use the cadavers of 26 guinea pigs from non-related experiments for this feasibility study to reduce the number of live animals in our experiments. In this *ex vivo* study, D-luciferin was added directly to the HFBSC suspension, which then was injected into several sites in the cochlea. However, for *in vivo* studies the application of D-luciferin has to be adapted, since direct application to the HFBSCs is not possible without an entryway, such as a tube or a minipump [10, 11]. Also, the lack of a tail (vein) complicates and significantly limits intravenous injections of substances in the guinea pig compared to mice and rats. Nonetheless, D-luciferin can be administered intraperitoneally, which should result in sufficient uptake for successful BLI. In addition, signal intensity could be improved by substitution of D-luciferin with the luciferin analogue AkaLumine-

HCl, which – aside from shifting the emitted light towards a near-infrared spectrum wavelength (λ_{max}: 677 nm) – generates a maximal signal at very low concentrations [12]. Furthermore, *in vivo* imaging could significantly enhanced by using the AkaBLI system, which combines AkaLumine-HCl with the newly developed Akaluc [13]. Akaluc is a mutated firefly luciferase that catalyzed AkaLumine-HCl ~7x more effective than wildtype firefly luciferase and emits light in the near-infrared spectrum (λ_{max}: 650 nm). In the near future, we hope to assess this in more detail in future *in vivo* studies.

Objective 5: Determine if the ototoxic drug ouabain results in selective type-I SGN degeneration in guinea pigs.

Ouabain has been reported to selectively destroy type-I SGNs in the cochlea of several rodent species. However, two conflicting papers about the possible effect of ouabain upon the guinea pig cochlea made us to re-investigate this deafening protocol. In Chapter 6 we describe our results and conclude that round window application of ouabain results in a rapid loss of type-I and type-II SGNs as well as OHCs in the guinea pig. In addition, affected aABRs do not implicate loss of SGNs. Thus, we conclude that round window application of ouabain is not limited to degeneration of type-I SGNs and, as a consequence, it is not a reliable model to investigate cell-based auditory nerve therapy in the guinea pig.

This non-selectivity together with a high variability presented an unexpected challenge, since literature on (most) rodents and cats was consistent. Also a recent study in guinea pigs underlined this consistency [14], which was in contrast to an older publication [15]. Since we expected an unambiguous effect of ouabain, this unforeseen situation and a limited number of animals resulted in small group sizes for two groups. We decided to investigate a 10-fold higher concentration of ouabain than initially intended since we expected unambiguous results. Due to a limited number of animals, these animals had to be taken from another group, which resulted in the small group sizes. However, this shortcoming in group size could be circumvented by a statistical analysis. Nevertheless, the effect of ouabain on the SGNs of the auditory nerve and the hair cells in the guinea pig cochlea is intriguing and should be investigated more closely in order to understand the exact mechanism of action.

Objective 6: Multimodality imaging of HFBSCs in a mouse model of traumatic brain injury.

In Chapter 7 we investigated the *in vivo* behavior of labeled HFBSCs in a traumatic brain injury (TBI) mouse model. To achieve this, we first transduced the cells with genes coding for reporter proteins and subsequently loaded them with SPIO ferumoxytol nanoparticles. HFBSCs were able to take up ferumoxytol nanoparticles and differentiate after genetic manipulation using a viral vector to introduce one or more reporter molecules. Finally, labeled HFSBCs were transplanted in mice with TBI and followed by means of BLI and MRI over the course of 49 days and 58 days, respectively. BLI data demonstrated survival and neuronal differentiation of HFBSCs. MRI data showed their exact location within the brain. Immunofluorescent staining underlined the differentiation towards a neuronal lineage after brain transplantation. This feasibility study is a first indication that HFBSCs might be used for cell-based therapy in TBI. Since isolation, expansion and neural differentiation of SCs from hair follicle is possible, these cells are a promising source for autologous cell-based therapy in neuronal degenerative disorders.

Future research

Future experiments have to be undertaken to visualize HFBSCs in the inner ear of guinea pigs in order to observe potential regeneration of the auditory nerve. To improve visualization of transplanted cells *in vivo*, the AkaBLI system could be employed. Due to a shift towards a near-infrared wavelength and an enhanced bioluminescent signal of the two components of this system, i.e. AkaLumine-HCl and Akaluc, cells can also be imaged in deep tissue.

Regarding the deafening of the guinea pigs, systemic administration of kanamycin and furosemide could be applied to observe the behavior of SCs in a model of acute SNHL. In this model no hair cells will be present, which is similar to deaf patients and to our *in vitro* experiments (Chapter 4), and could be used to study the regeneration of damaged tissue by HFBSCs and/or possibly their differentiation into neuronal or glial cells. This model of kanamycin/furosemide-induced deafness could be useful to study SGN regeneration in CI-implanted animals, since a CI circumvents the hair cells, which could present an advance towards clinical application.

The necessary steps to investigate the feasibility of SCs in such a model were performed in this thesis by stable transduction with reporter molecules that enable *in vivo* visualization, tracking of HFBSCs loaded with SPIO nanoparticles *in vivo* and *ex vivo*, and successful integration into tissue *in vitro* and *in vivo*.

In addition, a chemically defined, xeno-free cell culture method should be investigated to better simulate culture conditions of future regenerative therapies and prevent immune response reactions. Severe immune response reactions can be triggered by xenogeneic supplements, such as fetal bovine serum and (mouse) cell feeder layers, which would then lead to rejection of the transplant [16, 17]. Transplant rejection by the immune system of the host organism, a so-called graft-versus-host disease, would lead to inflammation, organ damage, and major health problems. This response of the immune system could also be induced by the transplantation of cells that were cultured in medium containing xenogeneic components. In this case the graft-versus-host reaction is triggered by xenogeneic antigens from the animal serum and/or feeder cells, which are presented to immune system cells, such as T cells, by the major histocompatibility complex class II on the membrane of the cells. Currently, xeno-free alternatives for media are available, which should facilitate the cultivation of stem cells under xeno-free conditions.

In this context, it is worth considering the use of iPSCs, which are autologous and can be cultured towards a defined cellular phenotype. However, although iPSCs are available from stock, the achievement of an iPSC population from each patient remains a challenge. Moreover, on their way from bench to bedside, the potential of iPSCs for regenerative therapy needs to be further investigated in animal models, such as the guinea pig in case of hearing research. Depending on which SC source gives better results for cell-based therapy, the transplantation of patient-derived, possibly pre-differentiated, iPSCs or ectodermal SCs into deafened guinea pigs during cochlear implantation may allow electrical stimulation of the SCs and conceivably facilitate neuronal differentiation, as shown in previous studies [18-20]. However, the optimal source for stem cell-based regenerative therapy needs yet to be evaluated.

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