From stem cells to Schwann cells
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Chapter 7

Summary and general discussion
SUMMARY AND GENERAL DISCUSSION

Stem cells continue to be a prominent subject in biomedical research, as more and more innovative stem cell-based technologies become available, and clinical application of stem cells for the treatment of a wide range of human disorders is becoming feasible. In this dissertation, diverse aspects of (neural) stem cell physiology and differentiation have been addressed that are relevant for their potential clinical application. The stem cells that have been studied in this dissertation are induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), neural crest stem cells (NCSCs), as well as a cell type ultimately generated by NCSCs, i.e. Schwann cells (SCs).

The specific aims of the various parts of this dissertation were:
1) to explore the use of iPSCs and NCSCs for translational purposes, in particular focused on the generation and potential clinical application of (autologous) SCs for peripheral nerve restoration;
2) to develop an in vivo model for examining the fate and efficacy of implanted SCs for the treatment of peripheral nerve injury;
3) to characterize and describe the distribution of a yet functionally undefined intracellular transport protein, UGS148, in the mouse brain; the gene encoding for this protein has been previously shown to be one of the most prominently expressed genes in embryonic NSCs.

In Chapter 1, we presented an extensive introduction addressing the background of the above research questions. In the subsequent 3 chapters, NCSCs and iPSCs were the main focus of research. The choice of cell type depends on its accessibility and reprogrammability, and is of major importance for the generation of iPSCs. Accessible sources of donor cells are keratinocytes, gingival fibroblasts, adipose stem cells, peripheral blood mononuclear cells, and epithelial-like cells from urine samples. In particular for young patients urine samples and pulpal cells from deciduous tooth are a safe and undisputed source. Another potential source are adult NCSCs, which are post-migratory cells of neural crest origin. They persist in isolated populations in several craniofacial structures in the adult vertebrates, and as such are a (relatively) accessible source of stem cells for transplantation studies and tissue engineering.

In Chapter 2, we showed that two types of NCSCs, i.e. hair follicle neural crest stem cells (HFNCSCs) and enteric neural crest stem cells (ENSCS), express the pluripotency genes Oct4, Sox2, and Nanog. These transcription factors, together with Klf4 and c-Myc, have been shown essential for inducing the reprogramming of somatic cells into iPSCs. Reduction of the number of exogenous reprogramming factors has been considered a strategy to simplify a standardized and clinically safe iPSC reprogramming procedure. Therefore, we argued that the endogenous expression of three major reprogramming transcription factors might substitute for exogenous factors and thus facilitate the reprogramming of NCSCs into iPSCs.

We described the generation of iPSCs from HFNCSCs isolated from the mouse whisker pad. Our results showed that these cells still do require a full set of reprogramming factors for iPSC induction: HFNCSC-derived iPSCs were created with efficiencies similar to fibroblasts. We concluded that high endogenous levels of pluripotency factors are no guarantee for facilitated induction of pluripotency in hair follicle NCSGs; it appears they do not significantly affect the specific combinations and transient concentrations of exogenous iPSC transcription factors.

iPSCs provide an unprecedented, theoretically unlimited source of autologous cells for cell based therapies. They have been differentiated in vitro into a range of neural cell types relevant for the potential treatment of CNS neurodegenerative diseases such as MS, Parkinson’s disease, or amyotrophic lateral sclerosis. In Chapter 3 we showed that iPSCs can be effectively differentiated into SCs, the myelinating and supportive cells for peripheral axons. We used fibroblasts from CNPase-GFP transgenic mice for the generation of iPSCs and demonstrated that they could be specifically differentiated into SCs without the use of stromal cells. Our specific differentiation protocol and the CNPase-GFP reporter system allowed us to generate a highly enriched population of mouse SCs. These iPSC-derived SCs could be used for future transplantation purposes.

An overview of current strategies and methods to generate SCs from pluripotent stem cells was presented in Chapter 4. SCs can be differentiated from ES cells and iPSCs by recapitulating their ontogeny, with neural crest formation as an intermediate step. During embryonic development, SC differentiation from NCSCs requires positive regulators of myelination, which are also mandatory for the in vitro differentiation of myelogenic SCs. The iPSC-derived SCs may be used for cell grafting, e.g. in the treatment of peripheral nerve injury or even CNS diseases, or as a tool to study SC-associated peripheral nervous system disorders.

Chapter 5 focused on peripheral nerve injury and the potential application of (eventually iPSC-derived) SCs in its treatment. Nerve guides coated with SCs have proven to promote axonal regeneration in peripheral nerve lesions. Transplantation of SCs into a nerve lesion may enhance recovery by means of remyelination and/or the secretion of neurotrophic factors. To examine the exact mechanisms that support axonal regeneration, tracking techniques might give valuable information. Labeling of SCs with chemical markers, fluorescent dyes, or quantum dots can be used for tracking implanted cells but do not give direct information of ongoing biological dynamics and cell behavior. Bioluminescent imaging (BLI) can track the in vivo behavior of the transplanted cells and thus might give more insight in the sequential steps of SC-sustained axonal regeneration. We examined the applicability of BLI for in vivo monitoring of the fate of SCs in implanted nerve guides. Rat SCs were transfected with the firefly luciferase (Fluc) gene and seeded in vitro in nerve guides that were subsequently implanted subcutaneously in rats. In vivo bioluminescence of the transfected SCs (Fluc-SCs) was assessed with a BLI system. Scans were validated ex vivo, using immunohistochemistry and electron microscopy. We could demonstrate that BLI allows longitudinal in vivo monitoring of Fluc-SCs, given that proper access of luciferin to the cells is assured. By coupling the luciferase reporter gene to promoters of genes encoding for proteins involved in peripheral nerve repair, BLI might enable in vivo evaluation of the implanted SCs and their biological contribution during different stages of axonal bridging.

The last part of this dissertation comprised more fundamental research and focused on recent developments in the field of adult neurogenesis and tanyocyte physiology. In Chapter 6 we introduced UGS148, an intracellular transport protein, and described its expression, distribution and potential...
function in neurogenic cells in the mouse brain. UGS148 is encoded by the RIKEN cDNA63330403K07 gene that has been shown to be one of the most prominently and characteristically expressed genes in embryonic NSCs and other multipotent stem cells. We generated an antibody against a specific epitope of this protein and used the antibody to study the expression of UGS148 with Western Blot and immunohistochemistry along with its mRNA expression using qPCR and in situ hybridization. We confirmed that UGS148 was highly expressed in (cultured) embryonic NSCs as well as, though at a low level, in adult NSCs located in the SVZ and the hippocampal dentate gyrus. Remarkably, we found high expression of UGS148 in the hypothalamic tanycytes of the adult mouse brain. Tanycytes of the third ventricle have been identified as having neurogenic capacity. The molecular function of UGS148 remains elusive. Based on its molecular structure and amino acid sequence, UGS148 contains several conserved domains such as a transmembrane region, domains associated with intracellular protein transport and sorting as well as with shuttling proteins between the ER and the nucleus or the ER and the cell membrane. Using electron microscopy, we confirmed previous observations that UGS148 is indeed also localized in the ER. In summary, UGS148 is a protein involved in intracellular sorting, trafficking and exocytosis of proteins, and/or membrane incorporation of receptor units. Our in vitro experiments suggested an indirect role for UGS148 in neurogenesis, in particular in the regulation of proliferation, likely via modifying the rate of membrane incorporation of growth factor receptor units, e.g. for FGF2. Apart from a role in neurogenesis, the typical high expression of UGS148 in the hypothalamic tanycytes may imply a function of UGS148 related to food intake and homeostasis, presumably also via regulating the proper incorporation of various types of receptor units for relevant hormones and cytokines. Mechanistic studies, such as in vivo silencing of the UGS148 gene, and generation of a conditional knockout mouse model, will be necessary to obtain more detailed information about the function of UGS148. Our data add up to recent studies which have shown that the adult hypothalamus contains cells with stem cell characteristics, which can generate neurons, which in turn play a role in the control of metabolism and energy balance. Future studies need to unravel how hypothalamic tanycytes, being cells with stem cell characteristics, which can generate neurons, which in turn play a role in the control of metabolism and energy balance. Future studies need to unravel how hypothalamic tanycytes, being cells with stem cell characteristics, which can generate neurons, might mediate physiological effects via their capacity to act as a NSC. 

Autologous iPSCs offer the promise of cell replacement therapies that do not require the use of immunosuppressive drugs to prevent immunological rejection. Targeted gene-repair strategies, such as homologous recombination using the CRISPR/Cas9 technology, can be used for repairing genetic defects in patient-derived iPSCs. Several studies on neurological, hematological, and metabolic disease models have given a proof of principle that human disease can be treated by iPSC technology. Essential for effective and safe clinical application of iPSCs will be 1) the drastic shortening of the generation and differentiation procedures, and 2) annihilation of the risks for iPSC-related complications, e.g. tumorigenesis.

Human iPSCs typically take 1 month to give rise to first-generation colonies, regardless of the reprogramming technique used. Characterization of the iPSCs will take several days to weeks. This process is still complicated by the fact that there is no consensus on the protocols to create the most reliable and safe clinically-grade iPSC lines, and the criteria to define them. Dependent on the gene-therapies required and the duration of cell-specific differentiation protocols, several weeks might be needed to subsequently differentiate the desired target cells. The final iPSC-derived cells may be of varying quality, and therefore again need extensive characterization before they can be applied clinically. Altogether, the entire process from source cell to target cell may take several months; iPSC cells may therefore intrinsically not be suitable for the treatment of acute or life-threatening conditions. The creation of iPSC HLA-haplotype banks, which will make matching HLA-specific iPSC-lines readily available for treatment of patients, may solve part of this problem, but does not address the issue of prolonged differentiation times.

Another major obstacle for therapeutic use are potential genomic modifications caused by integration of DNA viruses into the host genome, increasing the risk of insertional mutagenesis. Cre-mediated excision of transgene sequences in iPSCs created with retrovirus or lentivirus is elaborate and does not rule out the possibility of small amounts of remaining sequences, and might thus not be the proper way. Several alternative methods are being developed to enable generation of zero-footprint iPSCs without residual transgene sequences. These methods make use of cytoplasmic Sendai virus, episomal vectors, direct mRNA or protein expression of reprogramming factors, direct miRNA transfection, piggyBac transposons, or chemical reprogramming by means of small molecules. Drawbacks of most of these techniques are the extensive generation times, labor-intensiveness, low efficiencies, and special technical requirements needed. Episomal vectors and Sendai virus, having the most optimal efficiencies, are currently considered to be the most feasible techniques for the generation of clinically-grade iPSCs. Another important criteria for safe iPSCs is the use of xeno-free culture systems during the differentiation process. Non-human feeder cells and the use of factors produced from or in contact with animal products pose the risk of introduction of xenogenic pathogens or antigens in the patient, and should be avoided. Methods such as chemically defined medium (mTeSR) combined with Matrigel can substitute for the use of feeder cells. iPSC technology might offer promising possibilities in the field of peripheral nerve regeneration. Also, patient-derived iPSCs would offer unique possibilities to gain mechanistic insight into Schwann cell-related diseases. The ultimate proof to show the clinical potential of iPSC-derived SCs will have to be provided in clinical trials in patients with PNS or CNS diseases or injuries. However, as long as clinically-grade iPSCs are not available, autologous nerve grafting will likely continue to remain the gold standard for peripheral nerve regeneration.