

# Spinal Cord Injury: Tissue Engineering Using Neural Stem Cells

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## Abstract

Spinal cord injuries result in catastrophic dysfunctions that impair the quality of a patient's life. Following spinal cord injury (SCI), the cascade of cellular and biochemical reactions during primary and secondary injuries and a dense scar formation leads to a devastating physical and chemical barrier at the lesion site. These effects could not be reversed through conventional treatments. A multidisciplinary approach that includes materials science, engineering, biology, chemistry, and medicine is required to achieve a completely successful treatment for SCI. Tissue engineering, which integrates scaffolds, autologous (preferably) cells, and growth factors, is an encouraging development in the treatment of SCI which aims to replace and restore the anatomical and functional structure of the damaged spinal cord. Neural stem

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cells (NSCs), by differentiating into the cells of the nervous system, are a promising cell source for use in this challenging approach which has a great potential in the therapy of spinal cord injuries.

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## Introduction

Spinal cord injury (SCI) is among the most devastating disorders that impair the quality of a patient's life. The annual incidence of SCI in the United States is estimated to be 40 cases per million population according to the National Spinal Cord Injury Statistical Center (2011). The most common causes of SCI have been reported in 2011 as motor vehicle accidents (40.4%), falls (27.9%), acts of violence especially gunshot wounds (15%), and sports (8%) since 2005.

Spinal cord is an elongated cylindrical part of central nervous system (CNS) and contains bundles of nerves which carry nerve impulses along the spinal tract between the brain and the rest of the body (Snell 1992). It is composed of an inner core of gray matter, contains nerve cell bodies and associated nerve fibers, and an outer covering of white matter with descending and ascending tracts (columns) of myelinated axons. The spinal cord is surrounded by three meninges, the dura mater, the arachnoid mater and the pia mater, and is situated within the rings of bones, called vertebra, which constitute the spinal column. In addition to these protective structures, the cerebrospinal fluid found in the central canal and the subarachnoid space cushions and protects the spinal cord.

The degree of neurological defects varies depending on the extent and level of the SCI (Legos et al. 2002). A complete injury is defined by the total absence of motor and sensory function below the level of the injury which means that the patient has no sensation and voluntary movement. However, in an incomplete injury either the motor or the sensory function is present below the level of injury. Thus, a patient with an incomplete injury may be able to feel but can not move certain parts of the body. The other important criterion is the level of injury which is highly related with the parts of the body that might be

affected by paralysis and loss of function. According to their location the vertebra is classified from top to bottom (head-to-toe direction) as cervical vertebra (the eight vertebra in the neck; C-1 to C-8), thoracic vertebra (the twelve vertebra in the chest; T-1 to T-12), lumbar vertebra (in the lower back between the thoracic vertebra and the pelvis), and sacral vertebra (from pelvis down to the end of the spinal cord) (Snell 1992). Generally more dysfunction is expected in patient with SCI in the higher levels of the spinal column. SCI at cervical region usually results in quadriplegia or tetraplegia, and causes loss of function in the arms and legs or complete loss of sensation below the neck (Legos et al. 2002). Moreover, patients having injuries above C-4 level lose spontaneous respiration capability and may require a ventilator to breathe. Thoracic injuries usually affect the chest and the legs, and result in paraplegia, with the hands being not affected. Compared to the other parts, injuries at lumbar and sacral regions result in lesser effects, like decreased control of the legs and hips, and urinary system. Since 2005, the most frequent neurologic categories are reported as follows; incomplete tetraplegia (39.5%), complete paraplegia (22.1%), incomplete paraplegia (21.7%) and complete tetraplegia (16.3%) (National Spinal Cord Injury Statistical Center 2011).

In traumatic SCI, an external physical insult leads to neurological damage because of interruption of communication pathways between the brain and the periphery. The SCI is usually caused by blunt impact, compression, and penetrating trauma, and may result in complete transection. The complex pathophysiology of SCI starts with the mechanical damage to the neurons caused at the time of trauma, called primary injury, which triggers the secondary injury, a cascade of cellular and biochemical reactions (Legos et al. 2002). Formation of free radicals, lipid peroxidation, accumulation of excitatory neurotransmitters, disruption of ionic homeostasis, activation of inflammatory response are among the mechanisms that are parts of the secondary injury that leads to generation of an inhibitory environment for regeneration and repair. The resultant injury leads to necrosis and apoptosis of neurons and glia which in turn results in cystic cavity formation.

The loss of oligodendrocytes results in demyelination of axons, and impairs nerve impulse propagation. Following the tissue loss the cystic cavity is surrounded by reactive astrocytes due to astrogliosis (excessive increase in astrocyte numbers), and ends up with a dense scar tissue upon deposition of collagen. The growth of axons across the cavity is not sufficient, and the injured tissue is not able to achieve self-regeneration due to the presence of the inhibitory environment and the scar tissue which acts as a physical and chemical barrier.

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## Treatment Strategies in Spinal Cord Injury

Today, there still is no completely successful treatment of spinal cord injury. The routine clinical treatment for SCI is to stabilize the spine and restore its proper alignment along with surgical decompression of the cord. However, the decompressive surgery is a controversial issue regarding the appropriate time for surgical intervention and the ability of surgery to promote neurological recovery. The researchers have been trying to understand the neuronal behavior within the human spinal cord and the studies have been focused on functional training approaches. Neurorehabilitation is a method used for functional recovery of such disorders. For the therapy to be effective it is important to understand the reflexes and motor centers. So, it is necessary to evaluate neuronal function and biomechanical properties. Basically rehabilitation in functional training approaches should aim an improvement of function by taking advantage of the plasticity of the neuronal center. However, a single therapy as neurorehabilitation would not be sufficient to overcome the problems that arise from the secondary injury and scar formation in SCI; therefore, it should be accompanied by other inhibitory and/or regenerative therapies.

The scientific challenges for the treatment of SCI can be summarized under two main topics: neuroprotection and neural regeneration. Neuroprotection aims to prevent the secondary injury mechanisms and/or to minimize the extent of damage caused by autodestruction of neurons

and the tissue. Drugs have been used in spinal cord repair as neuroprotective agents to provide functional inhibition of molecules that prevent axonal regeneration. The most promising neuroprotective drug for SCI is methylprednisolone, a synthetic glucocorticoid steroid with anti-inflammatory activity, and is effective in attenuating particular secondary injury processes if administered properly and in time (Anderberg et al. 2007). There are also other drugs, like monosialotetrahexosylganglioside (GM-1), thyrotropin-releasing hormone (TRH), gacyclidine (GK-11), minocycline, and nimodipine, which have neuroprotective effects due to their antioxidant, anti-inflammatory or channel blockage properties. However, the dosage, timing and the way of administration are some technical constraints that should be considered when planning drug administration. Researchers have been investigating epidural drug delivery from injectable hydrogels as a more localized method that prevents the loss of dosage.

The present studies in SCI are particularly focused on neural regeneration. In neuroregeneration, the axonal growth and the neural plasticity are promoted to reorganize the injured spinal cord by forming new neural connections. A milestone in this field was the study carried out by David and Aguayo (1981) which changed the belief that injured axons are not able to regenerate after SCI. A peripheral nerve tissue graft was used to build a bridge as a guide along which axons could form connections and restore the function. It was observed that the axons could be regenerated by providing such a permissive environment, but they failed to elongate when faced with the native CNS tissue. Later, several more regeneration obstacles like nerve cell disability, an insufficient growth response by the injured neurons, and inhibitory environmental factors like NOGO-A and myelin-associated glycoprotein (MAG 13) produced by oligodendrocytes have been identified (Anderberg et al. 2007). In order to solve these problems various regeneration strategies have been developed to modify and/or eliminate the inhibitory properties of the injured spinal cord tissue. Several promising approaches, such as injection of macrophages to enhance the regeneration and removal of chondroitin sulfate proteoglycans to prevent scar

formation, were developed. Neuroregeneration strategies for SCI require the outgrowth of existing and new axons across the lesion site and their remyelination in order to achieve the ultimate goal, the functional recovery of the injured tissue. The potential strategies proposed to provide regeneration and to improve both the pathological and functional outcome in SCI mainly include the use of cell-free biomaterials, the therapy via cell transplants, and a combination of these strategies which is tissue engineering with cell seeded scaffolds.

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### **Tissue Engineering Approaches for Spinal Cord Injury**

Recent advances in nerve regeneration show that a combinatorial approach like tissue engineering, which integrates scaffolds, cells, and growth factors, is a creative strategy to achieve a completely successful treatment for SCI. The ultimate goal of tissue engineering in the treatment of SCI is to replace and restore the anatomical and functional structure of the damaged spinal cord. Cell transplantation, involving introduction of cells without scaffolds, encounters some problems like poor cell localization and survival, and uncontrolled differentiation following transplantation. Cell survival is enhanced when the cells are introduced within a cell carrier (scaffold), and thus, their integration and the success rate of their differentiation into appropriate cell types could be improved. This is to be expected because the regenerative process in the injured spinal cord is affected by the absence of extracellular matrix at the lesion site. In the presence of a scaffold, an extracellular matrix analog, the transplanted cells can be directed and organized to promote regeneration in the injured spinal cord. This is because these scaffolds serve as a bridge for the regenerating axons and guide them from one end of the injury site to the other end. In addition, the presence of cellular components in or on the scaffolds increases the healing effects and might shorten the healing time. Consequently, tissue engineering, a new perspective on SCI therapy, is an effective

strategy that combines cells, scaffolds and growth factors for proper regeneration of a damaged spinal cord.

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### **Cell Sources Used in the Treatment of Spinal Cord Injury**

In SCI, various cell sources are being tested in *in vitro* and *in vivo* studies for their neurogenic and/or neuroregenerative potential. The main goal of the cellular therapy is to fill the cavities (gaps) formed in the injured region by transplantation of cells with or without scaffold. The dead cells that result as a consequence of SCI can be replaced with new neuronal and support cells, such as Schwann cells and/or olfactory ensheathing cells, to restore myelination and/or for the release of neurotrophic factors for axonal regeneration and functional improvements. Another active area in the treatment of SCI is the use of stem cells. These cells are capable of self-renewal and proliferation, and therefore, have a great potential in cellular transplantation for the treatment of degenerative disorders. The stem cells of pluri- and multipotent nature are able to produce specialized, differentiated progeny, and having neurogenic differentiation potential and the plasticity of neurons make them an appropriate cell source for use in the treatment of SCI. These cells can be used to directly replace the damaged cells (neurons, astrocytes, and oligodendrocytes). The stem cells can also be used in undifferentiated state as support cells to release particular growth factors necessary for neural regeneration or are differentiated into neuron or support cells *in vivo* after transplantation. In addition, the stem cells become adapted to the environment, and consequently evolve with the pathology of the spinal cord to provide a sustainable treatment via neuroprotective and neuroregenerative mechanisms.

### **Embryonic Stem Cells**

Embryonic stem cells (ESCs) derived from inner cell mass of the blastocyst are pluripotent cells that are able to differentiate into derivatives of all

the three germ layers, like the neurons and the support cells in the CNS. However, there are some significant issues like teratoma formation that should be considered in cellular transplantation. There are a large number of studies on implantation of ESCs for SCI repair both *in vitro* and *in vivo*. The results have supported the idea that ESCs can survive, differentiate into neurons, and provide axonal regeneration and remyelination, and functional recovery. Since the surrounding of the injury site is not suitable for ESC survival and differentiation, the main strategy is the predifferentiation of cells into neuronal cells and thus improve their regeneration potential before transplantation for treatment of SCI (Cui et al. 2011). The neural cell adhesion molecule L1, a member of the immunoglobulin superfamily, enhances neurite outgrowth and survival. In a recent study, L1 molecule expressing ESCs, which were transfected with a plasmid encoding L1 molecule, were differentiated through SENA (substrate-adherent embryonic stem cell-derived neural aggregates) procedure before implantation (Cui et al. 2011). The results showed a better cell survival and improved locomotor function. Besides, regrowth of catecholaminergic nerve fibers distal to the injury site and recovery of endogenous spinal cord interneurons and motoneurons were achieved by SENAs. In addition to cellular therapy strategies, ESCs can be combined with biomaterials in order to provide regeneration via tissue engineering. It was shown that use of biodegradable polymer scaffolds carrying various growth factors, such as retionic acid, insulin-like growth factor (IGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ), can induce the differentiation of ESCs and promote cellular survival (Levenberg et al. 2003). Besides, when the cells were transplanted with the poly(lactic acid-co-glycolic acid) (PLGA)-poly(L-lactic acid) (PLLA) polymer blend scaffolds, cellular survival and differentiation continued *in vivo* for up to 2 weeks and a 3D vessel network like structure was observed. Furthermore, when the cell seeded scaffolds were compared with ESC cultures, it was confirmed that 3D cellular organization and a higher expression of differentiation-associated proteins could be supported by the scaffold. Considering these

findings, it can be concluded that ESCs with their great differentiation potential is an efficient cell source for cellular replacement in spinal cord injuries. However, it should not be ignored that, there are still ethical concerns and legal issues about the utilization of ESCs because these cells have to be obtained from early embryos.

## Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are found in the bone marrow, peripheral blood and umbilical cord blood. These stem cells are responsible for the formation of the blood and immune cells, therefore, they provide a regular renewal of the blood in the body. In addition, it was shown by various studies that HSCs can differentiate into muscle, bone and blood vessel cells, in addition to epithelium, intestine, liver and neural cells. Thus, HSC is a great source for cell therapy applications and there are various studies using engraftment of HSC in the treatment of SCI. It was proven that direct injection of HSCs 1 week after the spinal cord injury in a mouse model promoted functional recovery and cell survival for up to 5 weeks (Koshizuka et al. 2004). Furthermore, expression of astrocyte, oligodendrocyte, and neural precursor markers were detected, but, expression of neural markers could not be observed. In another study, implantation of human CD34+ HSCs isolated from bone marrow into lesions of the developing spinal cord in the chicken embryo resulted in differentiation of HSCs into neuronal cells (Sigurjonsson et al. 2005). Expression of high levels of the neuronal markers, neuronal nuclear antigen (NeuN) and microtubule-associated protein (MAP2), by the transplanted cellular population was observed in addition to axon extension and neuronal cyto-architecture formation.

## Mesenchymal Stem Cells

Mesenchymal stem cell (MSC) is the other cell source that can be utilized in the treatment of SCI. Their use in cell therapies does not raise ethical

concerns and debates due to their ease of isolation from various common sources such as bone marrow, umbilical cord blood and matrix, adipose tissues, etc. Each of these cells has different superiorities over the others. Even though the differentiation mechanism of transplanted MSCs in engraftment site is not clear, several *in vivo* studies showed that MSCs can migrate and differentiate into astrocytes and neurons depending on the extracellular matrix and the signal molecules in their environment. Most of the *in vitro* studies indicated that MSCs have a capability to transdifferentiate into neurons or glial cells upon treatment with appropriate combinations of growth factors and chemical stimulants, and several studies showed the functionality of these transdifferentiated neurons. Hu et al. (2010) reported that transplantation of umbilical cord MSCs (UC-MSCs) into rat spinal cord after a traumatic injury improved hind limb locomotor function recovery and achieved lengthening of neurofilament-positive fibers. It was found that the majority of the UC-MSCs were not differentiated into neuronal cells. However, the undifferentiated UC-MSCs released neurotrophin 3 (NT-3) and glial cell-derived neurotrophic factor (GDNF) after implantation. Thus, it was revealed that recovery was maintained through the production of the growth factors and cytokines by the UC-MSCs.

Initially, MSCs were isolated from the bone marrow and to date these cells have been tested in numerous tissue engineering studies. In one such study, the survival, adhesion and proliferation of rat bone marrow-derived MSCs on 3D gelatin sponges coated with a thin layer of PLGA *in vitro* were high, and upon implantation of these MSC seeded scaffolds into rat SCI lesions the inflammatory reactions and the lesion cavity formation were reduced (Zeng et al. 2011).

## Neural Stem Cells

During CNS development, after the gastrulation, neural plate is formed from the ectoderm. The proliferation and certain morphological changes of the cells within the neural plate lead to the closure of neural plate, and the neural tube and neural crest are formed. Neural crest gives rise to the

peripheral nervous system during embryogenesis while the neural tube develops into the central nervous system. Neural stem cells (NSCs) are derived from the inner epithelial periphery of the neural tube and these neuroepithelial cells are induced by intrinsic factors and the extrinsic soluble signals to differentiate into mature neuronal and glial cells. This whole process is called neurulation. Before the 1990s, it was believed that neuron production in the central nervous system ceased after birth. However, this argument was refuted by numerous studies and the presence of NSCs in the CNS was shown. It was first reported by Reynolds and Weiss (1992) that multipotent stem cells expressing nestin reside in the adult brain and these cells can be isolated through neurosphere formation assay. NSCs, which are self-renewable, are generally cultured as neurospheres. NSCs evolve into precursors committed to specific neural cell lineages, and these cells are capable of differentiation into functional neurons, astrocytes and oligodendrocytes. Therefore, transplantation of these cells into the injured region of the spinal cord can lead to the formation of new neurons and oligodendrocytes, expression of growth factors, and support regeneration and functional recovery. Thus, the role of NSCs in regenerative medicine is to become neural cells to repair communication pathways, or to do remyelination of the growing axons, or to release neurotrophic factors to stimulate regeneration.

NSCs derived from fetal tissue can preserve the full range of pluripotency, while the ones isolated from adult brain or spinal cord are more restricted to a neural phenotype. Fetal stem cells (FSCs) are used for cellular therapies in the treatment of SCI, and result in lower rates of tumor formation after transplantation, and this makes them more advantageous as a cell source compared to the ESCs. However, differentiation capacity of FSCs into motor neurons is lower compared to ESCs. Many studies suggest that induction of FSCs to become a neural cell type before implantation improves the outcomes of treatment with increased differentiation specificity. Tarasenko et al. (2007) reported that grafting of *in vitro* primed human fetal NSCs into the contused spinal cord 9 days after the injury improved the differentiation of cells into cholinergic neurons

with enhanced functional recovery. However, it was revealed that the time of the grafting is also crucial for the success of cell replacement therapy. The neural precursor cells can also be derived from ESCs to be used in the SCI, and result in behavioral improvement after transplantation to the lesion site (Hatami et al. 2009).

The evidence of the presence of NSCs in the adult CNS opens up a new era in the treatment of SCI via cellular therapies. These cells can be isolated and cultured *in vitro*. In the adult brain, NSCs are present in the subgranular zone (SGZ) of hippocampal dentate gyrus and in the subventricular zone (SVZ) of lateral ventricle where the new neurons are generated during adulthood. NSCs are most abundant in SVZ and the newly produced neurons migrate from SVZ to the olfactory bulb through the rostral migratory system. In SGZ, the number of NSCs is lower compared to SVZ. Besides, endogenous NSCs can be isolated from the central canal of the mature spinal cord. However, the NSCs isolated from different sources have different characteristics. They require different trophic factors and have different growth patterns. Thus, the environmental factors are important for specific differentiation of NSCs. Under *in vitro* conditions NSCs isolated from human adult brain were expanded in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF); however, upon removal of the mitogens from the culture NSCs differentiated into neuronal and glial cells. In an *in vivo* study by Lundberg and Bjorklund (1996) it was reported that the NSCs differentiated primarily into astrocytes after injection into adult CNS. Different studies also support that after transplantation of NSCs to the injury site, the cells differentiate especially into astroglial and oligodendroglial cells. For transplantation of NSCs after spinal cord injury, there is also a controversy about the ideal source of NSCs. One side advocates that all the stem cells are similar and they can be induced to differentiate into any type of cells through the signals of the local environment while others state that the local environment of a stem cell defines its fate and transplantation of these region-specific stem cells improves the outcome of the cell therapy. NSCs isolated from

both the spinal cord and the forebrain of the embryonic (day 16) rat were cultured under the same conditions with the medium containing either EGF, bFGF or both (Fu et al. 2005). It was shown that the cells could proliferate and expand in all three mediums, but more rapidly in the presence of both factors. It was also observed that the percentage of the neuronal differentiation of the NSCs isolated from the forebrain was higher compared to those from the spinal cord. This study suggests that use of a NSC source closer to the transplanted region could improve cell survival, engraftment and function. However, NSCs alone may not be sufficient for complete treatment of SCI. Because of the inhibitory environment and the glial scar tissue formation after the injury, neuronal differentiation and axonal extension of NSCs are limited. In the light of these facts, NSCs are also being tested with complementary strategies. Bioengineered scaffolds are utilized as a vehicle for NSC delivery. In addition, by entrapping bioactive agents in these scaffolds, graft performance could be improved. One of the first applications in this direction was in a rat SCI model in which the importance of using NSCs along with the scaffolds for best recovery was shown (Teng et al. 2002). Coordinated, weight-bearing hind limb steps were observed 70 days after the induction of injury and this recovery was stated to be related with the decrease of glial scarring. Besides, elongation of corticospinal tract fibers (from the injury region to the caudal cord) was recorded.

Consequently, each cell type referred to above could be a source for cell therapy and nerve tissue engineering. However, multipotent neural stem cells, as a result of their giving rise to the cells of the nervous system, appear to be the most promising cell source in nerve tissue engineering.

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### Scaffold Design for the Regeneration of Injured Spinal Cord

A human SCI can be extensive with massive disrupted tissue and scar formation which is represented by a serious lack of cells that would normally contribute to regeneration, and by a

physical barrier across which the axons are not allowed to grow. Therefore, the regeneration of functional axons across this large lesion would be achieved via a bridge like biomaterial scaffold through the injured area. These biomaterials can be used as cell-free structures; however, incorporation of cells and growth factors to such highly porous structures (scaffolds) creates a more permissive environment for healing and offers a longer term solution. In all tissue engineering approaches, the ideal scaffold should be three dimensional (to provide a site for cell attachment and to mechanically support tissue development), biodegradable with non-toxic degradation products and porous (for cell penetration, for nutrient supply and for removal of metabolic waste). Mechanical strength is an important parameter in the design of a nerve guide to prevent the collapse of the tube and the obstruction of regeneration. The chemical composition is critical for the mechanical properties of the material. In addition, crosslinking of the scaffold material or varying the composition of the material are approaches used to improve the mechanical strength of the scaffolds. For many years different biomaterials made of non-degradable and biodegradable polymers have been used in spinal cord repair. Generally, it is the regeneration strategy that determines the material choice.

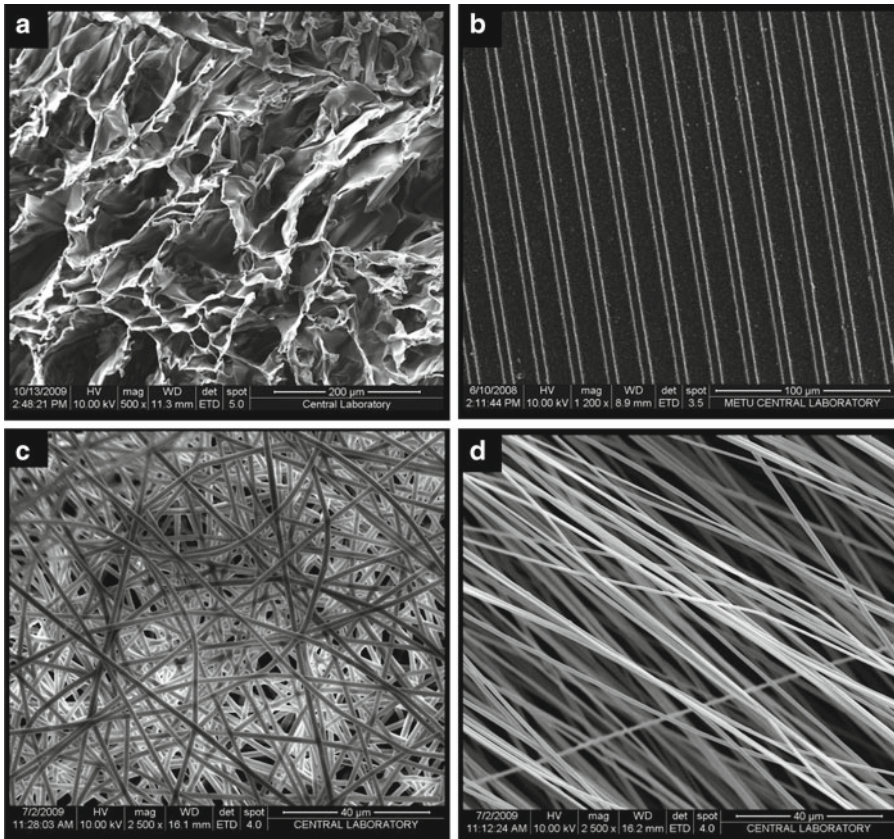
Non-degradable materials used in tissue engineering are generally of synthetic origin. Their synthesis processes are controllable and generally there is no need for complex protocols. Some non-degradable synthetic materials like silicone, poly(2-hydroxyethyl methacrylate) (PHEMA), polyacrylonitrile-polyvinylchloride (PAN-PVC), and poly(tetrafluoroethylene) (PTFE) are used in the design of nerve guidance tubes. These materials have some advantages over the degradable ones. For example, they do not require a control of the degradation rate or carry the risk of toxicity of the degradation products. However, their permanent presence creates a high risk of chronic inflammation and may result in nerve compression over time. Furthermore, the non-degradable synthetic materials are generally non-cell adhesive.

Biodegradable materials are preferred more for the demanding regeneration environment in SCI. There is no need for their removal since they would degrade as the new tissue regenerates. However, there is a risk of releasing toxic degradation products. Another concern when using biodegradable scaffolds is the degradation rate and time. The degradation time should not be less than the recovery time which is crucial for proper tissue regeneration. The rate of degradation of the construct should be tailored to match the rate of new tissue formation. The choice of polymer type and ratios and the fabrication method can alter the rate of biodegradability of the scaffold as well as its mechanical properties. The degradable materials could be of either natural or synthetic origin. However, materials from natural sources have some problems in uniformity, batch-to-batch variability, purity, and sometimes evoking immune responses. The degradable natural materials like collagen (particularly Type I), chitosan, alginate, fibrin and poly(hydroxyalkanoates) (PHA) and the degradable synthetic polymers like poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly(lactic acid-co-glycolic acid) (PLGA), poly( $\epsilon$ -caprolactone) (PCL), and some of their copolymers like poly(lactide-co-caprolactone) (PLCL) have been used in various nerve regeneration studies.

Electrically active materials are also used in the design of the nerve guidance channels. The electrical field in the natural extracellular matrix (ECM) is formed by electrically charged materials, like negatively charged proteoglycans that attract sodium ions, acts as a signal to promote axon regeneration. The applied electric field leads to a polarized rearrangement of the cytoskeleton of nerve cells via promotion of the microtubule disassembly locally along the neurite shaft. It is possible to deliver localized electrical stimulus at the injury site. The materials which can achieve these are electrically conducting, generally non-degradable polymers like polypyrrole (PP).

The structural and chemical versatility make biomaterials suitable to replace the scar tissue, fill the gap and serve as a bridge to carry regenerating axons across the gap. Most of these polymeric materials are implanted as solid scaffolds, though some are injected in sol or gel form. Gels,





**Fig. 29.1** Scanning electron micrograph of scaffolds which were fabricated in different forms (a) foam, (b) micropatterned film, and (c) electrospun random fibers and (d) electrospun aligned fibers

highly hydrated, crosslinked polymers, are viscous, and quite suitable to fill a small void preventing astrocytosis from expanding. Gels allow regeneration of axons, and are advantageous for drug delivery to the injured tissue. The property of hydrogels that are used as space filling agents makes these materials ideal for implantation at complex SCI sites. Hydrogels can also mimic the ECM environment with their polymeric network structure and high levels of water. On the other hand, use of a solid scaffold is more advantageous than injection of a hydrogel that sets or solidifies after injection. Polymeric scaffolds can be found in various forms such as sponges, fibers, and full or hollow cylinders that can be used to bridge the gaps. Sponges or foams, are porous and three dimensional, and are generally used for large tissue defects (Fig. 29.1a). They can be conveniently fabricated by freeze drying, solvent

casting or particulate leaching, or a combination of these methods and their pore size can be adjusted by various methods such as altering the size of crystals or changing the freezing temperature. However, like in the gels their mechanical strength is not high (mainly due to the high porosity and the inherent properties of polymers) making them unsuitable for long implantation durations. On the other hand, the tubular structures are used in the lesion site to serve as a bridge to cross large gaps. These can be generally porous, hollow tubes or multichannel tubes to provide a pathway for axonal regrowth. The roles of the tubular structures can be listed as to prevent scar formation, to concentrate neurotrophic molecules, and to guide the axon regeneration. Hollow single channel tubes provide limited guidance because of their large size and lack of oriented intraluminal substratum. However, tubular

constructs having anisotropic architecture such as multichannels or aligned textures are promising scaffolds in the unidirectional regeneration of neurons, which is essential for functional recovery after a spinal cord injury.

Upon SCI the damaged axons at the lesion site are misdirected which results in abortive regeneration. The advances in nerve tissue engineering aim to design scaffolds that mimic the architecture and the organization of the uninjured spinal cord. Random structures placed within the lesion site generally lead to a disorganized growth of axons (Stokols et al. 2006). By the incorporation of physical and chemical cues in the design, the oriented scaffold can imitate the anatomical structure of native tissue to provide contact mediated guidance for regeneration. The physical cues such as roughness and topography are important in the cell attachment via nonspecific adsorption. In topographical approaches, inclusion of internal matrices like channels, oriented micro/nanopatterned designs, and micro/nanofibers increase the surface area to promote cell adhesion and enhance the guided tissue regeneration via stereotropism. On the other hand, the surfaces modified with patterned ECM biomolecules, act as chemical guidance cues, facilitate cell attachment and facilitate unidirectional growth of axons, and even cells. In addition to cell attachment and guidance, some physical or chemical cues are used to promote cell survival and differentiation.

The cells adhere to ECM through interactions between ECM proteins and cell adhesion molecules like integrins and membrane receptors. These specific cell-matrix interactions modulate the organization of the actin filaments. The oriented actin cytoskeleton is the evidence of cell alignment via contact guidance. Thus, immobilization of biological molecules as ECM proteins or their constitutional motifs form chemical patterns on scaffolds to guide cells to mimic the oriented structure of the native tissue. The specific peptide sequence Ile-Lys-Val-Ala-Val (IKVAV), poly (D-lysine), and laminin are among the most common chemical cues for nerve guidance. Fibronectin can also serve as a chemical cue since it enhances attachment and proliferation of cells, and induces nerve regeneration.

Scaffold topography, as a non-biological approach to regulate cell behavior, could act as a biomimetic, cell stimulating cue. Even though the whole mechanism of cell response to topography is not clear, it has been proposed that cells adapt to physical, topographic substrates by conditioning growth environments through secretion and modulation of ECM proteins. Surfaces with micro and nano structures could be formed using a variety of techniques. Patterned surfaces could be obtained by lithography and subsequent transfer methods, or by other methods such as molding, surface grafting, ink jet printing, surface etching, and etc. The resultant surfaces could be used as is or as a template to transfer the patterns to films via solvent casting, hot embossing, or microcontact printing. Fibrillar scaffolds, on the other hand, are generated by pressure assisted injection through microsyringes, by self assembling or by electrospinning. Most of the polymers can be easily cast into different patterned films and fibers which are then used separately or in combination.

The most common technique to produce patterns or surface textures with controlled dimensions is microfabrication. Many groups have used the microfabrication techniques like photolithography to create microchannels or microgrooves (Fig. 29.1b). The dimensions of the micropatterned substrates have a strong effect on the behavior of cells that vary with the cell type and cell size. In one study microgrooved substrates of varying channel dimensions (e.g. 8  $\mu\text{m}$  wide groove, 20  $\mu\text{m}$  wide ridge, and 1 or 2  $\mu\text{m}$  depth) were produced to study the effect of these topographical cues on the behavior of neurons derived from chick embryo cerebral hemisphere (Clark et al. 1990). The shallower (1  $\mu\text{m}$  depth) grooves were not effective on the outgrowth of the neurites, with the growth cones crossing over many grooves and ridges. However, on 2  $\mu\text{m}$  deep patterns neurite outgrowth was significantly aligned along the groove axis with little crossing over the edges. It was also reported that the effect of the physical cues (such as micropatterns) on behavior and morphology of cells improved with the contribution of chemical and biological cues.

Recknor et al. (2006) showed that astrocytes and adult rat hippocampal progenitor cells were aligned along the pattern axis of laminin coated, micropatterned films. The co-culture of these progenitor cells on guided astrocytes, which served as the biological cues, enhanced neuronal differentiation and promoted neurite alignment on the patterned textures.

Fiber is one of the most suitable scaffold forms to reestablish the connection between the nerve fibers which is lost upon SCI. The fiber size, material, orientation, and fabrication methods can be altered as needed. Microfibers and nanofibers are the most common scaffold forms in neural tissue engineering. The most preferred techniques to obtain fibers are self assembly and electrospinning. In electrospinning, briefly the polymeric solution is ejected from a needle attached to the tip of a syringe under a high potential created between the needle tip and the collector plate. When the microsyringe is set in motion the solution is ejected through the needle tip in the form of a polymeric jet. The fibers are collected on the grounded collector. Concentration and flow rate of the polymeric solution, diameter of the needle, potential applied and the distance between the needle and the collector directly affect the morphology and diameter of the fibers. In standard electrospinning procedure, the randomly oriented fibers are collected on a metal plate (Fig. 29.1c). A rotating drum, disk, wire drum or a parallel pair of electrodes can be used as a collector to obtain aligned micro/nanofibers (Fig. 29.1d). Since it has been shown that the alignment of the fibers significantly alters the tissue engineered construct's performance, scaffolds with oriented fibers are commonly investigated in neural tissue engineering studies.

Nanoscale surface features can initiate the formation of focal adhesions, and might achieve a precise control of cell directionality and migration in implants. Therefore, cell attachment, proliferation, and differentiation are enhanced on the nanoscale patterned scaffolds. In addition, the nanofibers resemble the native ECM of the spinal cord more than the microfibers. The outcome of many studies involving fibrous scaffolds show that nano or low microscale fibers are quite

promising for the treatment of SCI. In addition, the alignment of the fibers is especially important in guided tissue engineering studies. In neural tissue engineering, the cells could be successfully aligned on guided textures like oriented parallel fibers. Moreover, the aligned fibers could direct the neurite growth along the fiber. Therefore, the aligned nanofibrous scaffolds are mostly selected to provide the topography suitable for achieving regeneration of SCI. Effects of both the diameter and the orientation of fibers on NSCs were investigated in *in vitro* studies by Yang et al. (2005). It was observed that NSCs were oriented along the direction of the parallel PLLA fibers, and neurite outgrowth was parallel to these fibers. However, the use of micro or nano scale fibers did not seem to have a significant effect on cell alignment. It was shown that the rate of NSC differentiation was higher on the nanofibers compared to the microfibers. In addition, the neurite extension was faster and longer on the highly aligned scaffold due to better contact guidance effects. The effect of the fiber orientation on NSC behavior was also studied by our group (Yucel et al. 2010). It was observed that mouse NSCs were randomly distributed in all directions on the nonguiding, random fibers. However, on the aligned fibers NSCs responded to the topography and were aligned in clusters. It was shown that the cytoskeleton and nuclei of the cells were also aligned and elongated along the axis of fibers.

In self assembly method, a molecularly designed bioactive matrix is used to provide treatment for SCI and regeneration of axons. A nanofibrous matrix composed of peptide amphiphile (PA) molecules is formed after injection into the lesion site. Thus, the scaffold of cylindrical nanofibers is formed by self assembly from aqueous solution, and bioactive epitopes are found on the surfaces of the nanofibers. In this strategy, neither the cells nor the exogenous proteins are combined with the scaffold, which completely differs from other approaches (Tashiro et al. 1989). The novelty of this study is the incorporation of the neuroactive pentapeptide epitope (IKVAV) of laminin into the negatively charged PA network. Incorporated IKVAV peptide mimicked laminin and supported ECM formation,

which was required for axon outgrowth. When this IKVAV PA solution interacted with the physiological fluid, PA spontaneously formed nanofibers both *in vivo* and *in vitro*. Previous studies on this system showed that the nanofibers including the IKVAV epitope promoted the outgrowth of cultured neurons and suppressed the astrocytic differentiation of neural progenitor cells.

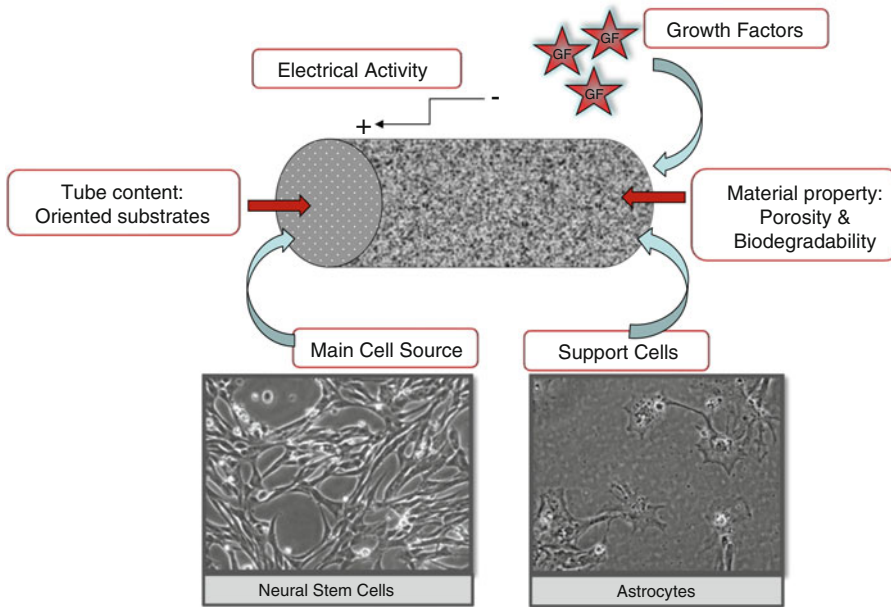
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### Growth Factor Incorporation into Scaffolds

Growth factors, especially neurotrophic factors, are essential in the development of nervous system, myelination of axons, and supporting survival and differentiation of neurons. In SCI treatment the application of these neurotrophic factors are crucial. These factors are able to stimulate the activation of the regeneration-associated genes to promote the regeneration of injured axon and to enhance the differentiation of NSCs to replace the injured neurons or neuroglia cells. In *in vitro* culture, the differentiation of NSCs is triggered by the withdrawal of mitogens like EGF and bFGF which are essential in NSC expansion. In addition, the potential of NSC differentiation can be enhanced by the use of neurotrophin molecules which improve the survival of postmitotic neurons, and so increase the number of newly committed neurons. The molecules of neurotrophin family promote the maturation process of newly formed neurons, increase the length and branching of neurons, and facilitate formation of functional neurons. Therefore, introducing the main growth factors into the lesion promotes recovery. There are two major approaches for growth factor administration; exogenous delivery and endogenous expression. Different growth factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) have been tested. However, the ways these factors influence the neural cells significantly differ. In addition to supporting survival and differentiation

of neural cells, these factors play a role on cellular migration and myelination, but with contrasting actions. Unlike endogenous BDNF, NT-3 supports Schwann cell migration but prevents myelination of cells (Yamauchi et al. 2004). As defined in here, the use of these antagonist growth factors after SCI is definitely a proper approach for the regulation of trophic environment (for both migration and myelination of neural cells to support regeneration). In addition to BDNF and NT-3, GDNF and NGF delivery after SCI are appropriate strategies for regeneration. It was reported that injection of genetically modified fibroblasts expressing NGF into the lesion site of rhesus monkeys achieved a greater extension of spinal cord sensory axons and putative coeruleospinal axons compared to the control group (Tuszynski et al. 2002). In addition, Schwann cell migration and spontaneous axonal plasticity were observed. It can be concluded that axonal plasticity can be enhanced by expression of trophic factors by the transplanted cells. In addition to NGF, Schwann cell migration to the injured region can be induced by the local expression of GDNF, and as a result, both remyelination and axonal regeneration can be promoted (Blesch and Tuszynski 2003). NT-4/5 is also candidate for growth factor delivery; however, this neurotrophic factor was less investigated compared to the others. NT-4/5 binds to the same receptor tyrosine kinase receptor B (TrkB) with BDNF, but their biological activities significantly differ. It was indicated that NT-4/5 may be more potent than BDNF in SCI treatment (Blesch et al. 2004). Thus, NT-4/5 expressing genetically modified fibroblasts were grafted into the lesion after thoracic spinal cord injury and axonal extension was observed as a result of the effect of NT-4/5. Moreover, remyelination was observed as a result of Schwann cell migration inside the graft, but functional recovery could not be observed.

Tissue engineering approaches with growth factor administration are utilized in order to improve the outcome and overcome the deficiencies in the treatment of SCI. The main strategy is genetic modification of cells in order to express or overexpress growth factors as mentioned above, and performing transplantation



**Fig. 29.2** Schematic presentation of an ideal tissue engineered construct seeded with neural stem cells

after seeding and culturing these cells on the scaffolds. On the other hand, growth factors can also be entrapped in the scaffolds (Fig. 29.2). These bioactive molecules can be released from the scaffold via controlled or sustained delivery, and they would support regeneration, cellular proliferation and differentiation after transplantation. Especially growth factor release from the scaffolds can induce the differentiation of the cells seeded on the scaffold, and cellular replacement in the lesion cavity can be provided.

### Tissue Engineering Applications Using NSCs for Spinal Cord Injury

There are various encouraging results obtained both in *in vitro* and *in vivo* that combine biomaterial scaffolds with NSCs. These NSCs are restricted to differentiate into neurons, a basic cell type found in the spinal cord, and supportive neuroglial cells, achieving remyelination and release of required neurotrophic factors to promote regeneration in the SCI.

The predetermined differentiation potential of NSCs in three dimensional matrices was demon-

strated in several studies. In one such study, NSCs were obtained from embryonic rat cortical or subcortical neuroepithelium, and cultured in collagen type I gels (Ma et al. 2004). It was shown that the differentiated neurons were excitable and ion channels/receptors, neurotransmitters and expression of specific proteins that characterize the polarity of neurons were present. In addition to these results, an active synaptic vesicle recycling among neurons entrapped in collagen presented the formation of functional synapse and neuronal network.

Investigators use different strategies to release the growth factors, especially neurotrophins, into the milieu of the tissue engineered NSC-scaffolds to improve the regeneration process at the site of the lesion of the spinal cord. The permissiveness of the environment can be further enhanced by incorporation of these factors directly into the scaffolds, or use the cells, like NSCs themselves or the support cells, as a source from which to deliver the neurotrophins. In one study, NT-3 and platelet-derived growth factor (PDGF) were incorporated into fibrin scaffolds which also contained mouse embryonic stem cell-derived neural progenitor cells, and these scaffolds were

embedded in a subacute rat model of SCI (Johnson et al. 2010). It was reported that the strategy of using growth factors within the fibrin scaffold enhanced the cell survival and proliferation in the spinal cord lesion 2 weeks after injury. Moreover, the scaffolds including a heparin-binding delivery system for the controlled release of growth factors directed the differentiation of progenitor cells into neurons. Moreover, NSCs by themselves can be used as a growth factor delivery vehicle via incorporation of a therapeutic target gene, particularly a gene that codes for a neurotrophic factor, into these cells via standard genetic material manipulation procedures to achieve the expression or overexpression of specific neurotrophic factors. NT-3 overexpressing NSCs generated via transduction were seeded on PLGA scaffolds and implanted into a canine SCI model (Kim et al. 2010). It was shown that NT-3 overexpression slightly enhanced the survival of transplanted cells. On the other hand, it was observed that it promoted the migration of NT-3 overexpressing NSCs to the spinal cord tissue, and also improved long-term survival of these cells. In the approach of incorporation of the supportive cells like Schwann cells, the cells could be used as a source of neurotrophic substances to improve the survival and axonal regeneration of injured neurons as well as take part in remyelination of axons (Chen et al. 2010). They observed that co-transplantation of NSCs and Schwann cells which were cultured on PLGA scaffolds could promote the functional recovery of the SCI of rats with the higher amplitudes of motor and somatosensory evoked potential of lower limbs compared to the same construct except Schwann cells. Moreover, it was suggested that Schwann cells promoted NSC differentiation into neurons to replace the degenerated counterparts in order to maintain the synaptic connections and to restore the neural pathways.

The other most commonly used strategy in tissue engineered NSC-biomaterial scaffolds for SCI is the use of guided scaffolds to orient axons and newly generated neurons in the same direction. Therefore, the scaffolds were designed by simulating the architecture of the healthy spinal cord. Even in the early studies the guided sub-

strates were constructed to emulate the white matter with longitudinally oriented textures for axonal and neuronal guidance (Teng et al. 2002). For this purpose, the outer part of the scaffold was fabricated via solid-liquid phase separation technique to produce long, axially oriented pores. In a similar approach alginate-based anisotropic capillary hydrogels were used to promote oriented axonal regrowth in the injured spinal cord (Prang et al. 2006). It was observed that this architecture induced guided axon regeneration across the scaffold after implantation into acute cervical spinal cord lesions in adult rats. Moreover, *in vitro* studies showed that neural progenitor cells could be introduced into these oriented constructs to encourage cell contact-mediated axon regeneration in the injured spinal cord. In another study, the multichannel porous PLGA scaffold was combined with a mixture of genetically modified NSCs transfected with either NT-3 or tyrosine receptor kinase C (TrkC), the NT-3 receptors, to obtain neuronal connections *in vitro* (Xiong et al. 2009). In the NT-3/TrkC group, a high percentage of NSCs were differentiated into functional neurons which established connections and exhibited synaptic activities, thus, these neurons could be activated at the molecular level in response to external stimuli. A biomimetic electrospun fibrous PCL/collagen tube was fabricated to be utilized as a delivery vehicle for NGF to facilitate regeneration after SCI by promoting NSC differentiation (Hackett et al. 2010). The gaps formed between nanofibers were small enough to entrap the growth factor inside the scaffold and allowed a slow release of these factors. It was observed that NSCs proliferated and differentiated effectively on nanofibers, even more favorably on the aligned fibers. It was revealed that the electrospun PCL/collagen tubes could have a great potential to serve as a scaffold with the highest proportion of neurons, astrocytes, and Nestin positive cells grown on them. In our study a tissue engineered, guided nerve tube with well defined topographical cues, aligned electrospun fibers and a micropatterned film, was developed by the use of NSCs and NSC derived astrocytes as support cells to repair the transected nerves in

the spinal cord (Yucel et al. 2010). NSCs were cultured both on the aligned, electrospun mat and micropatterned films for a while, then the cells on the micropatterned film were differentiated into astrocytes while the cells on the fibers were kept undifferentiated. The 3D tubular scaffold was formed by rolling the patterned film (with its micropatterns facing inside) over the fibrous mat, and the aligned fibers containing oriented NSCs and the microgrooves containing the aligned astrocytes were parallel to the tube axis. In this design, the aligned astrocytes on the film would serve as a growth and differentiation factor source for the NSCs on the aligned fibers in addition to enhancing their alignment. The success of *in vitro* study in cellular alignment and survival of both NSCs and astrocytes in the tubular scaffold after co-culture demonstrated the potential of the distinct tissue engineered nerve tube design to be used *in vivo* for the structural and functional regeneration of injured spinal cord.

In most of the studies for SCI the NSCs seeded on the biomaterial scaffolds were derived from mouse or rat; however, recently researchers have started using human NSCs. In one such study, human ESC-derived neural precursor cells (NPCs) were cultured in collagen scaffolds to promote recovery in injured rat spinal cord (Hatami et al. 2009). It was observed that human ESC-NPCs are able to differentiate into neurons and glial cells both *in vitro* and *in vivo*. The *in vivo* results showed that the recovery of hind limb locomotor function and sensory responses in an adult rat model of SCI were improved by implantation of collagen scaffolds seeded with human ESC-NPC, and these transplanted cells migrated toward the spinal cord. In another study, engraftment of NT-3 overexpressing human NSC on PCL scaffold, and combining chondroitinase treatment after implantation of cell seeded scaffold significantly promoted behavioral and electrophysiological recovery after SCI in a rat model (Hwang et al. 2011). In this strategy, the scaffold provided the mechanical support for the cells and also acted as a reservoir to provide migratory NSCs to the lesion. On the other hand, NT-3 overexpression of the cells increased the cell

survival, differentiation and migration. Results proved that, by combining all these tissue engineering components and performing chondroitinase treatment after implantation, neuroplasticity and axonal remodelling were improved, remyelination of contralateral white matter was promoted and functional recovery was ameliorated.

Through these strategies researchers are seeking to construct the ideal scaffold for SCI treatment (Fig. 29.2). The studies revealed that transplantation of guided, tissue engineered scaffolds seeded with NSCs combining with the developing approaches would be an effective way to achieve the regeneration and functional recovery of SCI.

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## Future Prospects

The unlimited potential in the scaffold design and the use of NSCs with current approaches open up the way for new treatments for SCI. Researchers are still facing the problem that regenerating axons fail to grow out of the scaffolds. To overcome this obstacle and initiate the entry of regenerated axons into the host environment, highly migratory cells, which replace degenerated cells or facilitate axonal growth, need to be transplanted within the scaffolds designed through advanced engineering approaches. Tissue engineering should be coupled with the manipulation of the milieu for the neutralization of inhibitory signals or the enhancement of effective signals so that significant neural regeneration is achieved in patients with SCI.

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