Cellular therapy after spinal cord injury using neural progenitor cells

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Chapter 7

Conclusions and general discussion

In this thesis, the possibilities and limitations of cell-based therapies after spinal cord injury are explored. Particularly, the potential of adult derived neural progenitor cell (NPC) grafts to function as a permissive Substrate for axonal regeneration was investigated. Therefore, NPC were isolated from the adult rat spinal cord, propagated and were grafted to animals that received an acute spinal cord injury. Furthermore, techniques that are essential for the application of cell-based therapy were developed, including the preparation of graft material, *ex vivo gene therapy* and the development of non-invasive *in vivo* imaging techniques.

1. GRAFT PROPERTIES ESSENTIAL FOR REGENERATION OF THE INJURED SPINAL CORD

The main rationale for the use of NPC grafts in the injured spinal cord is the ability of NPC to replace lost spinal cord cells with cells that originally are present within the central nervous system (CNS). Since the adult CNS milieu however does not allow substantial regeneration, a simple substitution of CNS cells at the tissue defect that typically develops due to the injury most likely is not sufficient. The objective therefore is to mimic the axonal outgrowth pattern of the developing CNS, by transplanting NPC grafts [1,2]. In order to reach this goal, grafted NPC first must be able to: 1) replace the lesion cyst that typically develops after the wire knife spinal cord injury, 2) offer trophic support and correct guidance cues to the regenerating axons in analogy to Schwann cell function in the injured peripheral nervous system and 3) replace lost spinal cord cells by differentiating into mature CNS cells that become functionally integrated into the spinal cord cytoarchitecture.

1.1 Lesion cyst replacement

The replacement of the lesion defect that develops after spinal cord injury (SCI) represents an important prerequisite to allow substantial axonal regeneration in the injured spinal cord. Since the used wire knife model of SCI induces a single cystic lesion cavity without inducing massive infiltration of peripheral cells, this model is very well suited to study the capacity of various cell grafts to restore lost spinal cord tissue. Although NPC survive transplantation into the acutely lesioned spinal cord, grafts of NPC alone are not able to replace the lesion defect since the NPC migrate away from the graft site (*Chapter 2*). Until so far, only the co-transplantation of NPC with fibroblasts allowed lesion cavity repair in combination with the successful delivery of NPC at the lesion site (*Chapter 3*). Experiments in which Schwann cells replaced the fibroblasts showed that although lesion cyst could be restored, the co-grafted NPC migrated into the spared spinal cord parenchyma, away from the Schwann cells that resided at the graft site (*Chapter 4b*).

1.2 Trophic- and guidance support for axonal regeneration by NPC

In the NPC-fibroblast co-grafted animals, regenerative sprouting of lesioned axons was induced when compared to

animals that received grafts of fibroblasts alone. Moreover, corticospinal tract (CST) regeneration was significantly induced by 170% in NPC-fibroblast co-grafted animals when compared to animals that received grafts of fibroblasts only (*Chapter 3*). The ability of NPC to induce regenerative sprouting of lesioned CST axons without the need of additional application of neurotrophic factors represents a unique property of NPC. This has only been unambiguously shown before with the grafts of olfactory ensheating cells [3, 4]. It has been reported that neural stem cells derived from the neonatal mouse cerebellum secrete by themselves various neurotrophic factors including nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor [5]. Whether the unmodified adult derived NPC that are used in the presented studies also secrete neurotrophic factors has not been investigated so far.

Regenerative sprouting of lesioned CST axons within the graft area of animals that were co-grafted with NPC and fibroblasts was observed along glial fibrillary acidic protein (GFAP) expressing grafted NPC (*Chapter 3*). These GFAP expressing cells are notably distinctive to the reactive astroglia that normally can be identified at the lesion site. First, both in the intact and the injured adult spinal cord, astrocytes mainly display a typical stellate morphology. The GFAP expressing cells that favor regenerative sprouting of lesioned axons however mostly appear as cells that possess a more elongated shape. Furthermore, a large proportion of the GFAP expressing grafted cells can be co-labeled with antibodies against brain lipid-binding protein (BLBP). BLBP is a marker for radial glial cells and immature astrocytes or astrocyte precursors [6]. We therefore postulate that the grafted NPC that differentiate into astroglila maintain an immature phenotype while serving as a scaffold for sprouting axons. This hypothesis is further validated by the fact that adult astrocytes are shown to be inhibitory to axonal regeneration [7, 8] It remains unclear whether all differentiating NPC are able to function as a permissive Substrate for CNS axon regeneration. Grafts consisting of homogenous populations of NPC derived cells could allow the identification of specific NPC phenotypes that augment axonal regeneration. Therefore further experiments need to be conducted that allow manipulation of NPC fate.

It remains unclear whether the co-grafted fibroblasts alter NPC function to stimulate outgrowth, or whether the fibroblasts are solely needed to retain the co-grafted NPC at the lesion site. In the peripheral nerve injuries, fibroblasts have been described to induce the production of extracellular matrix (ECM) and basal lamina components by Schwann cells [9]. Furthermore, the co-culture experiments of GFP expressing NPC with fibroblasts at least suggest that the morphology of the differentiating GFP expressing NPC is changed towards a more bipolar shape, a morphological phenotype that can be spatially associated with regenerating axons *in vivo* (*Chapter 3*).

The invasion of fibroblasts from the me-

ninges has been associated in the literature with astroglial scarring, a detrimental event for axonal regeneration [10]. Nevertheless, the presence of fibroblasts in co-grafts does not appear to prevent axonal regeneration in these experiments. Co-cultured fibroblasts do not induce the formation of a glia limitans like structure *in vitro*, which is in contrast to Schwann cells (*Chapter 3, 4b*). Therefore, the effect of the presence of fibroblasts within the lesioned area on axonal regeneration needs to be further investigated e.g. by grafting a NPC filled artificial matrix. If such a NPC-loaded matrix allows substantially more regenerative sprouting than the matrix only, the notion that undifferentiated NPC per se are capable of inducing regenerative sprouting of lesioned CNS axons would be further strengthened [11].

The direction of the observed regenerative sprouting of axons into the graft occurs randomized, which reduces the likelihood of axons to completely bridge the graft and reenter the spinal cord parenchyma distal to the lesion site and reinnervate target structures. Moreover, since the cytoarchitecture of the spinal cord axon projections is highly organized, oriented sprouting of lesioned axons is essential if the native organization of the spinal cord axon projections have to be reinstalled. Therefore, it is most likely that introducing directive cues are an important aspect in order to allow sprouting axons to align to their correct targets. Different methods have been proposed to induce a more oriented axonal sprouting, including the induction of a chemoattractive neurotrophic factor gradient or by transplanting an anisotropic matrix that consists of oriented pores that bridges the lesion site [12, 13]. Especially the use of an oriented anisotropic guidance matrix is of great interest, since it eventually also could replace the co-grafted fibroblast as a Substrate to retain the grafted NPC at the lesion site.

1.3 Appropriate replacement of lost spinal cord cells

In order to study fate and function of the used NPC upon transplantation, grafted cells need to be identified *in vivo* using a method that allows the reliable co-localization with phenotypical markers. Throughout this thesis, NPC are prelabeled using the thymidine analogue Bromodeoxyuridine (BrdU). BrdU is incorporated in the DNA of proliferating cells prior to transplantation, which later can be visualized using immunocytochemical methods [14]. Although BrdU prelabeling represents a reliable method for the identification of grafted cells, it is a far from ideal technique since the label is restricted to the cell nucleus. Therefore, to reliably co-localize the BrdU label with phenotypical markers that mostly are located in the cytoplasm can be problematic (*Chapter 2*). Furthermore, BrdU immunohistochemistry is preceded by an antigen retrieval protocol that is not compatible with immunohistochemical myelin stainings, which complicates the identification of grafted NPC that adapted to an oligodenroglial phenotype (*Chapter 4b*). A possible solution for this problem is the labeling of the grafted cells with a genetic marker. Therefore, it was tested

whether NPC could be genetically modified to express the cytoplasmatic marker green fluorescent protein (GFP) using various retro- and lentiviral vectors. Although undifferentiated NPC strongly expressed GFP, transgene expression was rapidly lost after transplantation of the GFP expressing NPC into the spinal cord (*Chapter 5*). The development of improved vectors that are able to induce stable transgene expression in adult derived NPC therefore is of great importance, in order to allow reliable cytoplasmatic labeling of grafted cells.

The grafted BrdU positive NPC were found to differentiate into glia only (*Chapter 2, 3, 4b*). At 3 weeks post transplantation, the grafted NPC mainly expressed the astroglial markers GFAP. Furthermore, the grafted NPC differentiate into glial cells that flawlessly integrate into the host tissue (*Chapter 2, 3, 4b*). The grafted NPC thus were able to build a reticulum that continues into the host parenchyma, without being sealed of by a rim of reactive astrocytes, which often can be observed when other cell types are grafted into the injured spinal cord [15, 16]. Whether substituted astroglial cells become functionally integrated as well has not been investigated yet.

The grafted NPC did not uniformly differentiate into GFAP expressing cells. NPC that adapted to oligodendroglial lineages could be detected at the lesion site as well (*Chapter 2, 3, 4b*). The introduction of new oligodendrocytes at the lesion site by NPC has several implications. First, it has been described in detail earlier that oligodendroglia and oligodendrocyte precursor cells can be associated with extracellular matrix molecules that are inhibitory to axonal sprouting [17, 18]. In the described fibroblast-NPC co-graft experiment, the negative effect of the present oligodendroglia on corticospinal axonal sprouting appears to be outweighed by the factors that instead induce axonal regeneration (*Chapter 3*). The newly formed oligodendrocytes further could have the potential to remyelinate both demyelinated spared axons and regenerating axons. Although it has been shown that grafted glial progenitor cells possess the capacity to remyelinate axons in the adult spinal cord [19], additional investigations are needed to analyze whether indeed remyelination occurs in the applied SCI model. Nevertheless, it is most likely that remyelination is a critical aspect of future therapies that aim to induce functional regeneration in the injured spinal cord [20, 21].

The phenotype in which the grafted NPC differentiate is primarily determined by cues in the micro-environment at the site of transplantation [22, 23], which not necessarily reflects the differentiation pattern desired and required for optimal axonal regeneration and functional recovery [11]. The over-expression of individual genes, which drive neural stem cells into specific phenotypes, has been demonstrated to generate homogenously differentiated cell populations [24, 25]. Neural stem cells modified in this way for transplantation might be able to override the differentiation inducing signals of the host environment at the injury site of grafted cells into specifi-

cally growth promoting phenotypes. It is most likely that only a transient expression of fate determining genes is preferred to induce the differentiation of NPC into a specific phenotype [11]. Therefore, the viral vectors that are described in this thesis may very well serve as a tool to reach this goal (*Chapter 5*).

Although at least a proportion of the used NPC have the capacity to differentiate into neuronal phenotypes (*Chapter 2, 3, 4b*), the micro-environment at the non-neurogenic regions of the adult CNS restricts the differentiation of the grafted NPC to glial lineages [22]. This however, does not impair the usefulness of the employed NPC since it was investigated whether NPC grafts are able to support regenerative sprouting of disrupted axons by providing a growth-permissive Substrate. Alternatively, functional recovery in the injured SC could be induced when NPC-derived neuronal cells function as a signaling relais that bridges the lesion site. This means that lesioned axons must project on the grafted neuronal cells that on their turn must innervate either spared spinal cord projections or the original target regions. Particularly embryonic stem cells have gained much attention to reach this goal since these cells appear to possess the potential to differentiate into neurons when grafted in the adult spinal cord [26]. Alternatively, fetal CNS tissue derived neurons also are shown to functionally integrate into the injured spinal cord cytoarchitecture [27]. The engraftment of neuronal cells by inducing neuronal differentiation of adult derived NPC in the adult spinal cord could serve as an interesting alternative to fetal derived graft material. Previous research however showed that neuronal differentiation of adult derived NPC that ere grafted to the injured spinal cord is extremely difficult to achieve [11].

2. ARE NPC GRAFTS ABLE TO INDUCE FUNCTIONAL RECOVERY AFTER SCI?

The promising results showing contact-mediated regenerative sprouting of corticospinal axons raise the question whether it is able to induce functional regeneration by grafting adult derived NPC in the injured spinal cord. Since the used dorsal wire-knife lesion of the CST at the cervical level only represents an incomplete lesion that does not lead to permanent functional deficits [28], the clinical relevance of the potential of grafted NPC to function as a scaffold for regenerating CNS axons needs to be tested in a SCI animal model that possesses a relevant functional correlate to the structural injury. Thoracic contusion models that are combined with various locomotor tests represents the most clinical relevant SCI animal model and is very well established in the literature [29-31]. The reduced reproducibility of the injury however has great implications for the application of cell-based therapy in contusion injuries. Especially in more chronic injuries, the size and the exact location of the rather diffuse injury site varies from subject to subject. It is evident that the exact placement of the cell graft is essential for the functional outcome. Non-invasive *in vivo* imaging of the lesion site

could serve as important tool to reach this goal, moreover since high field magnetic resonance imaging (MRI) was shown to allow the non-invasive, high-resolution visualization of pathomorphological changes such as hemorrhage, tissue degeneration and cyst formation in the rat spinal cord following contusion injury (*Chapter 6*). Unlike after transsection- or aspiration injuries of the spinal cord, contusion injuries only induce the development of a limited lesion cavity defect at the lesion epicenter (*Chapter 6*). The tissue defect at the lesion epicenter of contusion injuries therefore more likely represents a mixture of tissue debris, infiltrated immune cells and unorganized tissue. Since the lesion defect needs to be replaced by a graft in order to allow axonal regeneration over the lesion site, it is very well possible that a simple injection of large amounts of cells at lesion site induces an additional injury to the remaining spinal cord tissue that surrounds the lesion site and that is likely to contain spared axons.

3. ISOLATION OF AUTOLOGOUS NPC FOR SPINAL CORD REPAIR

The NPC that are used throughout this thesis are isolated from the adult spinal cord. Even though the grafted cells are typed as progenitor cells, at least a proportion of the used NPC possess stem cell characteristics including multipotency and self-renewal [22]. Although it remains unclear whether the NPC that are isolated from the adult spinal cord represent the same cell type as the NPC that are isolated from the subventricular zone (SVZ) or the hippocampus, both cell populations share most of their stem cell properties [32]. It should be noted the actual neural stem cell that is present in the SVZ and hippocampus of the adult brain expresses GFAP *in vivo* and possesses the properties belonging to astroglial cells [33, 34]. It is however very unlikely that the GFAP expressing cells that are shown to support regenerative axonal sprouting in this thesis represent undifferentiated neural stem cells, The expression of GFAP by the grafted NPC is only induced after transplantation, together with an immediate decline of cell proliferation of the grafted cells and the upregulation of other mature glial markers (*Chapter 2, 3, 4b*). Previous studies that investigated neural stem cell activity in the adult spinal cord did not describe the presence of GFAP expressing neural stem cells [22, 35].

Crucial for the applicability of a cell based therapy using progenitor / stem cells is the availability of sufficient amounts of cell material. In this respect, adult derived NPC have a decisive advantage over embryonic- and fetal derived progenitor cells. Even when the ethical concerns that are associated with the use of embryonic- and fetal derived progenitor cells are not considered, the limited availability of these cells most likely will result in logistic problems. Moreover, the use of adult derived NPC allows the advantageous autologous transplantation mode in which the receiving patient functions as his own tissue donor, preventing the occurrence of side effects that are associated with graft rejection or immune suppression. In a previous

study, we were able to show that in the rat, sufficient amounts of adult derived autologous NPC can be isolated using the minimal invasive brain biopsy technique, without inducing unacceptable additional damage. These NPC furthermore are able to induce axonal regeneration in the injured spinal cord of the donor animal [36]. The purification of specific sub-populations of glial progenitor cells from the heterogenous progenitor cell population represents a promising method to further enhance the capacity of NPC grafts to induce regeneration in the lesioned spinal cord. The Schwann cell purification method that employs magnetic-activated cell separation (MACS) as described in this thesis (*Chapter 4a*) can be adapted to enable the separation of specific NPC subpopulations for grafting purposes [37, 38].

4. COMBINING NPC GRAFTS WITH ADDITIONAL TROPHIC SUPPORT

An additional promising means to further enhance the sprouting response of lesioned axons is the local overexpression of neurotrophins such as neurotrophic factor-3 (NT-3), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic Factor (GDNF) or nerve growth factor (NGF), which previously has been shown to enhance regenerative sprouting of CST axons [16, 39-42]. A very attractive method to reach this goal would be to induce over-expression of NT-3 by grafting transduced NPC in an *ex vivo* gene therapy approach. The stable over-expression of transgenes in NPC however is very problematic due to gene silencing (*Chapter 5*). Of all vectors tested, only transient gene expression could be achieved that was rapidly downregulated upon transplantation of the transduced cells into the spinal cord. The local delivery of trophic factors in the CNS alternatively can be induced by direct injection of lentiviral vectors in the CNS parenchyma, which is sufficient to allow transgene expression for up to 16 months (*Chapter 5*).

In none of the observed cases, the regenerative sprouting of the CST was able to completely bridge the lesion site and sprout into the distal portion spinal cord parenchyma. Therefore, the observed regenerative sprouting must be further enhanced before a functional correlate of the observed regenerative sprouting is likely to evolve. Unlike the sprouting of lesioned axons from the spinal cord tissue into the graft, the reentering of sprouting axons from the graft into the distal spinal cord tissue is extremely difficult to achieve, which most likely is related to the development of the glial scar that surrounds the cellular graft (*Chapter 4b*). When the lesioned axons sprout into a graft that is more permissive for axonal regeneration than the surrounding spinal cord tissue, the sprouting axons are not very likely to reenter the distal spinal cord tissue[43]. This problem can only be solved partially by the local overexpression trophic factors at the graft site, since this creates an “oasis of trophic factors” inside the inhospitable spinal cord milieu [43, 44]. A possible solution could be the creation of a neurotrophic gradient over the lesion site. By using in-

jections of a lentiviral vector, a NT-3 gradient could be established, which promoted axonal regeneration beyond the lesion site and into the distal spinal cord tissue. [45]

5. FINAL CONCLUSIONS AND OUTLOOK

Syngenic adult derived neural progenitor cells are able to survive transplantation in the acutely lesioned spinal cord and differentiate into glial phenotypes. When co-grafted with fibroblasts, GFAP expressing grafted NPC are able to replace the lesion defect and are able to induce contact mediated axon guidance and regenerative sprouting, which is in analogy with the peripheral nervous system in which Schwann cells function as a guiding substrate. NPC that are co-grafted with highly purified Schwann cells however migrate away from the lesion site, which is paralleled with a reduced axonal outgrowth. A close investigation of NPC that are transduced to express ectopic genes by using different viral vectors revealed that *in vivo* gene expression in genetically engineered neural progenitor cells is temporally limited and mostly restricted to undifferentiated NPC.

Additional experiments in small animal models of SCI will have to be conducted in order to further evaluate the capacity of autologous NPC grafts to induce functional axonal outgrowth of the injured spinal cord before even considering the application in human patients. Moreover, it remains unclear in which extent promising results that are obtained using animal models of SCI are transferable to the human situation. It first must be unambiguously shown that neural progenitor cell mediated regenerative sprouting is able to induce target reinnervation and regain of function without inducing adverse side effects such as allodynia, chronic pain and muscle spasms. Furthermore, the risks that are associated with the isolation, propagation and transplantation of neural progenitor cells in human subjects remain to be established. The promising results that are partially described in this thesis nevertheless justify further research efforts in order to reveal the full potential of adult derived NPC. The combination of MRI imaging to monitor structural regeneration and longitudinal behavioral studies of spinal cord injured rats would greatly facilitate the verifications of the observed structural regeneration in functional relevant small animal models of SCI. This allows direct correlation of structure with function. Furthermore, the ability to visualize pre-labeled cells, at high resolution *in situ,* represents an important prerequisite for preclinical studies with the objective to develop an effective and safe therapy that is able to at least partially relieve the devastating consequences of spinal cord injury.

6. REFERENCES

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