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Neural Stem Cell Therapy and Inflammation Status of Post-traumatic Syringomyelia

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Stockholm 2022

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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022

ISBN978-91-8016-663-8
Cover illustration: Painting showing sagittal section of spinal cord with neurons (triangles) and the cyst in the center (red) surrounded by the reactive astrocytes. Stem cell transplantation reduces the cyst size (left panel) but not in case of vehicle showing that spinal cord cysts can be treated using neural stem cells. Illustration by Tingting Xu and Ipsit Srivastava.Painting by Ipsit Srivastava.

Neural Stem Cell Therapy and Inflammation Status of Post-traumatic Syringomyelia
THESIS FOR DOCTORAL(Ph.D.)

By

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20th June 2022 at 09:00

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To my family and all the people that helped me

POPULAR SCIENCE SUMMARY OF THE THESIS

The spinal cord is the critical path for the movement signals produced by the brain to control the body below the head, but also the other way around to sense all stimuli from the environment. In case of a traumatic injury when the spinal cord is crushed or cut partially or completely during an accident, the signal transmission is compromised. The consequence will depend on the extent of the damage to the spinal cord. The spinal cord injury is not repairable as many other tissues in our body, and the symptoms present after the acute stage are usually permanent. For some patients, the condition gets worse if they develop a chronic stage complication called post-traumatic syringomyelia (PTS), characterized by formation of cysts in the spinal cord, with more tissue loss as the cyst(s) expand. For these patients motor and sensory symptoms, neuropathic pain and spasticity are aggravated. The exact mechanism is not clear although disturbed fluid balance after SCI is probably important, and the surgical treatment is not always effective. Therefore, our aim was to explore other potential mechanisms of PTS and investigate new therapies to improve treatment efficiency for PTS.

Inflammation plays an essential role in tissue degeneration, therefore we explored its possible role in the development of PTS. We demonstrated with multiple methods that there is more pronounced inflammation in PTS than its primary disease, SCI, and identified a number of cell types that may be important in the disease progression. These mechanisms may be useful targets for new therapies.

Neural stem cells have been very promising in experimental studies of SCI, and there is safety observed in clinical trials. Considering the similarity between PTS and its primary disease SCI, we investigated the effects of human neural stem cells on preventing the progressive tissue loss and also recover the damaged tissue. We found that two types of neural stem cells effectively reversed cyst expansion. We also showed that one type of them indeed stimulated tissue repair in PTS, partly by inhibiting inflammation but also by other mechanisms.

Therefore, our study demonstrate that neural stem cells can potentially be an effective treatment for PTS which could bring new hope for PTS patients and also lay the groundwork for further mechanistic explorations.

ABSTRACT

Post-traumatic syringomyelia (PTS) is a severe complication of chronic spinal cord injury (SCI) with intraspinal expanding cysts as the major pathology. PTS occurs in up to 30 % of SCI patients, and time of diagnosis is usually years after the primary injury. The sign of PTS is the deterioration of existing SCI symptoms, with further loss of motor, sensory and autonomic functions and increased neuropathic pain and spasticity. PTS used to be overlooked due to the limited availability of MRI, which is the critical diagnostic method.

The exact mechanism behind PTS is not clear yet, but disturbance of CSF flow by subarachnoid scar tissue with imbalance of in- and outflow of CSF in the spinal cord parenchyma is the most recognized factor. The surgical treatment, releasing the tethered spinal cord, with or without shunting to drain the fluid-filled cyst is not always effective. Therefore, it is essential to further investigate pathophysiological mechanisms and possible risk factors for the development of PTS, to identify therapeutic strategies in addition to surgery and improve the efficiency of treatment.

Neural stem/progenitor cell (NPC) with the ability to proliferate and differentiate into multiple lineages in CNS have been the focus of regenerative research in CNS disorders and insults such as SCI. NPCs can protect injured tissue, promote tissue repair and axon regeneration by releasing trophic factors, providing supportive tissue, modulating inflammation and differentiating into neurons, oligodendrocytes and astrocytes to replace the lost cells, and modulate the pathophysiology at the lesion site. NPCs can be transplanted to the injured recipient, or similar endogenous cells may be stimulated by various factors. In P**aper I**, we tested if NPC transplantation could improve tissue repair in PTS. We first developed a clinically relevant rat model of PTS mimicking the clinical situation by injecting blood into the subarachnoid space in combination with a mild traumatic SCI. The injection of blood was used to induce tethering of spinal cord without introduction of any foreign neurotoxic materials which could be toxic to the transplanted cells. Our rat model showed pathology similar to clinical PTS, including the appearance in MRI. With this model, we demonstrated the safety and efficacy of two types of human NPCs, embryonic/fetal NPCs and induced pluripotent stem cells (iPSC) derived NPCs-neuroepithelial stem cells (NESCs). We found that both of them can completely prevent the expansion of cysts in PTS and showed no sign of tumor formation after long-term survival. The therapeutic effect on cyst expansion did not correlate with the survival of transplanted cells and occurred in spite of limited differentiation of grafted cells.

To move further to clinical translation, in **Paper II**, we transplanted GMP-compliant iPSC-derived NESCs to the same rat model as in **Paper I**. We observed considerably better survival of transplanted cells than **Paper I**. Importantly, the transplanted cells not only prevented the cyst expansion but also modulated major pathophysiological SCI process, reducing astrocytic scar and inflammation, but also stimulating endogenous regeneration, enhancing proliferation of oligodendrocyte progenitor cells (OPCs) and supporting regeneration of serotonergic axon regrowth.

A better understanding of mechanism behind PTS will hopefully bring better therapeutic strategies for clinical treatments. In **Paper III**, we investigated and compared the pathophysiological processes focusing on inflammation and glial cell reaction to find additional mechanisms behind the development from SCI to PTS. We found signs of more pronounced inflammation at molecular and cellular level, less astrocyte-associated repair in PTS than SCI, and more severe tissue degenerative processes in PTS than SCI.

Taken together, with all the studies in this thesis, we have gained better understanding of the factors that could be related to the progression of PTS, and application of cell therapy which pave the way for improving the therapeutic strategies for PTS patients in a near future.

LIST OF SCIENTIFIC PAPERS

I. Ning Xu[*], **Tingting Xu[*]**, Raymond Mirasol, Lena Holmberg, Per Henrik Vincent, Xiaofei Li, Anna Falk, Eirikur Benedikz, Emilia Rotstein, Åke Seiger, Elisabet Åkesson, Scott Falci, Erik Sundström
Transplantation of Human Neural Precursor Cells Reverses Syrinx Growth in a Rat Model of Post-Traumatic Syringomyelia
Neurotherapeutics, Volume 18,2021

II. **Tingting Xu[#]**, Xiaofei Li, Yuxi Guo, Elias Uhlin, Lena Holmberg, Sumonto Mitra, Dania Winn, Anna Falk, Erik Sundström[#]
Multiple therapeutic effects of human neural stem cells derived from induced pluripotent stem cells in a rat model of post-traumatic syringomyelia
eBiomedicine, Volume 77, 2022, Article 103882.

III. **Tingting Xu**, Yuxi Guo, Lena Holmberg, Xiaofei Li[#], Erik Sundström[#]
Inflammatory status of the chronic spinal cord injury and post-traumatic syringomyelia.
Manuscript

(* Co-first authorships, # Co-correspondence)

CONTENTS

LIST OF ABBREVIATIONS

PTS	Post-traumatic syringomyelia
SCI	Spinal cord injury
CSF	Cerebrospinal fluid
BSCB	Blood spinal cord barrier
NESCs	Neuroepithelial stem cells
NPCs	Neural stem/progenitor cells
MSCs	Mesenchymal stem cells
snRNA-seq	Single nuclei RNA sequencing
NSC	Neural stem cells
BBB	Basso, Beattie and Bresnahan
CNS	Central nervous system
GMP	Good manufacturing practice
ESCs	embryonic stem cells
iPSCs	induced pluripotent stem cells
OPCs	Oligodendrocytes progenitor cells
NRP	Neuronal restricted precursors
GRP	Glial restricted precursors
SAS	Subachroid space
ROS	Reactive oxygen species
MMPs	Matrix metalloproteinases
NMDA	N-methyl-D-aspartate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ALS	Amyotrophic lateral sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
MPO	Myeloperoxidase
siRNA	Small interfering RNA
STAT3	Signal transducer and activator of transcription 3
CSPGs	Chondroitin sulfate proteoglycans
UC-MSC	Umbilical cord mesenchymal stem cell
BMSCs	Bone marrow stromal cells
ADSCs	Adipose derived stem cells
ANOVA	Analysis of Variance
MRI	Magnetic resonance imaging
EAE	Experimental autoimmune encephalomyelitis
CST	Corticospinal tract
MS	Multiple sclerosis
ChABC	chondroitinase ABC

1 INTRODUCTION

1.1 POST-TRAUMATIC SYRINGOMYELIA

1.1.1 Overview of PTS

Post-traumatic syringomyelia (PTS) is a severe complication of spinal cord injury with the pathological characteristics of intraspinal cord cyst formation and slow expansion. With this regard, PTS is different from SCI in terms of pathophysiology and the corresponding principle of treatment, with neural circuit reconstruction as the main goal in SCI whereas the cessation of cyst expansion in PTS(1, 2). PTS can happen up to 30% of the SCI patient months to years after the primary injury (3, 4). The classical sign of the onset of PTS is the aggravation of existing symptoms from SCI, such as motor, sensation, neuropathic pain(5, 6). The proposed mechanism for PTS is the disturbed CSF flow in the parenchyma due to scar formation in the subarachnoid space after traumatic injury and lead to the net inflow of CSF via perivascular space after the break of flow balance between in and out of spinal cord(7, 8). Other possible mechanisms also could contribute to the development of PTS, inflammation for example which is the major pathophysiology in SCI even in chronic stage(9-11). In addition, the current surgery in clinic, detethering usually do not lead good efficiency and the success rates varies between clinics since it is usually challenging to completely remove the scar tissue(12, 13). Therefore, it is necessary to explore the other possible mechanisms and other alternative or assistant treatments to gain better therapeutic effects for PTS patients.

In summary, to gain better therapeutic efficacy for PTS patients, the exploration of its mechanism and alternative or assistant treatment is very essential.

1.1.2 Mechanism of PTS

1.1.2.1 The change of CSF fluid dynamic

The main theory about the mechanism behind PTS concerns the dynamic changes of cerebrospinal fluid flow inside and outside of the parenchyma. Scar tissue form in the subarachnoid space after primary SCI, probably mainly because of hemorrhage, free blood and iron in the subarachnoid space which triggers arachnoiditis(14-16). In addition, the scar tissue tethers the spinal cord to the surrounding dura mater and vertebral column. Consequently, the tethering and the connective tissue interfere with the CSF flow, which lead to a pressure gradient from the outside into the cord parenchyma. This affects the balance between inflow and outflow of CSF in the spinal cord parenchyma via perivascular space. In the end, there is a net inflow into the parenchyma at the lesion site (**Fig 1**). The tissue with

degenerative changes such as micro cysts due to the primary SCI provides the lowest resistance to this flow from the subarachnoid space(17, 18).This theory is supported by a recent published paper, the extradural restriction leading to the net inflow of CSF with the confirmation of CSF tracer via perivascular space even though there is cyst formation at the time frame of study(19).Furthermore, the tethering of spinal cord by the scarring can lead to shearing force and damage to spinal cord.

However, one previous study suggested that the pressure in the cyst is higher than the subarachnoid space, hence there could be other sources of cyst fluid. The most possible source is the fluid coming from the blood vessels due to the breakdown of blood-spinal cord barrier (BSCB)(20).

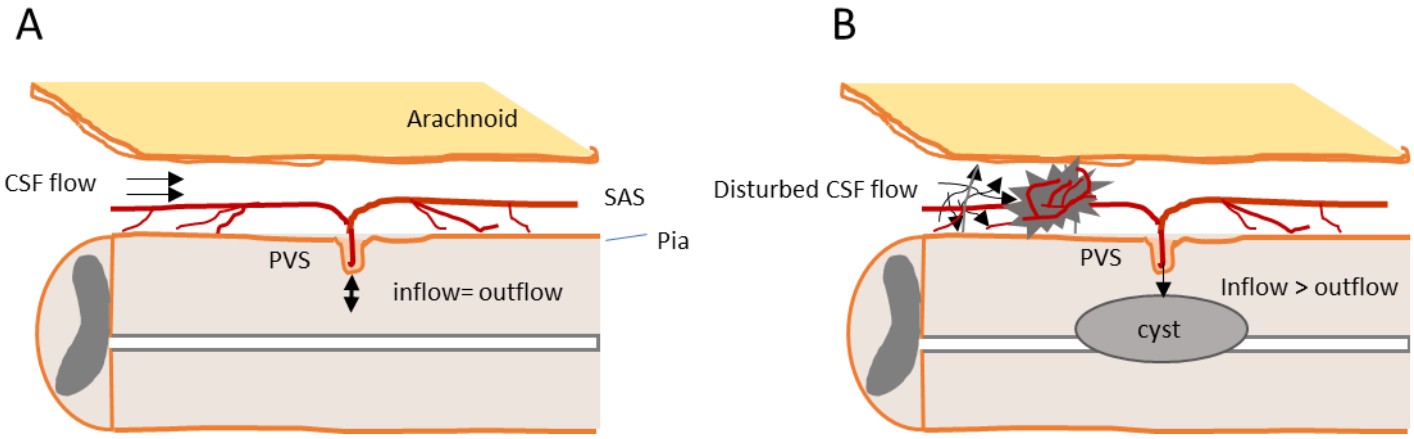

Fig1. A: Normal CSF flow in the subarachnoid space (SAS). Normally, CSF flows in the SAS and is thought to there is balance between inflow and outflow of the spinal cord parenchyma via perivascular space. B: Disturbed CSF flow via subarachnoid scarring lead to the development of intraspinal cyst. Eventually, the flow balance is disrupted and result in net inflow.

1.1.2.2 The extracellular fluid homeostasis in PTS

The homeostasis of fluid in the parenchyma of CNS is maintained by the integrity of blood spinal cord barrier(21), the water channel, particularly aquaporin-4 on astrocytic end-feet(22) in addition to the balance between inflow and outflow of CSF via perivascular space. BSCB consists of endothelial cells, basal lamina, pericytes and astrocyte end feet(23). The persistent structural and functional impairment of BSCB in SCI could be related to the development of PTS(24, 25). One study applied electronic microscope found the superstructure changes of BSCB in PTS, including the decrease of tight conjunction and increase of transcytosis, also observed the enlargement of perivascular space further confirmation of the fluid dynamic

theory(26). In addition, one study reported that the pressure of the cyst is higher than the subarachnoid space, hence there could be other source of cyst fluid other than CSF, it could be from blood vessel. Hemley et al. demonstrated that the functional and structural damage of BSCB corresponds with the time course of PTS, in comparison with a control group (20).

In addition, astrocytes as the essential component of BSCB express water channel aquaporin-4 and ion channel, Kir4.1 both mainly are related to the homeostasis of fluid in the CNS (27, 28). Aquaporin-4 is mainly expressed on the end-feet of astrocytes and was increased in tissue surrounding the cyst in an animal model of PTS induced by combining injection of quisqualic acid to parenchyma and kaolin to subarachnoid space. This suggests that there is a relationship between aquaporin-4 and the development of PTS. But it is not clear if this is a cause or a consequence of PTS. In addition, glymphatic system with aquaporin-4 as an essential component, which is proposed important in the waste clearance in the extracellular space in CNS, could also be involved in the development of PTS(29, 30). Similarly, the same group studied the expression of Kir4.1 as well, which is a potassium channel, in the same animal model of PTS, and there was down-regulation of kir4.1 channel on astrocytes around the cysts, suggesting that disturbance of Kir4.1 could contribute to the pathophysiology of PTS.

With all these studies, deficits in the BSCB could contribute to the formation and expansion of a syrinx in PTS.

PTS as a complication of SCI, share some pathophysiology with SCI, such as demyelination. However, if other well studied secondary injuries in SCI could participate in the development of PTS is not clear yet, inflammation for instance. Therefore, the main secondary injuries and tissue degeneration processes in SCI will be discussed in the following section.

1.2 SPINAL CORD INJURY

1.2.1 Overview of spinal cord injury

With spinal cord injury (SCI) we usually refer to traumatic spinal cord injury, which mostly results from mechanical trauma, such as car accidents and fall from heights(31). SCI leads to permanent impairment or loss of motor, sensory and autonomic functions(32). In addition, the loss of functions is often combined with neuropathic pain and spasticity(33, 34). To have good management and treatment in clinic, SCI is divided into different phases depending on the major pathophysiology processes and corresponding main treatment strategies, acute phase(0-48h), subacute phase (48h-14d) and chronic phase (>14d)(35). While the primary injury – the physical laceration, compression and shearing of the spinal cord tissue – is the

immediate consequence of the trauma, and results in necrotic cell death, a series of secondary injuries triggered by primary injury contribute to the tissue damage(36-38). These secondary injury processes include hemorrhage, ischemia, inflammation, overproduction and/or release of toxic molecules, which lead to death of different cell populations through apoptosis and necrosis, as well as other forms of cell death(35, 39). In the acute phase when the primary injury is dominating, the most critical treatment is to decompress the spinal cord by stabilizing the spine and removing bone fragments in clinic(40). In the subacute phase when secondary injury become dominant, the most important treatment is to target these secondary injuries and begin the regenerative interventions rehabilitative training(41). Lastly, at chronic phase when the secondary injuries become less intensive, the key treatment is to restore the function of spinal cord such as rehabilitate training to increase the plasticity of spinal cord(42). It is worth noting that NPCs transplantation has been shown could improve the regeneration the most when transplanted in the subacute phase in experimental studies, therefore, they are promising therapy for SCI when all the safety and technical issues are addressed(43, 44).

In addition to main secondary injury processes, the damage induced by primary injury, such as vascular disruption and cellular death which result in or facilitate secondary injuries will be discussed as well.

1.2.2 Pathophysiology of SCI

1.2.2.1 Vascular events

The vascular damage can lead intraparenchymal hemorrhage with the leakage of red blood cells and the leakage of plasma molecules and along with the damage of BSCB integrity induce vasogenic edema and facilitating inflammation(45-47). The hemorrhage size is related to the severity of trauma, and it reach the maximum at the injury site and extend both rostrally and caudally. Hemorrhage can further lead to cell damage in the following ways, 1) the production of reactive oxygen species (ROS) during the degradation of hemoglobin(48), 2) the ischemia due to the compression from hematoma(49). Some studies showed that the cavity at late stage is corresponding to the site of hemorrhage at early stage, therefore which could be one of the reasons lead to the formation of cyst in PTS(25, 46). For the therapeutic strategies targeting on hemorrhage, the heme oxygenase could be beneficial since it can degrade heme into bile pigment which act as antioxidant(50). In addition to hemorrhage, vasogenic edema is common vascular response in SCI, which can be derived from the direct

damage of vasculature and the impairment of BCSB. And vasogenic edema can further aggravate ischemia in addition to hemorrhage(51, 52).

BSCB damage not only limited to the lesion site, the increase of BSCB permeability have been reported rostral and caudal to the lesion site, which could be mediated by pro-inflammatory cytokines(53, 54). Therefore, therapeutic studies in SCI also focus on improving the integrity of BSCB by targeting on each essential component of BSCB, including increasing the tight junctions between endothelial cells, reducing the degradation of basal lamina, targeting the aquaporin-4 channel on astrocytes end feet. Some studies showed that reactive oxygen species (55-58) and pro-inflammatory cytokines(59, 60) can lead to degradation of tight junction protein between endothelial cells, and therefore the application of antioxidant and anti-inflammatory drugs may protect tight junction protein from degradation(61). Moreover, the matrix metalloproteinases (MMPs) that are mainly released by infiltrating neutrophils, can degrade the basal lamina and then contribute to the impairment of BSCB(62-65). Therefore, the inhibition of MMPs by drugs is under development, such as Flufenamic (66). The end-feet of astrocytes play an important role in the homeostasis of water via aquaporin-4 which is a bidirectional water channel. Impairment or depolarization of aquaporin-4 could aggravate vasogenic edema since aquaporin-4 was suggested play a beneficial role in vasogenic edema and the inhibition of depolarization by trifuoperazine could help inhibit edema(67). Therefore, the alteration of aquaporin-4 polarization and the damage of BSCB contribute to the fluid accumulation in the extracellular space, which could be related to cyst formation in PTS.

1.2.2.2 Cellular damage from toxic molecules

In addition to necrosis which directly results from the primary injury, there are cell death derived from secondary injuries, such as the glutamate mediated excitotoxicity. Several detrimental cascades occur following the excessive release of excitatory glutamate, which through activation of NMDA receptors and AMPA receptors cause unmanageable increases in intracellular and mitochondrial calcium levels(68). The intracellular increase of calcium concentration can activate the apoptosis cascade via the phosphorylation of calcineurin or via release of cytochrome c and apoptosis-inducing factors from mitochondria(69). In addition, the calcium overload in mitochondria can interfere the normal oxidate phosphatase and then generate ROS and nitric oxide(70). The ROS can further lead to the oxidative damage of cellular components, lipid membrane, cytoskeletal and DNA(71). A number of experimental studies applying NMDA and AMPA receptor antagonists have shown reduced injury and improved functional recovery in SCI(72, 73). Riluzole, a drug that reduce extracellular

glutamate concentrations or act as a sodium channel blocker, has shown neuroprotective effects (74)and is used clinically to slow down progression of amyotrophic lateral sclerosis (ALS)(75). Some calcium channel blockers could help with the neurological recovery. Inflammatory cells, such macrophages and neutrophils can produce ROS when they activated(76) and the production of ROS is via the enzymes, such as NADPH oxidase (77)and nitric oxide synthase(78). Therefore, these cellular damage mechanisms could be involved in the development in PTS since inflammation is a potent risk of PTS(79).

1.2.2.3 Inflammation in injured spinal cord

In response to traumatic injury, the resident cells, including microglia and astrocytes react immediately with changing morphology and releasing cytokines and chemokines to boost inflammation and recruit periphery immune cells to the lesion site(80). Different immune cells peak at different time points, but they overlap and then cooperate to promote the tissue repair at acute-subacute phase by clearing tissue debris and promoting the formation of glial scar (**Fig 2**)(81). The first infiltrated immune cell type is neutrophil which peaks around 1-3 days after injury and come down to basic level at chronic phase(82-84). Infiltrated neutrophils have been shown have both detrimental and beneficial roles, by releasing toxins such as ROS and MPO and promoting debris clearance by phagocytosis to promote tissue repair respectively(82, 85). The second peak is around 7 days after injury and formed by the resident microglia and infiltrated macrophages and they usually last until chronic phase(86). They play the role in clearing tissue debris and also interact with astrocytes to induce the formation of glial scar tissue and neurotoxic astrocytes via TNF-α, IL-1α and C1q(87, 88). The infiltration of lymphocytes come later(89). B cells is suggested playing a negative role in tissue repair but T cells playing dual role in both tissue repair and injury depending on their phenotypes(90). Regulatory T cells, one subtype of T cells is usually beneficial in tissue repair by releasing anti-inflammatory cytokines IL-10 and they can last till chronic phase due to long life span(91, 92).

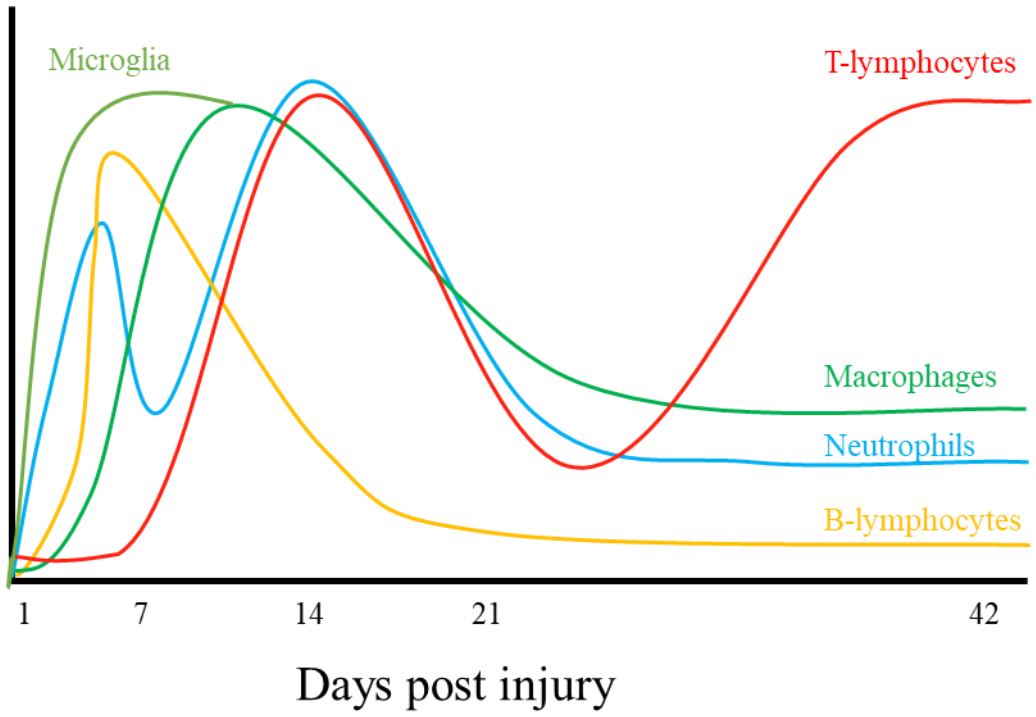

Fig 2. The time course of resident immune cells and infiltrated periphery inflammatory cells after spinal cord injury.

Even though inflammation in general is protective in acute phase, the long-lasting high-level inflammation could play detrimental role in tissue degeneration especially microglia and macrophages which are relatively abundant at chronic phase(93). First, they could contribute to cell death of neurons and oligodendrocytes via inducing apoptosis and necroptosis by releasing cytokines, TNF-α for example(94, 95), with studies showed that neutralizing antibodies or receptor antagonist targeting TNF-α can promote functional recovery at the chronic stage of SCI(96-98). Second, activated microglia and macrophages can induce the neurotoxic astrocytes which is harmful to neurons and oligodendrocytes by saturated lipids release(99). Lastly, macrophages can lead to dieback of injured axons(100). Taken together, inflammation mediated cell death and tissue degeneration could be associated with the progression of PTS from SCI.

Furthermore, the distribution of inflammatory cells is not very clear at chronic phase, limiting to or extending beyond lesion site for instance. More importantly, it is essential to distinguish the subtypes of inflammatory cells considering the heterogeneity of them(101-103). Recent studies even showed that some unclassical inflammatory cells such as oligodendrocytes and OPCs could participate in inflammation via antigen presentation in multiple sclerosis(104, 105). Therefore, to precisely and specifically target the inflammatory cells in anti-

inflammatory therapeutic study, one should take the heterogeneity into consideration and cover all the potential inflammatory cells.

1.2.2.4 Glial scar

In addition to inflammation, the reactive astrocytes, fibroblasts and scar-forming microglia also play a major role in tissue repair by forming scar after SCI(106). However, the role of scar-forming cells in PTS have not been investigated yet.

Astrocytes become active when respond to inflammation and the extracellular matrix remodeling in SCI(107). The major downstream signaling pathway in astrocytes during scar formation is STAT3 which mediates proliferation and morphological change of astrocytes(108, 109). The formation of tight astroglia scar can encase the lesion site to restrict the spread of inflammation and promote tissue repair at the subacute-chronic phase of SCI(110). Moreover, studies proposed that two polarization types of reactive astrocytes exist, A1 and A2 in analogy to M1 and M2, with A1 is the neurotoxic type induced by M1 and A2 is the neuroprotective type induced by M2(88). In SCI, A1 is more dominant than A2, different from ischemic stroke in which A2 astrocytes are more dominant(111). Therefore, the response of astrocyte to injury is context dependent and knowing which phenotype play the leading role could provide clues for developing treatment targeting astrocytes.

Moreover, microglia also form the essential part of glial scar as interface between infiltrated macrophages and astrocytes(112). The depletion of microglia results in defective glial scar and the widespread of infiltrated macrophages in spinal parenchyma. Therefore, the efficient glial scar formation relies on the interaction different scar forming glial cells. Similarly, investigation of the origin of scar-forming subtypes of microglia will give more precise therapeutic targets.

In addition to astrocytes and microglia, fibroblasts also participate in scar formation in SCI. They form a major part of the inner core of scar tissue(113). The cellular origin of fibrotic scar tissue is debatable, with study showed they derived from type A pericytes(perivascular) using transgenic mice and more recent studies showed that perivascular fibroblast is the major source of fibrotic scar in injured spinal cord(114, 115). Although the identity of the origin is controversial due to the lack of specific markers, fibrotic scar tissue is important for the gapping the lesion by producing extracellular matrix molecules, collagen for example, in SCI(116).

All scar forming cells together are essential for the inflammation containment and tissue repair in SCI, therefore, it is essential to know the scar formation condition in PTS which is lack of tissue repair.

1.2.2.5 Demyelination

Demyelination is an important factor of functional loss after SCI, and it can be derived both from primary and secondary injury. The primary injury could lead to the striping of myelin and also the death of oligodendrocytes at the lesion site (117, 118). However, the secondary injury mediated demyelination can also extend from the injury site to the adjacent tissue and from acute phase to chronic phase(119). Oligodendrocytes are sensitive to glutamate-mediated excitotoxicity(120) and other toxic chemicals, such as ROS (121)and inflammatory cytokines(122). In addition, the axon damage can aggravate demyelination since there is mutual support between myelin-forming oligodendrocytes and axons(123, 124).

Spontaneous remyelination occurs in SCI and is mainly contributed by OPCs, infiltrating Schwann cells and even mature oligodendrocytes though with controversial results (125-127). However, the endogenous remyelination is usually very limited and the newly formed myelin sheath are thinner than the ones formed during development(128). Therefore, there have been many studies targeting factors related to improving remyelination, mainly through OPCs. Among these studies, antibodies to proinflammatory cytokines antibodies and siRNA that interferes with apoptosis have shown efficacy (129). In addition, small molecules such as FGF and EGF can improve myelination by protecting the survival of OPCs from depletion at the lesion site and differentiation of OPCs (130). Cell transplantation therapy is a very promising option, and different types of immature cells of the oligodendrocyte lineage as well as neural stem cells may have the ability to replace the lost myelin forming oligodendrocytes(131).

It is worth noting that remyelination alone can only improve the function of the demyelinated but spared axons. Therefore, strategies targeting both remyelination and axon regeneration could be a more effective strategy.

1.2.2.6 Axon degeneration

After SCI, the proximal ends of severed axons undergo different degrees of retrograde degeneration that may include the cell body, while the distal ends undergo complete degeneration(132). The retrograde degeneration is the result of the axon dieback that starts immediately after injury and continues for months after injury. The axon dieback is quite fast in the first hours during which 200-300 μm from the lesion site is involved and is then

followed by a slow dieback in the next 48 hours during which the degenerative front only moves around 30 μm(133). The acute phase axon dieback is calcium dependent(134). After the acute phase, there is a continuous dieback of axons mediated by macrophages(135, 136).

In addition to the axon degeneration there is also some axonal regrowth. About 30% of axons formed growth cone early after injury but the axon elongation is rapidly lost by 48 hours post-injury. Therefore, some strategies to improve axon regrowth have focused on stabilizing the axons undergoing dieback (with the microtube stabilizing agent, taxol)(137), promoting endogenous axon growth by improving the intrinsic growth ability (neurotrophin) (138)or reducing extrinsic inhibiting molecules, CSPGs for example, using ChABC(139). More importantly, one of the critical processes for functional improvement in SCI is the synaptic connection between the regenerative axons and its appropriate targets(140). Therefore, neural stem cells with their ability to improve axon regrowth and reconstruct the neural circuits by differentiation into neurons is very promising in SCI.

Furthermore, Wallerian degeneration of the distal ends result in myelin debris which may inhibit axon regrowth. The less efficient clearance of myelin debris by microglia in CNS compared to macrophages in the peripheral nervous system could be a factor in axon regrowth failure in CNS(141).

Altogether, PTS shared these processes with SCI but how are they related to the progression of PTS need further study, especially inflammation which last until chronic phase after injury.

1.3 ANIMAL MODELS OF SCI AND PTS

1.3.1 Animal models of SCI

In most cases, different disease models serve different purposes. A good animal model should allow not only the analysis of the pathophysiological mechanisms, but also studies of therapeutic interventions. There are several commonly used SCI models in pre-clinical studies that mimic the clinical condition, including contusion, compression and transection. These models in total represent more than 90% of the studies(142, 143). The contusion model is the most used model, followed by the transection and compression models. In general, contusion and compression models are suitable for the translational studies such as the secondary injuries and cell therapy due to the similarity to the clinical condition. However, the transection model with precise and specific damage, is often used in mechanistic studies for regeneration, such as axon regrowth and reparative role of exogenous material or endogenous progenitor cells, ependymal cells for instance(144). Compression and contusion

models are not suitable for these studies, since the injury of the axon of interest is not well defined, and the ependymal cells are usually damaged in these two injury models(145).

For each SCI model there are variants based on the device used. For contusion injuries, the NYU and Infinite horizon impactors, which are more advanced versions of the Allen weight drop, have been used extensively(146). Compression injuries have been made using aneurysm clips or similar devices as well as intrathecal balloons, while transections either is complete or target specific parts of the spinal cord(147). Moreover, the severity of injury also can be chosen depending on the purpose of a particular study, mild, intermediate or severe in a contusion or compression model and complete or incomplete in a transection model. Moreover, there are options in terms of species. Rats have been the most used species, and most of the functional assessments were developed for rats(142). But the use of mice has increased enormously during the last 20 years due to the use of genetic technique and molecular tools(148). However, the anatomical differences between rodents and human must be taken into consideration(149). Some important features of the different types of SCI models induced by contusion, compression and transection are listed in **table 1**.

Table 1. The comparison across different types of SCI models

	Contusion	Compression	Transection
Pathology	Dorsal white matter and central grey matter	Central lesion, regular shape	Open cavity
Uniqueness	Precise control of injury severity	Simple and reliable	Precise control of injury site
Variability	Medium	High	Low
Application	Translational studies	Translational studies	Neuroscience research
Examples of application	Studies of neuroprotective mechanism, pathology and secondary injury mechanisms	Studies of pathology and secondary injury mechanisms	Studies of specific pathway function and regeneration

1.3.2 Animal models of PTS

PTS animal models are usually produced based on SCI models and rats instead of mice in rodents were used in the PTS model for a few reasons. First, mice do not form cysts after SCI(115, 150), but it is not known if mice can develop PTS when adopting the rat models used. Second, spinal surgery and therapeutic procedures such as cell transplantation are much

more difficult in small animals like mice. Lastly, magnetic resonance imaging (MRI) is commonly used to monitor the cysts in live animals, and the resolution of many MRI equipment favors the use of large animals.

Regarding PTS, the combination of intraspinal injection of quisqualic acid and subarachnoid injection of mineral kaolin was first used. Quisqualic acid produced necrotic cavities in the parenchyma by inducing severe excitotoxicity, and the mineral kaolin as a strong irritant which triggers inflammation and thereby induces arachnoiditis, believed to be a key process in the formation of cysts in PTS(79, 151). The intraspinal pathology in this animal model is rather similar to clinical condition, so it has been used for many years by several groups for pathology studies(152). However, it is far from a clinical condition in terms of etiology.

To better mimic human PTS, a combination of mechanical injury of the spinal cord and an injection of the mineral kaolin into subarachnoid space was used as a more accurate model for studies of the mechanisms(152). Studies in rabbits and rats have compared the efficiency of SCI alone, and combination with subarachnoid space injection of kaolin, which demonstrated that the combination is much more reliable in creating intraspinal cysts(153). Although this model shows high efficiency and is closer to the clinical condition than the quisqualic acid-kaolin model, it is still quite different from PTS in human patients. Therefore, there are limits when it comes to the use of this model for therapeutic intervention studies. Kaolin – which we found can remain in the vertebral column for months – will maintain a chronic inflammation, and will affect the efficiency of any treatment in a way that is not clinically relevant.

Another PTS model used is extradural restriction to mimic the narrowing or obstruction of the subarachnoid space in PTS. Although there was no cyst formation in this rat model within the observation time, an imbalance of inflow and outflow of CSF via the perivascular space was identified, which is believed to be one of the main mechanisms behind PTS(19).

1.4 CELL BASED THERAPY IN SCI AND PTS

The main therapeutic goal for PTS is to prevent further expansion of the intraspinal cysts, and if possible, eliminate the cyst. The current treatment to surgically release the spinal cord from tethering is still challenging with variable therapeutic effect(12, 13). In addition, although the surgical treatment can completely prevent the cyst expansion in many patients, the loss of spinal cord tissue during the cyst expansion is permanent, and the associated functional loss cannot be recovered. Therefore, stem cell-based therapy with the promising results from the experimental studies in SCI could bring new hope for PTS patients as well.

With the limited regenerative capacity of the CNS, the neural circuit in the spinal cord cannot be recovered once damaged after SCI. Research in CNS regeneration has focused either on stimulating endogenous stem cells or transplanting exogenous stem and progenitor cells to replace primarily the lost neurons and oligodendrocytes(154). However, triggering endogenous stem cells in experimental research mainly involves genetic methods, which are not easily transferred to the clinic. Although other methods may be used to induce endogenous stem cells, to replace the very large number of cells that are lost in a PTS spinal cord will be very challenging. Therefore, neural stem cell therapy with or without scaffold is preferable. Notably, neural stem cell therapy is a broad term that includes different cell types, such as neural stem cells (NSCs), neural stem/progenitor cells (NPCs) from different sources (e.g. embryonic stem cells, iPSCs). In this thesis, NPCs is used as the general term for neural stem/progenitor cells as they are usually derived from a mixture of stem and progenitor cells. NPCs can reduce the acute degenerative processes, differentiate into neurons, provide neurotrophic factors and modulate inflammation(155). Consequently, they have become a promising strategy for SCI treatment studies. Indeed, studies have shown functional synaptic connections between host and transplanted cells which could be correlated to functional recovery(156). In addition, the few clinical trials performed have shown acceptable safety, and some even showed some efficacy that paves the way for further development of cell therapy for SCI(157). However, for PTS in which the priority is cyst expansion suppression or elimination, the goals are partially different from SCI. Whether the neural stem cells can prevent cyst expansion, replace lost neural tissue and promote tissue repair still needs exploration. In addition to neural lineage stem cells, mesenchymal stem cells, with the potent ability to modulate inflammation via releasing extracellular vesicles have also been tested in SCI and also could work when administrated intrathecally as an alternative to local injection in the spinal cord parenchyma(158). However, there is not much convincing data that mesenchymal stem cells can replace neural tissue.

Even with the success in the field of stem cell therapy for SCI, there are some major issues one needs to consider for acquiring the best treatment effects: a) the choice of stem cells, e.g. embryonic stem cell (ESC)- and induced pluripotent stem cell (iPSC)-derived NPCs, fetal spinal cord NPCs, mesenchymal stem cells (MSCs), is not only related to ethical issue, safety and specificity, but also can determine the efficacy. For example, NPCs have good differentiation potency into neural cells while MSCs have the ability to reduce inflammation, therefore, they can be chosen depending on the major goal of the treatment; b) the developmental stage (NPCs or more committed progenitor cells) which is related to differentiation capacity, safety and accessibility. Committed progenitor cells are more

restricted and thereby limited compared to earlier stage stem cells and also have less risk of acquiring unwanted fated. In the following part, we will discuss the properties of the most used cell types, including different sources of NPCs and MSCs to explore their potential for treatment of PTS, with cyst reduction and cell replacement as the priorities in PTS. Each cell type has its own characteristics even though they have similarities. Therefore, understanding of their characteristics can help us to choose the optimal cell types in particular situations.

1.4.1 Neural stem/progenitor cells

1.4.1.1 ESCs-derived NPCs

ESCs are derived from the inner mass of cells of the blastocyst and can differentiate into any cell type in the body(159). They have advantages and disadvantages to be utilized for transplantation therapy comparing to other optional cell types. One of the biggest advantages of ESCs is that they have high proliferation rate in vitro, and consequently are easy in sufficiently large numbers for transplantation. However, much more time and effort are required for differentiation into neural lineages compared to somatic NPCs, for which the neural trait is already set. On the other hand, the longer culture and differentiation time of cells in vitro, the more factors with uncertain effects will be involved which could introduce tumorigenicity despite with lots of efforts(160). There is always a risk of tumor formation using pluripotent cells. Purification of the more committed NPCs from the contamination of undifferentiated pluripotent ES cells before transplantation can remove unwanted pluripotent cells, and more robust differentiation protocols(161) are also making the use of ESC-derived cells safe enough for clinical trials. Still, ethical issue must also be considered when working with ESCs. ESC-derived NPCs have been shown to improve functional recovery(162). However, no clinical trial data regarding ESC-derived NPCs in SCI available so far.

1.4.1.2 iPSC-derived NPCs

Induced pluripotent stem cells (iPSCs) are usually derived from skin fibroblasts from the donors, and then these fibroblasts are programmed with transcription factors including, OCT4, SOX2, KLF4 and MYC(OSKM) with or without additional NANOG and LIN28 (NL) which could improve the efficiency of reprogramming(163). Like ESCs, the pluripotent iPSCs can be expanded in large quantities which is difficult to achieve with somatic NPCs. In addition, they can be a source of autologous transplant without immunogenicity problem, and with minimal ethical concerns. However, reprogramming and safety test are time consuming which makes these cells unsuitable for acute phase treatment. To deal with this issue, there

are national initiatives to make iPSC banks with HLA profiles matching most of the local population, to give the possibility of allogeneic transplantation with graft tolerance after transplantation.

iPSC-derived stem and progenitor cells share the same risk of tumors as ESC-derived NPCs(161), and similarly there has been a lot of efforts to reduce this risk by developing the differentiation protocols. There has been evolution of the reprogramming technique to minimize the risk of tumor formation mainly by avoiding the integration in the host genome. The technique has been developed from the integrative virus or vectors to non-integrative mRNA, protein and small molecules, increasing safety but decreasing efficiency(164, 165). Therefore, to get sufficient efficiency and at the same time decrease the risk of tumor, synthetic modified mRNA has gained popularity(166). In addition to the improvement in reprogramming, there are extra considerations for the following steps after reprograming, such as the purification of the derived cells before application to remove undifferentiated pluripotent iPSCs before application, or pretreatment of cells by gamma-secretase inhibitors to increase the differentiation of iPSC-derived NPC into neurons(167). To further reduce the risks of tumor growth after transplantation, suicide genes that can eliminate proliferating, immature cells after transplantation(168).

Studies with iPSC-derived NPCs therapy in SCI have shown promising results in experimental studies, with reduced pathological changes , such as attenuation of inflammation and fibrosis(169). Studies have shown that the functional recovery are related to the differentiation of the transplanted iPSC-derived NPCs, either as integrated neurons(170) or myelin-forming oligodendrocytes (171). As ESCs-derived NPCs, there is no clinical trials with iPSCs-NPCs so far. Hopefully, a clinical trial may not be far away since a research group in Japan has published the protocol for a clinical transplantation trial on iPSC-derived NPCs for transplantation in the subacute phase of SCI, with the approval from the local government (172).

1.4.1.3 Somatic NPCs

Two types of somatic NPCs, derived from adult and embryonic/fetal tissue respectively, have been investigated in the treatment of SCI. Adult tissue-derived NPCs can be obtained from the biopsy of CNS surgery, although a very limited source compared to the other one. Embryonic/fetal tissue-derived NPCs are derived from the CNS of embryos or fetuses after clinical abortion. They are relatively safer than the other two sources of NPCs, the ESC-derived and iPSC-derived NPCs, in that there are no pluripotent cells present. Our group and

other researchers have shown safety of human fetal NPCs with no apparent risk of tumor formation for as long as 5 months after transplantation(173). However, in somatic NPCs, the major issue is that they do not proliferate as extensively as pluripotent stem cells, which restrict the potential expansion during cell banking. Therefore, it may be difficult to acquire enough cells for clinical transplantation therapy. There are also ethical issues concerning the use of abortion tissues in several countries. Despite this, with the differentiation efficacy to neurons and the ability to modulate inflammation(174) somatic NPCs are good candidates for SCI. Furthermore, some studies showed that NPCs have the potential to reconnect the neural circuitry by forming synaptic connections between the corticospinal tract (CST) and the denervated endogenous neurons below the injury in mouse SCI model(156).The synaptic connections with CST were demonstrated in the same lab in the non-human primate SCI model as well(175). However, the differentiation potency of NPCs varies , probably due to differences in the original CNS region of NPCs and is very limited in some cases(176, 177). Therefore, the application of more restricted progenitor cells could allow better differentiation to certain cell types after transplantation, especially for some diseases in which a certain cell type is impaired, such as Parkinson's disease.

1.4.1.4 Restricted precursors

a. Neuronal-restricted precursors

Compared to NPCs, neuronal-restricted precursors (NRPs) have better differentiation capacity which provide more opportunity to replace the lost neurons after transplantation. However, they grow much slower and cannot proliferate as extensively as more primitive progenitors. In addition, their ability to migrate is more limited which could restrict their administration to local one(178). Moreover, studies showed these restricted progenitors have region specific bias in differentiation, and they tend to differentiate into the neurons they were intended to be in the original site(179). Therefore, it seems not suitable to establish a general bank for CNS disorders in general, but they could be ideal to treat specific diseases especially with loss of one cell type.

b. Glial-restricted precursors

Glial restricted precursor (NRPs) will differentiate into either oligodendrocytes or astrocytes(180). There are a few types of GRP have been identified in both fetal and adult brains in human and rodents, including oligodendrocyte-astrocytes precursors. GRPs are more likely to differentiate into astrocytes or oligodendrocytes compared to NPCs. Therefore, they are more suitable for the disease which glial cells are required for recovery,

such as MS, in which the remyelination by myelin-forming oligodendrocytes is critical for the functional recovery. Moreover, GRPs have been suggested to play a protective role in SCI after transplantation mainly by providing supportive environment, such as reducing glial scar and promoting the axon regrowth. They also can promote the survival and differentiation of NRPs during co-transplantation(181-183). OPCs with more committed cell fate than GRPs have better potential to differentiate into oligodendrocytes, which is required for the remyelination in SCI. Studies have shown that remyelination by transplanted OPCs improve functional outcome in animal experiment (184).

Taken together, neural lineage stem cells with the ability to replace the lost cells by differentiation, and to modulate inflammation have been demonstrated experimentally to be effective in the treatment of SCI. For instance, axon regrowth and reconnecting neural circuitry have been demonstrated. However, immunogenicity is an issue for all of these cell types, except autologous iPSC derived NPCs used in chronic injuries. In contrast, mesenchymal stem cells have low immunogenicity and even immune-modulatory effects and could therefore be an alternative for the treatment of SCI.

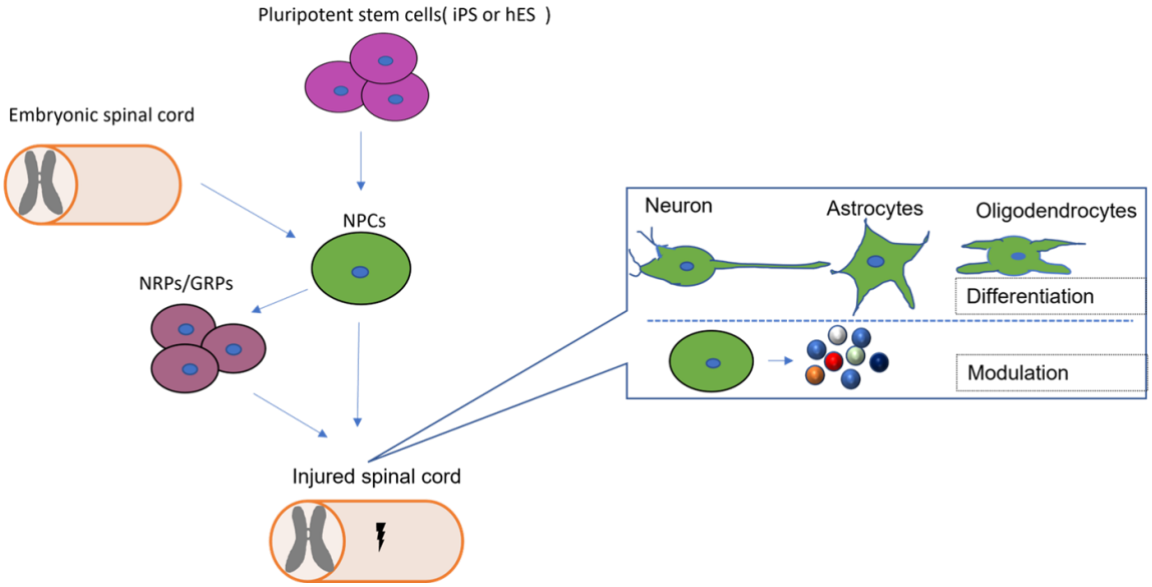

Fig 3. Different sources of NPCs and NRPs/GRPs transplantation therapy for SCI. They play their role mainly by differentiation into neural lineage cells, including neuron, astrocytes and oligodendrocytes. And can release trophic factors to modulate local environment.

1.4.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are multipotent stromal cells that can differentiate into a variety of cell types, such as muscle cells and fat cells(185). There are several different types of MSCs from different tissue, including bone

marrow mesenchymal stem cells (BMSCs), umbilical cord mesenchymal stem cells (UC-MSCs) and adipose derived stem cells (ADSCs) with BMSCs are the most used one. MSCs can responds to inflammation by releasing growth factors which can promote tissue remodeling and angiogenesis and by producing cytokines to modulate inflammation in turn (186). Therefore, a feature of MSCs in SCI is their capacity to modulate the local environment rather than to differentiate into neural lineage cells. Studies showed that these modulating factors are released by MSCs via secretion of extracellular vesicles and the contents vary with the sources of MSCs(187). Knowing the similarities and differences among the different sources of MSCs allow the appropriate choice for particular disease. There is still no direct comparison between the efficacy of different sources of MSCs in SCI. However, a study showed that the ADSCs had better survival than BMSCs(188). Nonetheless, their differences are relatively minimal compared to other stem cells, NPCs for example, see **table 2** for details.

Table 2. The comparison across different types of MSCs

	BMSCs	UC-MSCs	ADSCs
Harvesting procedure	Invasive	None-invasive	Invasive
Easiness to isolate and culture	Easy	Easy	Easy
Effect of age on cell quantity and quality	Quantity and quality decline with age	Unaffected	Quantity and quality decline with age
Cell renewal	Lower proliferative potential	Higher proliferative potential	Lower proliferative potential
Expression of embryonic markers	Lower	Higher	Lower
Risk of tumorigenicity	Very low	Very low	Very low

There are many clinical trials on transplantation of MSCs from different sources in SCI. In general, safety and efficacy have mainly been demonstrated with MSCs administrated intrathecally mostly. About 20 clinical trials used BMSCs, but the results vary with from 10% to 75% on the rate of efficacy. The efficacy outcome from US-MSCs and ADSCs appears to be relatively more consistent but with far fewer trials than BMSCs(189).

2 RESEARCH AIMS

1.To develop a clinically relevant rat model of PTS and demonstrate the feasibility of neural stem cell therapy

2.To optimize cell therapy for PTS with GMP-compliant iPSC-derived NPCs and identify their therapeutic effects

3. To explore the molecular and cellular mechanism of PTS

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

3.1.1 Neural stem/progenitor cells derived from human embryos

In order to isolate human embryonic/fetal NPCs, remains of human embryos and fetuses from first trimester clinical routine abortions were acquired from local gynecology clinics. The human embryos/fetuses were only used for research purposes after oral and written consent signed by the abortion patients and then neural stem/progenitor cells were dissociated from the embryonic spinal cord. Patients' identity information is hidden from everyone else. The entire procedure regarding the use of abortion tissue was approved by the Regional Human Ethical Authority, Stockholm (Dnr :2007/1477-31,2011/1101-32 ,2013/654-32 and 2018/2497-32) and the National Board of Health and Welfare (Socialstyrelsen)

3.1.2 Human Induced Pluripotent Stem Cells

To further explore neural stem/progenitor cell treatment in PTS, human skin biopsies were taken to produce induced pluripotent stem cells after obtaining the written informed consent from healthy volunteers. All the procedures with induced pluripotent stem cells follow the ethical permit from Regional Ethical Committee, Stockholm (Dnr: 2012/208-31/3).

3.1.3 Animal experiments

In vivo experiments are necessary to investigate neural stem/progenitor cell treatment in PTS considering the complexity of the CNS and interacting peripheral tissue.As described above, rats were chosen for serveral reasons for these studies. We minimized the number of rats as much as possible based on our previous experiment, and all the procedures were performed strictly according to the ethical permit approved by the Regional Committee on Research Animals Ethics Committee, Stockholm (Dnr: 1733) to minimize the suffering of rats, before, during and after treatment.

3.2 HNPC CULTURE

hNPCs were cultured as free-floating cell aggregates, so called neurospheres as previously described. Human embryonic spinal cord, 5.5–7 weeks post-conception, was retrieved from elective routine abortions. Dissociated tissue was cultured in DMEM/F12 with glucose (0.6%, Sigma),Hepes (5 mM, Life Technologies), heparin

(2 µg/ml,Sigma), N2 supplement (1%, Life Technologies), basic fibroblast growth factor (FGF2, 20 ng/ml, R&D Systems), epidermal growth factor (EGF, 20 ng/ml, R&D Systems), and ciliary neurotrophic factor (CNTF, 10 ng/ml, R&D Systems) at a density of 40,000–50,000 cells/cm2 in 20 ml of medium.The NPC were maintained at 37 C in 5% CO2, adding fresh medium twice a week. Neurospheres were passaged every 7 to 10 days by enzymatic dissociation using TrypLE Express (Life Technologies) and mechanical dissociation. Neurospheres at passage 5–10 were used for all experiments.

3.3 NESCS CULTURE AND DIFFERENTIATION

NESCs were cultured on PLO and laminin2020-coated flasks in growth medium (DMEM/F12 Glutamax supplemented with 1% N2, 0 1% B27, FGF 10 ng/µl, EGF 10 ng/µl, Peprotech #AF-100-15, and 1% Penicillin-Streptomycin Thermo, #15140-122). When confluent, cells were detached from the bottom of the flask using TrypLE select (Invitrogen # 12563) and split 1:3. NESCs were differentiated as an adherent culture in differentiation medium (DMEM/F12 Glutamax supplemented with 1% N2 and 1% B27) without any supplement of growth factors. Trypan blue was used to assess the viability of NESCs

3.4 EXPERIMENTAL ANIMALS AND TRAUMATIC SURGERY

Female Sprague-Dawley rats (Charles River, 180-220 g) were used for injury model and cell transplantation treatment. Rats were given atropin (0.05 mg/kg, NM Pharma AB) i.p. 10–15 min prior to anesthesia. Anesthesia was maintained for the entire surgical procedure by isoflurane inhalation (3% for induction and 1.75% for maintenance, oxygen rate 500 ml/min). Core body temperature was maintained at 37°C using a rectal probe and a heating pad. 6 ml of Ringer's solution with 5% glucose was administrated subcutaneously both before and after injury to reduce the risk of dehydration. PTS was induced as previously described. Briefly, a laminectomy was performed at spinal level Th9, followed by mild contusion injury inflicted by the Infinite Horizon impactor (Precision Systems and Instrumentation, LLC) (force = 100 kdyn) on the exposed dorsal spinal cord at spinal cord level Th10-11, followed by subarachnoid injection of 100 µl venous blood from the tongue vein. Then artificial dura (Lyoplant, B/Braun Aesculap AG) was placed to cover the opening of dura

before the wound was closed with sutures layer by layer. Temgesic (buprenorphine, 7 µg/kg s.c., Reckitt& Colman) was administered subcutaneously twice daily for 4 days and the bladder was emptied manually until spontaneous voiding was stable.

For subacute transplantation, rats were randomly divided into NESC and vehicle groups . For chronic transplantation, rats with cyst volumes larger than 2 mm^3 based on the pre-transplantation MRI were selected, since cysts smaller than 2 mm3 usually heal spontaneously. Rats were assigned to cell group (hNPCs or NESCs in Paper I ; NESCs in Paper II) and vehicle groups based on the pre-transplantation MRI in order to achieve similar cyst volumes in the two groups.

3.5 BEHAVIORAL ASSESSMENT

To assess motor and sensory functions the rats were habituated and/or trained for the test situation. All test sessions were recorded by video. To monitor motor performance (i.e. muscle strength, muscle control and proprioception) we used 1) the Basso-Beattie-Bresnahan locomotor rating scale (BBB) for open-field locomotion (190, 191), 2) the KSAT for motor functions during swimming, 3) beam walk test to assess the ability to traverse flat beams, and 4) grid walk to evaluate hindpaw placing. For the BBB scale rats were left for 4 min on an elevated 65 x 150 cm platform and the use of hindlimbs, tail and trunk were video recorded and scored according to the BBB scale. Motor performance during swimming was scored using the Karolinska Institutet Swim Assessment Tool (KSAT)(192), which focuses on the intensity and frequency of limb and tail movement during swimming in a 150 cm long tank. The tank was filled with water at 30°C. Swim parameters according to the KSAT scale were evaluated. In the beam walk test, rats were trained to traverse 1 m long beams with a rubber surface, with widths from 6 to 0.7 cm. Rats were given 3 trials on each beam, and for each trial the performance was scored from 0 (unable to traverse the beam), 1 (traversed the beam with > 5 misplaced steps), 2 (traversed with 1–5 misplaced steps), or 3 (no misplaced steps). The average score for each beam was added to give a total score between 0 and 18. The grid walk test was performed using a 150 x 20 cm mesh work of 1.5 mm steel thread with 3 x 3 cm holes, and the number of times a hindpaw slipped through a hole, up to the ankle joint, during three trials was counted. The maximum number of mistakes was 47.

Thresholds for pressure/mechanical pain were assessed using von Frey filaments (Stoelting) ranging from 0.4 to 447 g. The rats were tested by applying increasing pressure to the sole of the hindpaws using successively thicker filaments, until an avoidance response was triggered. This was repeated for each hindpaw until the same result was acquired three times. Thermal pain tresholds were determined using the Plantar test (Hargreaves') analgesia meter (Ugo Basile). A beam of infrared light was directed to sole of the hindpaws, and the delay until the rat lifted the paw while the intensity increased was recorded. The light was automatically turned off after 15 s to avoid tissue damage. Each paw was tested 5 times and the average delay was used as the result.

3.6 CELL TRANSPLANTATION

For transplantation of hNPCs, the capillary was loaded with a suspension of 25–30 neurospheres, diameters 180–400 μm in 7–10 μl of cell culture medium without any growth factors or mitogens. The number of cells in each neurosphere was calculated based on the diameter of the neurospheres, as previously described , and a vial with neurospheres containing ≈ 300,000 hNPCs prepared for each rat to be transplanted. For the transplantation of NESCs, cells were thawed in a water bath and then washed and resuspended twice with DMEM/F12 medium and their viability was tested with trypan blue. Then NESCs were resuspended to 100,000 viable cells/μl with injection medium, DMEM/F12.Ciclosporin (Sandimmun, 10 mg/kg s.c., Novartis) was administrated to all rats (cells and vehicle groups) daily beginning from 1 day before transplantation until the endpoint. Before transplantation, the laminectomy area was incised and 3 μl NESCs suspension, hNPC neuorospheres suspenstion in DMEM/F12 medium or 3 μl DMEM/F12 alone was slowly injected into the parenchyma at the lesion site (subacute) or into the cyst (chronic) guided by pre-operative MRI. The preparation before and the care of the rats after the operation were the same as previously described.

3.7 MRI SCAN AND IMAGE ANALYSIS

MRI was performed using a 9.4 T horizontal magnet (Varian, Yarnton, UK) with a 31 cm bore. A 72 mm volume coil in conjunction with a four-channel phased array surface receive coil (RapidBiomed, Würtsburg, Germany) was used for transmission.

Rats were anesthetized with isoflurane and body temperature was maintained at 37 °C during anesthesia. The rats were placed in the micro-MRI machine, with monitoring of heart and respiratory rates being monitored. Acquisition of the MRI images and analysis of the images to measure the length and volume of the intraspinal cyst was performed as previously described. Briefly, using ImageJ software (NIH), the T2-weighted transverse images with hyperintense signal in the spinal cord were converted to 8-bit format images and then threshold based on the grayscale to include and highlight the pixels representing cystic fluid (white) in the spinal cord and exclude the remaining pixels representing tissues. The area of each image was measured, summed, and multiplied by the thickness of the transverse image (0.5 mm) to get the total cyst volume.

3.8 TISSUE AND CELL PREPARATION FOR IMMUMOSTAINING

The rats were anesthetized with pentobarbital (60 mg/kg) before decapitation and then the spinal cord was dissected, collected, and then immersed in 4% PFA for 24–36 h followed by immersion in 10% and 30% sucrose, each for 24 h. The injury site of spinal cords was embedded in O.C.T compound (Tissue-Tek), snap frozen and sectioned longitudinally or coronally with a section thickness of 10 µm. Cultured cells were fixed with 4% PFA for 10 min, followed by 5 quick rinses with MilliQ water before primary antibody incubation

3.9 IMMUNOBIOLOGICAL STAINING AND IMAGING

For immunofluorescent staining, sections or cells were washed in PBS for 10 min and then incubated in primary antibody diluted in blocking buffer (PBS with 10% normal donkey serum and 0·3% triton X-100) overnight at room temperature. After incubation, sections were washed once in PBS and then incubated with the corresponding donkey-derived secondary antibodies (Alex488, Cy3 or Alexa647 conjugated) for 1 hour. After secondary incubation, sections were washed with PBS-T (PBS containing 0.05% Tween 20) for 10 min, counterstained with DAPI (1:2500) for 1 min before being finally rinsed in PBS-T and then mounted with PVA-DABCO.

Tissue sections were imaged with a Zeiss LSM 700 confocal microscope with Plan Apochromat 20X/C-Apochromat 40X objectives using Zen software (Zen Black 2010 v6.0.0.309). Cell cultures were imaged with a wide-field microscope (Zeiss Cellobserver) and images were acquired with a Zeiss AxioCam camera and created

with Zen software (Zeiss Zen 2 (Blue) v2.3). All images were processed and quantified using ImageJ.

3.10 MULTIPLEX ANALYSIS

Multiplex analysis was performed with the Bio-Plex Pro Rat Cytokine 23-plex Assay Kit (#12005641, Bio-Rad) according to the manufacturer's instructions using 100 µL of the sample homogenate. The samples were set in duplicate. The total protein concentration of each sample was measured using the BCA kit (Pierce™ BCA Protein Assay#23227, Sigma) and cytokine levels were normalized to total protein for analysis.

3.11 ISOLATION OF SINGLE NUCLEI

Spinal cord nuclei were isolated using the 10x Genomics protocol (CG00124) with modifications. In brief, the thawed spinal cord was rinsed with wash buffer (2% BSA and RNase inhibitor in PBS) and then ground with a grinder about 10-15 strokes on ice. The homogenate was lysed in lysis buffer (10x protocol, CG00124) until the nuclei were dissociated (checked in microscope). 5 ml hibernate medium was added to the lysis solution followed by filtering with a 30 µm cell strainer. Then the filtered nuclei solution was centrifuged at 500 x g for 5 min and rinsed twice with wash buffer. Myelin was separated from nuclei using gradient centrifugation with Optiprep (Sigma, D1556), 25% Optiprep on top of 29% Optiprep centrifuged at 10000x g for 40 min and then the floating myelin was removed. All procedures were performed on ice or at 4°C

3.12 SINGLE NUCLEI RNA SEQ AND BIOINFORMATIC ANALYSIS

3.12.1 Single nuclei RNA sequencing (snRNA-seq)

Single-cell suspensions were prepared as described above. A total of 4 samples, each containing spinal cord tissue from 3 rats with similar treatment, were sequenced with a median of 1703 genes per cell, ranging from 230 to 6500 genes per cell. Sample libraries were prepared according to Chromium Single Cell 3′ Library and Gel Bead Kit (v3) instructions. All samples were indexed with Chromium i7 Multiplex Kits (10X Genomics; PN-120262), and single-cell suspensions were processed according to the manufacturer's instructions to create final libraries for Illumina sequencing. For sequencing, the libraries were loaded onto an Illumina NextSeq 500 flow cell at recommended loading concentrations and paired end sequenced under recommended

settings (R1: 28 cycles; i7 index: 8 cycles; i5 index: none; and R2: 91 cycles). After sequencing, the Illumina output was processed using the CellRanger's (v2-4) recommended pipeline to generate gene-barcode count matrices. In brief, the base call files for each sample were demultiplexed into FASTQ reads and then aligned to the rattus norvegicus reference genome using the STAR splice-aware aligner. Reads that confidently intersected at least 50% of an exon were considered exonic and further aligned with annotated transcripts. The reads were then filtered to remove UMIs and barcodes with single base substitution errors and finally used for UMI counting. The output was a count matrix containing all UMI counts for each droplet. Sequence alignment and transcript counts were performed using CellRanger.

3.12.2 Standard pre-processing workflow

Before data analysis, we processed the snRNA-seq data according to the with Seurat 4.0 workflow(193, 194). First, we excluded the empty droplets or doublets/multiplets by filtering the gene counts either above 2500 or below 200 per cell. In addition, we also applied the DoubletFinder(195) package to predict and exclude doublets. After filtering unwanted nuclei, we performed sequential steps of data processing including normalization, running PCA, data integration with harmony(196). The integrated dataset was then analyzed using the function of FindNeighbors() and FindClusters(). When clusters were identified, the corresponding maker genes (Differentiated Expressed Genes, DEGs) were acquired by using the function of FindAllMarkers() and each cluster was annotated based on these markers. Furthermore, GO term were established with the input of marker genes based on the database on https://biit.cs.ut.ee/gprofiler/gost.

Subclustering was done individually, and the resolution of each cluster was decided based on both the package Clustertree, the umap and DEGs.

3.13 STATISTICAL ANALYSIS

The statistical comparison included two-tailed Student's t-test, one-way ANOVA, two-way ANOVA, and Fisher's exact test. All the data analyses above were used as indicated. Statistical significance was indicated as * $p < 0.05$ and **$p < 0.01$. Shapiro-Wilk test (GraphPad Prism) was applied for the normality test.

4 RESULTS AND DISCUSSION

4.1 WE DEVELOPED A RAT MODEL OF PTS AND DEMONSTRATED THE EFFICACY AND SAFETY OF NPC THERAPY FOR PTS.

PTS, with its severe symptoms and challenging surgery, has prompted us to explore other treatments surgery. With the successful and promising outcome of stem cell therapy in SCI, we investigated the potential of human NPCs as a treatment for PTS. However, we concluded that the commonly used PTS rat model applying subarachnoid injections of kaolin is not suitable for the cell therapy experiments. Our research group previously found that kaolin was present months after the injection, and could also be found in the cysts, which could directly affect any transplanted cells. Therefore, we first developed a rat model that avoids introduction of potentially toxic foreign materials, and instead used subarachnoid injection of autologous blood to induce arachnoiditis (**Fig 4**). After characterizing the new rat PTS model we studied the efficacy of two types of NPCs transplantation treatment for PTS, and the fate of transplanted cells.

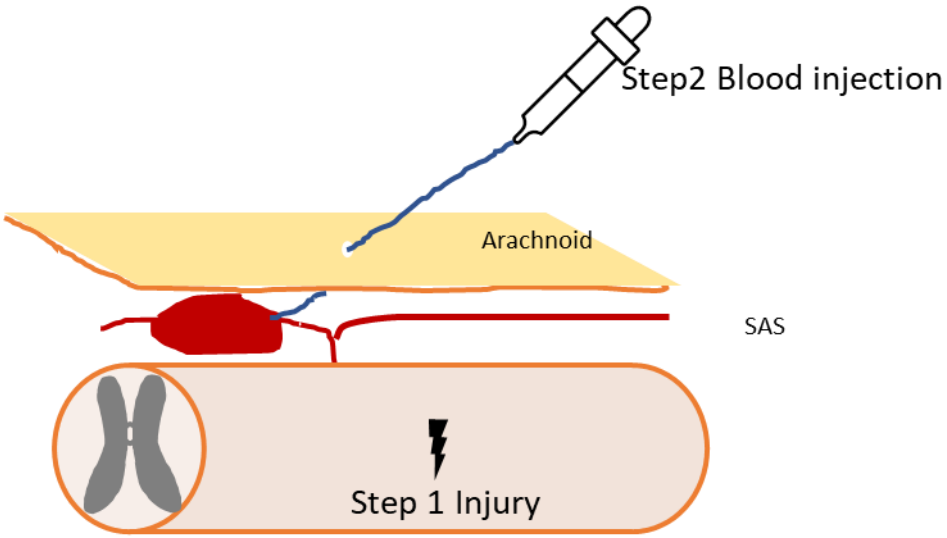

Fig 4. PTS rat model with two steps: Step1. mild contusion injury on the exposed spinal cord after laminectomy. Step2. 100ul autologous blood taken from the vein in tounge was injected to the subarachonid space slowly.SAS= Subarachnoid space.

We began by characterizing the pathology of the rat spinal cord of the PTS rat model. We monitored the tapering cyst with the use of serial coronal sections of spinal cord 10 weeks after injury, as the cyst area decreased from the center of lesion to both rostral and caudal side. In addition, we found the cysts were mainly localized in the

grey matter, and lined with GFAP positive astrocytes, which is similar to PTS patients. Importantly, the cysts were not a result of a widened central canal, but rather due to the loss of spinal parenchyma. Most of the cysts did not communicate with the central canal, unless the lost grey matter included the region with the central canal. Furthermore, when the spinal columns were dissected we found clear tethering of the spinal cord to the surrounding tissue, again resembling the clinical situation. As described above, tethering is most likely the critical factor for the development of PTS from SCI.

To assess PTS in vivo we applied MRI, which is the common and critical method for diagnosing and monitoring the progression of PTS in clinic. With the high magnetic field 9.4 T micro-MRI we could evaluate the cysts in detail three-dimensionally. We observed hyperintense signals in T2-weighted MRI, representing to fluid at the epicenter. In addition, the T2 hyperintense signal reached over 3-4 spinal segments long. We compared the hyperintense signal with the signal from the subarachnoid space containing CSF to ensure that we identified fluid-filled cysts and not edematous tissue, and also confirmed the results of the micro-MRI with histological analysis that T2-weighted MRI hyperintense signal was corresponding to the cyst filled with fluid rather than solid tissue. We were therefore able to use micro-MRI to monitor the change in cyst volume over time, and analyze the effect of cell transplantation in living animals. Regarding the MRI analysis of the subarachnoid space, it is noteworthy that the strands of connective tissue that represented the tethering could be clearly identified with MRI.

In order to see if our PTS model would show similar worsening of neurological symptoms as in PTS patients, we assessed motor and sensory functions in PTS rats. For motor function we analyzed open-field motor behavior using the BBB-scale, as well as a swim test, beam walk and grid walk, with SCI as control. Overall all motor tests showed similar functional profiles in PTS and SCI model although the SCI rats performed worse in some motor tests (swim test, beam walk). There was a rapid recovery 2-3 weeks after the injury, and the performance stabilized up to 20 weeks. The most important finding was that we saw no indication of any worsening of functional loss during the time when the cysts developed and expanded (8–20 weeks post-injury). The probable explanation is that the affected areas are mainly located in the grey matter at the thoracic level while the descending axons innervating the

hindlimb motorneurons are mainly located in the white matter at the thoracic level. The tests we used were designed primarily to assess hindlimb motor. To our knowledge there are no functional tests to assess motor functions of the thorax and abdomen innervated by the thoracic motor neurons that are lost as the cyst expands. Another reason for the lack of functional worsening could be be that the slow expansion can be compensated for by the plasticity of the spinal cord which is also have been observed in PTS patients with large cysts(197), in which no functional impairment is seen until the tissue loss is beyond compensation. For the sensory tests, the results were similarly negative, but with a unilateral difference in thermal sensitivity compared to the SCI rats. The PTS rats showed increased thermal sensitivity towards the end of the observation period which was not seen in the SCI rats. It is not obvious if this is due to improved sensory function, or to thermal hypersensitivity due to the loss of tissue in PTS rats. However, both motor and sensory functions need to be analyzed further with more sensitive methods.

After establishing the reliable rat model, we tested the efficacy and safety of two types of NPCs, human fetal NPCs and iPS cell-derived NPCs-NESCs, in the rat model. To easily localize the cells histologically after transplantation, we used GFP-labeled cells. To localize the cysts as preparation for the cell transplantation, and to assess the change in cyst volume over time with or without cell transplantation, we performed micro-MRI before transplantation and 10 weeks after. We found that most of the transplanted cells (both NPCs and NESCs) remained in the cyst where they were originally injected, and most of them remained as stem cells labeled by Sox2, and not as mature neural cells, such as neurons or astrocytes. The result is different from a previous NPCs transplantation study to rat SCI performed in our research group, where differentiation was pronouced with 10% of surviving cells expressing neuronal marker at 20 weeks after transplantation while the majority of cells were positive for GFAP(173). The reason for the differences could be the different animal model or the duration of differentiation. We observed a decrease of 46.9% and 18.9% of the cyst volumes in NESCs and NPCs, respectively, while a 100% increase in cyst volume was observed in the sham transplantation group. The cyst reduction is not by filling effects of transplanted cells since the total volume of transplanted cells is much less than the volume of cyst reduction. In addition, we observed no ectopic clones from transplanted cells that showed no signs of tumor formation.

Taken together, the rat model we developed very well represented clinical phenotype of PTS, even the mechanism on PTS in addition to the pathological characterization. Importantly, this model allows further exploration of cell therapy as no toxic reagents or foreign substances are required to induce cyst formation and expansion. Furthermore, the ability of NESCs and NPCs to reverse cyst expansion without tumor formation and other observed side effects make them feasible candidates for the treatment of PTS in the future. However, the detailed mechanism behind the therapeutic effects needs additional exploration. There are probably several mechanisms interacting, and related to 1) release of growth factors to rescue dying cells, 2) suppression of the inflammation that presumably continues when cysts expands, and 3) modulation of the reactive astrocytes and other cells associated with fluid homeostasis in the spinal cord.

4.2 CLINICALLY RELEVANT NEURAL STEM CELL TRANSPLANTATION HAS MULTIPLE THERAPEUTIC EFFECTS ON PTS.

With the feasibility of neural stem cell therapy for PTS achieved in **Paper I**, we transplanted GMP-compliant iPSC-derived NPCs-NESCs, to further advance neural stem cell therapy for PTS into clinical translation. Therefore, the entire process from induction of the iPSCs to transplantation was optimized, by complying with GMP in the production of iPSCs and by using the cryopreserved cells directly after thawing to reduce the time before transplantation and the waiting time for the patients in clinic. Furthermore, in addition to transplantation NESCs into cysts in the chronic phase (10 weeks post-injury), we also transplanted cells in the subacute phase (1 week post-injury) before cysts developed to further explore the therapeutic potential of NESCs. We found that we could replicate our previous results since the transplantation of GMP-compliant iPSCs prevented and reversed cyst expansion. In addition, we found that NESCs modulate local environment, and promote endogenous regenerative capacity.

The cryopreserved NESCs showed high viability after thawing. They also had the expected capacity to differentiate *in vitro* into neurons and astrocytes immediately after thawing. Similarly, we analyzed the scenario of the transplanted NESCs 10 weeks after transplantation in both acute phase and chronic phase transplantations, for their survival and differentiation. We found that large numbers of cells survived 10 weeks after transplantation and their proliferation rates dropped abruptly to about 2% from 30% 1 week after transplantation, indicating a low risk for tumor formation.

Furthermore, when we quantified the state of differentiation condition using markers of different neural lineages, we found that many of the surviving cells expressing glial cells marker, Sox9 while small portion expressing neuronal marker, NeuN. The direction and rate of differentiation differed from a study of similar cells in the mouse SCI model, in which they observed that most of the grafted cells expressed neuronal markers(170). A possible explanation for the inconsistency is that the microenvironment in the cysts of our PTS model is different from the local environment in the mouse SCI model. One parameter of importance is inflammation, and whether the differences in differentiation of grafted cells is due to the extent of inflammation needs further exploration (198).

In **Paper II** we found that treatment with NESCs not just prevented cyst progression, but also decreased the cyst size, in agreement with the results in **Paper I.** Cyst volumes decreased by 84.4% in NESCs group while it increased by 75.3% in the vehicle group. In addition, the length of the cyst decreased by 25.6% the NESC treatment group while it increased by 2% in the vehicle group. This verified the effectiveness of cell treatment for PTS that we showed in **Paper I** and strengthens the potential for a future clinical use of NESCs in PTS patients. In addition, transplantation of NESCs in the subacute phase (pre-cyst stage) into the injured spinal cord effectively prevented the formation of cysts, with only 9.3% developing into PTS in the NESCs group 10 weeks after transplantation while 83.3% developing into PTS in the vehicle group. Although it is unlikely that NESC will be used to prevent PTS in SCI patients before it appears, it is interesting from a mechanistic aspect that NESC therapy is effective in both subacute and chronic stage transplantation.

In order to better understand the possible mechanism of the treatment effects by NESCs, we examined the cellular players of the major pathophysiological processes which occur from the subacute to the chronic phase in SCI, such as microglia/macrophages and reactive astrocytes. We found that the density of active microglia/macrophages marked by ED1 was much lower in the NESC group than in the vehicle group around the lesion site after subacute and chronic transplantations. In addition, we found the expression of pro-inflammatory cytokines, IL-1β was much lower in the NESCs group than in the vehicle group. The expression of IL-1β was mainly seen in astrocytes. Our data suggest that one of the mechanisms by which NESC transplantation prevent cyst formation in the subacute-chronic stage and prevent cyst expansion after transplantation to cysts in PTS is attenuation of the

inflammation. Studies have shown that pro-inflammatory cytokines can induce neuronal cell apoptosis(199), and microglia can induce the phenotype of neurotoxic astrocytes which can also contribute to cell death(88). The molecular mechanism is not clear yet, could be that NESCs act in the same way as MSCs in immunomodulation via the secretion of extracellular vesicles containing anti-inflammatory cytokines. Moreover, we found that aquaporin-4, an important protein that can regulate CNS fluid homeostasis, has high expression in the astrocytes around the lesion site in the vehicle group but became less expressed in the NESCs group. Aquaporin-4 has been shown play a role in neuroinflammation such as in the EAE animal model, and it was confirmed by the *in vitro* experiment in which proinflammatory cytokines, TNF- α and IL-6 reduced in the *aquaporin-4* knockout astrocytes(200). Therefore, the reduction of aquaporin-4 supports the suppression of inflammation by NESCs again.

In addition, we found that NESCs promote endogenous regeneration. They trigger proliferation and migration of OPCs and improve regrowth of serotonergic axons. At 10 weeks post-transplantation, there were large numbers of endogenous OPCs ($Olig2^{+}/HuNu^{-}$) in the graft, suggesting that NESCs can stimulate OPCs' proliferation and migration from the host tissue into the region of transplantation. We further confirmed this by showing that there were more double-positive Ki67 and Olig2 cells in the host tissue in the NESCs than in the vehicle group. Mouse NPCs have previously been shown to enhance OPC proliferation in a mouse model of multiple sclerosis (MS) and improve remyelination, an effect mediated by platelet-derived growth factor AA (PDGFAA) and fibroblast growth factor-2 (FGF2)(201). Furthermore, migration of OPCs is also mediated by PDGF(202).OPCs proliferate mainly in the acute phase but not in the chronic phase in SCI in response to injury(203). Interestingly, we found that NESCs can stimulate the proliferation and migration also after transplantation in the chronic stage, which indicated that NESCs may promote remyelination of the demyelinated axons seen in chronic SCI. However, further investigations are needed to examine whether these OPCs would be able to differentiate into oligodendrocytes, which are more important for functional recovery, and demonstrate that they engage in remyelination.

We also observed less thick astrocytic scar tissue in the NESC group than in the vehicle group consistent with previous stem cell transplantation studies in SCI(203). Since astrocytes are quite plastic and can change their phenotype in response to the

local environment they are in, the scar-forming astrocytes are likely to decrease with the modulation of local environment by NESCs. Considering the barrier role of the astrocytic scar in axon regrowth, we wondered whether there might be regrowth of serotonergic axons along with the decrease in astrocytic scar thickness in the NESC group. Serotonergic axons are particularly suitable to analyze regeneration since they are all descending bulbospinal axons, and also has important functional roles. As expected, we found a denser serotonergic axon below the lesion site in the NESCs group than in the vehicle group, indicating either regrowth of severed axons or sprouting from axons in adjacent spared tissue.

All in all, we believe that NESCs with all the therapeutic effects on PTS is a candidate for PTS therapy in clinic after the optimization of NESCs and the transplantation procedure.

4.3 CHRONIC INFLAMMATION CONTRIBUTE TO THE DEVELOPMENT OF PTS

The currently dominating theory regarding the formation of PTS is that scarring in the subarachnoid space changes local hydrostatic pressure and the flow of CSF, and also exerts shear forces on the tethered spinal cord. This leads to an imbalance between the inflow and outflow of fluid in the spinal cord, with cyst formation in the parenchyma already affected by the SCI(7, 8). However, the surgical treatment, detethering, is technically challenging in some cases due to widespread scar attachment, and PTS may recur even after seemingly successful surgery. Therefore, a better understanding of the cellular and molecular factors that correlate with the development of PTS from SCI could help to design efficient therapeutic or even preventive strategies. In the current study, we termed the animals with progressive cyst expansion as PTS and those with no obvious cysts visible on MRI or upon postmortem histological examination as SCI. We found evidence that inflammation is associated with the progression of PTS from SCI using a combination of multiplex analysis, immunohistochemistry and snRNA-seq

Inflammation has been shown to be a major contributor of secondary injuries after traumatic SCI, usually persisting into the chronic stage. Therefore, we studied the course of inflammation during the development of PTS by analyzing a panel of 23 cytokines including pro-inflammatory cytokines such as TNF-α, IL-1β and IL-2, and anti-inflammatory cytokines such as IL-4 and IL-10. We found that most cytokines including

both pro-inflammatory and anti-inflammatory cytokines increased in the acute phase, with a second increase in chronic phase after a slight decrease in the sub-acute stage in both PTS and SCI groups. However, even though most cytokines displayed the same temporal pattern between PTS and SCI, the concentrations were higher in PTS than in SCI. Furthermore, we observed no apparent increase of TNF-α and IL-1β, which has been seen in a recent study on the acute-subacute phase of rat SCI mode (204). One possible explanation could be that they were inhibited by increased anti-inflammatory cytokines shown in both our study and the study above. Due to their major role in the initiation of inflammation, the lack of apparent increase of TNF-α and IL-1β is worth further investigation. In addition, we quantified the density of active microglia/macrophages using ED1 to probe the potential origin of the elevated cytokines. We found that dense ED1 positive cells were present at the lesion site in both PTS and SCI conditions suggesting microglia/macrophages are still active at chronic phase similar to a study showing active macrophages at 6 weeks after injury(205). However, we did not observe a significant density difference of ED1 positive cells between PTS and SCI, indicating that either other cells involved in the inflammation or that active/macrophages are molecularly different between PTS and SCI despite their similar density.

To analyze the cellular and molecular component of inflammation in the development of PTS, we analyzed all cell types in spinal cord using snRNA-seq and compared uninjured control to SCI 2 months after injury, PTS 2 and 4 months after injury. As expected, the cells in the spinal cord are heterogenous in all conditions with a large number of different cell types that could be identified. The proportion of immune cells increased after injury, particularly in PTS, while the proportion of astrocytes increased in SCI but decreased in PTS, these results indicate a general difference between SCI and PTS. To further investigate the differences and the contribution to the progression of PTS from SCI, we further analyzed the subtypes of each major cell type. First, after subclustering and GO term analysis, we found that the proportion of inflammatory microglia and inflammatory macrophages was higher in PTS than SCI, and so was the expression of inflammation-related genes such as *Cd68* and *Gpnmb*. Moreover, by integrating a previously published scRNA-seq dataset from acute phase mouse SCI(101), we observed that the percentage of proliferating immune cells were smaller in all the chronic conditions in our study, suggesting there were other cell types that contributed to inflammation. Interestingly, we found the expression of inflammation related genes, *Cd68*, *Gpnmb* and *Ptprc* in other non-inflammatory cell types, endothelial cells, fibroblasts, oligodendrocytes and OPCs. In addition, the proportion and expression of *Cd68* and *Gpnmb* were higher in PTS than

SCI. Overall, the results show that inflammation contributed by classical inflammatory cells, microglia and macrophages, and non-classical inflammatory cells, endothelial cells, fibroblasts, oligodendrocytes lineages were all involved in the progression of PTS. The inflammatory function of oligodendrocyte lineages was supported by a recent study, in which a subtype of oligodendrocytes can express *MHC II* genes in multiple sclerosis patient samples(104). In our data, these immune cell-like oligodendrocytes do not express high level of *MHC II* genes, suggesting that these immune-like oligodendrocytes might have different molecular signatures in different types of pathological conditions. Moreover, the inflammatory role of endothelial cells and fibroblasts in the CNS has not been well studied. We further confirmed these results by double staining of Cd45 (Ptprc) with markers for endothelial cells, fibroblasts and oligodendrocyte lineage respectively, and determined their colocalization in all cell types.

Astrocytes respond to spinal cord injuries in different ways. First, they are involved in inflammation by releasing inflammatory cytokines during the inflammatory process, and they can be induced to a neurotoxic phenotype by inflammatory cells(88, 99). Second, astrocytes can promote tissue repair as they form glial scars to curb the spread of inflammation(110). We found three subtypes of astrocytes across all four groups, 1 subtype of homeostatic astrocytes and 2 subtypes of reactive astrocytes. After comparing PTS and SCI, we observed that potential neurotoxic gene expression in a reactive astrocyte subtype was higher in PTS than in SCI. Given the important role of homeostatic astrocytes in maintaining CNS homeostasis, the reduced proportion of homeostatic astrocytes in PTS could also be related to the chronic tissue damage that takes place in PTS.

To determine the overall status of tissue degeneration in PTS, we analyzed the myelination-related genes of oligodendrocytes, OPCs and the axon structure-preserving genes. We found that the expression of myelin-related genes *Mog*, *Mobp* in oligodendrocytes was much lower in PTS than SCI. Similarly, the expression of *Sox6*, transcription factor related to the differentiation of OPCs, is lower in PTS than in SCI. Myelination was obviously more affected in PTS than in SCI. One explanation might be the presence of the generally stronger inflammation in PTS than SCI, since inflammatory macrophages could mediate the demyelination by damaging affecting oligodendrocytes and OPCs and inflammatory cytokines can induce the apoptosis of oligodendrocytes (100, 122).Furthermore, astrocytes could also contribute to the demyelination process shown an ischemic stroke model despite the lack of identification of the phenotypes of

astrocytes(206). Furthermore, we examined structural neurofilament producing genes of axons, including *Nefl, Nefm, Nefh*, since the impairment of myelination could result in damage to axon structure. As expected, these genes were lower in PTS than SCI, suggesting that the capacity of neuron to form normal axon structure is more affected in PTS but need further investigation. Overall, the more severe tissue degeneration will probably make the regenerative treatment more challenging in PTS than in SCI.

Taken together, inflammation contributed by microglia, macrophages and inflammatory subtypes of endothelial cells, fibroblasts, OPCs and oligodendrocytes, as well as neurotoxic astrocytes most likely leads to more severe tissue degeneration in PTS, although we cannot determine to what extent these processes are driving the degeneration of grey matter in PTS. We provided a detail characterization of different cell types in the uninjured spinal cord, in SCI and in PTS, and reveal the potential cellular and molecular targets for reducing inflammation and ultimately prevent the development of PTS.

5 CONCLUSIONS

PTS is a serious complication of SCI. Moreover, PTS is still not a well-studied disease despite the main theory of dynamic change of fluid in the parenchyma and the others, such as damage to BSCB. In the projects of this thesis, I and my colleagues have examined the therapeutic effects of NPCs on PTS, and we explored other potential mechanisms contributing to the development of PTS. The main conclusions of each paper of this thesis are given below.

1. In **paper I**, we developed a rat PTS model with the same pathology and imaging characteristics as patients by closely mimicking the clinical situation, and showed that it is safe and feasible to apply human neural stem cell therapy. We could effectively reverse the cyst expansion without showing signs of tumor formation. We believe that this model will allow further exploration of the mechanism of PTS.
2. **In paper II**, we identified multiple therapeutic effects induced by GMP-compliant human iPS cell-derived NPCS transplanted in both pre-cyst stage PTS and cyst stage PTS. These effects included 1) prevention of cyst formation in the pre-cyst stage and reversing cyst expansion in the cyst stage, 2) modulation of the local environment to a more permissive milieu (attenuation of inflammation astrocytic scar) which enhanced axon regrowth, and 3) triggering the endogenous regenerative capacity by promoting proliferation and migration of endogenous OPCs. Thereby we have further advanced neural stem cell therapy for PTS towards clinical translation.
3. **In paper III,** with the comprehensive analysis of cytokines and creating the cell atlas of chronic SCI and PTS, we showed that a large number of parameters of inflammation is more pronounced in PTS, which suggests that inflammation contributes to the progression of PTS. We also found that inflammation is not only induced by the classic inflammatory immune cells (microglia and macrophages), but also by several non-inflammatory cells, including a subtypes of endothelial cells, fibroblasts, OPCs and oligodendrocytes. Hence, we provide additional cellular and molecular targets for future anti-inflammatory therapeutic approaches for PTS.

Overall, with the studies included in this thesis, we have advanced the therapeutic research of PTS, and gained a better understanding of the molecular and cellular processes in PTS and their potential role in the development of PTS.

6 POINTS OF PERSPECTIVE

Despite the promising results of neural stem cell therapy for PTS treatment that we provide, future work should be undertaken to investigate the detailed mechanisms of the treatment effects, in order to develop pharmacological treatment that could be more suitable to prevent PTS. To achieve this goal, further studies on transplanted cells are needed to know their potential function after transplantation either by in situ RNA-seq or the sn RNA-seq after cell sorting, and then further confirm it by manipulating relevant genes and examine the loss of function. The secondary question is whether or not the therapeutic effects of cells are multifaceted, since cells might provide the mechanical support important for axon regrowth. To give the answer, the comparison of the therapeutic effects between the pharmacological molecule obtained above and the cells should be made. However, as long as PTS cannot be prevented, and there are patients with loss grey matter due to cyst expansion, neural stem cell therapy is the only strategy to replace lost tissue.

Furthermore, we are still not sure if inflammation is involved in triggering PTS or driving the expansion of cysts, although we know that it is intimately related to the progression of PTS. To define the role of inflammation, intervention studies in a suitable PTS model should be conducted. From these results available drugs could be tested in clinical trials, or new drugs could be developed to specifically target the inflammatory cells that we identified in the paper III. Such drugs should also be tested in the pre-cyst stage of a PTS model to determine if such an intervention would prevent the development of cysts.

Taken together, this thesis lays the groundwork for future research into PTS from both a therapeutic and mechanistic perspective.

7 ACKNOWLEDGEMENTS

To **Erik Sundström**, my main supervisor, I am deeply grateful for your patient guidance throughout the project and this work is the result of many constructive discussions with you. Your support and encouragement help me to keep going when I'm feeling down. In the past few years, I have learned a lot from you, not only in the aspects of science research, like the discussions of research projects and the wise comments, ideas and inspirations you gave during the Journal Club in our group but also philosophy of life, you are always generous and nice to everyone. In a word, I really enjoyed the short long journey of my PhD at Karolinska Institutet with your supervision and I believe that everything I learned will positively help me in my life including research in the future.

To **Elisabet Åkesson**, my co-supervisor, thank you for the genuine feedback and comments on my presentations and writings. Thank you for always being thoughtful for organizing some fika for memorable events in our group. Your comfort and encouragement keep me warm and optimistic in the long dark winter. It is a pity that we have not been able to meet as much as we have before ever since you have become more involved in your new position, but I can still feel your care and support.

To **Xiaofei Li,** my co-supervisor**,** thank you for the research support in this thesis. Even though you are late to join our team, but you are really one of the essential components in my PhD journey. Without you I could not complete my PhD with confidence. Thank you for all the fun and scientific suggestions you gave. You are more of a friend to me than supervisor, and I really enjoyed the time when we had fun chatting and project discussions at the ramen place, Thai place and lunchroom. Your insightful way of looking at scientific questions really helps me to broaden my horizons, which will benefit my future research work.

To **Marianne Schultzberg**, my co-supervisor. Thank you for your support in my research projects with your tremendous knowledge about neuroinflammation and the encouragement to make me more energetic. You are kind enough to be willing to help me if needed. I really appreciate your supervision.

To **Zhu Jie,** who introduced me to Karolinska Institutet. I still remember the first day we met in China in your office and how nice you were to encourage me to practice my spoken English. With your help, my start in Sweden was not too difficult. You are like a mentor to me, who not only care about my study, but also supported and advised me on my career planning. Again, I really appreciate the great help and hope see you in China.

To **Lena Holmberg**, is the official name for Hullan. Thank you so much for your help and support in my PhD study, you are always there when I need your help. We're teammates when we worked together at AKM, we're friends when we enjoyed chatting, skating and other fun we've had together. For me you are more like a family member, you take care of my feelings, my mood and give me advice on living in Sweden easily and happily. I really cherish the time with your company very much.

To **Eva-britt Samuelsson**, the person I first learnt how to culture the delicate cells from. With all the skills you passed down to me, I was able to keep my cells alive and happy. Even if you could not accompany me to the end of my PhD when your retirement started you help me to start my journey which is very important for a student who had little experience in research experiments.

To current and previous members in Sundström group: I really enjoyed spending time with all of you. Without your support and help, this thesis could not be completed. **Yuxi Guo**, a vigorous master student, although you have been only joining our group less than a year, but with your excellent computer skills and amazing ability to learning new experiments, you have already contributed to two of my papers in this thesis. Good luck with your new life as a PhD student. **Zjanna Alekeenko**, thank you for being nice all the time and willing to help. I enjoyed Journal Club with your excellent comments and insights. **Lydia Liu**, Thanks for answering all my programming and statistics questions. I enjoyed activities we had together, hiking, eating hotpot and sharing our thoughts on relationships. I also thank **Eva Wärdel**, **PH**, **Pablo Bonilla Villamil**, **Chenhong Lin**, **Åke Seiger**.

To all PIs and colleagues in Neurogeriatrik

Thank you **Per Nilsson** for being supportive and I really appreciate your encouragement and enlightenment. Thank you, **Bengt Winblad**, for being so nice and supportive. I also thank **Maria Ankarcrona**, **Eva kallstanius** and **Johanna Wanngren** for all the helps I got from you.

To our collaborators

Thank you, **Patrik Jarvoll** for being kind and I enjoyed the fun conversations we had during the MRI run. It is still nice to think how we've seen each other's improvement since we first worked together with tricky procedures. **Perter Damberg**, thank you for sharing your expertise and your knowledge of mechanics etc. **Anki,** thank you for being kind and willing to help. **Kicki,** thank you for your help and the funny jokes you made.

Thank you, **Anna Falk**, **Dania Winn** and **Elias Uhlin**, for your contribution to the papers in this thesis.

I would like to thank all the members in **Mikeal Svensson**'s lab, **Yongtao** for coordinating the CNS-repair seminar so well, **Mikeal Svensson, Lou Brudin** and **Jacob Kjell** for your helpful comments, enlightening discussion and nice presentations.

All members of the big family on the 10th floor

To my gym buddies **Richeng Jiang,** I feel so lucky to have your company over the past few years, there was sharing and supporting. How nice it is that we complete the PhD journey at the same time , and **Makoto Shimozawa**, thank you so much for being so kind and sharing your scientific ideas not only in biology but also in physics. All in all, I really enjoyed the lovely time training together at the KS Gym, **Simone Tambaro**, for being supportive and sharing your expertise in immunostaining, **Luis Enrique Arroyo-Garcia** for creating a lively atmosphere in the office, **Ipsit Srivastava** for the fun chatting during lunchtime and sharing your insightful thoughts on science, more importantly for using your talent to create the amazing cover for my thesis. **Johanna Mayer, Hao Li and Hongjing Zhao** for all the nice chats.

To My friends in Stockholm

Yang Yu, Gefei chen, Yang gao, Zhulin Zhou, Zhang Bo, Xinyue Zhang you have been like a family, and we had lots of nice time sharing delicious food together. Thank you all for being supportive.

To you, **Zhenqing**, thank you for being generous and supportive all along. I like it when you can always help me see things optimistically.

To my **mom** and **papa**, your unconditional love and support enables me to pursue my own dream. With your support, I have the confidence and courage to fly as high as I can.

In the end, I would like to thank all the financial support for my PhD study and projects in this thesis, **China Scholarship Council**, **Vinnova**, **Stratneuro Karolinska Institutet.**

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