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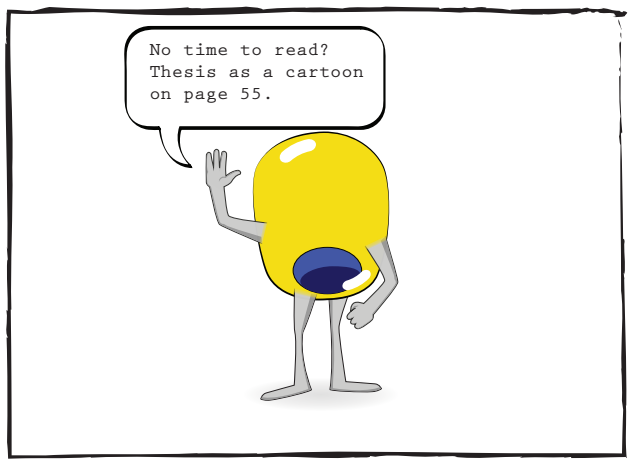
Repair and Reconstruction of Peripheral Nerve Injuries

Treatment with G-CSF and Stromal Vascular Fraction

HANNA FROST | FACULTY OF MEDICINE | LUND UNIVERSITY



Peripheral Nerve Injuries

Can your love handles heal your injured nerves? This doctoral dissertation aims to explore the possibilities of mobilizing autologous cells in order to improve regenerative outcomes after a peripheral nerve injury, in conjunction with surgery. Peripheral nerve injuries are a clinical problem and could use a quick fix.

I set a time frame of one hour.

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Repair and Reconstruction of Peripheral Nerve Injuries

Treatment with G-CSF and Stromal Vascular Fraction

Hanna Frost

LUNDS UNIVERSITET

DOCTORAL DISSERTATION
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Title and subtitle Repair and Reconstruction of Peripheral Nerve Injuries: Treatment with G-CSF and Stromal Vascular Fraction

While surgery is a cornerstone in treatment of peripheral nerve injuries, it is not a comprehensive approach, and outcome is unsatisfactory, especially sensory function. The present aim was to translate recent findings about stem- and progenitor cells to improve regenerative outcome, where the cells have to be autologous, available within the same surgical procedure, and minimally manipulated.

Granulocyte colony-stimulating factor (G-CSF) mobilizes hematopoietic stem cells from the bone marrow. Post-traumatic G-CSF therapy, evaluated in a rat sciatic nerve injury model with immediate repair, showed a 13% local decrease in Schwann cell apoptosis at the site of lesion, and a similar trend in the distal nerve segment in healthy rats, and at the site of lesion in diabetic Goto-Kakizaki rats. G-CSF had no effect on axonal outgrowth in short- or long term experiments.

Stromal vascular fraction (SVF) of adipose tissue is a heterogenic mixture of cells, including small amounts of adipose derived stem cells. Electrospun multi-channeled nerve conduits, designed to mimic a native nerve, with longitudinal nanofibers inside the channels for axonal guidance +/- delivered SVF to the nerve conduit was used to bridge a 10 mm sciatic nerve gap in healthy rats. The nerve conduit supported axonal outgrowth and acted as a cell delivery vehicle during the observation time (four weeks). SVF did not improve axonal outgrowth, and adverse effects – gross encapsulation – was observed in 9/30 implants after SVF therapy. Schwann cell infiltration was inferior in nerve conduits supplemented with SVF cells, with a partially enhanced inflammatory response.

Co-culture of SVF cells and peripheral nerve segments performed on aligned nanofibers, recreating *in vitro* the environment above, showed no change in expression of Schwann cell marker S-100 in SVF cells, but increased Sox10 in SVF cells exposed to a nerve segment compared to baseline. Pilot experiments with mass spectrometry indicated a SVF-nerve interplay in the local microenvironment.

In conclusion, G-CSF and SVF therapy affected glial cells, but did not improve axonal outgrowth. G-CSF decreases Schwann cell apoptosis, but does not improve regenerative outcome. An electrospun nerve conduit can be used to bridge a nerve gap and act as a cell delivery vehicle. SVF delivered in micro-channels interferes with ingrowth of Schwann cells by unknown mechanisms.

Key words peripheral nerve injuries, nerve regeneration, reconstructive surgical procedures, diabetes mellitus, rat sciatic nerve, granulocyte colony-stimulating factor, stromal vascular fraction, electrospinning, Schwann cells, rats

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Repair and Reconstruction of Peripheral Nerve Injuries

Treatment with G-CSF and Stromal Vascular Fraction

Hanna Frost

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Lund 2019

To my fiercely intelligent mother
who had to spend her high school years
cleaning the municipal dentist´s clinic
instead of doing her homework
and never made it to medical school

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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. G-CSF prevents caspase 3 activation in Schwann cells after sciatic nerve transection, but does not improve nerve regeneration

Frost HK, Kodama A, Ekström P, Dahlin LB. Neuroscience. 2016 Oct 15;334:55-63. doi: 10.1016/j.neuroscience.2016.07.045. Epub 2016 Aug 4.

II. Electrospun nerve guide conduits have the potential to bridge peripheral nerve injuries in vivo

Frost HK, Andersson T, Johansson S, Englund-Johansson U, Ekström P, Dahlin LB, Johansson F. Sci Rep. 2018; 8: 16716. Published online 2018 Nov 13. doi: 10G.1038/s41598-018-34699-8. Author correction in: Sci Rep. 2019; 9: 10017.

III. Effects of Stromal Vascular Fraction on a rat peripheral nerve segment in an aligned nanofiber in vitro setting

Frost HK, Johansson F, Dahlin LB, Ekström P. In manuscript

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Abbreviations

ADS	Adipose tissue-derived Stromal Cells
ADSC	Adipose Derived Stem Cells
ATF-3	Activated Transcription Factor-3
ATMP	Advanced Therapy Medicinal Product
BB rats	Biobreeding rats
BMSC	Bone Marrow Stromal Cells
CAT	The Committee for Advanced Therapies
DRG	Dorsal Root Ganglia
EMA	European Medicines Agency's
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
G-CSF	Granulocyte-Colony Stimulating Factor
GK rats	Goto-Kakizaki rats
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
MSC	Mesenchymal Stem Cells
PBS	Phosphate-Buffered Saline
PCL	Polycaprolactone
PLLA	Poly-L-Lactic Acid
SVF	Stromal Vascular Fraction
SFI	Sciatic Functional Index
VEGF	Vascular Endothelial Growth Factor

Thesis at a glance

The aim of this thesis was to find new treatments for peripheral nerve injuries, by translating new knowledge about stem- and progenitor cells to clinical practice. I used a rat model of sciatic nerve injury and repair, and attempted to mobilize autologous cells to improve regenerative outcome. The time frame to execute the therapeutic intervention was set to one hour. Hematopoietic stem cells and adipose derived stem cells were identified as potential cell sources to augment peripheral nerve repair.

Paper I

Healthy and diabetic Goto-Kakizaki (GK) rats were treated with daily injections of Granulocyte-colony stimulating factor (G-CSF) after a sciatic nerve transection injury and immediate repair. A 13% local decrease in Schwann cell apoptosis was detected after seven days in healthy rats, with a similar trend in diabetic GK rats. G-CSF therapy did not affect axonal outgrowth after seven days or after twelve weeks. G-CSF treatment had no positive effect on regenerative outcomes after twelve weeks.

Figure 1. Specimens were treated with G-CSF for five days after sciatic nerve injury and repair. The observation times were one and twelve weeks. Illustration by Peregrin Frost.

Paper II

A 10 mm sciatic nerve gap injury in healthy rats was bridged with an electrospun nerve conduit, designed to mimic a native nerve. Selected groups had cell therapy with autologous stromal vascular fraction (SVF) transplanted into the conduits. The observation time was 28 days.

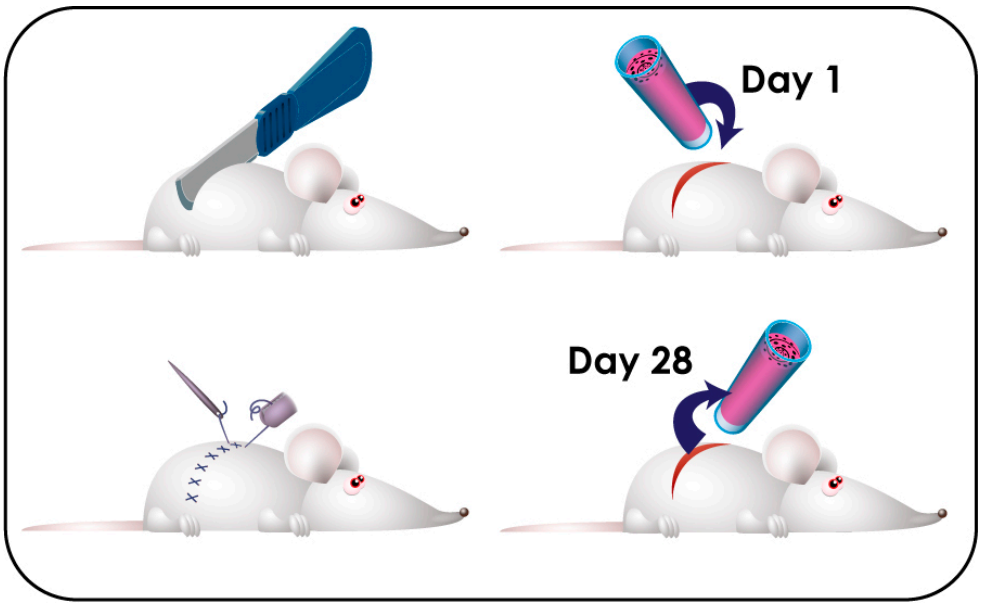


Figure 2. An electrospun nanofiber nerve conduit was used to bridge a 10 mm sciatic nerve gap, and SVF was added to selected groups. Hollow tube and autograft were used as control. Illustration by Pergrin Frost.

PLLA nerve conduits supported axonal regeneration, but did not outperform autologous nerve grafts. SVF therapy did not enhance axonal regeneration, and there was a partially enhanced inflammatory response. After SVF therapy, some rats had gross encapsulation of the implants. Schwann cells were poorly distributed in the nerve conduits with SVF cells.

The electrospun nerve conduit can function as a cell delivery vehicle, but SVF therapy did not improve outcomes. The demands on multi-channeled nerve conduits for cell therapy are unknown.

Paper III

An *in vitro* environment was created to study the interactions of SVF cells and a rat sciatic nerve segment in the particular microenvironment above (Fig. 3). The observation periods were two and five days.

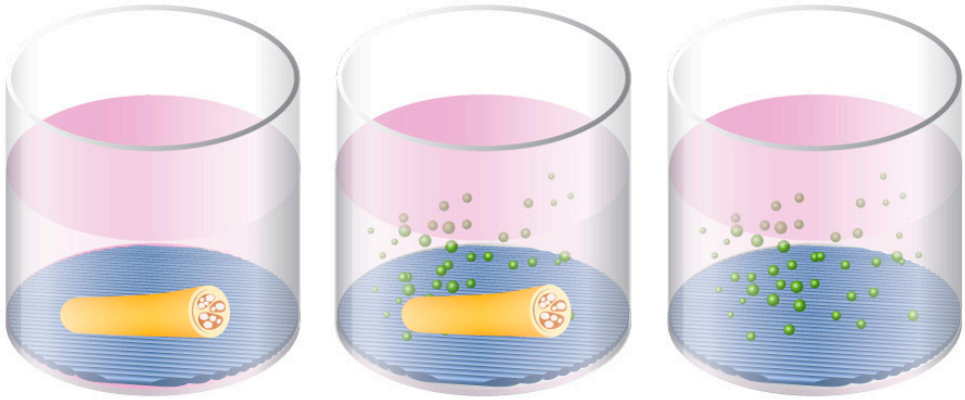


Figure 3. The *in vitro* setup to study SVF-nerve interactions in serum free SVF-nerve co-culture on aligned nanofibers. Nerve segment and SVF alone were used as control. Illustration by Peregrin Frost.

In SVF cells co-cultured with a sciatic nerve segment, the expression of the Schwann cell marker S-100 was unchanged, but the pan-Schwann cell marker Sox10 was increased over time compared to baseline. After two days, only nerve segments co-cultured with SVF could be stained for two transcription factors normally present during nerve regeneration (ATF-3 for activation and cleaved caspase 3 for apoptosis, respectively). A pilot mass spectrometric analysis of aspirated medium, also containing floating cells, revealed changes in the microenvironment.

SVF and a peripheral nerve segment exert biological effects on one another. The nature and effect of these interactions remains to be clarified.

"Science exists only because there are limits to what Nature permits. The laws of Nature and the unchanging 'constants' of Nature define borders that distinguish our Universe from a host of other conceivable worlds where all things are possible. In those imaginary worlds of unlimited possibility there can exist neither complexity nor life."

John D. Barrow: Impossibility.
The Limits of Science and the Science of Limits.

Introduction

Providing the best treatments known, peripheral nerve injuries still result in unsatisfactory functional recovery in adults with sensory deficit, motor dysfunction and a variety of other symptoms, such as cold sensitivity and sometimes pain [1-3]. The majority of traumatic peripheral nerve injuries in Sweden occur at wrist and hand levels (63%), followed by the forearm (10%) and the upper arm and shoulder (7%) [4]. Commonly among young professionals, injuries of the hand occur early in their careers, at a high cost to society and themselves [5].

The peripheral nervous system

The peripheral nervous system (PNS) includes sensory, motor and autonomic nerves that lie outside the brain and spinal cord. It is mainly composed of two major cell types: neurons and glia. Sensory neurons have their soma (cell body) in the dorsal root ganglia, and extend specialized processes – axons - to the target organ. The axon end can be more than one meter away from its cell soma, placing high demands on the intraneuronal communication and production machinery.

Glial cells were historically believed to be passive and supportive in function, but play an active role. The most known glial cells of the peripheral nervous system are Schwann cells, named after the German physiologist Theodor Schwann. In nerve trunks, non-myelinating (Remak) or myelinating Schwann cells ensheath the axons [6]. Myelinating Schwann cell wrap around a single axon, forming myelin sheets, facilitating electrical conduction [6], whereas Remak Schwann cells ensheath multiple small-calibre axons [7]. Sensory and motor axons are associated with distinctive subtypes of Schwann cells with different protein expression [8], which can alter during a regeneration process [9].

Another type of specialized glial cell guides fine sensory fibers into the epidermal skin layer [10]. The paradigm was that the glial-axon connections end at the level of the basement membrane into the epidermis, where free nerve endings of non-myelinated axons are known to act as nociceptive sensors. Very recently, however, a research group discovered these cutaneous Schwann cells to be specialized nociceptive cells that initiate pain sensation with direct excitatory functional connection to sensory neurons [11].

The role of Schwann cells is not, however, restricted to general physiological functions, since the regenerative potential of the peripheral nerve system depends on Schwann cells, and their ability to convert to cells devoted to repair after injury [12]. Schwann cells are intimately involved in all stages of peripheral nerve repair [13] and their ability to support the regeneration process is diminished if a repair or a reconstruction is delayed [14-16].

Wallerian degeneration and axonal outgrowth

Wallerian degeneration is an intricate progression of events following a peripheral nerve injury. It was observed by the physician August Waller in 1850, and a more detailed description of the morphological changes was provided by Ramón y Cajal in 1928, under the name "trophic degeneration".

After axonal transection, 200-300 µm of the nerve ends quickly fragment. This acute axonal degeneration appear within ten to twenty minutes, and is followed by a latent period ranging within 25-45 hours [17], after which the axonal cytoskeletal proteins are disassembled [18]. The injured neurons execute vast gene expression changes to transit from cell-signalling to building a new axon, a response referred to as "the cell body reaction" or "signalling to growth mode switch" [12].

The Schwann cells execute a repair program. They switch from myelin maintenance, to shed and actively break down their own myelin [19,20]. Signs of this process, myelin autophagy, can be seen as the formation of myelin ovoids, emerging within the first 24 hours. The Schwann cell proliferation, a characteristic feature of Wallerian degeneration, peaks two to three days after injury [13]. Macrophages are recruited, activated, and digest myelin by phagocytosis in a second phase of myelin breakdown [19]. Later, the macrophages are cleared by apoptosis, or migrate out across the basal lamina and enter the lymphatic circulation [21].

Schwann cells engage in cellular elongation and branching to form regeneration tracts: Bands of Büngner, selectively guiding regrowing axons [19,22]. The distal tip of the regenerating axons form a growth cone, described by Cajal in 1890 [23].

Cell signalling or signal transduction

Signal transduction was poetically described as "whispers between cells" by Nobel Prize laureate Martin Rodbell at his speech at the Nobel Banquet 1994. Cells sense external stimuli, and communicate and respond to their environment. The discovery of Nerve Growth Factor (NGF) in 1952 has been described as a Rosetta Stone for uncovering the common principles that underlie the diverse mechanisms of signal transduction to regulate cell behaviour [24].

Cells express different sets of genes in response to their environment, and at different times during development. In eukaryote cells, most genes are silent unless they are switched on by the binding of transcription factor proteins to DNA at multiple control sites. Transcription factors involved in peripheral nerve injury and regeneration include c-Jun and Activating Transcription Factor 3 (ATF-3) [25-27]. Addition of the transcription factor Vascular Endothelial Growth Factor (VEGF) was early shown to promote neuron survival, increase axonal outgrowth, and

promote proliferation of Schwann cells in explanted dorsal root ganglia [28]. Experimental signal transduction studies have unravelled a variety of other molecules [29].

Current treatment of peripheral nerve injuries

Early surgical nerve repair or reconstruction is considered to be a well established treatment of nerve transections and lacerations in the upper extremity [84], where the same principles can be adapted for the less frequently seen nerve injuries in the lower extremity. In sharp transection injuries, nerves are directly repaired by end-to-end approximation and suture of the endoneural sheets of the nerve trunk, i.e. epineurial or perineurial suture technique, using visual magnification devices and microsurgery instruments. In situations where a nerve defect is present, a nerve reconstruction procedure is required. Outcome procedures have been enhanced by rehabilitation programs for sensory re-education [30,31]

Nerve grafts, nerve conduits and nerve transfers

If coaptation of the nerve ends is not possible without tension, because of tissue loss or when repair is delayed and the nerve has retracted and lost its elasticity, the nerve gap must be bridged. Autologous nerve graft with a less important donor nerve is gold standard [32], though it requires a second surgical site and results in mild but permanent donor site morbidity [33].

In extensive peripheral nerve injuries, however, there may not be enough donor material available for autologous nerve grafting. For these situations, nerve conduits in various materials (including the biodgradable polymers PCL and PLLA) and human acellular nerve allografts have been developed [34-39]. Another option is a nerve transfer, a procedure more common nowadays, where an expendable nerve is used to reinnervate a more important nerve [40,41].

Causes of poor functional recovery

Due to the biological predisposition for apoptosis in injured PNS neurons, particularly the small diameter neurons [42,43], advanced surgery with axonal guidance alone is not enough to promote complete post-traumatic nerve regeneration. For reasons that are not understood, neurons and Schwann cells commit apoptosis following axotomy [42,43], which exacerbates the damage. It has been speculated that this is part of a homeostatic program that removes neurons, axons and synapses upon limited injury [44].

For motor fibers, axonal misdirection and reinnervation results in axons projecting to new muscle fibers, different muscles and sometimes even antagonistic muscles. Motor axons can also grew into sensory targets [45]. Long term follow up in humans, at a mean 14 years after a peripheral nerve injury at the wrist level, showed recovery of motor function, but profound sensory deficits in adults [46].

Misdirection of the axonal outgrowth of sensory fibers results in new afferent signalling patterns, which are difficult for the brain to interpret. Those injured in childhood can regain almost full sensory and motor recovery [3], though electrophysiology shows persistent pathology irrespective of age at long term follow up [47]. The mechanisms for the excellent sensory recovery in children are probably located in the CNS.

Sprouting axons can regenerate with directionless growth, and result in local neuroma formation. The painful condition is sometimes treated surgically by burying or capping the nerve end in a more protected place, if nonsurgical treatment fails [48].

Delayed surgical nerve repair or reconstruction results in impaired nerve function in humans [49]. Rat studies have shown that axonal outgrowth is inferior in delayed nerve repair [15], and that Schwann cell apoptosis is increased after delayed nerve repair [14]; studies highlighting the need for urgent nerve repair and reconstruction.

Figure 4. Post-traumatic neuronal cell death aggravates peripheral nerve injuries, and terapeutic interventions to increase neuronal survival may improve regenerative outcomes. Retrograde transport of signals is involved in the response of both neuronal survival and death [27]. Not further covered in this thesis, the feature of neuronal death was the topic of my first project as a member of the hand surgical research group. Illustration by Peregrin Frost.

The diabetic peripheral nerve

The incidence of diabetes has increased globally over the past decades, and was estimated to affect 451 million people worldwide in 2017, with an expected increase to 693 million by 2045 [50]. The peripheral nerve in diabetes is already provoked and the microenvironment modified.

Rat studies have shown impaired axonal outgrowth after nerve end-to-end repair at six days in diabetic rats, while diabetic rats have been shown to develop a thicker regenerative matrix after three weeks after a 10 mm nerve gap reconstruction, as compared to healthy rats [51-53].

With this knowledge, it may be rational to include diabetic specimens in the basic studies of nerve injury and repair, to anticipate a scientific knowledge gap. We used Goto-Kakizaki rats with a diabetes type II-similar phenotype.

Stem cell therapy - a translational approach

The differentiated state of adult cells is less stable than historically assumed [54]. The discovery of induced pluripotent stem cells in 2006 showed that the transition between different cell types can be effected by the overexpression of a few transcription factors [55].

Much of the spear-heading research on stem cell lineages, their developmental potential, and plasticity of adult stem cells was undertaken on bone marrow residing hematopoietic stem cells (HSCs) [56]. HSCs have been observed to migrate to and engraft in experimentally injured non-hematopoietic tissue, with functional effects [57,58], though they maintain their hematopoietic nature [59,60].

Not knowing the mechanisms underlying the functional improvement following stem cell therapies, this project seized the finding that there *was* a positive effect. The poor outcome after a peripheral nerve injury warranted the attempt to investigate somatic cell therapy to augment the surgical repair and reconstruction.

Schwann cells would be the ideal candidates for cell therapy in this setting, and have been evaluated in rodent models after short *ex vivo* expansions [61,62]. The problem is that Schwann cells are notoriously difficult to isolate in a viable and proliferative state, and they are slow to grow [63,64]. Hence the interest to use other cell populations, including stem- and progenitor cells.

Considering the therapeutic window, when surgical treatment is still possible and effective, the ideal time to harvest and deliver cells would be in the same surgical

procedure, preferably within one hour. Considering these factors, hematopoietic stem cells and adipose derived stem cells were identified as interesting candidates.

Hematopoietic stem cells and G-CSF

The HSCs appear to be under the control of several factors, among which Granulocyte-colony stimulating factor (G-CSF) can be clinically used to mobilize HSCs from the bone marrow to the peripheral blood [65]. G-CSF has been widely used in clinical practice, most commonly to support the delivery of chemotherapy by treating drug induced neutropenia. Moreover, G-CSF has been reported to have anti-inflammatory and anti-apoptotic neuroprotective effects in the central nerve system [66,67], and has been evaluated for spinal cord injury in clinical (phase II) trials [68,69].

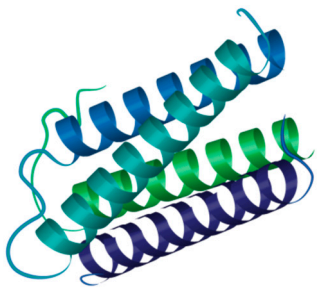


Figure 6. The glycoprotein G-CSF had previously been investigated in sciatic nerve crush injury models in mice, a clinically less relevant model, since peripheral crush injuries often recover, even without treatment.
Illustration by Peregrin Frost.

G-CSF and peripheral nerve injury

G-CSF injections have been shown to result in engraftment of bone-marrow derived cells in the peripheral nerve after a sciatic nerve crush injury in a mouse model, with improved nerve conduction latency and sciatic functional index (SFI), i.e. walking pattern, in short term experiments [58]. Another mice study used double gene therapy with VEGF and G-CSF in a sciatic nerve gap model. They showed improved myelination, vascularization, and sensory and motor neuron survival [70].

Overexpression of G-CSF in mice has been showed to give better motor neuron survival at four days following a sciatic nerve axotomy [71]. Keiner *et al.* (2019)[72] reported a study in male rats with a sciatic nerve lesion caused by a high pressure waterjet device. The rats were treated with post-traumatic G-CSF and observed up to14 days. The authors report higher α-motor neuron survival in the G-CSF treated group [72]. A weakness of that study is that a nerve crush injury is not clinically significant, and the crushing mechanism was unorthodox.

Published after my study, Jia *et al.* (2017)[73] reported a rat study investigating the synergistic effect of five day *ex vivo* expanded bone marrow stromal cells (BMSC) plus G-CSF [73]. A single-cell suspension was injected into acellular nerve xenografts, obtained via the chemical extraction method developed by Sondell-Kanje [74], to bridge a 10 mm sciatic nerve defect in rats of either gender. G-CSF was injected daily for seven days in selected groups. The observation time was eight weeks [73]. The control group with G-CSF intervention showed improved electrophysiology, morphometry and weight ratio of the tibialis anterior muscle as compared to nerve xenograft alone. Co-treatment with G-CSF+BMSC produced even better regenerative outcomes [73].

Adipose tissue and adipose derived stem cells

HSCs are not the only stem cells available in adults. Adipose tissue contains mesenchymal stem cells, termed adipose derived stem cells (ADSC). ADSC can be easily harvested and *in vitro* expanded with a broad differentiation potential [75]. The web-based registry of clinical trials information, ClinicalTrials.gov, has shown 280 clinical trials using ADSC for a wide array of indications, including central nervous system diseases, collagen diseases, joint disease, immune disease and diabetes.

The use of unprocessed fat for peripheral nerve injury

Though dermal fat grafts or vascularised flaps [76] are sometimes used in the clinical setting to cover scarred or exposed peripheral nerves, unprocessed adipose tissue seems to have limited use for peripheral nerve regeneration. Dumanian *et al.* (1999)[77] reported that crude fat grafting of peripheral nerves subjected to circumferential epineurectomy, a model for perineural scar, resulted in diminished neural scar stiffness, but also "unexpected findings of substantial postoperative neuropathy". They observed asymmetric toe spreading and associated limb atrophy, limping with gait, or autophagy when crude fat grafts were used [77]. The authors speculated that this could be due to slower revascularization of the nerves, toxic by-products released from poorly preserved fat grafts, or local mechanical effects.

Papalia *et al.* (2013)[78] filled vein grafts with lipoaspirate-derived entire adipose tissue, bridging a 10 mm median nerve defect in rats, and found significantly worse outcome in the adipose tissue-enriched group. They concluded that the transplanted tissue negatively interfered with regenerating axons and migrating Schwann cells both mechanically, by occluding a large part of the conduit lumen, and metabolically, by consuming nutrients and oxygen; they suggested that adipose tissue must first be processed to stromal vascular fraction (SVF) [78].

Stromal Vascular Fraction and peripheral nerve injury

A cell source available for my study is the minimally manipulated SVF of the adipose tissue. The term "stromal cells" refers to connective tissue cells of any organ. In one hour – a clinically applicable time to harvest cells in the same surgical procedure - SVF is obtained by enzymatic or mechanical digestion of adipose tissue, followed by centrifugation to remove mature adipocytes. SVF cell pellet contains a heterogeneous mixture of cells. Uncultured SVF contains hematopoietic-lineage cells [stem and progenitor cells <0.1%, granulocytes 10–15%, monocytes 5–15%, lymphocytes 10–15%]; endothelial cells 10–20%; pericytes 3–5%; and stromal cells 15–30% [79].

SVF has been used in hollow conduits to bridge sciatic nerve gaps in rodents with positive results. Suganuma *et al.* (2013) [80] described the use of uncultured SVF in two week experiments, with more S-100 stainable cells and improved axonal regeneration as detected by staining for protein gene product (PGP) 9.5 protein, as well as increased levels of Neu-1 and VEGF expression on mRNA level [80]. Mohammadi *et al.* (2014) [81] used a chitosan conduit filled with SVF for rat sciatic

nerve gap in streptozotocin induced diabetic male rats (n=18 per group). For control, they used the chitosan conduit with PBS. The observation times were four, eight and 12 weeks. The authors report improved functional outcome (sciatic functional index), gastrocnemius muscle weight, and nerve morphology after SVF therapy [81].

Ozkan *et al*. (2016) [82] filled vein grafts with SVF to bridge 8 mm defects in the tibial nerve in a rat model (n=10 per group). They compared to vein graft alone and to nerve graft; the observation time was three months. Morphometric analysis was performed using light microscopy. A weakness of the study is the lack of information about systematic random sampling, or the number of observations used to calculate statistics. The authors observed no differences in myelin diameter or axonal diameter between the groups, but the number of myelinated axon in the group with vein graft+SVF was higher than in vein graft alone. They found no difference between vein graft +SVF as compared to nerve graft in the number of myelinated axons [82].

In a recent study, Shimizu *et al.* (2018) [83] report adding SVF into a polyglycolic acid-type I collagen tube as a scaffold, bridging a 7 mm facial nerve gap in a male rat model (n=8 per group). The observation time was 13 weeks [83]. The SVF group had improved compound muscle action potential amplitude and decreased latency as compared to the nerve conduit alone. Axon diameter, fiber diameter and myelin thickness was increased in the SVF group as compared to control. Moreover, for some parameters, SVF therapy showed a similar level of improvement as did undifferentiated, *ex vivo* expanded ADSCs [83].

Clinical use of cell therapies today

Any prospective clinical treatment must consider possible adverse events by the treatment in itself. The Committee for Advanced Therapies (CAT) at the European Medicines Agency's (EMA) regulate Advanced Therapy Medicinal Products (ATMPs), including somatic-cell therapy medicines and tissue-engineered medicines.

Various cell therapies have a long record of safe and effective clinical translation. The bone marrow transplant, later understood to be a hematopoietic stem cell transplant, has been explored in humans since the 1950s to treat haematological malignancies [85,86], and has been expanded to treat non-malignant diseases. Bone marrow concentrate and iliac crest bone marrow-derived mesenchymal stem cells have been described in clinical use to treat hip osteonecrosis, non-unions and augment rotator cuff tear repair since the late 1990s [87,88].

In vitro fertilization, sometimes with substantial manipulation of cellular tissues, has been employed worldwide since 1986.

In the context of peripheral nerve injuries, it can be speculated that the pedicled hypothenar fat flap (i.e. fat tissue lifted from the donor site, and moved along with its blood supply), used to cover the median nerve in recurrent carpal tunnel syndrome [89,90] may partly exert its effect as an adipose stem cell therapy, aside from serving as a cushioning and sliding layer [76]. Likewise, the practice of microfracture in attempt to repair articular cartilage [91], and surgical bone drilling of cortical holes to enhance fusion between the two bony surfaces, may partly act their therapeutic functions as a stem cell treatments.

Specific aims

This research study is of the translational experimental kind, with the purpose of finding new treatment methods for injured peripheral nerve trunks (including the brachial plexus), by using recent discoveries in basic science about stem- and progenitor cells.

I wanted to explore a therapeutic intervention with autologous cells, mobilizing cells surgically or pharmacologically, without *ex vivo* expansion, and also investigate the effect of G-CSF alone. I set a time limit of one hour for the mobilization procedure - a clinically reasonable time for an additional procedure in conjunction to peripheral nerve surgery.

More specifically, the aims were:

- To investigate the effect of G-CSF treatment on peripheral nerve injury in a rat model of sciatic nerve injury and repair in healthy rats

- To investigate the effect of G-CSF treatment on peripheral nerve injury in a rat model of sciatic nerve injury and repair in diabetic Goto-Kakizaki (GK) rats

- To investigate the delivery of autologous SVF treatment on peripheral nerve injury bridged by a new electrospun conduit designed to mimic a native peripheral nerve

- To understand the effects of SVF and the interactions between SVF and a nerve end, including crucial Schwann cells, in an *in vitro* model of the *in vivo* system above

“We experimented day and night, holidays not excepted.”

Nikola Tesla

Methods

Below is a summary of the methods used including comments. For details, please refer to the experiment section of paper I, II and III.

Sciatic nerve injury model

The domestic Norway rat, *Rattus norvegicus*, is one of the most common research subjects worldwide, and falls in the small animal category. The rat model of experimental sciatic nerve injury and repair as well as reconstruction is well established in the research society including in our research group [92]. The rat sciatic nerve has a size comparable to a digital nerve in humans, and is suitable for surgical manipulation. As four-legged animals, rats quickly compensate for the motor deficit caused by a sciatic nerve injury: they develop drop foot on the operated hind limb. Nerve function can be evaluated with functional tests.

For most experiments, I used healthy female Wistar rats. Female Goto-Kakizaki (GK) rats were used as a diabetic model. GK rats were originally derived from Wistar rats, bred and selected for naturally occurring genetic variants causing diabetes. GK rats display a type 2 similar phenotype with glucose intolerance, insulin resistance, hyperlipidemia and signs of diabetes complications [93]. GK rats have a moderate increase in blood glucose.

Under anaesthesia, the sciatic nerve was exposed and sharply transected under an operating microscope. The nerve was repaired by end-to-end anastomosis using standard 9-0 Ethilone sutures. For some experiments, a 10 mm nerve section was measured and excised to create a nerve gap. The gap was reconstructed by suturing the nerve ends to a 14 mm nerve conduit, 2 mm into each end. For selected groups, a 10 mm segment was excised and re-sutured in the reverse direction as autograft.

After 3 months, most sciatic nerve defects of 10 mm in rats will be bridged by newly regenerated axons, regardless of treatment. The critical gap length (the length of a nerve defect that cannot be bridged using a hollow nerve conduit) is about 15 mm in the rat model.

The axonal outgrowth rate in humans, based on clinical signs, is 1-2 mm/day after a nerve transection and repair [94]. In the rat sciatic nerve injury model, regeneration speeds as estimated by pinch test (pinching the nerve until a reflex is elucidated) is 3.3-4.3 mm/day after a nerve crush, with an initial delay of 1-1.5 days [95,96]. After transection and suture, the speed is estimated to 3.2 mm/day, following an initial delay of four days [97]. For autograft, the regeneration speed in the rat sciatic nerve model is estimated to 1.5 mm/day after an initial delay of 3-6 days [98].

Other small animal experimental regeneration models include mice, and rabbits. Mice have a smaller size, making them less suitable for studying nerve conduits

with the same conduit dimensions as in humans. Rabbits are larger than rats, and can accommodate longer nerve gaps, of up to 8 cm. Rabbits are sometimes used for screening implants before moving to large animal studies [99].

In Sweden, animal research can only be performed after an ethical permit granted by an ethical committee, and research staff must have necessary formal training. When using animals in research, the principles of replacement, reduction and refinement (the 3Rs) must be deliberated in every aspect of the project. The 3Rs were formulated 1959 by William Russel and Rex Burch in the book *The principles of humane experimental technique*, and are implemented in Swedish and European law.

The homologous model of cell transplantation

The Committee for Advanced Therapies (CAT) at European Medicines Agency has recommended the homologous model for cell therapy studies [116]. Cell transplantation between individuals and between species can give non-representative results, and mask adverse effects if transplanted cells are cleared by the immune system of the recipient [116].

von Frey monofilament test

Sensory changes can be detected by applying monofilaments against a target area, for quantitative touch–pressure threshold detection. Such monofilaments are made from different materials and come in different thicknesses. They exert calibrated forces when applied towards skin until the monofilament bends or until the area is withdrawn. The method is subject to anticipation bias [100]. The use of one single filament with increasing pressure was described by von Frey in 1922 [101,102].

In paper one, I used an Ugo Basile Dynamic Plantar Aesthesiometer to mechanically stimulate the paw of experimental rats. It stimulates with a von Frey-type 0.5 mm filament with increasing force and speed. The device is equipped with an adjustable, angled mirror, used by the researcher to manually position the filament under the rat hind paw of the specimen to be examined. The device automatically detects and records latency time and actual force at the time of paw withdrawal. To minimize variability, all measurements were performed by one researcher, in the same room, and at the same time of the day.

Muscle wet weight

Following a peripheral nerve injury, denervated muscle undergoes atrophy and degeneration. In nerve crush injury models in rats, the muscle wet weight is decreased from day three [103]. During peripheral nerve regeneration, the muscle is reinnervated and the weight can be determined as a measurement of motor recovery. To eliminate differences between specimens, it is related to the non-injured side. The muscle weight is well correlated with tetanic force values [104], an invasive method to determine motor recovery, which can be performed only on research animals under anaesthesia.

The sciatic nerve innervates the gastrocnemius muscle. By harvesting and weighing the gastrocnemius muscle and relating it to the muscle weight on the non-injured side in the same specimen, one can thus assess motor recovery after a sciatic nerve injury.

Tissue processing and sectioning

Sciatic nerves were harvested, fixated in Stefanini solution, and cryoprotected by incubation with 20% sucrose overnight. Samples were stored at -20ºC until sectioning. A cryotome was used to cut 8µm sections of the sciatic nerves. For the electrospun nerve conduits in paper II, the rigidity of the PCL conduit made it very difficult to cut longitudinal sections. Instead, transverse sections were collected proximally, at the proximal suture line, at 2mm, 5mm, 8mm; at the distal suture line, and distally. The PLLA conduit was softer and was possible to cut longitudinally. In paper II, the regeneration matrix in the hollow tube after 28 days was very thin, and thus difficult to cut in the correct plane. For the hollow tube, the longitudinal sections were thus very sparse.

Immunofluorescence microscopy

For immunofluorescence, antibodies are visualized by a fluorescent tag (a fluorochrome). Fluorescence occurs when an electron is excited to a higher energy level, makes a permitted decent to a lower energy level, emitting the released energy as light of a specific wave length. The emission can be detected using a fluorescence microscope, or other devises such as a fluorescence-activated cell sorting machine (FACS).

The advantage of immunofluorescence microscopy is the high contrast and molecular specificity. Limitation includes its dependence on the capability of the

selected antibody to bind its target with specificity and consistency. Some non-specific binding is unavoidable, and can be limited by using blocking solution.

The incorporation of electrospun nanofibers in the specimens studied by a fluorescence sensing device encounters the phenomena that the fibers can support light containment. This was observed in the cross sections and longitudinal sections of the electrospun nerve conduit (paper II), and in the nanofiber beds (paper III). The light scattering yielded a faint background within the fibers themselves.

During the experiments described in paper III, the nanofiber beds shrunk during fixation and processing, and they obtained the shape of a shallow bowl, akin to a contact lens. Here, images had to be sampled at different focal planes, incrementally stepping through the sample, and later reconstructing the focus-stacks, also called z-stacks, using the computer software. A disadvantage of focus stacking is the slow rate of acquisition, and an increased amount of light exposure to the sample.

Tabel 1. Stainings for cellular imaging used in this thesis

For specific details on the antibodies used, please refer to the individual papers.

Stain	Biological purpose
mouse monoclonal anti-neurofilament protein + Alexa Fluor tag	NF proteins are intermediate filaments, present in axons as a component of the cytoskeleton. Used to visualize newly regenerated axons.
mouse monoclonal anti-S-100 α β + Alexa Fluor tag (paperI, II)	A commonly used Schwann cell marker. S-100 is a dimeric calcium sensor protein.
rabbit polyclonal anti-S-100 β + Alexa Fluor tag (paper III)	The S-100 β subunit is expressed in Schwann cells and in adipocytes.
mouse monoclonal anti-Sox-10 + Alexa Fluor tag	A pan-Schwann cell marker. Sox10 is expressed in SC at all stages of development.
rabbit Anti-cleaved caspase 3 + Alexa Fluor tag	An activated form of caspase 3, considered to be the major executioner enzyme in the apoptotic machinery.
mouse monoclonal anti-ATF-3 + Alexa Fluor tag	ATF-3 is a transcription factor expressed after a peripheral nerve injury, during activation of regeneration
mouse monoclonal anti-ED1+ Alexa Fluor tag	ED1/CD68 is a transmembrane protein commonly used as a marker for macrophages
goat polyclonal anti-CD34 + Alexa Fluor tag	CD34 is a cell surface marker present on cells of hematopoietic origin
DAPI	DAPI binds DNA and emitts a bluish-white fluoresence after excitation. Used as a nuclei stain.
Hoechst 33342	Binds DNA and emitts a blue/cyan fluorescence light after excitation. Used as a nuclei stain.
Lipid ToxRED	Binds neutral lipid droplets with high affinity. It can be detected by fluorescence microscopy
May Grunewald Giemsa	A dye combination commonly used to identify different blood cell types.

Image analysis

Though more time consuming, manual counting and measuring of microscopic images has higher accuracy than automatic image analysis. For axonal outgrowth, I measured the length of neurofilament stainable axons from the suture line to the front of three parallel axons [105]. The sutures are easily recognized as broad, homogenous, curved structures. For paper one, a senior researcher, experienced in the technique, performed blinded measurements.

To estimate the number of cells in an image, I used counting by visual detection and counterstaining with DAPI, which stains cell nuclei. A counting frame was applied with a random placement, and objects were counted unless they crossed the so called forbidden line. For systematical random sampling, I rolled a dice to determine the starting frame. Often counting objects on images of longitudinal nerve sections, I chose a circular counting frame to avoid interfering with the longitudinal nature.

Images can be challenging to count if there is cell overlap, variability of shape, and focus problems. In paper III, focus problems were overcome by focus stacking (see above).

An electrospun vehicle for cell delivery

Electrospinning was originally developed for the textile industry. An electric field is applied to a polymer, producing electrically charged jets directed towards a charged collector. The polymer dries by evaporation of the solvent while travelling through the electric field, and produces nanofibers. The fibers are deposited on collecting surface or a drum [106]. The setup is economical and simple. The technique can be used to create complex three dimensional structures, and to incorporate topographical cues, such as aligned nanofibers for axonal guidance [107]. Seeking biomimicry, electrospinning has been used to manufacture an architecture resembling the structure of a decellularized nerve, but with larger micro-channels to promote cell infiltration [108].

In vitro culture of primary SVF cells

In vitro cell culture can be used to observe cells in an isolated, artificial environment. The first successful culture of mammalian cells was observed in 1908, when Margaret Reed cultured guinea pig bone marrow cells on nutrient agar intended for amoebae [109]. A limitation of the *in vitro* system is the isolation and lack of contact with other cells and structures normally present in an organism, such as the absence of a systemic immune response and inflammation.

Cell culture is normally performed in plastic containers and incubators with regulated heat (+37°C) and controlled humidity, oxygen and carbon dioxide levels. Cells are added under sterile conditions, and grown in a cell medium, a solution (liquid or gel based) supplemented with salts, nutrients and often a buffer system to maintain physiological pH and osmolality. The addition of antibiotics is often necessary, since the culture conditions are also favourable for pathogens.

To optimize culture conditions for animal cells, the medium is often supplemented with a serum, a cocktail of growth factors and many other active substances, necessary for the proliferation and survival of animal cells. Fetal bovine serum (FBS) is the most commonly used serum. A downside of FBS is that its xenogenic, and can modify (mask or enhance) the behaviour of cells *in vitro*. As a natural serum, FBS is also undefined, and its composition may vary from time to time, thus making it preferable to use a chemically-defined serum. In view of these considerations, I chose in our experiments to run serum-free cultures. This option can be used for short term experiments only, in my case five days

Electron microscopy

The development of electron optics and the first electron microscope was awarded the Nobel Prize in Physics 1986. It relies on the irradiation of a surface with electrons, detecting them on an electron-optical lens to produce an enlarged image. The sample must first be prepared by fixation, dehydration, drying, and coating to increase conductivity. The resolution level of electron microscopy is extremely high.

In my study, electron microscopy was used to visualize the electrospun nerve conduit in paper II and determine its porosity. It was also used to visualize the nanofiber co-culture constructs in paper III.

Mass spectrometry

Mass spectrometry can be used to precisely identify, and today also quantify, also subfractions of proteins from complex samples. For a long time, mass spectrometry could only be done on the smallest proteins, because of problems to get proteins volatile and fly. The research breakthrough that permitted mass spectrometry for biological macromolecules was awarded the Nobel Prize in Chemistry in 2002.

In a mass spectrometer, a sample is vaporized and ionized by an electron beam. It fragments and positively charged ions reach a detector. The data can provide the molecular weight of the compound, and be related to such information in a protein

atlas. Proteins must be extracted from the raw material, and the protein concentration determined, prior to loading the sample into the mass spectrometer machine.

For this project, the protein preparation and mass spectrometric analysis was performed at the Local Mass Spectrometry Support at the Medical Faculty, Lund University.

Statistical methods

For cell counting and measurements of axonal outgrowth in paper I-III, values were expressed as median (min–max). The gastrochnemius muscle wet weight (paper I) was presented by the medium value and the inter-quartile range. For the von Frey monofilament test (paper I), data was presented as mean +/- two standard deviations.

Statistical differences between treatment and control groups were determined by the non-parametric method Kruskal-Wallis (KW), with the post hoc Mann-Whitney (MW) U-test to detect differences at the specific time points and at specific locations (paper I, II). Statistical analysis for within-group analysis over time (von Frey monofilament test, paper I) was performed using Friedman's two-way analysis of variance by ranks. Spearman correlation test was used to analyse associations between axonal outgrowth and cleaved caspase 3-stained cells (paper I). The significance level was set to 0.05.

The IBM SPSS Statistics version 22 SPSS software was used for numerical and statistical analysis of data.

"Give me a fact, said Carlyle,
and I will prostrate myself before it."

Santiago Cajal: Advice for a Young Investigator

Results and comments

Paper I

Short term experiments (seven days) showed that post-traumatic G-CSF therapy with G-CSF injections of 50 µg/kg a day during five days resulted in a local 13% decrease in Schwann cell apoptosis (cleaved caspase 3 staining) at the site of lesion in healthy rats. A similar trend was observed 12mm distal to the lesion. In diabetic GK-rats, there was merely a trend towards decreased Schwann cell apoptosis at the site of lesion, while the distal nerve segment was not assessed. In long term experiments (90 days), there was no difference between the amounts of cleaved caspase 3-stained cells at the site of lesion (expressed as % of total number of cells).

Axonal outgrowth was analyzed by the presence of neurofilament protein in longitudinal sections as visualized by immunofluorescence microscopy. G-CSF therapy did not affect the length of axonal outgrowth in short term experimentation (seven days) in neither healthy nor diabetic GK rats. A repetition study on healthy rats was performed by another experienced surgeon, confirming the result. In long term experiments on healthy rats (90 days), the axons were regenerated, and the area fraction of neurofilament protein was used to determine axonal outgrowth, again with no differences between groups.

As for the inflammatory response, there was no difference between groups in the quantity of ED1-stained cells 5 mm distally to the lesion (no/mm^2).

There was no difference in functional recovery after 90 days for G-CSF treated specimens as compared to placebo (saline). The motor function was evaluated by the wet weight of the gastrocnemius muscle. von Frey monofilament was performed weekly to evaluate perception of touch. Only the non-treated rats presented a significant change in withdrawal threshold over the course of the experiment, but no significant differences in withdrawal threshold could be detected between groups.

Thus, G-CSF treatment resulted in a local decrease of apoptotic Schwann cells in short term experiments, but did not improve axonal outgrowth or functional recovery.

Paper II

I investigated the potential of a bio-inspired electrospun nerve conduit mimicking a native peripheral nerve, with longitudinal nanofibers inside the channels for axonal guidance, and its use as a SVF cell delivery vehicle in selected groups. Two polymers, PCL and PLLA were used for fabrication of two similar conduits.

As for construct performance, it was possible to manufacture a conduit from PCL with fair resemblance to a nerve, while the PLLA conduit had less well defined pores. For the nanofiber construct to maintain its structure during surgical handling, it had to be inserted into a protective shell; we used a silicon tube. This was a weak point, as some nerve guides relocated within the silicon shell. Further, silicone has no porosity, thus not allowing the exchange of oxygen, nutrients, and growth and differentiation factors through the wall; in spite of its clinical use in randomised clinical studies [110,111]. The mechanical stability and the outer shell are areas of development.

My study showed that the PCL nerve conduit was poorly tolerated by the rats, with a high degree of automutilation (n=8/43). This is in accordance with previous studies [114,115].

The PLLA nerve conduit supported axonal outgrowth *in vivo*, but did not outperform autograft. It supported the ingrowth of Schwann cells, as identified by the Schwann cell marker S-100. The PLLA nerve conduits and their content were analyzed through longitudinal sections. There was no difference in the migrating length of S-100 stainable cells between PLLA nerve conduit alone, autograft and the hollow tube. The PCL conduit, because of its rigidity, had to be analyzed in transverse sections. At 8 mm from the site of lesion, there were more S-100 stainable cells in the PCL nerve guide than in the autograft. This indicates that Schwann cells might respond and migrate or proliferate differently into a nerve guide from the proximal and the distal ends; an observation also seen in tendon autografts [112].

I noted several effects after the SVF therapy. There was no difference in axonal outgrowth as compared to the nerve conduit alone. I observed an increase in ED1 stainable cells (i.e. macrophages) proximally and at 5mm in PLLA nerve conduits endowed with SVF cells. Moreover, Schwann cell distribution was inferior in the PLLA nerve conduits supplemented with SVF cells.

A large number of specimens who underwent SVF therapy (n=9/30) had their conduits grossly encapsulated; in this group, some nerves were continuously integrated with the nerve guides, some were not. A similar situation has been previously reported in the context of SVF in a goat model of intervertebral disc degeneration, where the specimens developed adverse effects manifested as sterile inflammation following the SVF injection [113].

To our knowledge, our paper was the first report of an *in vivo* study of a porous multi-channeled, electrospun nanofiber nerve conduit enriched with supporting cells. A strength of the study is the use of the homologous model for cell transplantation, though this limits the amount of available adipose tissue for transplantation purposes.

Paper III

Based on the results in paper II, I set up a serum-free *in vitro* model to study the nerve-SVF interplay in our specific microenvironment. I wanted to look into the nerve end-SVF interaction, rather than DRG-SVF, to better reflect the biological setting with a long distance between the axonal end and the cell soma.

The expression of the Schwann cell marker S-100 in SVF cells was unchanged during the observation period of five days, while the appearance of the pan-Schwann cell marker Sox-10 in SVF cells was increased after co-culture. There were, however, no changes between SVF+nerve and SVF alone at baseline (t=2h, to allow cells time to adhere to the nanofiber matrix) or at five days.

In sciatic nerve segments, I found that cleaved caspase 3-stainable Schwann cells (as an index of apoptotic cells) could only be detected after co-culture with SVF at two days, but not at five days. ATF-3, related to activation of the regenerative response, could only be detected after co-culture with SVF at two days. The observations were made from a small number of specimens, and no statistics were calculated.

The lack of protein expression of ATF-3 and cleaved caspase 3 in nerve segments without SVF at two days may imply that our serum free cultures lacked the cell signals that can affect Schwann cell activation following a nerve transection. Interestingly, it also implies that part of the signals for Schwann cells to express ATF-3 and cleaved caspase 3 at two days did not come from the nerve cell soma, but from the microenvironment including the SVF.

We attempted to model the environmental cues in paper II, with *in vitro* culture on a bed of aligned nanofibers, similar to those used inside the channels of the electrospun nerve conduit. This *in vitro* model, however, does not reflect the conditions of nerve-SVF interaction a constrained space.

To obtain information on the microenvironment, pilot experiments were conducted analyzing the culture media, also containing any floating cells, with mass spectrometry. The analysis identified more than 50 proteins present in the nerve+SVF co-culture situation, but not in SVF alone. Addition of SVF to a sciatic nerve segment is likely to alter the local microenvironment at the contact position. Analysis of more situations, also including the sciatic nerve segment alone, is expected to provide more information.

General discussion

In the beginning - that is 2010 – this thesis was labelled "the one hour cell therapy project". It aspired to explore the possibility to mobilize and harvest the body´s own stem cell sources, embracing – in a very practical sense - the limitations of time and space.

It went against the tradition of the time, when various *ex vivo* expansion and differentiation strategies were explored. However, previous work with cell lineages had led to awareness of their limitations. There was also a strong influence by a paper written by the Committee for Advanced Therapies about the risks with Advanced Therapy Medical Products, including cell therapy [116]. The road to implement cell therapy in a clinical setting for a non-lethal disease seemed very long.

Perhaps one day we may have personalized medicine, and a personalized cell bank with capacity to grow, transfer and harvest large amounts of cells, readily available in case of emergency. Meanwhile, the use of available autologous cell resources could strike a balance between do no harm and do good.

The increasingly complex map of signal transduction

Substantial effort have been made to uncover the molecules that can influence the growth, survival and differentiation of neurons and Schwann cells [6,19,29,117,118]. VEGF is one of the many molecules shown to have neurotrophic and mitogenic activity on cells in the PNS [28]. Glial cell line derived neurotrophic factor (GDNF) has been investigated after delayed nerve repair [119]. Nerve Growth Factor (NGF) Brain Derived Neurotrophic Factor (BDNF), Neurotrophins-3 and 4/5, Ciliary Neurotrophic Factor, Leukemia Inhibitory Factor, Oncostatin M, Hepatocyte Growth Factor, Cardiotrophin-1, Bone Morphogenetic Proteins, Epidermal Growth Factor, Fibroblast Growth Factor and insulin are other molecules shown to have neurotropic effects [120].

When studies on signal transduction have showed such a large amount of players interacting in a complex environment, any attempt to artificially alter and orchestrate the regenerative process by the addition of a few molecules seems like an illusion of grandeur. Hence, the idea to "let the cells do the job".

What is the role of cleaved caspase 3 expression and Schwann cell apoptosis?

Because of the intimate relationship between Schwann cells and axons, strategies to enhance Schwann cell function could benefit regenerating axons. In my first paper, I found that G-CSF caused a significant 13% decrease of cleaved caspase 3 stainable Schwann cells at the lesion site in healthy rats, and a similar trend in diabetic rats, as well as in the distal nerve segments of healthy rats. However, G-CSF treatment did not improve early or late axonal outgrowth or functional recovery.

Perhaps the degree of Schwann cell apoptosis is not relevant. Perhaps the difference was too small to make any significant difference. Perhaps the amount of cleaved caspase 3 stainable Schwann cells reflects something else, such as a change in total number of cells.

A study on diabetic Biobreeding (BB) rats showed a higher number of both cleaved caspase 3 and ATF-3 (a marker for survival and regeneration) in the diabetic BB nerves after nerve transection and repair [53]. The authors found no correlation between neither cleaved caspase 3 nor ATF-3 expression in Schwann cells, and axonal outgrowth [53]. In paper I, I did not examine ATF-3 expression.

Following a peripheral nerve injury in mice, *in vitro* and *in vivo*, Schwann cells have been shown to become apoptotic as determined by nick end labelling technique (TUNEL) [121], and the apoptotic nature has been confirmed by electron microscopy [122]. Thus, it is less likely that Schwann cell expression of cleaved caspase 3 in our studies would be non-apoptotic.

In CNS, however, a non-apoptotic increase in cleaved caspase 3 expression was recently described in glial cells, but not in neurons, following exercise in a rat model. TUNEL staining was performed and was negligible in all groups, not reflecting the pattern of cleaved caspase 3 [123]. TUNEL, like cleaved caspase 3 staining, provides a snap shot image of the apoptotic status of the cells. Especially in astrocytes, the CNS glial cells, cleaved caspase 3 has been detected in the absence of cell death [124-126].

The delivery of cells for cell therapy

In my first paper, I explored the effects of G-CSF therapy by daily injections. The use of a drug approved for hematopoietic stem cell mobilization to the peripheral blood, albeit for other indications than a peripheral nerve injury, is clinically applicable.

The intravenous route may be also used to deliver cells for cell therapy. Allogenic bone-marrow derived, cultured mesenchymal stem cells have been shown to migrate and engraft in a peripheral nerve after injury [127]. The intravenous route has also been used to deliver mesenchymal stem cells to treat a recurrent laryngeal nerve crush injury in rats [128].

Injecting cells straight into tissues has been associated with poor retention and dramatically low survival rate of transplanted cells [129]. Some kind of implant or scaffold can be used to dock and support the cells in the target area. In the field of peripheral nerve injury research, this comes along with the need to develop nerve conduits that can bridge a critical gap.

Acellular nerve allografts have been developed [74,130,131], and are commercially available. Lacking Schwann cells, and with non-therapeutic levels of neurotrophic factors, acellular nerve grafts are less effective than autologous nerve grafts in supporting axonal regeneration. Attempts have been made to improve their function by adding polymeric microspheres of a tropic factor [132].

Electrospinning can be used to manufacture a structure mimicking a native nerve [108]. The design with aligned nanofibers inside the channels to guide the axons had previously been fabricated, but not investigated *in vivo* [108]. Their original design of the three-dimensional nerve guide had micro-channels with slightly wider diameter than a native nerve, to encourage cell infiltration.

It is a challenge to stimulate cells to migrate into a conduit, and along with the axons. The electrospun nerve conduit allowed for infiltration of Schwann cells, as S-100 stainable cells could be observed extending at a similar depth in the PLLA nerve guides, the autologous nerve grafts, and the hollow silicone tubes. However, I found limited Schwann cell ingrowth into our nerve conduit when SVF cells were added. Perhaps even wider micro-channels would have been preferable for local cell transplantation *in vivo*.

I used PCL and PLLA, two of the few polymers on the FDA list of approved materials, for smooth clinical translation [133]. Chitosan, another biomaterial of interest for peripheral nerve regeneration [38], is a sugar, and difficult to manipulate by electrospinning technique because of its chemical properties [134].

Any use of nerve conduits must take into account the cellular and molecular interaction between the PNS and the artificial device in the context of injury and

regeneration, demanding an interdisciplinary approach [38]. Cells carry with them a complex microenvironment, and the interaction between transplanted cells at the zone of injury surrounding a nerve end yields yet a new microenvironment (paper III). While earlier scaffolds attempted to replace lost tissue, the new approach in materials science is to provide a correct environment for cells, and – also here - "let the cells do the job".

The risks of cell transplantation

Cell transplantation has disadvantages, such as a secondary surgical site and possible presence of enzyme remains and inhibitory molecules, which may reduce the efficacy of any therapeutic cells present in the heterogenic cell pellet that constitutes SVF. To enhance the quality of the cell therapy product, *ex vivo* expansion is a better method to provide a clean, well characterized cell population with higher viability. The accepted viability rate of SVF is >70%, while the culture of adherent adipose tissue-derived stromal cells (ASCs) should yield viability rates >90% [79]. Such cells, however, cannot be isolated in one hour, or in the same surgical procedure.

Toxic effects from cell transplantation also includes migration to unwanted sites, and unexpected behaviour at a distant site [116]. I attempted to restrict ectopic engraftment by delivering the cells in our electrospun nerve conduit (paper II).

While much has been learned and solid foundation is based on the purity of *ex vivo* expanded and differentiated cells, recent years have witnessed an increased interest in minimally manipulated cells [135]. Because of rigorous demands on cell therapy products, and because many mesenchymal cell therapies seem to exert their therapeutic potential by secretion, researchers have even started to investigate cell-free regenerative medicine, relying on secretome derivatives, such as conditioned media or exosomes, rather than cells [136].

Exosomes are released by different cell types, and their composition varies depending on the source from which they are derived. Exosomes from Schwann cells, and exosomes from ADSC differentiated towards a Schwann cell-like phenotype, both enhance neurite outgrowth *in vitro* [137]. Exosomes from cultured, undifferentiated ADSC have anti-apoptotic effects on cultured Schwann cells *in vitro* (expression of Bcl-2 and Bax on mRNA level) [138].

Is there a place for G-CSF therapy?

My G-CSF study, published in 2016, showed no effects of axonal outgrowth or functional recovery of post-traumatic G-CSF therapy after twelve weeks. This is in contrast with a study by Jia *et al.* (2017)[73], where G-CSF therapy in a rat model of sciatic nerve injury bridged by an acellular nerve xenograft showed improved muscle weight after G-CSF therapy as compared to control (the nerve xenograft alone) at eight weeks. The study by Jia *et al.* examined a more severe injury, a nerve gap, and it also incorporated xenotransplantation of acellular nerve tissue. In this setting, it is possible that the positive effects of G-CSF therapy may reflect its anti-inflammatory effects.

The key finding of my G-CSF study is the 13% local decrease in Schwann cell apoptosis. Other studies have shown increased motor neuron survival after a sciatic nerve lesion or axotomy [71,72]. For a sciatic nerve lesion, G-CSF could possibly exert a more important effect on motor neurons in CNS, rather than in PNS. While G-CSF is clinically applicable, and the drug is well tolerated, the results of G-CSF therapy alone may be insufficient to motivate therapy.

Will the real stem cells please stand up?

Clinically mobilized hematopoietic stem and progenitor cells (HSPCs) from the peripheral blood have basically replaced bone marrow as a source of stem cells for transplantation purposes, but G-CSF therapy may not be an equal option after all. While clinical trials showed no difference in long-term survivability between peripheral blood grafts and bone marrow-derived cells, this may be explained by a larger stem cell yield [139]. Studies in mice have indicated that the G-CSF-mobilized HSPCs are not the "true" stem cells, or that G-CSF mobilization generates HSPC transplantation defects [139,140]. Given the new knowledge about the limitations of G-CSF mobilization, the applicability of primary bone marrow cells for peripheral nerve injury is still open to question.

Is SVF therapy good for the peripheral nerve?

The usefulness of SVF therapy is unclear. There are reports on positive effects of SVF in hollow tubes, but hollow tubes are not appropriate for many grafting applications. When delivered in our electrospun multi-channeled nerve guide conduit, SVF therapy did not improve axonal outgrowth and impaired Schwann cell infiltration. The multi-channeled conduit must be further optimized for cell therapy.

Sterile inflammation has been reported following SVF therapy in a goat model [113], and we observed gross encapsulation in 9/30 specimens (paper II). Some caution is advised.

A philosophical reflection: Two is company, three is a crowd?

Is the delivery of SVF to the zone of injury a collaboration opportunity for the cells involved? The delivery of cells for somatic cell therapy is not a naturally occurring phenomena, though it has been successfully explored in many disease models [141]. The pilot mass spectrometry analysis in paper III showed that the microenvironment changes when SVF and a sciatic nerve segment are brought together, and future data will cast further light on this matter.

An interesting finding in paper III is that expression of ATF-3 and cleaved caspase 3 was detected only in nerve segments co-cultured with SVF. Following a nerve injury, the neurons respond by profound changes in protein expression, a response referred to as "the cell body reaction" or "signalling to growth mode switch" [12]. In our *in vitro* setting, however, the explanted sciatic nerve segments, disconnected from their cell nucleus, could not possibly have provided the Schwann cells with any cell body response. This indicates that some of the key signals for the Schwann cell expression of ATF-3 and cleaved caspase 3 came from the local micro-environment related to the SVF cells, and not from the injured neuron.

The proliferation of Schwann cells and myelin phagocytosis, features of Wallerian degeneration, is not a response originating in the Schwann cells alone, but involves non-resident cells [142,143]. Beuche *et al.* (1984) [142] "encaged" sciatic nerves from mice in Millipore chambers and kept them in the peritoneal cavity of a host animal for up to eight weeks, restricting the entry by other cells [142]. The authors reported absence of Schwann cell proliferation, and no signs of active removal or digestion of myelin. Further, co-culture with macrophages in the same Millipore chambers did not result in myelin phagocytosis [142]. A situation similar to Wallerian degeneration could only be seen when nerves were kept in diffusion chambers of larger pore size, and were invaded by leukocytes and monocytes [142]. Hence, non-resident cells – albeit not SVF cells – already have a role in peripheral nerve repair.

What do SVF cells do when they, after an enzymatic bath and centrifugation, enter the zone of injury? What do Schwann cells do when they meet a SVF transplant? Is a sciatic nerve end a highly localized force also for SVF cells? These are question of connectedness versus identity.

Cells respond to their environment, and long-term contact between bone marrow derived mesenchymal stem cells (MSC) with endothelial cells has been shown to alter gene expression profiles and differentiation [144-147]. Undifferentiated *ex vivo* expanded bone marrow derived MSC express S-100 two weeks after transplantation into a 10 mm nerve conduit in the rat sciatic nerve [148]. I looked for signs of *in situ* differentiation of SVF cells into a Schwann cell like phenotype in the co-cultures (paper III). I found unchanged S-100 expression, but increased Sox10 levels compared to baseline. It indicated that the interaction with the peripheral nerve segment modulated the protein expression in SVF cells in short term experiments. Long-term contact, and contact *in vivo*, may have more pronounced effects.

The regenerative outcome after a peripheral nerve injury depends on collective dynamics between different cell types, and between CNS and PNS. We can alter this dynamics by G-CSF and by SVF transplantation. Future research can improve our understanding on molecular and cellular levels, and the delivery, effects and mechanisms of transplanted cells.

"Let ut haste to hear it"
Fortinbras
(from Shakespeare: Hamlet)

Conclusions

This thesis investigates the use of G-CSF and SVF improved outcome after a peripheral nerve injury and repair or reconstruction.

Based on the experimental data, I conclude the following:

- G-CSF therapy does not improve axonal outgrowth or functional outcome after a peripheral nerve injury in healthy or diabetic rats.
- G-CSF therapy has anti-apoptotic effects on Schwann cells at the site of lesion at seven days in healthy rats. In diabetic GK-rats, there is only a similar trend.
- An electrospun multi-channeled nerve conduit supports axonal outgrowth *in vivo.* The nerve conduit supports Schwann cell infiltration. The nerve conduit can serve as a cell delivery vehicle.
- SVF therapy delivered in the conduit does not improve axonal outgrowth at four weeks. SVF therapy delivered in the conduit resulted in a partially enhanced inflammatory response, poor Schwann cell infiltration, and a large number of grossly encapsulated implants.
- PCL implants were poorly tolerated.
- Co-culture of SVF and a sciatic nerve segment on a nanofiber bed results in an increased expression of Sox10 in SVF cells at five days as compared to baseline. The addition of SVF to a sciatic nerve segments alters the microenvironment *in vitro*.

Sammanfattning på svenska

Vid nervskador i händer och armar får patienter, trots bästa behandling, bestående förlust av känsel och muskelstyrka. I det här projektet undersökte jag om läkningen skulle kunna förbättras genom att tillföra kroppsegna stamceller till skadeområdet.

Stamcellsforskningen har gjort stora framsteg, men för att kunna utnyttja dem inom sjukvården, för t.ex. celltransplantation, krävs fler studier om effektivitet och säkerhet. Det kan vara en fördel att använda kroppsegna celler, som inte påverkas av kroppens immunsystem, även om koncentrationen är låg. Benmärg innehåller stamceller som kan mobiliseras ut i blodcirkulationen genom stimulering med läkemedel. Fettvävnad innehåller också stamceller och vävnaden är lätt att skörda. En generell utmaning med celltransplantation är hur man ska kunna försäkra sig om att cellerna stannar kvar i rätt område, och inte vandrar iväg eller sköljs bort.

I första delen visar jag att användning av läkemedlet G-CSF, som bl.a. mobiliserar kroppsegna stamceller från benmärgen ut i blodet, leder till bättre överlevnad av nervernas stödjeceller, men behandlingen förbättrade inte utväxten av nervtrådar eller återhämtning av känsel och muskelstyrka efter en nervskada.

I andra delen undersökte jag användningen av en ny nervguide av nanofibrer, tillverkad genom en metod som kallas elektrospinning – något som blivit mycket populärt inom regenerativ medicin. Nervguiden har ett kanalsystem specialdesignat för att likna kroppens egen nervstruktur, med kanaler klädda med parallella nanofibrer, vilket hjälper nervtrådarna att hitta rätt under utväxten efter en skada. Tillverkningsmetoden är billig.

I försök gjorda på råttor fann jag att nervtrådar kunde växa i nervguiderna, och att celler transplanterade från det kroppsegna bukfettet kunde överleva och stanna i nanofiberguiden under studieperioden som var 28 dagar. Materialet, en bionedbrytbar polymer som också används i kirurgiska suturer (trådar), tolererades väl och orsakade ingen nämnvärd inflammation.

Tillförsel av cellblandning utvunnen från bukfettet förbättrade dock inte nervutväxten. Viktiga stödjeceller (Schwannceller) verkade heller inte vandra in i nervguiden i lika hög utsträckning om cellblandningen från bukfettet tillsattes, vilket kan bero på att stödjecellerna och de transplanterade cellerna konkurrerade om samma utrymme. Jag drog slutsatsen att modellsystemet kan vara av betydelse för framtida sätt att behandla nervskador, men att fler studier krävs för att undersöka och optimera hur kanalsystem ska designas för att användas tillsammans med celltransplantation inom sjukvården.

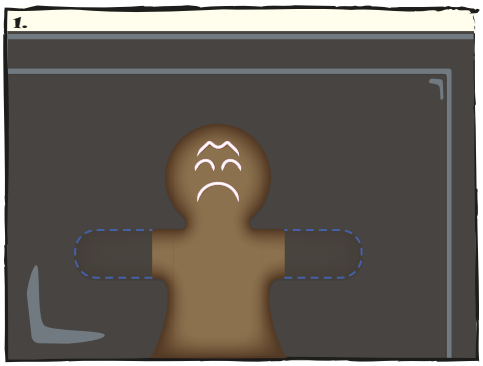
I tredje delen gjorde jag en provrörsmodell av celltransplantationssystemet med nanofiberguiden. En nervbit odlades tillsammans med cellblandningen från bukfettet på en bädd av samma sorts nanofibrer. Jag hittade vissa förändringar både i cellblandningen och hos nervbitarna. Analys av odlingsvätskan visade att tillförsel av cellblandningen resulterat i biologiska förändringar även i cellernas närmiljö.

Sammanfattningsvis har avhandlingen visat att behandling med ett läkemedel (G-CSF) minskar andelen döende stödjeceller i en skadad och reparerad nerv, men påverkar inte slutresultatet efter nervskadan. Med nanoteknik kan man tillverka en konstgjord nerv som kan stödja celltransplantation som behandling. Behandlingen med celler från det egna bukfettet förbättrade inte utväxten på kort sikt, och i flera fall kapslade kroppen in nervguiderna med transplanterade celler. Provrörsförsök kan ge mer information om vad som händer när celler från bukfettet transplanteras till en skadad nerv.

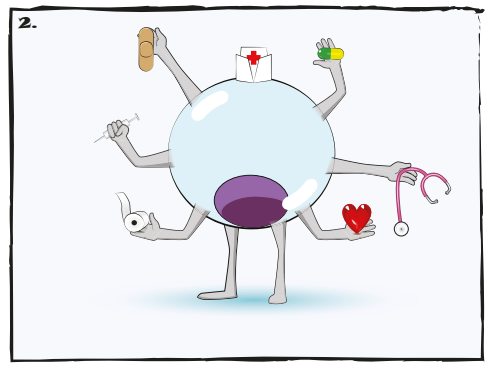
“Only those who will risk going too far
can possibly find out how far one can go.”

T.S. Eliot

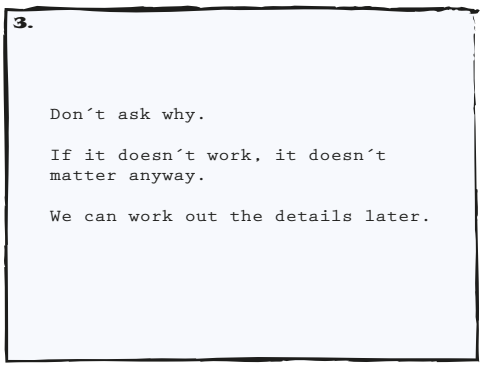
Thesis as a cartoon

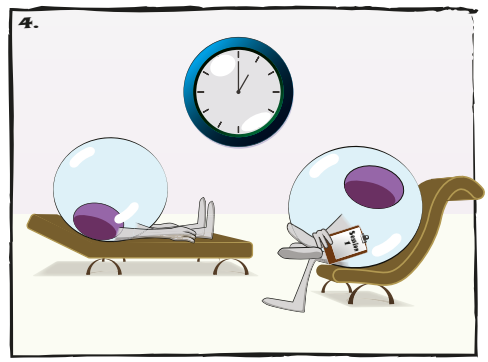


Peripheral nerve injuries are still really problematic.

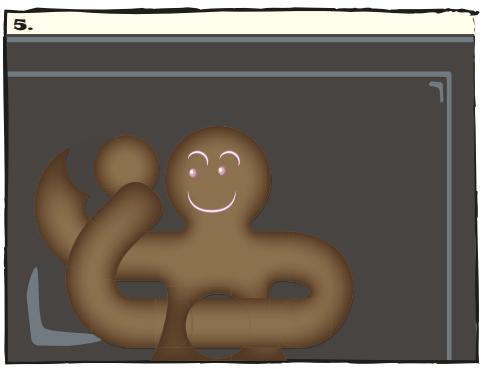


Stem cells seem to have some kind of unspecific healing potential.

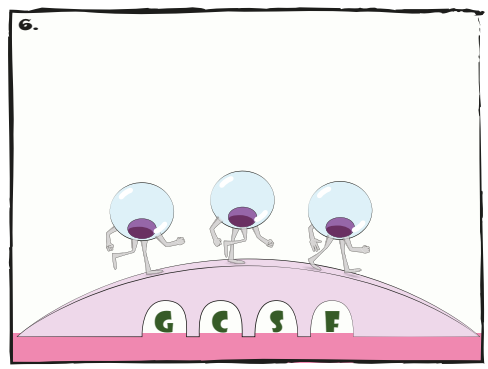




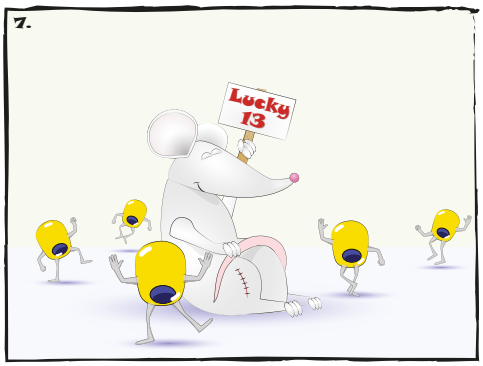
One hour is a reasonable time limit for cell therapy as an additional procedure.



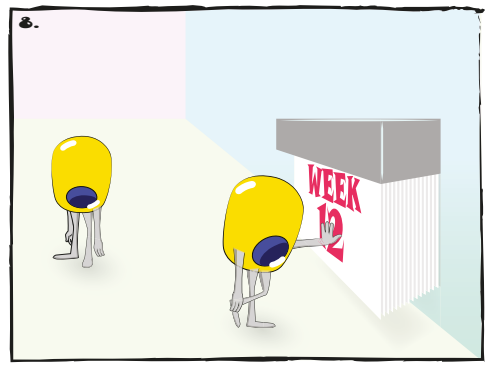
Minimally manipulated cells from your own body are safer and easier to implement as a new therapy.



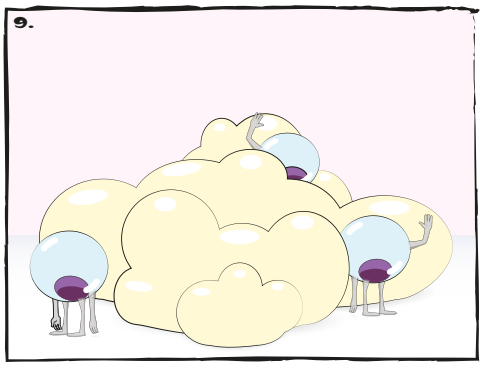
G-CSF is a drug with many effects, among them mobilisation of hematopoietic stem cells from the bone marrow. They migrate to injured areas, also to nerves.



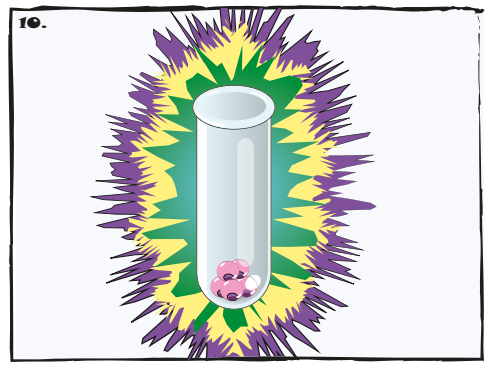
G-CSF decreased the death of Schwann cells (supporting cells) by 13% in short term experiments in healthy rats.



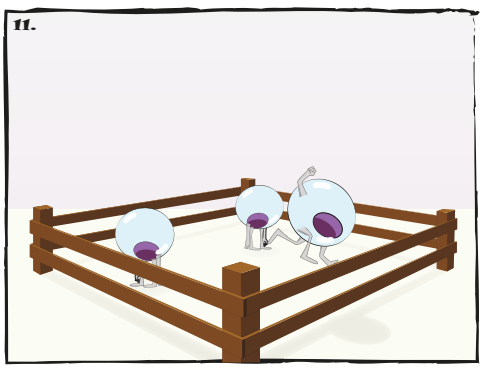
But there was no effect on long term recovery at 12 weeks.



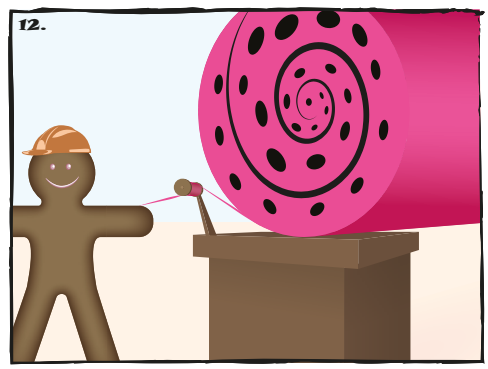
Adipose tissue, too, contains stem cells.



In one hour, fat can be processed to Stromal Vascular Fraction (SVF) which contains many types of cells, including stem cells.



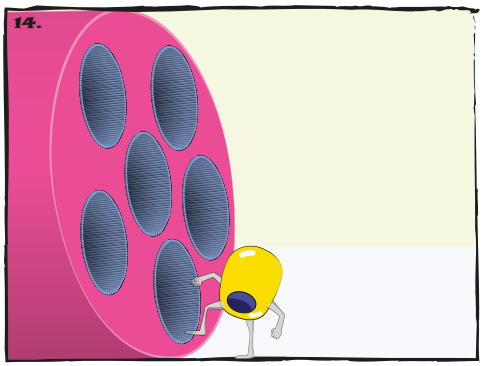
A general problem is how to dock transplanted cells to the area of injury.



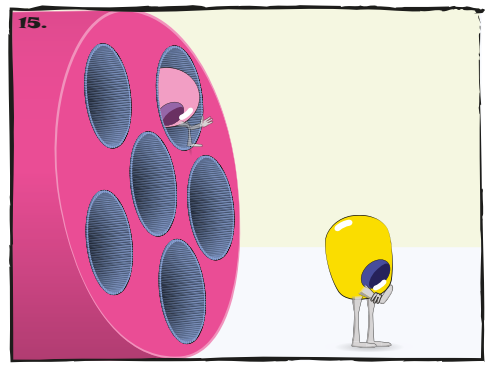
Nano engineers used electrospinning to create a nerve conduit mimicking a real nerve, boosted with aligned nanofibers to guide regrowing axons (nerve threads).

13.

Stromal vascular fraction,delivered in a nanofiber nerve guide,was investigated during 28 day experiments in rats.



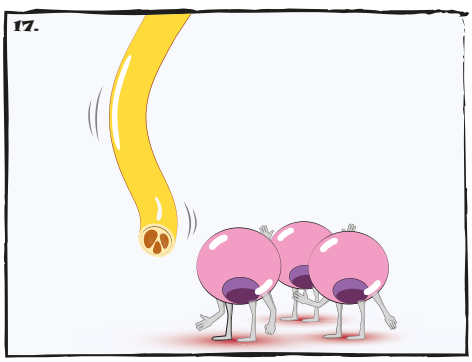
New axons grew into the nerve guide.
Schwann cells entered!



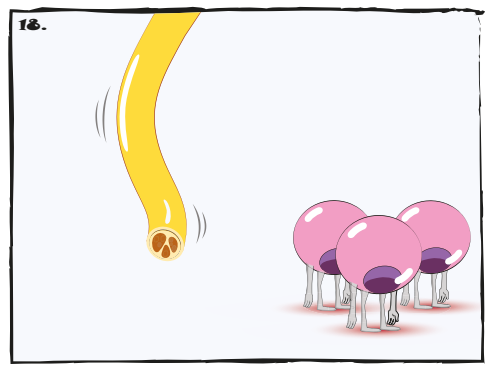
But not so much when SVF cells had been added.

16.

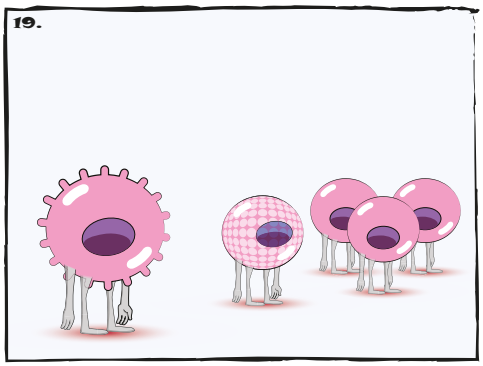
In vitro experiments will cast light on SVF-nerve interactions!
We cultured SVF cells with nerve segments on nanofiber beds in a 37ºC incubator for five days.



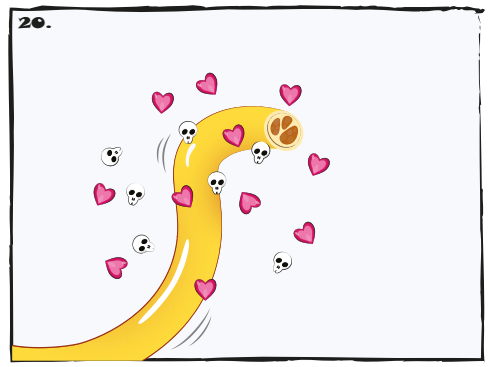
Would the SVF cells be thrilled to interact with a nerve segment?



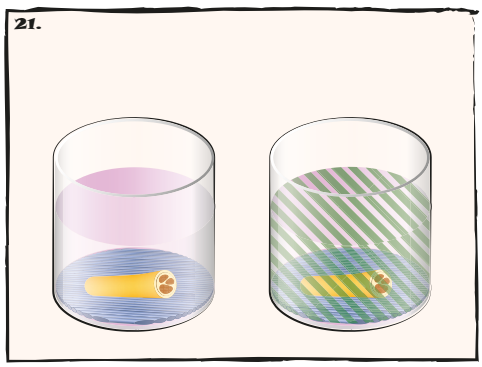
Or would they be indifferent?



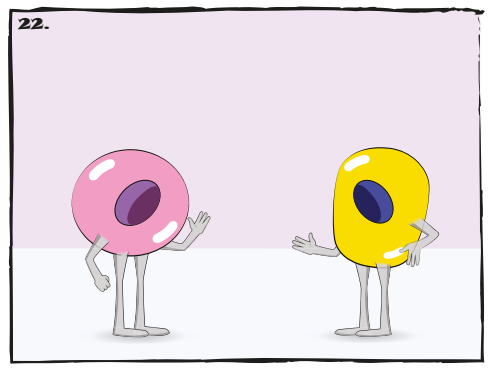
There was some change in the SVF cells, but they were mostly unchanged.



There were changes in the nerve segments too, after two days with SVF, but this is very preliminary.



Changes could also be detected in the culture media (the liquid supporting the cells outside the body).



SVF cells and the nerve segment have effects on eachother and on their surrounding. For better or for worse? The end.

Illustrations by Peregrin Frost

“The level of disorder in the universe is steadily increasing.”

Second Law of Thermodynamics

Acknowledgements

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"At one time, hot red wine served as a sterilizing fluid through which tortoise tendon was passed before it was used."

Morton Spinner: Development of Modern Technique of Nerve Repair

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