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PREFACE

Consensus exists among both basic and clinical scientists that peripheral nerve repair is no longer a matter of only surgical reconstruction, but rather a matter of tissue engineering which brings together several interdisciplinary and integrated treatment strategies.

In 2009, we edited a first thematic issue of the *International Review of Neurobiology* entitled "Essays on Peripheral Nerve Repair and Regeneration" (Volume 87) that collected a number of reviews on various and broad aspects of peripheral nerve regeneration research (including also several methodological papers). Following the interest raised by that book, and considering the growing scientific interest on nerve repair and regeneration, we have edited this new thematic issue of the *International Review of Neurobiology* which is intended to address more specifically some of today's hot topics on peripheral nerve's tissue engineering, namely stem cells and regeneration promoting factors.

Tissue engineering is an emerging science that finds its roots in various and complimentary disciplines (from molecular biology and biomaterials to transplantation and reconstructive microsurgery) and, in order to reflect its interdisciplinary and multitranslational spirit, this thematic issue of the *International Review of Neurobiology* brings together 10 reviews which aim to cover some of the most promising innovative strategies for promoting peripheral nerve repair and regeneration that emerge from basic research in the different relevant scientific areas.

After a couple of introductory reviews that set up the stage, three papers address the issue of cell transplantation for nerve reconstruction, with special emphasis on the potential use of stem cells from mesenchymal origin.

The book continues with four reviews that address the perspectives of growth factor-mediated therapies for improving peripheral nerve repair, and is concluded by two other papers on the role of the local environment (extracellular matrix) in nerve regeneration.

Although the papers included in this book address topics that are more specific in comparison to those addressed in the *International Review of Neurobiology* thematic issue published in 2009, all reviews have been written avoiding excessive technical details and in order to be accessible to a broad

and interdisciplinary audience. It is thus expected that this collection of papers will stimulate the interest of many interdisciplinary researchers (both with basic and clinical background) and will eventually contribute to the scientific progress in tissue engineering of the peripheral nerve as well as to its successful future applications with patients suffering from nerve injury.

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CHAPTER ONE

Tissue Engineering and Regenerative Medicine: Past, Present, and Future

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Contents

Abstract

Tissue and organ repair still represents a clinical challenge. Tissue engineering and regenerative medicine (TERM) is an emerging field focused on the development of alternative therapies for tissue/organ repair. This highly multidisciplinary field, in which

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bioengineering and medicine merge, is based on integrative approaches using scaffolds, cell populations from different sources, growth factors, nanomedicine, gene therapy, and other techniques to overcome the limitations that currently exist in the clinics. Indeed, its overall objective is to induce the formation of new functional tissues, rather than just implanting spare parts. This chapter aims at introducing the reader to the concepts and techniques of TERM. It begins by explaining how TERM have evolved and merged into TERM, followed by a short overview of some of its key aspects such as the combinations of scaffolds with cells and nanomedicine, scaffold processing, and new paradigms of the use of stem cells for tissue repair/regeneration, which ultimately could represent the future of new therapeutic approaches specifically aimed at clinical applications.

1. INTRODUCTION

In 1993, Langer and Vacanti defined tissue engineering (TE) as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer & Vacanti, 1993). On the other hand, regenerative medicine has been defined as "the process of replacing or regenerating human cells, tissues or organs to restore or establish normal function" (Mason & Dunnill, 2008).

Since its start, TE has been relying on three pillars (Salgado, Coutinho, & Reis, 2004a): scaffolds, cells, and growth factors. On the other hand, regenerative medicine uses other strategies to induce organ regeneration including cell-based therapies, immunomodulation, gene therapy, nanomedicine, and TE itself. In fact, because of their similar objectives, these two fields have been merging in recent years, originating the broad field of tissue engineering and regenerative medicine (TERM).

This chapter focusses on deciphering some of the essential elements and hot topics on TERM, and the ways in which they can be applied to the development of regenerative therapies.

2. TERM: BASIC RESEARCH

Within the human body there are tissues with a limited capability of repair/regeneration, posing a challenge that is often difficult for clinicians to overcome. Therefore, in order to achieve its proposed objectives, TERM has been relying on different strategies, among which the triad referred to in Section 1 (scaffolds, cells, and growth factors) stands out. Scaffolds are biodegradable templates that act as temporary matrices for cell and tissue

growth/differentiation (Salgado, Coutinho, & Reis, 2004b). On the other hand, cells are also extremely important as they will act (in most cases), along with their extracellular matrix (ECM) molecules, wherever applicable, as the biological trigger that will stimulate the endogenous regeneration. The purpose is to culture cells on the scaffolds over a period of time after which the scaffold/cell tissue construct will be implanted *in vivo*. Initially, differentiated cells from the tissues to be regenerated were used; however, with the expansion of the stem cell field, most of the strategies used nowadays are based on stem cells. Finally, growth factors are also of extreme importance, as they trigger crucial processes such as cell differentiation, vascularization, and others, which are relevant for the process of tissue regeneration.

The culturing conditions are another important topic to be considered. As tissues are typically 3D constructs, it is important to use appropriate culture conditions that mimic those found *in vivo*. Bioreactors play an important role for this purpose, and a wide variety of systems, including spinner flasks (Ruottinen, Vasala, Pospiech, & Neubauer, 2007; Sucosky, Osorio, Brown, & Neitzel, 2003), recirculation bioreactors (Jun, Yongsheng, Henry, & Mei, 2007; Mahmoudifar & Doran, 2005), rotating wall vessels (e.g., uni- and bi-axial bioreactor rotation) (Ayyaswamy & Mukundakrishnan, 2007; Manley & Lelkes, 2006; Singh, Teoh, Low, & Hutmacher, 2005), have been developed.

In the following sections, different examples of how TE can be used for tissue repair are presented and discussed.

2.1. Bone TE

Bone is a vascular and highly specialized form of connective tissue composed of 50–70% mineral, 20–40% organic components, and 5–10% water. The organic matrix is mainly composed of collagen type I (~95%), while nanohydroxyapatite is a constituent of the inorganic mineral portion. Bone has a limited ability to heal, and to circumvent this problem, autografts (Merkx, Maltha, Freihofer, & Kuijpers-Jagtman, 1999; Saint-Cyr, Miranda, Gonzalez, & Gupta, 2006), allografts, or xenografts (Merkx et al., 1999; Su-Gwan, Hak-Kyun, & Sung-Chul, 2001) have been used for treating extensive bone lesions. As a viable alternative, hydroxyapatite (Hap, $Ca_{10}(PO_4)_6(OH)_2$) ceramic scaffolds with controlled architecture have been developed (Oliveira, Silva, et al., 2009). Despite their interesting properties and biological performance, the HAp implants lack osteoinductive capacity (Boyde, Corsi, Quarto, Cancedda, & Bianco, 1999; Woodard et al., 2007). Therefore, the combination of bone marrow stromal cells (BMSCs) with

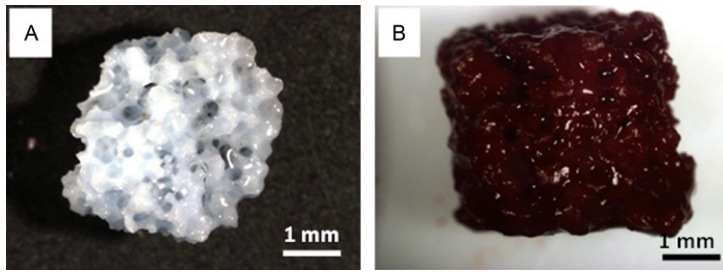

Figure 1.1 Optical microscopy images of the HAp scaffold (A) and HAp scaffold seeded with rat bone marrow stromal cells (1×10^6 cells scaffold^{-1}), which were stained with Alizarin red (mineralization) after culturing (14 days) in MEM medium with 0.01 mg ml^{-1} Dex-loaded CMCht/PAMAM dendrimer nanoparticles, 0.28 mM ascorbic acid, and 10 mM β-glycerophosphate (B).

HAp scaffolds (Kruyt et al., 2007; Nishikawa et al., 2004; Uemura et al., 2003) is promising, as it affords a superior *de novo* bone formation. Composite materials have also been proposed for applications in bone regeneration as they can combine the best of two worlds, polymers and ceramics, while making it possible to best mimic the bone composition (Oliveira, Costa, et al., 2009). One of the main problems in the use of stem cells in bone TE is the need to control stem cell differentiation *in vivo* and avoid de-differentiation. Oliveira, Sousa, et al. (2009) and Oliveira, Sousa, et al. (2011) have been making advances with novel nanotechnology-based strategies to enhance osteogenesis *in vitro* and *in vivo*. The studies have demonstrated the efficacy of intracellular delivery of dexamethasone by using dendrimer-based nanoparticles in order to induce the osteogenic differentiation of BMSCs and enhance *de novo* bone formation (Fig. 1.1). However, a major challenge in the field of bone TE remains to be met, that is, promoting vascularization in order to allow nutrients access into large TE constructs, thereby maintaining their viablility at the implantation site.

2.2. Cartilage TE

Hyaline cartilage has a high water content (75 wt%) but has no nerves or blood vessels (Aigner & Stove, 2003; Temenoff & Mikos, 2000). The cartilage ECM is rich in collagen type II and includes a low number of chondrocytes (<10 wt%) with a low proliferation rate. For these reasons, it has a limited capacity to regenerate when cartilage lesions occur at a critical

size (Hunziker & Rosenberg, 1996). Although a fibrocartilage tissue can be produced at the lesion site during the process of repair (Apprich et al., 2010; Erhart-Hledik et al., 2012; Zbýň et al., 2012), this newly formed tissue has different biomechanical and biological performance in comparison to native cartilage, which can lead to progression of cartilage degeneration, reinnervation, and appearance of pain after a few years. Gellan gum hydrogels (Oliveira, Santos, et al., 2009), chondroitin sulfate, chitosan, and hyaluronic acid (Kikuchi, Yamada, & Shimmei, 1996; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012) have shown great potential in cartilage TE. Further details on the potential use of polysaccharides in cartilage TE can be found elsewhere (Oliveira & Reis, 2011).

2.3. Osteochondral (OCD) TE

Thinning of cartilage or progression of cartilage lesions into the subchondral bone can lead to the appearing of OCD defects (Grade IV Outerbridge classification, Fig. 1.2A) (Sanders & Crim, 2001). OCD treatment possibilities are mainly based on clinical assessment and pain, but management comprises patient age and extension of the lesion requirements (Menetrey et al., 2012). Autograft transplantation (Espregueira-Mendes et al., 2012), microfracture (Kuo et al., 2006), subchondral drilling (Bouwmeester, Kuijer, Homminga, Bulstra, & Geesink, 2002), autologous chondrocyte implantation (ACI) (Ellender, Gomoll, & Minas, 2008), and matrix-associated autologous chondrocyte transplantation (Domayer et al., 2012) are examples of clinical approaches that have been exploited as primary/secondary treatments.

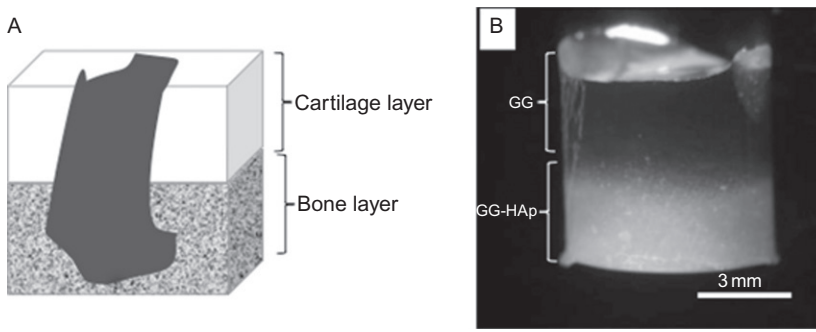

Figure 1.2 Illustration of an osteochondral defect (Grade IV) (A), and photograph of the gellan gum/gellan gum-hydroxyapatite (GG/GG-HAp) bilayered scaffold (B).

OCD reconstruction is particularly challenging as it requires addressing simultaneously, the regeneration of two different tissues. Thus, OCD treatment options should be able to preserve the OCD tissue. In this sense, strategies involving the use of cartilage- and bone-like (bilayered) scaffolds are advantageous (Fig. 1.2B). Oliveira et al. (2006) have pioneered the development of bilayered scaffolds for treating OCD lesions.

2.4. Meniscus TE

Meniscus TE has been attracting a great deal of attention in the last few years because of the increasing interest in preserving this tissue. Meniscus is a fibrocartilaginous tissue mainly composed of collagen type I, and it plays an important role in knee-joint stability and homeostasis. It is a complex tissue since it has a grading of vascularization, that is, it has vascularized (red–red), poorly vascularized (red–white), and avascular (white–white) regions, and it is known that the lesion site within the meniscus is cr for its repair (Pereira, Frias, Oliveira, Espregueira-Mendes, & Reis, 2011). Treatment possibilities include total and partial replacement (Monllau, González-Lucena, Gelber, & Pelfort, 2010), and application of biological factors (Pereira, Frias, et al., 2011). Acellular scaffolds have been used in a clinical setting for partial replacement of meniscus (Pereira, Frias, et al., 2011; Verdonk et al., 2012). Collagen-based (Menaflex®, ReGen Biologics, USA) (Hirschmann et al., 2013; Linke, Ulmer, & Imhoff, 2006; Zaffagnini et al., 2011) and polyurethane-based implants (Actifit®, Orteq Ltd, UK) have been used in a clinical setting, but the final repaired tissue is not an ideal one (Pereira, Frias, et al., 2011). In this sense, other biomaterials have been proposed for use in meniscus TE. Interestingly, silk scaffolds derived from high-concentration silk fibroin solutions (10–16 wt%) have been developed by Leping et al. (Yan et al., 2012). Their study revealed that the mechanical properties of the silk scaffolds are concentration dependent, and suggested that the 10–12 wt% scaffolds may be applied for meniscus regeneration, while 16 wt% silk scaffolds may find application in cartilage TE. A systematic review of the preclinical and clinical studies related to meniscus TE has been reported by Pereira, Frias, et al. (2011). Scientists are still facing many challenges in meniscus TE, but future research directions should be targeted for controlling segmental vascularization and reinnervation of the meniscus TE scaffolds.

2.5. Intervertebral disc (IVD) TE

IVD degeneration is another important topic that has been addressed in TE. The IVD is a cartilaginous structure located between two vertebral bodies. The IVD is composed of the nucleus pulposus (NP), annulus fibrosus (AF), and cartilaginous endplates (Coventry, Ghormley, & Kernohan, 1945). During progression of IVD degeneration, the avascular NP, located in the central region and containing type II collagen (mainly) and minor quantities of other types of collagens (e.g., IX, VI, and III) (Urban, Roberts, & Ralphs, 2000), starts to lose its properties, which leads to an increased risk for disc herniation. Management of IVD degeneration in the long term requires new treatments based on regenerative approaches. Preclinical studies include the use of stem cells (Vadalà, Denaro, Sobajima, Kang, & Gilbertson, 2005) and chondrocyte transplantation (Meisel et al., 2007), gene therapy (Cassinelli, Hall, & Kang, 2001), and TE-based strategies (Richardson, Mobasheri, Freemont, & Hoyland, 2007). Injectable biomaterials such as hydrogel systems are also attractive since it is possible to administer them through minimally invasive techniques. Enzyme-, ionic- and photo-crosslinked hydrogels have been tested to repair NP (Baer, Wang, Kraus, & Setton, 2001; Bron, Vonk, Smit, & Koenderink, 2011; Jeon, Bouhadir, Mansour, & Alsberg, 2009; Pereira, Silva-Correia, Oliveira & Reis, 2013; Reza & Nicoll, 2010; Roughley et al., 2006; Silva-Correia et al., 2011; Silva-Correia, Oliveira, Oliveira, Sousa, & Reis, 2010; Su, Chen, & Lin, 2010; Thomas, Fussell, Sarkar, Lowman, & Marcolongo, 2010). Hyaluronic acid (Su et al., 2010), carboxymethylcellulose (Reza & Nicoll, 2010), and poly (*N*-isopropyl acrylamide) (Thomas et al., 2010) are some of the biomaterials that have been reported for IVD regeneration. Recently, gellan gum-based hydrogels have also been proposed as NP substitutes (Silva-Correia et al., 2010, 2012, 2011). Similar to other ionic-crosslinked polymerized hydrogels (e.g., alginate), gellan gum may not present the required stability *in vivo*, with possible misplacement of structural integrity (Oliveira, Santos, et al., 2009; Oliveira et al., 2010). Chemical modification and photo-polymerization strategies can improve the stability of hydrogels. Silva-Correia et al. demonstrated that methacrylated gellan gum hydrogel possesses improved mechanical properties in comparison to gellan gum hydrogel (Silva-Correia et al., 2012). For prevention of angiogenesis and the symptoms associated to progression of IVD degeneration, the development of advanced materials such as anti-angiogenic scaffolds seems to be an alternative. Complementarily, other advanced strategies that

make use of drugs, peptides, or growth factors with the ability to control innervation and blood vessel infiltration during tissue repair should also be addressed. But besides the need to control angiogenesis, the biomechanical performance of NP substitutes is the major challenge when treating IVD (Reitmaier et al., 2012), and therefore new biomaterials that can promote the interaction with AF are also in demand. Closure of AF is another important challenge that remains to be tackled, namely when considering only NP replacement, but the focus is still on finding the best procedure to avoid NP extrusion.

2.6. Neural TE

2.6.1 Spinal cord injury

Spinal cord injury (SCI) usually results in dramatic neurological deficits and disabilities. A traumatic injury in the spinal cord leads to the interruption of the neural connections between the brain and the rest of the body, resulting in paralysis and loss of sensation below the injury level, as well as frequent infections in bladder, kidneys, bowel problems, and cardiac and respiratory dysfunctions. Currently, there is no clinical solution to treat SCI patients; therefore, it is very essential to find novel strategies that can lead to the regeneration of SCI-affected individuals. Because of the complexity of the SCI pathophysiology (Donnelly & Popovich, 2008), only regenerative strategies based on multidisciplinary and integrative approaches such as those presented by TE concepts will most likely help in solving the problem. In view of the irregular geometries of the cavity formed after an SCI and the mechanical properties of the neuronal tissue, injectable hydrogels are the biomaterials that better fill the prerequisites to successfully repair the spinal cord. In fact, the use of more than 35 different hydrogels has already been described with the aim of treating the spinal cord (Perale et al., 2011). For instance, Lee et al. (2009) demonstrated that the delivery of human neural stem cells (NSCs) encapsulated into the hydrogel Matrigel promotes sensory axonal regeneration and functional improvements of animals with SCI. However, although hydrogels are the most promising biomaterials to treat the spinal cord, there are also some challenges: most hydrogels are poor substrates for supporting the growth and migration of axons. In order to overcome this drawback, researches are "functionalizing" the hydrogels with active peptides. For instance, Silva and colleagues demonstrated that the addition of a fibronectin-derived peptide to the backbone of the gellan gum hydrogel drastically improved the cell behavior of encapsulated NSCs (Silva, Cooke, et al., 2012). It is important to note that hydrogels are not the

only construct engineered with the aim to treat the SCI; for example, the use of rigid tubes or channels loaded with cells are also often used to fill large gaps created by partial or complete transections (Reynolds et al., 2007; Silva et al., 2010; Silva, Sousa, Pires, et al., 2012). Moreover, many researchers have also engineered nanoparticles in order to deliver therapeutic agents that can protect/repair the injured spinal cord (Cerqueira et al., 2013; Shi et al., 2010). Finally, recent evidence has demonstrated that a rapid prototyped scaffold (Fig. 1.3A) can be used to promote spine stabilization of SCI rats and, at the same time, vertebral bone repair (Oliveira et al., 2012; Silva, Sousa,

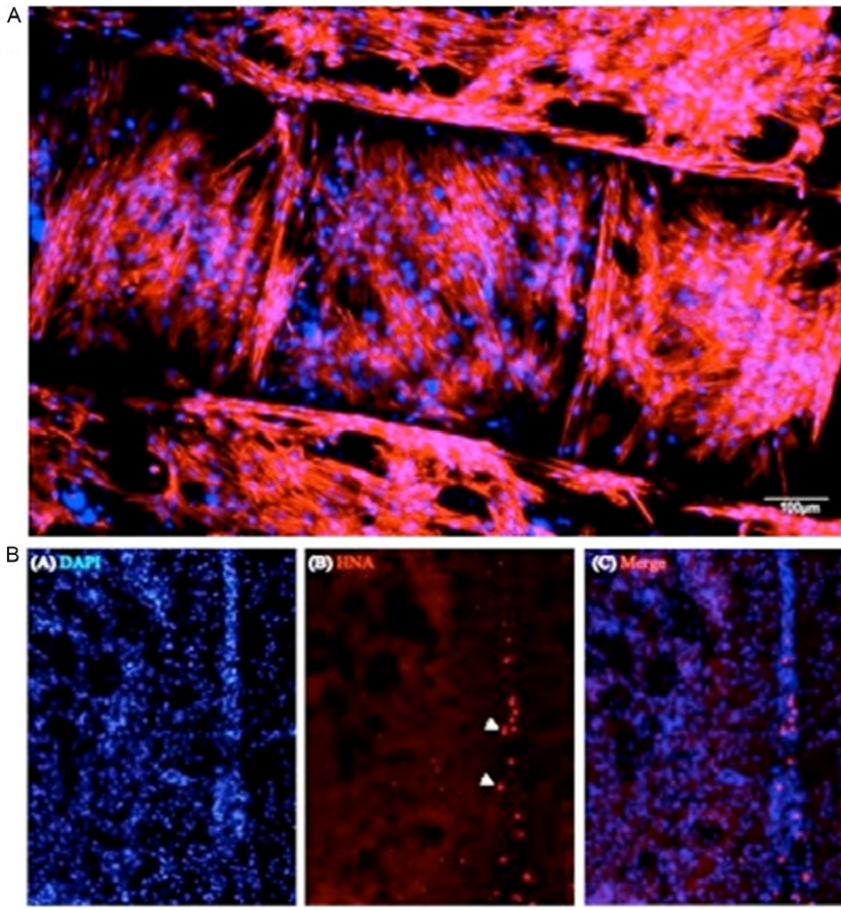

Figure 1.3 Mesenchymal stem cells growing on a rapid protoyped scaffold aimed at spine stabilization (A), and in the brain parenchyma 7 days after transplantation (B).

Fraga, et al., 2012). Though mostly ignored by researchers, spine stabilization after an SCI seems to be imperative, not just in humans but also in rodents (Silva et al., 2013).

2.6.2 Peripheral nerve injury

Following an injury, the axons of the peripheral nervous system (PNS) and the central nervous system (CNS) respond differently. While the CNS regeneration is very limited, severed PNS axons are able to re-extend and re-innervate their targets. However, a complete recovery of nerve function is very unusual and the clinical results are still unsatisfactory, especially for large gaps. In fact, injuries to the PNS are one of the major sources of disability in the United States (Noble, Munro, Prasad, & Midha, 1998), impairing the ability to move muscle and/or resulting in painful neuropathies. The standard clinical procedure after a nerve trauma is the simple suture, without generating tension, of the nerve ends; however, the absence of tension is impossible when suturing large gaps. In these cases, a nerve autograph is typically employed (Mukhatyar, Karumbaiah, Yeh, & Bellamkonda, 2009). Although the use of autographs is currently the best clinical treatment available, this procedure has many drawbacks, including the need for a second surgery, the loss of donor site function, limited availability, and the presence of inhibitory molecules in autographs, which may reduce its efficacy (Groves et al., 2005; Mukhatyar et al., 2009). Therefore, the pursuit of alternative therapies that can successfully repair peripheral nerves is still essential. Neural TE is one of the most promising alternative therapies being studied, mainly because with the use of 3D constructs, it will be possible to combine several key elements to successfully promote neuronal repair. They include the protection of the nerve stumps from the entrance of external scars, specific topographic administration of trophic and tropic elements, guiding of growth cues, and the addition of exogenous pro-regenerative mediators such as drugs and cells (Hart, Terenghi, & Wiberg, 2011). A wide range of biomaterials have been assessed experimentally (Chiono, Tonda-Turo, & Ciardelli, 2009), poly-glycolic acid (PGA) being the one with the most success. PGA tubes were shown to support successfully nerve regeneration both experimentally (Dellon & Mackinnon, 1988) and clinically (Mackinnon & Dellon, 1990). Collagen tubes have also been widely tested for bridging nerve defects. For instance, Karup and colleagues have shown that collagen scaffolds are able to successfully bridge a 5-cm nerve gap in nonhuman primates (Krarup, Archibald, & Madison, 2002). Moreover, Rodríguez, Verdú, Ceballos, and Navarro (2000) found

that the combination of Schwann cells, matrigel, and polylactate caprolactone (PLC) appears to be a promising alternative to traditional therapies. Despite some encouraging results, researchers working in this area have still to respond to several critical questions: (i) what the ideal combination of polymer/cell/drug to successfully repair the PNS axons is; (ii) whether there is the need for mechanical tension to promote axon proliferation and if this can be provided by a TE construct; and (iii) which experimental model will best reflect the clinical behavior.

In summary, basic studies dealing with the preclinical validation of TERM strategies have been growing considerably in the last few years; however, the clinical translation of TE products has been very scarce and mainly limited to repair/regeneration of bone, cartilage, OCD, and meniscus lesions.

3. NEW TRENDS IN SCAFFOLDING TECHNOLOGIES

Scaffolds are considered an essential element in the common TE strategy, as substitutes for ECM. Several biomaterials, in the form of 3D scaffolds (natural-based and synthetic), have been investigated including polymers, ceramics, and composites. It is widely accepted that in order to be used for TE-based purposes a scaffold must: (1) be biocompatible; (2) possess a controlled degradation rate; (3) have mechanical properties similar to those of the tissue to be replaced; (4) be porous, with an open network of pores easily processed into 3D shapes and adaptable to the clinical problem under treatment; and (5) allow the growth and differentiation of cells from the tissue to be replaced (Salgado et al., 2004a). Moreover, they can also be used as a vehicle for the delivery of drugs/growth factors, while being simultaneously used as a matrix for cells and tissue ingrowth.

Several methods have been used to produce such interconnected porous structures, such as fiber extrusion and bonding, 3D printing, emulsion freeze-drying, porogen leaching, particle aggregation, electrospinning, and supercritical fluids technology, among others. These methods enabling the fabrication of biodegradable porous scaffolds may be classified as solvent-based or melt-based (Liu Tsang & Bhatia, 2004). Both methods have strengths and limitations in developing devices for biomedical applications, in particular for porous scaffolds. Solvent-based methods require very careful processes and verifications to ensure that no harmful residual solvents are retained in the scaffolds after processing. Conversely, melt-based processing, although not involving the use of solvents, requires the use of mild

temperatures to ensure that no thermal degradation is involved during the processing of sensitive biomaterials.

The fibrous nature of the natural ECM has led many researchers to focus on the development of fiber-based scaffolds. Consequently, fiber-basedscaffolds were among the first templates proposed for TE (Freed et al., 1993; Mikos et al., 1993). The fibers can be produced by wet (Pashkuleva, Azevedo, Lopez-Perez, & Reis, 2008) or dry–wet (Lazzeri, Cascone, Quiriconi, Morabito, & Giusti, 2005) spinning from polymeric solutions or by melt spinning (Pavlov, Mano, Neves, & Reis, 2004). The fiber mesh scaffolds can be further obtained by a great variety of processing methods involving knitting (Chen, Qi, et al., 2008), physical bonding (by the combined application of pressure and temperature) (Gomes et al., 2008), and electrospinning (Martins, Araujo, Reis, & Neves, 2007).

3.1. Fiber bonding

The production of microfibrous scaffolds can be achieved by the fiber bonding technique. Basically, a predefined quantity of microfibers produced by the extrusion process is randomly arrayed in a customized Teflon mold and heated at a predefined temperature and time period, under compression. This method helps to retain the spatial random arrangement of the fibers so that the fiber structure does not collapse (Mikos & Temenoff, 2000). By controlling the compression of the bundles and the amount and size of fibers, it is possible to obtain scaffolds with different morphologies regarding porosity and pore size (Chung et al., 2008). The most important advantage of this processing method is the production of scaffolds with high surface area and high porous interconnectivity for cell attachment and rapid diffusion of cells and nutrients. Several studies reported in the literature have shown that scaffolds produced from fibers have adequate porous interconnectivity and mechanical properties for various TE applications (Gomes, Holtorf, Reis, & Mikos, 2006; Gomes, Sikavitsas, Behravesh, Reis, & Mikos, 2003).

Oliveira et al. (2007) tested fiber mesh starch-polycaprolactone (SPCL) scaffolds and PGA non-woven scaffolds with bovine articular chondrocytes. The results have shown that SPCL scaffolds can support bovine articular chondrocyte adhesion and proliferation, contrary to PGA scaffolds that presented a central area of cell depletion. It has also been demonstrated that the use of starch-based scaffolds, in conjunction with fluid flow bioreactor

culture, enhanced the differentiation of marrow stromal cells, leading to the development of bone-like mineralized tissue (Gomes et al., 2006). Aiming at the development of functional vasculature in bone TE applications, Santos et al. (2007) demonstrated that starch-based fiber mesh scaffolds are an excellent substrate for the growth of human endothelial cells (ECs) required for the vascularization process. Several other studies have shown that chitosan-based fiber mesh scaffolds obtained by fiber bonding processes have an adequate structure for bone (Costa-Pinto et al., 2009; Martins, Pinho, et al., 2010) (Fig. 1.4A) and cartilage (da Silva et al., 2011; Oliveira, Crawford, et al., 2011) applications.

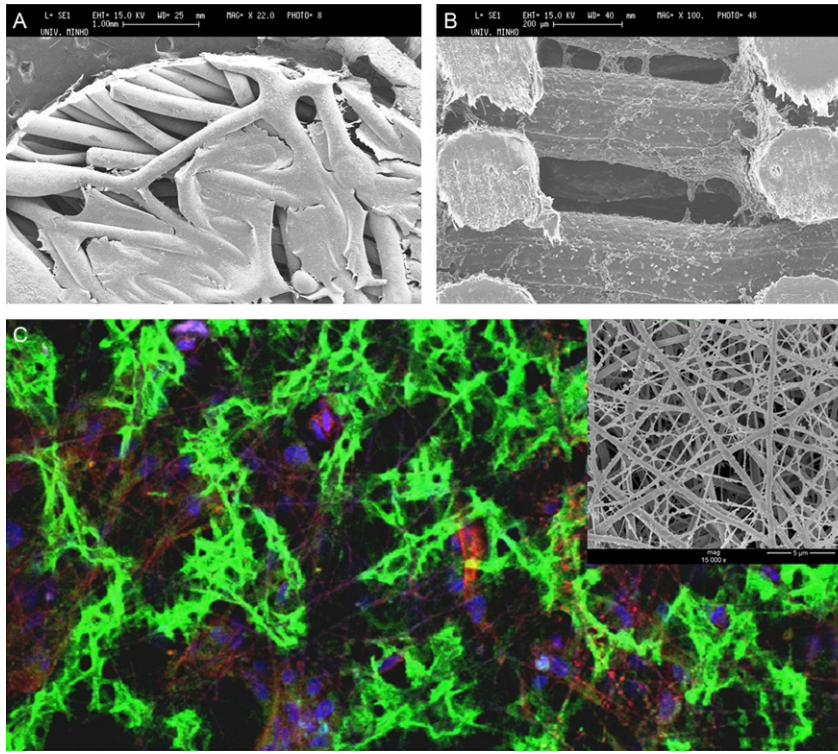

Figure 1.4 Human Wharton's Jelly-derived mesenchymal stem cells cultured on rapid prototyped scaffolds (A); Human bone marrow–derived mesenchymal stem cells cultured on microfiber mesh scaffolds (B); Human articular chondrocytes cultured on electrospun polycaprolactone nanofibers functionalized with chondroitin sulfate (C).

3.2. Wet-spinning

Wet-spinning is a non-solvent-induced phase inversion technique allowing for polymer fiber production through an immersion–precipitation process. In detail, a continuous polymer fiber is produced by precipitation of a polymer solution filament in a coagulation bath composed of a poor solvent (non-solvent) or a non-solvent–solvent mixture with respect to the processed polymer. A homogeneous solution filament, composed of polymer, solvent, and possible additives, solidifies because of polymer desolvation, caused by solvent–non-solvent exchange (Puppi et al., 2011).

A number of studies have reported the development of wet-spun fibers made of natural polymers, such as chitosan (Tuzlakoglu, Alves, Mano, & Reis, 2004) and regenerated silk fibroin (Yao, Masuda, Zhao, & Asakura, 2002). In recent years, some research has proposed wet-spun fibers as carriers for drug release, such as either chitosan (Denkbas, Seyyal, & Piskin, 2000) or PLLA fibers (Gao, Gu, & Ping, 2007) loaded with 5-fluorouracil, and PCL loaded with clodronate (a bisphosphonate that has demonstrated efficacy in the treatment of various bone diseases and as an anti-inflammatory drug) (Puppi et al., 2011). This technique was also proposed to develop scaffolds for TE applications, based on PCL fibers (Williamson & Coombes, 2004), braided PLLA or chitosan fibers (Zhang, Hua, Shen, & Yang, 2007), and starch-based fibers (Tuzlakoglu et al., 2010).

3.3. Rapid prototyping

Conventional processing methods to produce scaffolds have some limitations: reduced control of size and geometry of the pores, with eventual reduced control of the interconnectivity of the porosity and spatial distribution of pores within the scaffold. However, the advent of rapid prototyping or solid free form fabrication enabled obtaining a sufficiently higher degree of control in the scaffold morphology and of the final shape of an implantable device (Yeong, Chua, Leong, & Chandrasekaran, 2004).

Rapid prototyping has emerged as a powerful polymer processing technique for the production of scaffolds in the TE area (Peltola, Melchels, Grijpma, & Kellomaki, 2008; Yeong et al., 2004). The biggest strength of these techniques is that a defined structure can be built with customized shapes linked with computer-aided design (CAD) that provides more flexibility, versatility, and reproducibility in creating scaffolds with precise control (Landers et al., 2002; Peltola et al., 2008). Models can be derived from computed tomography scans, magnetic resonance imaging scans, or model

data created from 3D object digitizing systems. Among the several rapid prototyping systems, based on laser, printing, and extrusion technologies (i.e., selective laser sintering, stereolithography, 3D printing, fused deposition modeling, and 3D plotting), 3D printers and plotters are generally faster, more affordable, and easier to use than the others (Pfister et al., 2004).

Nevertheless, a limitation of the rapid prototyped scaffolds is the cell-seeding efficiency, because pore size is relatively large in comparison to cell dimensions (Fig. 1.4B). This drawback of rapid prototyped scaffolds could be overcome by the integration of electrospun nanofibers (Martins et al., 2009) or by the creation of pore size gradients (Sobral, Caridade, Sousa, Mano, & Reis, 2011). Recently, SPCL SEMI-tubular structures (Fig. 1.3A) produced by 3D Bioplotting have also been used to ensure mechanical stability of TE strategies for recovery from spinal cord injuries (Silva et al., 2010, 2011).

3.4. Electrospinning

Electrospinning has emerged as a straightforward, efficient, inexpensive, and versatile polymer process technology enabling the production of ultrafine fibers. Electrospun fibers present remarkable properties, namely a high specific surface area due to the sub-micrometer range of the fibers' diameter (i.e., frequently below 1 μm). Typically, the electrospun nanofibers are randomly organized in a mesh-like structure characterized by a high interconnectivity and porosity, with pore sizes in the micrometer range (Martins, Reis, & Neves, 2008).

Conventional electrospinning consists of drawing a polymer solution droplet, produced by a syringe pump, from a capillary. The solution undergoes extensional flow and deposits into a collector by the application of an external electrostatic field. Basically, when a pendant droplet of the polymer solution in the tip of the capillary is subjected to an electric field strong enough to overcome its surface tension, a tiny jet is ejected in the direction of the collector. Before reaching the collector, the solvent evaporates partially leading to the deposition of long and thin fibers, sometimes at the nanoscale. In the electrospinning process, the solvent performs two crucial roles: solvating the polymer molecules ready to form the electrified jet, and carrying the solvated polymer molecules toward the collector and then leaving the polymer fibers by rapid vaporization of the solvent molecules. Therefore, the appropriate selection of a solvent system is a prerequisite for successful electrospinning.

The most typical morphology corresponds to a randomly aligned porous non-woven mesh. Indeed, this nanofibrous structure physically resembles the collagen fiber network existing in the natural ECM of most organs of the human body. Therefore, electrospun biodegradable nanofiber mesh (NFM) provides an appropriate environment for cell attachment, proliferation, and, when using progenitor/stem cells, their differentiation (Martins et al., 2007). Moreover, the previously referred properties render electrospun nanofiber meshes useful for many applications, particularly those related to the field of biomedical engineering such as bone and cartilage tissue scaffolding (Alves da Silva et al., 2010; Araujo et al., 2008; da Silva et al., 2009; Martins et al., 2011) (Fig. 1.4C), and drug delivery systems (Martins, Duarte, et al., 2010; Puppi et al., 2011).

3.4.1 Compression molding combined with particulate leaching

Methods based on the leaching of soluble particulates are amongst the most commonly used in the fabrication of 3D porous scaffolds. Compression molding combined with particulate leaching is one of those methods and consists on mixing a polymer or a polymeric blend with leachable particles. This mixture is further loaded into a mold and heated above the glass transition temperature of the polymer and pressure is applied, for a certain period of time, aiming to maximize packaging. After cooling, the molded polymer–porogen composite is immersed in a solvent for the selective dissolution of the porogen. The porosity and pore size of the scaffold is controlled, respectively, by the amount and particle size of the porogen used (Correlo et al., 2009).

This methodology was applied by Gomes, Godinho, Tchalamov, Cunha, and Reis (2002) to develop starch-based scaffolds exhibiting pore sizes between 10 and 500 μm and porosity of about 50%. In a different study, Correlo et al. (2009) produced scaffolds from chitosan/polyester blends and composites using for the first time melt-based compression molding followed by salt leaching. Studies conducted with these scaffolds and bovine articular chondrocytes demonstrated that pore size and geometry of the pore had an effect on cell proliferation and ECM production (da Silva et al., 2010). Other *in vitro* studies confirmed that the developed chitosan/polyester-based scaffolds support adhesion, viability/proliferation, and osteogenic or chondrogenic differentiation of a mouse mesenchymal stem cell line (BMC-9) (Costa-Pinto et al., 2008; Oliveira et al., 2008). More recent studies have shown that chitosan-based scaffolds prepared by compression molding combined with particulate leaching, besides supporting *in vitro*

proliferation and osteogenic differentiation of hBMSCs, induced bone formation *in vivo* (Costa-Pinto et al., 2012). Therefore, these results indicate that these scaffolds can be used for cell-based approaches in the bone TE field.

3.4.2 Supercritical-assisted phase inversion

For the last nearly two decades, there has been considerable progress in supercritical fluid processing, leading to the discovery of new processes that offer various possibilities to prepare materials in different forms or formulations. Supercritical fluid technology offers a clean and environmentally friendly, single-step operation, which provides an attractive platform to meet the demands of the industry. Supercritical-fluid phase inversion offers an attractive and alternative process to obtain solvent-free structures. This method involves casting of a polymer solution onto an inert support followed by immersion of the support with the cast film into a bath filled with a non-solvent for the polymer. The contact between the solvent and the non-solvent causes the solution to be phase-separated. Supercritical carbon dioxide can be used as a non-solvent and this presents several advantages, namely because carbon dioxide can dry the structure rapidly without causing its collapse; cell morphology and shape can thus be tuned by changing the operating conditions (Duarte, Caridade, Mano, & Reis, 2009; Duarte, Mano, & Reis, 2009d).

Several studies report different polymers processed using this technique, both synthetic (e.g., P_LLA, PMMA, Nylon 6, PS, cellulose acetate, polysulfone, poly(ethylene-*co*-vinyl acetate), poly(vinylidene fluoride), polycarbonate, polycarbonate/PEG, and poly-vinyl-alcohol) and natural polymers (i.e., starch and chitosan) (Duarte, Mano, & Reis, 2009b, 2009c, 2010). The possibility to prepare drug delivery systems in a single step operation has also been reported in the literature (Duarte, Mano, & Reis, 2009a).

4. STEM CELLS: LOOKING BEYOND DIFFERENTIATION

One of the key elements in most of TERM approaches is the use of a cell population that will induce new tissue formation through the interaction of the resident cells of the tissue to be regenerated. The nature as well as the differentiation stage of these cells may vary according to the TERM strategies to be used and the total number of cells needed for transplantation, as well as the available tissue for biopsy and posterior cell isolation. The initial

rationale for the use of cells in TERM approaches was based on the premise that they would replace the cells lost during the injury/degenerative process, and at the same time contribute to the formation of new tissue. An example of this was the initial use of fully differentiated osteoblasts for the regeneration of bone and cartilage tissue (Salgado, Oliveira, Pedro, & Reis, 2006). Another typical example of the use of fully differentiated cells in repair/regeneration strategies is the use of Schwann cells for peripheral nerve applications (Hall, 2005; Hu et al., 2013; Kingham et al., 2007). The great potential of these cells has been associated with their capacity to form Bünger bands and to release neurotrophic factors, leading in this way to the regeneration of the damaged nerves (Hall, 2005). Nevertheless, the use of cells from fully differentiated tissue, although logical and often functional, has always faced a problem, which in most cases is the limited amount of tissue available for cell isolation.

In this sense, stem cells of either pluripotent or multipotent origin have been proposed for the regeneration of tissues. Among the different stem cell populations, one stands out in particular—the mesenchymal stem cells (MSCs) (Chen, Shao, Xiang, Dong, & Zhang, 2008). According to the definition introduced by the International Society for Cell Therapy (ISCT), there are some minimal criteria for the identification of MSC populations, such as the adherence to plastic in standard culture conditions; positive expression of specific markers such as CD73, CD90, and CD105, and negative expression of hematopoietic markers such as CD34, CD45, HLA-DR, CD14 or CD11B, and CD79α or CD19; and *in vitro* differentiation into, at least, osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). Most of the time, cells with this phenotype can be isolated from the bone marrow (BM-MSCs), adipose tissue (adipose tissue stem cells—ASCs), or the Wharton Jelly of the umbilical cord (WJ-MSCs/HUCPVCs) (Salgado et al., 2006; Silva, Gimble, Sousa, Reis, & Salgado, 2013; Teixeira, Carvalho, Sousa, & Salgado, 2013). The great potential of MSCs has been associated with their widespread availability throughout the human body, along with the fact that, when isolated, they display great proliferative potential with minimal senescence through multiple passages (Uccelli, Benvenuto, Laroni, & Giunti, 2011).

In terms of action, the multi-differentiation of MSCs is still considered a possible explanation to some authors. For instance, Tohill, Mantovani, Wiberg, and Terenghi (2004) showed that bone marrow mesenchymal stem cells (BM-MSCs) are able to express glial markers (GFAP, S100) *in vitro* and that after transplantation, they were also able to maintain the expression of

these markers, enriching the surrounding environment and enhancing the nerve regeneration. In a recent study, Ladak, Olson, Tredget, and Gordon (2011) demonstrated that BM-MSCs are also able to differentiate into Schwann-like cells, and able to support the peripheral nerve regeneration. These authors also verified that Schwann-like cells derived from MSCs were able to exert similar neurotrophic effects like normal SC, leading to the neurite/axon outgrowth (Ladak et al., 2011). Other authors have also shown that stem cells with a mesenchymal-like phenotype isolated from the adipose tissue, known as adipose tissue stem cells, yielded promising results in the regeneration of damaged nerves (Kingham et al., 2007; Liu, Yang, & Shen, 2012; Liu, Cheng, Guo, et al., 2011). Kingham and colleagues demonstrated that using a specific protocol of differentiation through the use of a mixture of glial growth factors such as GGF-2, bFGF, platelet-derived growth factor (PDGF), and forskolin, ASCs were also able to acquire a phenotype similar to SC that expressed glial cell markers such as Stro-1, S100, p75, and Nestin (Kingham et al., 2007).

In recent years, there has been a paradigm shift in the rationale for the use of MSCs in tissue repair and regeneration (Baglio, Pegtel, & Baldini, 2012; Lai et al., 2010), as these cells are able to survive for different periods of time upon transplantation (Fig. 1.3B). In fact, the regenerative and immunomodulatory properties of MSC secretome have already been demonstrated in different tissues and disease contexts of PNS or CNS, both *in vitro* and *in vivo* (Ankrum & Karp, 2010; Azari et al., 2010; Bonfield, Nolan Koloze, Lennon, & Caplan, 2010; Caplan & Dennis, 2006; Joyce et al., 2010; Meyerrose et al., 2010). Concerning the effects of MSC secretome in some disorders of the CNS (e.g., Parkinson's disease), studies have already shown that after transplantation of MSCs, they are able to induce improvements in the motor function of the animals (Blandini et al., 2010; Chao, He, & Tay, 2009). With BM-MSCs, Cova and colleagues demonstrated that after transplantation the cells were able to interact with the surroundings of the lesion site of Parkinson's Disease (PD), while maintaining their phenotype even under non-physiological conditions (Cova et al., 2010). At the same time, they also showed that BM-MSCs were able to secrete a broad panel of trophic factors such as EGF, Vascular endothelial growth factor (VEGF), NT-3, FGF-2, and Brain-derived neurotrophic factor (BDNF), proving that BM-MSCs did not need to differentiate into a neuronal phenotype to exert a neuroprotective action in the dopaminergic populations (Cova et al., 2010). Also with ASCs and WJ-MSCs, studies have shown that similarly to BM-MSCs, these two populations were also

able to exert neuroprotection in PD through the secretion of trophic factors, leading to the recovery of TH-positive cells and behavioral amelioration (McCoy et al., 2008; Weiss et al., 2006). Additional evidence of the therapeutic relevance MSC secretome derives from animal models of, and patients with, SCI (Arboleda et al., 2011; Karamouzian, Nematollahi-Mahani, Nakhaee, & Eskandary, 2012; Shang et al., 2011). Indeed, it was demonstrated that besides the fact that MSCs are modulators of the immunoresponse, they are able to modify the SCI milieu as a result of the secretion of a different panel of trophic factors such as BDNF, Nerve growth factor (NGF), and VEGF, promoting axonal regeneration, glial scar reduction, and neurite outgrowth (Neuhuber, Timothy Himes, Shumsky, Gallo, & Fischer, 2005; Park et al., 2010). For instance, with BM-MSCs, Lu, Jones, and Tuszynski (2005) demonstrated that these cells were able to secrete NGF, NT-3, and high levels of BDNF, which was correlated with the extent of host axonal growth. With ASCs and WJ-MSCs, Lopatina et al. (2011) and Zhang et al. (2009) demonstrated that both populations were able to potentiate the levels of regeneration through the secretion of trophic factors, thus providing neuroprotection and trophic support for preventing cell death and axonal degeneration.

Regarding peripheral nerve repair/regeneration, Liu, Cheng, Guo, et al. (2011) showed that after implantation of ASCs into a rat model, there was an improvement in the walking performance measured by a footprinting test, as well as an increased conservation of muscle-mass and nerve conduction velocity associated with the increased number of myelinated and re-myelinated fibers after ASC graft. In line with this, another study showed that associating the transplantation of ASCs with acellular nerve allografts also improves the regeneration of peripheral nerves (Liu, Cheng, Guo, et al., 2011). In addition to this, they found that ASCs were able to increase the expression of BDNF, NGF, NT-3, and GDNF, relating the secretion of neurotrophic factors as a stimulus to the repair of the nerve damage (Liu, Cheng, Feng, et al., 2011). As in the case of ASCs and BM-MSCs, the use of umbilical cord mesenchymal stem cells (UC-MSCs) has also been suggested as another source of MSCs with promising results in the regeneration of damaged peripheral nerves (Lee et al., 2011; Peng et al., 2011; Wang et al., 2012). Using an approach similar to that of Kingham and colleagues, Peng et al. (2011) demonstrated that Wharton jelly MSCs (WJ-MSCs) were also able to acquire the phenotype of an SC cell, exhibiting a high expression of glial markers (GFAP, p75, S100, and MBP). At the same time, they also verified that WJ-MSCs

were able to secrete bioactive neurotrophins, and they associated this phenomenon to robust *in vitro* neurite outgrowth (Peng et al., 2011). Similar outcomes were also shown by Lee et al. (2011), demonstrating that UC-MSCs are, in fact, able to reestablish the conduction velocity of the nerves; however, in addition to this fact, the same authors found that a combinatory approach (UC-MSCs + SC) achieved better outcomes than with the UC-MSCs alone.

Today, the use of induced pluripotent stem cells (iPS cells) constitutes a new potential tool to be used as therapy in the regeneration of damaged nerves (Wang et al., 2011). The purpose of iPS cells is based on their unlimited potential of expansion and differentiation (Lee et al., 2007). Wang and colleagues (Wang et al., 2011) demonstrated that it is possible to differentiate iPS cells into neural crest stem cells (NCSCs) and, after transplantation of these NCSCs, they were able to accelerate axonal myelination and differentiate into SC cells without any teratoma formation (Wang et al., 2011). Although the application of iPS cells has yielded promising results so far and shown a tremendous potential for the regenerative medicine applications in different kinds of disorders such as nerve injuries, more studies are needed to precisely define the best protocol of differentiation and the best method for their application (Miura et al., 2009).

5. CONCLUSIONS

Recent advances in different areas such as materials engineering, scaffold processing, bioreactor technology, gene therapy, and stem cell basic and applied biology have allowed TERM to be placed on the forefront of new therapies for tissue/organ repair and regeneration. In this sense, the upcoming years will be decisive for the affirmation of TERM and for recognition from both the general public and the medical community. For this purpose, it is of utmost importance that the multidisciplinary profile of this exciting field is reinforced in the near future.

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CHAPTER TWO

Tissue Engineering and Peripheral Nerve Reconstruction: An Overview

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Contents

Abstract

Nerve repair is no more regarded as merely a matter of microsurgical reconstruction. To define this evolving reconstructive/regenerative approach, the term tissue engineering is being increasingly used since it reflects the search for interdisciplinary and integrated treatment strategies. However, the drawback of this new approach is its intrinsic complexity, which is the result of the variety of scientific disciplines involved. This chapter presents a synthetic overview of the state of the art in peripheral nerve tissue engineering with a look forward at the most promising innovations emerging from basic science investigation. This review is intended to set the stage for the collection of papers in the thematic issue of the *International Review of Neurobiology* that is focused on the various interdisciplinary approaches in peripheral nerve tissue engineering.

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The higher regeneration potential of the peripheral nervous system is at the basis of the usually higher degree of recovery after peripheral nerve trauma provided that the continuity of the nerve is maintained or, if lost, adequately reconstructed (Geuna, Fornaro, Raimondo, & Giacobini-Robecchi, 2010; Geuna et al., 2009; Raimondo et al., 2011). However, complete recovery is only occasionally achieved after a nerve lesion and, in many cases, the clinical outcome is rather unsatisfactory (Battiston, Raimondo, et al., 2009; Siemionow & Brzezicki, 2009). Today, there is a growing consensus that further improvements in peripheral nerve repair and regeneration are no more a matter of developing new microsurgical tools and techniques, but rather one of a multitranslational regenerative medicine approach aimed at reaching a new level of innovation that brings together different scientific disciplines. The aim of this chapter is not to carry out an extensive review of the enormous number of papers published on nerve repair and regeneration, but rather to provide an overview of the state of the art in peripheral nerve tissue engineering with a look forward at the most promising innovations emerging from the recent advancements originating from basic and clinical research in the main scientific disciplines involved: reconstructive microsurgery, transplantation, biomaterial science, physical therapy, and pharmacotherapy.

1. RECONSTRUCTIVE MICROSURGERY

Reconstructive microsurgery is the key discipline among the various ones that have enriched the world of peripheral nerve tissue engineering over the recent years. In fact, the surgeon is the key ring of the chain that brings scientific and technological innovation to the patient's bed. Yet, the surgeon should participate in the design of the basic science experiments in order to optimize the whole process of research and development (Battiston, Papalia, Tos, & Geuna, 2009).

Although surgical nerve reconstruction has been attempted since the ancient times (Battiston, Papalia, et al., 2009), its main improvements have been made over the last few decades (Siemionow & Brzezicki, 2009). Techniques for microsurgical nerve reconstruction include direct suture (end-to-end neurorrhaphy), neurolysis, nerve autografts, and nerve transfers (Siemionow & Brzezicki, 2009). Particularly noteworthy is the latter surgical approach, which has seen widespread application over the very recent years (Teboul, Kakkar, Ameur, Beaulieu, & Oberlin, 2004; Tung &

Mackinnon, 2010; Zhang & Gu, 2011) and has widened the surgical options in the repair of very severe nerve traumas, including brachial plexus lesions.

Microsurgical techniques for nerve repair have improved very much, making it possible to foresee that further improvement in peripheral nerve tissue engineering would not depend mainly on a further implementation of the single surgical techniques; nonetheless, improvement might still be achieved from technological innovation and the development of new reconstructive procedures.

For instance, the use of glue instead of nerve suturing is very promising since experimental studies in animal models have indicated that its performance might be equal, or even superior, to epi/peri-neurial microsuturing (Sameem, Wood, & Bain, 2011; Whitlock et al., 2010).

Another area of potential technological advancement is represented by robot-assisted surgical reconstruction (Liverneaux, Nectoux, & Taleb, 2009; Nectoux, Taleb, & Liverneaux, 2009; Zorn et al., 2008) although the use of robots in peripheral nerve reconstruction is still low in comparison to other surgical fields. Results from experimental studies on robotic nerve reconstruction are very encouraging (Latif et al., 2008) and it can be foreseen that robot-assisted technologies will be favored more and more by peripheral nerve surgeons over the next years.

Finally, development of innovative microsurgical acts and techniques can also be foreseen and the history of the last decades teaches us that progress can be derived from revisiting and/or modifying an old surgical technique, rather than by a complete innovation. The history of end-to-side neurorrhaphy is an example since this surgical technique had already been described in the eighteenth century (Papalia et al., 2007) and was rediscovered by Viterbo, Trindade, Hoshino, and Mazzoni Neto (1994); today, it represents an interesting innovation in peripheral nerve repair (Geuna, Papalia, & Tos, 2006; Papalia et al., 2003).

2. TRANSPLANTATION

Among the different pillars of tissue engineering, transplantation is definitely the approach that is drawing the most interest in regenerative medicine. While at the beginning transplantation strategies were based on whole organ transplantation, today they are evolving to more sophisticated approaches based on the employment of only parts of an organ (tissue transplantation), or even single-cellular (cell transplantation) or sub-cellular constituents (gene transfer).

2.1. Organ/tissue transplantation

Organ/tissue transplantation for peripheral nerve gap repair is represented by autografts, that is, the transplantation of an autologous nerve segment harvested from the sacrifice of another "less precious nerve." Nerve autografts were introduced by Millesi (1981) and Millesi, Meissl, and Berger (1972), on the basis of the evidence that suturing the nerve stumps under tension hinders nerve regeneration and represents the "gold standard" for nerve gap bridging (Siemionow & Brzezicki, 2009). However, the harvesting of a healthy nerve represents a clear limitation of this technique, and therefore alternative nerve conduits have been sought over the last decades.

Veins are the most commonly used biological alternative to nerve autografts, in clinical practice as well (Terzis & Karypidis, 2009). This type of tissue autotransplantation had been introduced as early as 1909 by Wrede (1909), who reported functional recovery after reparation of the median nerve by means of a 45-mm-long vein tube. The interest in this surgical technique revived with the clinical studies by Chiu and Strauch (1990) and Walton, Brown, Matory, Borah, and Dolph (1989) who showed that sensory nerve repair by vein autografts may lead to satisfactory return of sensibility comparable to the nerve grafting technique and, since then, vein conduits have seen a discrete spread among nerve surgeons (Chiu, 1999).

Another alternative to nerve autografts that has received attention among surgeons is the use of skeletal muscle guides (Fawcett & Keynes, 1986; Keynes, Hopkins, & Huang, 1984; Kong, Zhong, Bo, & Zhu, 1986). This technique, which was first reported in 1940 (Kraus and Reisner, 1940), finds its rationale in the similarities between the muscle basal lamina and the endoneurial tubes of degenerating nerves that guide Schwann cell (SC) migration and axonal regrowth (Fawcett & Keynes, 1986). Various experimental studies showed that both fresh and denatured muscle conduits have the potential for bridging peripheral nerve defects (Meek & Coert, 2002; Mligiliche, Tabata, Endoh, & Ide, 2001), and clinical studies showed that muscle grafts are effective in obtaining some degree of functional recovery in most patients (Fawcett & Keynes, 1986; Norris, Glasby, Gattuso, & Bowden, 1988; Pereira, Bowden, Gattuso, & Norris, 1991; Pereira, Bowden, Narayanakumar, & Gschmeissner, 1996; Pereira, Palande, et al., 1991; Rath, 2002).

Since the effectiveness of both vein and muscle grafts is limited to short nerve gap repair, because long vein segments tend to collapse while regenerated axons tend to grow outside long muscle grafts without reaching

the distal nerve stump (Battiston, Tos, Cushway, & Geuna, 2000; Battiston, Tos, Geuna, Giacobini-Robecchi, & Guglielmone, 2000), the possibility of combining the two approaches, that is, filling up vein tubes with muscle fibers, has been explored (Brunelli & Brunelli, 1993). This muscle-vein-combined technique for nerve reconstruction has been extensively investigated in experimental models (Fornaro, Tos, Geuna, Giacobini-Robecchi, & Battiston, 2001; Geuna, Tos, Battiston, & Giacobini-Robecchi, 2004; Raimondo et al., 2005; Tos et al., 2007) over the last two decades and papers reporting its successful clinical employment in both sensory and mixed nerves (also in the case of gaps longer than 30 mm) have already been published (Battiston, Geuna, Ferrero, & Tos, 2005; Battiston, Tos, Cushway, et al., 2000; Battiston, Tos, Geuna, et al., 2000; Marcoccio & Vigasio, 2010; Tos, Battiston, Ciclamini, Geuna, & Artiaco, 2012). It can thus be expected that its use with patients will increase over the next years.

Finally, the use of acellularized nerve allografts is receiving much attention because of the ability of these conduits to bridge large nerve defects (Glaus, Johnson, & Mackinnon, 2011; Rivlin, Sheikh, Isaac, & Beredjiklian, 2010; Stefanescu, Jecan, Badoiu, Enescu, & Lascar, 2012). Very recently (Brooks et al., 2012), the results of a large clinical trial were published showing an excellent functional outcome similar to that of traditional autografts and although the high costs of commercially available processed nerve allografts is a concern, this approach to nerve gap reconstruction holds promise as a successful alternative to traditional nerve autografts.

2.2. Cell transplantation

While enrichment of nerve guides with different cell types has been explored, the most reasonable approach seems to be the use of glial cells because of their key role in axonal regeneration (Geuna et al., 2009).

During the regeneration process, glial cells support axonal regrowth not only mechanically, by forming the Büngner bands that guide axons to the distal innervation targets, but also by secreting a number of growth factors and, together with macrophages, removing necrotic tissue and myelin debris (Geuna et al., 2009; Hall, 2001). For these reasons, their absence inside an artificial conduit is likely to be a limiting factor that can be overcome by enriching the conduit with these types of cells or their precursors.

It has been shown that SC transplantation inside different types of nerve scaffolds leads to the improvement of both quality and rate of axon regeneration (Goto, Mukozawa, Mori, & Hara, 2010; Hadlock, Sundback,

Hunter, Cheney, & Vacanti, 2000; Mosahebi, Woodward, Wiberg, Martin, & Terenghi, 2001). Significantly, this tissue engineering approach has also proved to be effective in bridging long nerve gaps where the use of the vein conduit alone is known to be ineffective (Strauch et al., 2001; Zhang et al., 2002).

As an alternative to SCs, several studies have explored the possibility to enrich nerve guides with olfactory ensheathing cells (OECs). Results showed that these glial cells provide trophic/tropic support to regenerating axons (Dombrowski, Sasaki, Lankford, Kocsis, & Radtke, 2006; Guntinas-Lichius et al., 2001; Radtke et al., 2005; Verdu et al., 1999). It has also been shown that OECs can integrate into the host repaired nerve and contribute to the myelination of the regenerated axons (Dombrowski et al., 2006; Radtke & Vogt, 2009).

In spite of the promising experimental results, the employment of autologous glial raises some concerns in the perspective of clinical application, especially in case of acute nerve injuries, because of the time required for expanding autologous glial cells in culture and the risk of fibroblast contamination (Moreno-Flores et al., 2006; Mosahebi et al., 2001). Therefore, the use of neuro-glial precursors, which have the potential to differentiate into both neurons and glia (Bithell & Williams, 2005), has been proposed as an alternative to primary glial cell autotransplantation. However, experimental studies carried out so far have led to conflicting results: while some studies have shown that artificial nerve guides enriched with neuro-glial stem cells promote axonal regeneration (Heine, Conant, Griffin, & Hoke, 2004; Murakami et al., 2003), other studies have reported no effects, not even a negative one (Amado et al., 2010, 2008).

Another option for cell transplantation in peripheral nerves is the use of mesenchymal stem cells (MSCs) as they can be easily obtained, purified, and expanded in culture, offering a potentially unlimited source of cells for tissue engineering (Caplan & Dennis, 2006; Geuna, 2001; Tohill & Terenghi, 2004). Another advantage of MSCs is that they can be obtained from various adult stem cell niches, such as bone marrow, adipose tissue, tooth pulp, and umbilical cord blood (Alhadlaq & Mao, 2004). MSCs are thought to be able to differentiate into multiple cell lineages including neuron-like and glial-like cells (Alhadlaq & Mao, 2004; Kingham et al., 2007; Mantovani et al., 2010; Raimondo, Penna, Pagliaro, & Geuna, 2006) and it has been shown that human MSCs can be differentiated into neural cells *in vitro* and transplanted in the injured facial nerve of the guinea pig for improving nerve regeneration (Cho et al., 2010).

2.3. Gene transfer

Gene transfer represents one of the pillars of tissue engineering in various biomedical fields including peripheral nerve regeneration (Haastert & Grothe, 2007; Hoyng, Tannemaat, De Winter, Verhaagen, & Malessy, 2011; Mason, Tannemaat, Malessy, & Verhaagen, 2011; Zacchigna & Giacca, 2009). Gene therapy has been used to promote nerve regeneration through the local supplying of neurotrophic factors since their systemic administration might lead to side effects that are almost avoided by local delivery. Yet the development of nontoxic, nonimmunogenic viral vectors driving long-term transgene expression makes their use much safer today (Zacchigna & Giacca, 2009).

In particular, viral vectors based on the adeno-associated virus (AAV), a nonpathogenic and widespread parvovirus, are attracting much interest because they are incapable of autonomous replication and are able to transduce both dividing and non-dividing cells, showing a specific tropism for post-mitotic cells including neurons. Because these vectors do not contain any viral genes—which are transiently transfected in trans for the packaging process—they elicit virtually no inflammatory or immune response. As a consequence, transgene expression from these vectors persists for several months in a variety of animal tissues *in vivo* (Monahan & Samulski, 2000). The high effectiveness of skeletal muscle infection by AAVs makes it possible to use them for transferring genes for nerve regeneration either through the infection of the muscles surrounding nerve lesion site, or even by fashioning muscle-vein-combined scaffolds previously potentiated by AAV gene transfer (Fornaro et al., 2001; Geuna et al., 2003; Zacchigna & Giacca, 2009).

3. BIOMATERIAL SCIENCE

Definitely, the search for new peripheral nerve substitutes is one of the issues that has received the most attention in the context of peripheral nerve repair and regeneration research. The considerable progress in material science in recent years (Williams, 2009) has stimulated the design and experimental testing of a considerable number of new nerve guides and it is far beyond the aim of this chapter to review that enormous body of literature in detail (Cunha, Panseri, & Antonini, 2011; Daly, Yao, Zeugolis, Windebank, & Pandit, 2012; de Ruiter, Malessy, Yaszemski, Windebank, & Spinner, 2009; Deumens et al., 2010; Jiang, Lim, Mao, &

Chew, 2010; Nectow, Marra, & Kaplan, 2012; Pfister et al., 2011; Siemionow, Bozkurt, & Zor, 2010; Steed, Mukhatyar, Valmikinathan, & Bellamkonda, 2011).

Biomaterials for tissue engineering can be classified using various approaches (Pfister et al., 2011; Williams, 2009) and, regarding nerve repair applications, a simple three-category classification can be adopted according to the three generations of biomaterials that have been developed in this area (Geuna, Tos, & Battiston, 2012).

The *first generation* is represented by nonabsorbable materials. The first attempts, which led to rather poor results, were based on the employment of polyethylene, polyvinyl, and rubber tantalum metal cuffs (Campbell, 1970; Ducker & Hayes, 1968; Fields, Le Beau, Longo, & Ellisman, 1989). Other nonabsorbable nerve guides that have been used, also in clinical practice, albeit with contrasting results, are polytetrafluoroethylene (Stanec & Stanec, 1998) and Gore-Tex (Pitta, Wolford, Mehra, & Hopkin, 2001). In more recent years, the use of silicon conduits led to the first clinically positive results (Dahlin, Anagnostaki, & Lundborg, 2001), especially for repairing short nerve gaps (<5 mm), leading to the concept that intentionally leaving a short gap between the two nerve stumps can enhance nerve regeneration by allowing the accumulation of cells and extracellular matrix, which can stimulate correct axonal regrowth (Dahlin & Lundborg, 2001). However, the main concern regarding the clinical employment of nonabsorbable synthetic material in humans is the occurrence of complications caused by local fibrosis, triggered by the implanted material (Dahlin et al., 2001; Merle, Dellon, Campbell, & Chang, 1989).

Therefore, the *second generation* of nerve guides has been focused on bioabsorbable tubes that have been tested both experimentally and in clinical practice (Dellon & Mackinnon, 1988; Luis et al., 2007; Mackinnon & Dellon, 1990a, 1990b; Meek et al., 1999; Navarro et al., 1996; Nicoli Aldini et al., 1996; Robinson et al., 1991; Tountas et al., 1993; Valero-Cabre et al., 2001; Yannas & Hill, 2004; Young, Wiberg, & Terenghi, 2002). Nerve conduits made of polyglycolic acid were shown to be effective for restoring nerve defects (Mackinnon & Dellon, 1990a) and approved by the FDA for use in humans. In a multicentric randomized prospective study on digital nerve reconstruction with this type of nerve guides (Weber, Breidenbach, Brown, Jabaley, & Mass, 2000), it was shown that it provides superior results both for short gaps (<4 mm), in comparison to end-to-end repair, and for longer defects (up to 30 mm), compared to nerve autografts.

Finally, the *third generation* of nerve guides has been developed, within the absorbable material category, a represented by biomimetic biomaterials, that is, components of the extracellular matrix. Among the most promising biomimetic biomaterials for nerve regeneration, collagen proved to lead to functional recovery similar to nerve autografts in the rat and the primate (Archibald, Shefner, Krarup, & Madison, 1995; Li, Archibald, Krarup, & Madison, 1992). More recently, particular attention has been directed toward chitosan, a derivative of chitin, which has shown notable effectiveness in promoting nerve regeneration in experimental animal models (Amado et al., 2008; Lauto et al., 2008; Matsumoto, Kaneko, Oda, & Watanabe, 2010; Simoes et al., 2010; Yamaguchi, Itoh, Suzuki, Osaka, & Tanaka, 2003).

Since living tissues are complex structures, in tissue engineering not only is the type of material important but also its 3D structure and thus the design of the artificial tissue/organ (Cui, Boland, D'Lima, & Lotz, 2012; Yang, Leong, Du, & Chua, 2001). Yet it appears that future progress in nerve tissue engineering will develop from a combination of different approaches rather than the optimization of a single approach (Battiston, Raimondo, et al., 2009). For these reasons, it is expected that their implementation in nerve prosthesis will not emerge only from the introduction of new materials or improvement of the existing ones, but rather from the combined use of other complementary tissue engineering tools.

So far, various peripheral nerve prostheses have been translated to the clinical employment. In all cases, artificial nerves are represented by hollow tubes. The first nerve guide that has been introduced to the clinical employment is made of polyglycolic acid (Neurotube®). Other materials that have been used so far include poly-DL-lactide caprolactone (Neurolac®), polyvinyl alcohol hydrogel, in the form of tube (SaluTunnel™) and wrap (Salubridge™), resorbable porcine small intestinal submucosa (AxoGuard™), and resorbable collagen (Neuragen®, NeuroMatrix™, NeuroFlex™, RevolNerv®) (de Ruiter et al., 2009; Kehoe, Zhang, & Boyd, 2012; Meek & Coert, 2008).

Although the application of artificial hollow tubes for nerve reconstruction has proven to lead to successful functional recovery in several clinical trials (Lundborg, Rosen, Dahlin, Danielsen, & Holmberg, 1997; Rinker & Liau, 2011; Weber et al., 2000), it appears that surgeons are still waiting for a new generation of nerve guides that may guarantee similar (or even better) results in comparison to traditional nerve autografts.

4. PHYSICAL THERAPY

The usefulness of physical therapy for functional rehabilitation to prevent muscle atrophy and drive cortical remodeling after nerve injury and repair is widely acknowledged (Lundborg, 2003). Much less consensus exists about the possibility to use physical therapy to directly improve the effectiveness of nerve tissue engineering. Actually, clinical application of physical therapy approaches for improving nerve regeneration is anecdotal and much of the research is still at the experimental/preclinical level.

Electrical stimulation has been widely experimentally investigated as a therapeutic strategy in addition to microsurgery to improve functional recovery (Gordon, Brushart, & Chan, 2008; Haastert-Talini, 2014; Wang et al., 2009). Electrical stimulation has been shown to speed up axonal growth, increase the number of regrowing axons through the graft (Gordon et al., 2008), and enhance SC proliferation (Huang et al., 2010) and neurotrophic factor levels (Wang et al., 2009). Different electrical stimulation techniques have been successfully used to stimulate denervated muscles or proximal nerve stumps such as transcutaneous electrical stimulation (Gigo-Benato et al., 2010), percutaneous stimulation (Chen et al., 2001), direct low-frequency electrical stimulation (Gordon, Sulaiman, & Ladak, 2009), and electrical stimulation via synthetic nerve guidance channels (Ghasemi-Mobarakeh et al., 2011). Also, in clinical trials, electrical stimulation resulted in an improvement in functional recovery (Gordon et al., 2009; Goto et al., 2010).

Another physical therapy approach that is receiving increasing attention is phototherapy. The first experimental data showing that light can exert a positive effect on axonal regrowth and nerve regeneration are old, and it is only over the last few years that an increasing number of papers have begun providing a body of evidence in support of the effectiveness of phototherapy in improving peripheral nerve regeneration (Anders, Geuna, & Rochkind, 2004; Gigo-Benato, Geuna, & Rochkind, 2005; Rochkind, Geuna, & Shainberg, 2009). The possibility of combining phototherapy with other nerve tissue engineering strategies is very promising (Hsieh et al., 2012; Jin, Prabhakaran, Liao, & Ramakrishna, 2011) and, although clinical studies are still limited (Chow, Johnson, Lopes-Martins, & Bjordal, 2009), it appears that the time has come for larger clinical trials.

Another promising approach for improving the outcome of nerve tissue engineering is physical exercise. Various studies have shown that active

exercise improves nerve regeneration and enhances functional recovery (Armada da Silva, Pereira, Amado, & Veloso, 2014; Asensio-Pinilla, Udina, Jaramillo, & Navarro, 2009; English, Cucoranu, Mulligan, & Sabatier, 2009; Malysz et al., 2010; Marqueste, Alliez, Alluin, Jammes, & Decherchi, 2004; Sabatier, Redmon, Schwartz, & English, 2008; van Meeteren, Brakkee, Helders, & Gispen, 1998). The motorized walking or running treadmill test, a technique used to exercise rodents following injury, demonstrates that active exercise enhances axonal elongation (Sabatier et al., 2008), increases the number of regeneration axons (English et al., 2009), and improves the functional outcome (Ilha et al., 2008; van Meeteren, Brakkee, Hamers, Helders, & Gispen, 1997; van Meeteren et al., 1998). Passive exercise, commonly used in rehabilitation, has been reported to stimulate nerve regeneration and functional recovery (Ilha et al., 2008; Udina, Puigdemasa, & Navarro, 2011).

Other interesting approaches include stimulation by magnetic fields (Wang & Zhao, 2010), shock waves (Hausner & Nógrádi, 2014), manual stimulation (Bischoff et al., 2009), and neural interfaces (del Valle & Navarro, 2014; Herrera-Rincon, Torets, Sanchez-Jimenez, Avendano, & Panetsos, 2012).

5. PHARMACOTHERAPY

In spite of the great progresses of pharmacology in many other fields of medicine and surgery, there is still not any established drug treatment protocol for specifically improving nerve regeneration after trauma and reconstruction. Although, of course, these patients may be given various medicaments along with the postoperative, with the aim of treating concurrent conditions (e.g., antibiotics for infections) and sometimes as alimentary integrators (such as acetil-carnitine), no dedicated drug is usually administered after nerve surgery with the goal of improving the degree of nerve regeneration and maturation.

While no specific nerve regeneration–promoting drug has still entered the clinics, on the experimental side, many studies have suggested that various pharmacological approaches may have a positive effect on this complex healing process.

It is far beyond the goal of this chapter to revisit all the drugs that can have a potential effect on nerve regeneration. Just to mention some of the most promising molecules, particular interest is being given to immunosuppressants (Yan, Sun, Hunter, Mackinnon, & Johnson, 2012) and various hormones, such as melatonin (Odaci & Kaplan, 2009) and erythropoietin

(Yin, Zhang, Bo, & Gao, 2010). Evidence has also been provided recently that corticosteroids exert a positive effect on nerve regeneration (Mohammadi, Amini, & Eskafian, 2013).

Other drugs that have shown positive effects on peripheral nerve regeneration are etifoxine, a ligand of the translocator protein (18 kDa), which modulates inflammatory responses (Girard et al., 2008); flunarizine, a calcium channel antagonist and vasodilatator (Patro, Chattopadhyay, & Patro, 1999); cilostan, an antiplatelet and vasodilatation agent (Yamamoto, Yasuda, Kimura, & Komiya, 1998); and GM1 gangliosides (Lopez et al., 2010; Silva-Neto, Vasconcelos, Silva-Junior, & Beder-Ribeiro, 2009).

It appears thus that the time is also ripe for clinical trials with candidate nerve regeneration–promoting drugs.

6. CONCLUDING REMARKS: COMBINING THE DIFFERENT TISSUE ENGINEERING APPROACHES, THE MAIN CHALLENGE FOR IMPROVING NERVE REPAIR OUTCOME

An emerging consensus among basic and clinical scientists is that in order to optimize the strategy for tissue engineering of the peripheral nerve, a new level of innovation is needed that brings together in a multi-translational approach the different pillars of tissue engineering. Figure 2.1 illustrates this concept.

Reconstructive microsurgery is definitely the key element in this web, not only because it represents the link between innovative research and the patient but also because surgeons must interact with all scientists from other cultural backgrounds. In particular, interaction with biologists and biotechnologists is very important especially when transplantation approaches are concerned since transplantation is progressively evolving from whole organ transplantation to more sophisticated forms of tissue engineering based on the employment of only parts (tissue transplantation), or even single-cellular (cell transplantation), or sub-cellular constituents (gene transfer), of an organ.

Moreover, surgeons must interact with engineers and material scientists in the light of the recent enormous advances in nanotechnology that makes it possible to develop and design very complex synthetic scaffolds to repair neural defects. Finally, surgeons must interact with pharmacologists and physical therapists in order to define combined therapeutic strategies that are more and more effective in improving the outcome of nerve tissue engineering.

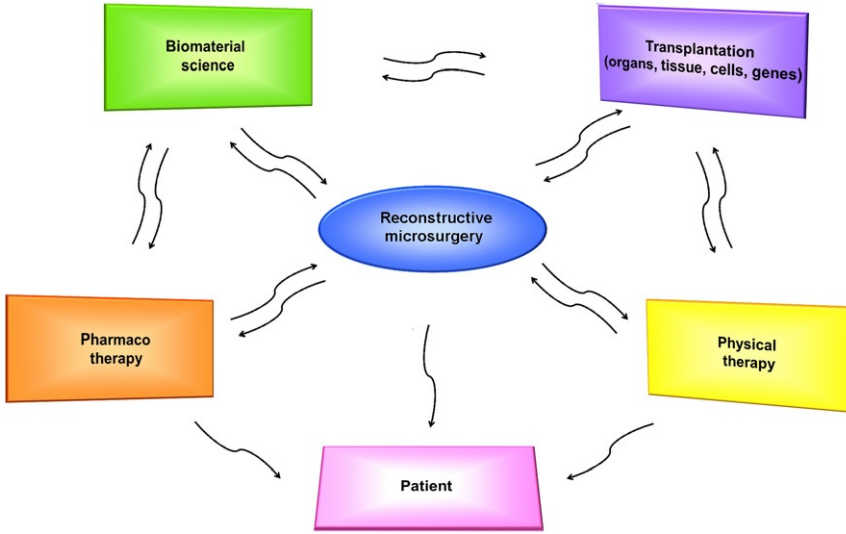

Figure 2.1 Schematic drawing depicting the various disciplines involved in tissue engineering of the peripheral nerve.

In conclusion, it clearly appears that future progress in regenerative medicine will not develop from the improvement of a single strategy, but rather from the optimized combination of many different approaches. The multilevel and interdisciplinary approach thus appears to be the main challenge for peripheral nerve tissue engineering since different competences and expertise need to be brought together. Though challenging, this approach represents an exciting opportunity for researchers to explore new scientific fields with the hope that it will allow us to make significant clinical advances in the forthcoming years.

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CHAPTER THREE

Bone Marrow Mesenchymal Stem Cell Transplantation for Improving Nerve Regeneration

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Contents

Abstract

Although the peripheral nervous system has an inherent capacity for regeneration, injuries to nerves still result in considerable disabilities. The persistence of these disabilities along with the underlying problem of nerve reconstruction has motivated neuroscientists worldwide to seek additional therapeutic strategies. In recent years, cell-based therapy has emerged as a promising therapeutic tool. Schwann cells (SCs) are the main supportive cells for peripheral nerve regeneration; however, there are several technical limitations regarding its application for cell-based therapy. In this context, bone marrow mesenchymal stem cells (BM-MSCs) have been used as alternatives to SCs for treating peripheral neuropathies, showing great promise. Several studies have been trying to shed light on the mechanisms behind the nerve regeneration–promotion potential of BM-MSCs. Although not completely clarified, understanding how BM-MSCs exert tissue repair effects will facilitate their development as therapeutic agents before they become a clinically viable tool for encouraging peripheral nerve regeneration.

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1. INTRODUCTION

Although significant advances have been achieved in the peripheral nerve repair field, nerve trauma is still one of the most challenging microsurgery reconstructive problems, leading to disabilities and pain and representing a devastating impact on patients' quality of life. The peripheral nervous system is provided with an intrinsic growth capacity and a favorable microenvironment that guides and facilitates axonal outgrowth after a minor lesion. However, after a complete lesion, functional recovery is poor and seldom achieved. In recent years, nerve trauma has attracted great interest from clinicians, microsurgeons, and neuroscientists worldwide.

Great efforts have been devoted to the development and improvement of therapies that could potentially lead to nerve tissue regeneration and satisfactory retrieval of function. Among the therapeutic strategies developed so far, there is direct nerve repair, implemented when there is no or minor tissue loss; nerve grafting, which is the gold standard microsurgical technique applied in small to moderate-sized gaps, but this often leads to donor site morbidity for autologous transplants or graft rejection for heterologous transplants; and the use of conduits to bridge large nerve gaps, which can also serve as vehicles for delivery of growth-promoting molecules or cells, but which still represents a challenge to the peripheral nerve repair field. Electrical stimulation is another investigated modality seeking the optimal goal of nerve regeneration; however, the appropriate biophysical parameters are not well defined and its effectiveness falls short of achieving adequate reinnervation and complete functional recovery.

More recently, gene and cell therapy have emerged as promising therapeutic approaches for promoting nerve reconstruction. In particular, cell-based therapy as a source of graft replacement and/or growth-promoting molecules delivery has been greatly studied, showing significant nerve regeneration outcomes and, therefore, translational potential.

This review discusses the problems associated with nerve trauma with the overall aspect of achieving neuroplasticity. In addition, cell therapy for improving nerve regeneration, focusing on the promising potential of bone marrow mesenchymal stem cells (BM-MSCs) and mechanisms of how they might exert beneficial effects and aid in the process of nerve tissue repair. It also addresses the implementation of this cell-based therapy in basic, preclinical, and clinical trials to date.

2. CELLULAR AND MOLECULAR BASES OF NERVE DEGENERATION AND REGENERATION

After damage to the peripheral nervous system, complex and orchestrated cellular and molecular events take place in an attempt to support nerve regeneration. These events that were first described by August Waller in 1850 are well known as Wallerian degeneration, which comprise the degeneration of the distal nerve stump while the proximal nerve stump is capable of elongating and regenerating (Geuna et al., 2009; Stoll, Jander, & Myers, 2002). The onset of this process is marked by calcium influx, and consequent activation of proteases, such as calpains, which trigger the disintegration of axon cytoskeleton. Schwann cells (SCs) become active cells in charge of axonal and myelin debris clearance, and macrophages are recruited to the injury site, cooperating and playing a major role in this phagocytic process, which is pivotal for allowing nerve tissue regeneration. SCs dedifferentiate, reverting to a proliferative progenitor-like phenotype, and align within the basal lamina tubes, called bands of Bügner, providing a guidance substrate for growing axons. Once the SCs attach to axons, the remyelination process takes place to form the compact myelin sheath. Upon injury, the delicate balance between growth-promoting and inhibitory factors, serving as molecular cues and acting to activate signaling pathways, plays a key role in the fate of the regeneration process.

Despite the permissive growth environment of the peripheral nervous system and its intrinsic growth capacity, functional recovery after nerve surgical reconstruction is often suboptimal. As already well described, following a nerve injury, the peripheral nerve will try to repair itself by outgrowing and/or by making collateral sprouts in an attempt to reinnervate the muscle or skin. However, the rate of nerve growth is critical for the functional outcome, because the regeneration potential tends to decrease in the course of time. In addition, mismatching of sensory and motor fibers and the nerve trapping in inappropriate tissues along the way can potentially contribute to neuroma formation and atrophy of target organs, thus impairing full functional recovery. Several other factors can influence the success of the regeneration process, such as age, the nerve trunk affected, the surgical repair approach employed, the interval between the trauma and nerve repair, and above all, the type and extent of the injury (Navarro, Vivó, & Valero-Cabré, 2007).

Despite several therapeutic approaches developed so far seeking for the optimal nerve reconstruction and functional recovery (Oliveira, Almeida, & Martinez, 2012), in the following paragraphs we will consider cell-based therapy focusing on the promising BM-MSCs, which have been proven to possess great translational clinical potential.

3. CELL-BASED THERAPY FOR IMPROVING NERVE REGENERATION

Following a peripheral nerve injury, the SCs play the main supportive role in promoting tissue regeneration, either by secreting growth-promoting molecules or by axonal remyelination. Evidence demonstrates that transplantation of SCs is able to support axonal outgrowth, thus promoting regeneration, both *in vitro* (Schlosshauer, Müller, Schröder, Planck, & Müller, 2003) and *in vivo* (Keilhoff, Goihl, Langnäse, Fansa, & Wolf, 2006). This evidence in addition to the inherent properties of SCs, would make these cells the ideal source as cell therapy in the peripheral nerve regenerative medicine if there were not technical limitations hampering their clinical implementation. SCs have restricted mitotic activity, expanding poorly under culture conditions, which make them an insufficient source for treatments. In addition, donor site morbidity is an important issue concerning this cell system, as another nerve has to be sacrificed for harvesting material for transplantation. All these technical limitations have motivated efforts to seek for alternatives to SCs for cell-based therapy and stem cells have been considered a potential candidate in their place.

4. BM-MSC-BASED THERAPY

Stem cells are undifferentiated cells found in all multicellular organisms having two unique and indispensable features: the ability of self-renewal, going through numerous rounds of mitosis while maintaining an undifferentiated state; and potency, the capability of differentiating into a diverse range of specialized cell types. The main types of stem cells are embryonic, fetal, and adult stem cells.

The primary role of adult stem cells in humans is to repair or replace damaged tissues in which they are found. Although considered to have limited potency, giving rise only to cells related to their tissue of origin, there is a growing body of evidence demonstrating their versatility, indicating that they are able to differentiate into a diverse range of cell types, beyond their

tissue of origin. Unlike the controversial embryonic stem cells, the use of adult stem cells should reduce ethical concern as they do not require human embryo destruction for their procurement. Other advantages of their application in the tissue regenerative medicine field are the possibility of autotransplantation, avoiding the risk of tissue rejection, and also the remote possibility of teratoma formation that embryonic stem cells treatments face (Bjorklund et al., 2002).

Umbilical cord, hematopoietic, and mesenchymal cells are the most well-studied adult stem cell source in stem cell therapy-based research. Similar to hematopoietic stem cells, there is a bone marrow-derived nonhematopoietic stem cell subpopulation, called mesenchymal stem cells. These cells have the ability to differentiate into mesodermal lineages, such as myocyte, chondrocyte, osteocyte, adipocyte, tenocyte, and stromal fibroblast (Muraglia, Cacedda, & Quarto, 2000). In addition to their natural differentiation pathway, there is evidence of their capability to transdifferentiate into endodermal and ectodermal lineages such as hepatocyte, and neuron and glia, respectively (Bossolasco et al., 2005; Dezawa, Takahashi, Esaki, Takano, & Sawada, 2001; Kim, Seo, Bubien, & Oh, 2002; Krampera et al., 2006; Woodbury, Schwarz, Prockop, & Black, 2000) (Fig. 3.1).

Within the scope of cell therapy, the BM-MSCs represent a feasible and promising system for improving nerve regeneration, as has been demonstrated by several studies (Dezawa et al., 2001; Oliveira et al., 2010; Pereira Lopes et al., 2006). In addition to being endowed with great plasticity, owing to their capability of turning into nonmesenchymal lineages, BM-MSCs can meet many of the needs and requirements of a cell system delivery for nervous tissue transplantation and repair. Besides their easy accessibility, the BM-MSCs have the ability to rapidly and extensively expand *in vitro* under culture conditions. Thus a small amount of aspirate can yield sufficient cells for transplantation. Moreover, the BM-MSCs are immune privileged cells, which make them so far the best choice for allogeneic transplantation, avoiding tissue-rejection effects and the use of immune suppressive drugs. There are several other advantages, such as their capability of releasing paracrine factors, acting as a cellular protein factory; the ability of homing to injury sites, as well as integrating and surviving within the host tissue; suitability for stable transfection by exogenous genes; the lack of important ethical issues impeding their use compared to embryonic stem cells; and their safety and efficacy (Keilhoff & Fansa, 2011).

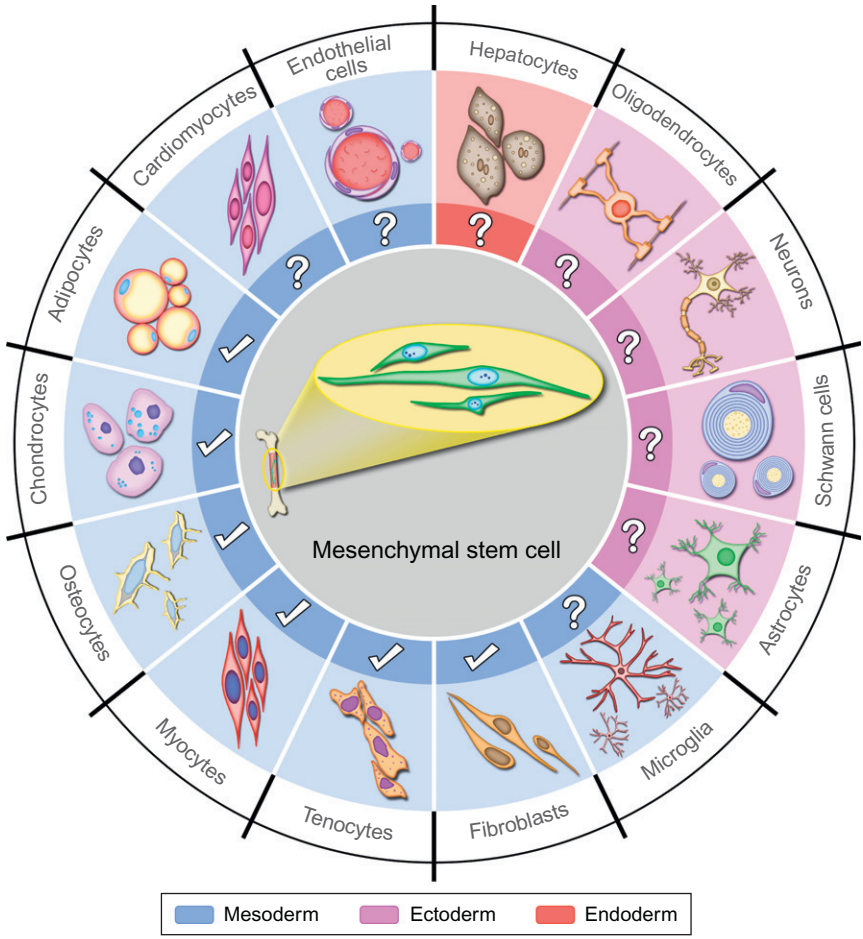

Figure 3.1 The transdifferentiation potential of BM-MSCs. An illustration of plasticity of BM-MSCs and their capacity to transdifferentiate into several lineages in addition to the mesengenic lineage.

The BM-MSCs possess broad features, which make them multifunctional cells. Thus, several studies have been trying to shed light on the possible mechanisms that give these cells a nerve regeneration–promotion potential (Wang, Ding, Gu, Liu, & Gu, 2009). Such mechanisms may occur by paracrine, neuro/axonoprotective, or immunomodulatory means; transdifferentiation into SCs; genetic manipulation; cell-to-cell contact; resident cellular fusion; mitochondrial DNA or mitochondrial transfer; or even a combination of the above (Fig. 3.2).

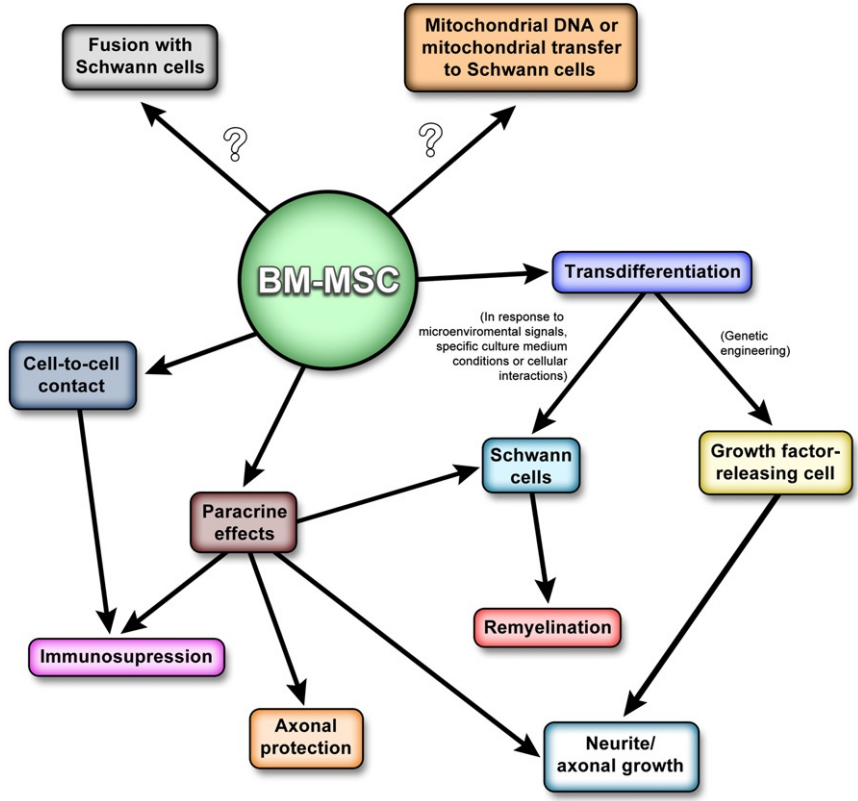

Figure 3.2 Mechanisms of BM-MSC action. A schematic diagram showing the possible mechanisms behind the nerve regeneration–promotion potential of BM-MSCs.

5. PUTATIVE MECHANISMS BEHIND THE NERVE REGENERATION–PROMOTION POTENTIAL OF BM-MSCs

Following a peripheral nerve lesion, bioactive factors are credited to play a critical role in the nerve regeneration process. In particular, a wide range of growth factors and cytokines can be secreted by cells present in the nerve injury site in response to stress and inflammation signals (Gu et al., 2010; Kassis et al., 2008). The growth factors that promote neuronal survival are called neurotrophic factors, and they are of clinical significance since they are also capable of supporting axonal outgrowth, remyelination,

and endogenous cell proliferation and differentiation. As previously mentioned, there is a growing body of evidence that BM-MSCs exert beneficial effects on nerve tissue regeneration (Ribeiro-Resende et al., 2009; Oliveira et al., 2010) and these effects are strongly correlated with the production of neurotrophic substances.

Recently, Gu et al. (2010) demonstrated that dorsal root ganglion (DRG) explants and neurons cocultured with BM-MSCs showed enhanced neurite outgrowth and neuronal cell survival via production of an array of soluble factors. The proteomic analysis revealed a wide list of secretory proteins, including four proteins belonging to the family of neurotrophic factors: basic fibroblast growth factor (bFGF), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and brain-derived neurotrophic factor (BDNF). Interestingly, the influence of BM-MSCs on the behavior of DRG explants/neurons was likely induced by the release of soluble factors into the culture, since no direct contact between DRG explants/neurons and BM-MSCs was present in the coculture system. In another similar *in vitro* assay, DRG explants treated with BM-MSC-conditioned medium presented increased neurite outgrowth and when anti-NGF neutralizing antibody was added the effect was blocked, suggesting that the NGF trophic activity was mediated by the BM-MSCs (Ribeiro-Resende et al., 2009). The *in vivo* assay also achieved this effect when BM-MSCs were implanted at the injury site immediately after a rat sciatic nerve lesion. The results were encouraging, since the BM-MSC-based therapy also improved the regeneration of motor and sensory axons. However, the implanted cells presented low potential to differentiate into SCs. These data reinforce that the substantial beneficial effects exerted by the implanted cells is mainly dependent on their trophic activity rather than their plasticity or stemness potential (Ribeiro-Resende et al., 2009). In agreement with these findings, our group also demonstrated that, following a transection lesion, the mouse sciatic nerve repair by means of a collagen conduit filled with BM-MSCs is capable of enhancing axon regeneration and remyelination, which might have contributed to the return of motor function observed. In addition, high levels of NGF-β were detectable in the regenerated nerve tissue, suggesting that the BM-MSCs are also able to express this potent neurotrophic factor *in vivo*. Interestingly, BDNF expression could not be observed, at least at 6 week post injury (Pereira Lopes et al., 2006). Our group obtained similar results when we investigated BM-MSC-based therapy combined with a polycaprolactone conduit approach on the mouse median nerve transection model. We observed a significant increase in the number of both myelinated

and unmyelinated nerve fibers. Moreover, muscle atrophy was prevented in the BM-MSC-treated animals and functional analysis revealed an improved performance. Thus, it is possible that the positive effects of nerve regeneration were mainly due to the paracrine potential of the BM-MSCs rather than to the transdifferentiation potential, since few implanted cells expressed the S-100 SC marker (Oliveira et al., 2010). In addition to the expression of NGF, it has been demonstrated that the BM-MSCs are also capable of expressing several other important neurotrophic factors *in vivo*, such as glial-derived neurotrophic factor (GDNF), CNTF, FGF, and even BDNF (Chen et al., 2007; Yang et al., 2011). An experiment of DRG explants from mice cocultured with human BM-MSCs demonstrated that the human cells expressed β-NGF and BDNF in different donor populations. In addition, the human BM-MSCs were capable of promoting neurite outgrowth in these primary neurons under culture conditions and this effect was attributed not only to the NGF and BDNF, but also to other neuro-regulatory factors (Crigler, Robey, Asawachaicharn, Gaupp, & Phinney, 2006). It is likely that the lack of standardized BM-MSC-based therapy experiments regarding the animal model, length of nerve gap, time points of analysis, and the vehicles of cell delivery, account for the differences in the BDNF expression pattern of BM-MSCs observed in the literature.

Another relevant issue concerning cellular behaviors related to the paracrine effects is that the BM-MSCs can be direct neurotrophic mediators, as discussed above, as well as indirect mediators, by inducing the SCs to produce them. An investigation involving a coculture of rat SCs and BM-MSCs demonstrated cell–cell interaction despite no direct contact between them. The BM-MSCs not only favored the survival and proliferation of the SCs but also modulated cellular behaviors, revealed by the high expression of mRNA and protein levels of NGF, BDNF, and high- and low-affinity NGF receptors for the SCs in the BM-MSC-conditioned media (Wang et al., 2009). The proliferative effects of the BM-MSCs on the glia cell line are not restricted to the SCs, but also to DRG satellite cells following a rat sciatic nerve injury and tubulization, demonstrating that these effects extended far beyond the site of cell implantation (Ribeiro-Resende et al., 2009). These results provide evidence that BM-MSC-based therapy improves peripheral nerve regeneration possibly through relay and magnification of neurotrophic function from stem cells to glia cells in addition to direct secretion of neurotrophic factors by BM-MSCs.

The paracrine effects exerted by the BM-MSCs do not seem to be the unique mechanism that accounts for their nerve regeneration–promotion

potential. Among other positive features, BM-MSCs are endowed with a great plasticity, which make them more versatile and prone to turn into different phenotypes in response to specific culture conditions, genetic manipulations, or microenvironmental signals *in vivo*. Scientists are currently working on methods to effectively transdifferentiate adult stem cells into functional specialized cells. As already pointed out, after a peripheral nerve injury, the SCs play a pivotal role in the process of tissue regeneration. If the BM-MSCs are capable of significantly exerting beneficial effects on nerve regeneration, either by direct and/or indirect paracrine signals, turning into the SC-like phenotype would make them a powerful tool in the regenerative medicine field.

The growth factors and cytokines released in the injured microenvironment are of great importance for determining cell traits since they act as epigenetic regulators of cellular differentiation. Although controversial, these environmental signals, in response to injury or inflammation, could also play a crucial role on the mechanism of transdifferentiation of adult stem cells into other specialized cells types, beyond their germ layer of origin. BM-MSCs injected at the distal stump of the rat sciatic nerve after a transection lesion were capable of surviving and migrating in addition to differentiating into an SC-like phenotype, even in low percentages (Cuevas et al., 2002). In this work, there was no manipulation in the BM-MSC medium for induction toward an SC phenotype, suggesting that the transdifferentiation took place in a "physiological" manner, likely due to the environmental signals in response to the nerve injury. It is possible that the great plasticity of the BM-MSCs, in addition to the need for recovery of the nerve tissue contributed to the system's adaptation. Although not quantified, our group also showed that very few transplanted BM-MSCs into the injury site were capable of transdifferentiating into an SC-like phenotype (Oliveira et al., 2010).

The transdifferentiation of the BM-MSCs into a SC-like phenotype without induction does not seem to be as efficient as the addition of cell type-specific small molecules to the culture medium, which can trigger cell-signaling pathways and consequently induce the cell towards a different status or phenotype. In 2001, Dezawa and collaborators described a cellular culture protocol for the induction of BM-MSCs toward a SC-like phenotype. The induced cells expressed several SC markers and also morphologically resembled SCs. The next step was to test the influence of the transdifferentiated BM-MSCs on axonal regrowth and remyelination by transplanting the cells into the cut end of the rat sciatic nerve. The results suggested that the BM-MSCs were not only able to acquire an SC-like

phenotype and morphology but also to differentiate into myelinating cells, capable of supporting nerve fiber regrowth. Since then, several other neuroscientists have tried to induce BM-MSCs into an SC-like phenotype based on this protocol for investigating the nerve regeneration process. Recently, Ladak, Olson, Tredget, and Gordon (2011) investigated the purity of the induced cells using a modified protocol from Dezawa et al. (2001). They found that ~50% of the induced BM-MSCs acquired an SC phenotype and they attributed the higher SC marker expression compared to previous works to the use of glial growth factor-2 rather than heregulin, as a neuregulin source. Mahay, Terenghi, and Shawcross (2008) performing a DRG coculture, without direct contact, demonstrated that BM-MSCs transdifferentiated into an SC-like phenotype upregulated BDNF and NGF, thus displaying trophic influences like those of SCs. Moreover, they observed neurite outgrowth, even when the BDNF and NGF responses by DRG neurons were inhibited, suggesting that the functional characteristics of BM-MSCs similar to SCs were not restricted to the releasing of NGF and BDNF alone, but also to other bioactive neurotrophins. This leads to the question, in addition to the biological function of efficient growth-factor releasing cells, would BM-MSCs converted into glia have myelinating capacity as well? Indeed, Dezawa et al. (2001) showed that transdifferentiated BM-MSCs transplanted to the cut end of rat sciatic nerve, colocalized with the myelin-associated glycoprotein antibody signal, potentially suggesting that BM-MSCs are able to differentiate into myelinating cells. In agreement with these findings, Keilhoff et al. (2006) also demonstrated the myelinating capacity of BM-MSCs transdifferentiated into an SC-like phenotype, but interestingly, in some cases, the cells were able to wrap more than one axon, a phenomenon characteristic of myelination by oligodendrocytes in the central nervous system.

Intriguingly, a two-step medium based on transdifferentiation protocol for inducing BM-MSCs into neurotrophic secreting cells was accomplished successfully, resulting in the production and release of high amounts of neurotrophic factors, such as BDNF and GDNF. By using the novel protocol, the transdifferentiated cells demonstrated an astrocyte-like morphology and also expressed characteristic astrocyte markers. The transplanted cells were capable of preserving the number of myelinated fibers, as well as improving muscle reinnervation and consequently restoring motor function in the rat sciatic crush injury model (Dadon-Nachum, Sadan, Srugo, Melamed, & Offen, 2011). Independent of the glial cell fate through the transdifferentiation induction protocol, it is important to notice that the

converted BM-MSCs are capable of rescuing neurons in addition to acting as pathfinders for the outgrowing fibers, displaying a myelinating function, which is pivotal to the nerve regeneration process. Based on the protocol described by Dezawa et al. (2001), human BM-MSCs are also capable of acquiring morphological characteristics of SCs *in vitro* and *in vivo*, consequently supporting regenerating axons (Shimizu et al., 2007).

The cell transdifferentiation mechanism is still a subject of controversy, even though several scientists believe that it might be a potential tool for adult stem cell transplants. As mentioned previously, cell induction by means of adding specific growth factors and small molecules in the culture medium can lead to the direct conversion of a somatic cell type into other lineages. In addition, there could be other less complex mechanisms of inducing direct cell conversion into another cell lineage fate, for example, by intercellular interactions.

Coculture of DRG neurons in contact with BM-MSCs leads to transdifferentiation of the stem cells to present morphological and phenotypic characteristics of SCs. Thus, it was suggested that the intercellular interaction between DRG neurons and BM-MSCs might have played a role in the transdifferentiation process, likely through the release of cytokines and molecules by neurons on the axonal surface. However, the converted cells failed to form compact myelin, probably because the transdifferentiation process was incomplete, or a local host environment or specific extracellular environment is required for BM-MSCs to transdifferentiate into compact myelin-forming cells (Yang, Lou, Huang, Shen, & Chen, 2008).

It has been well described in the literature that undifferentiated BM-MSCs have the ability to release growth-promoting molecules *in vitro* and *in vivo*, and that this BM-MSCs feature can be enhanced by their transdifferentiation into SCs. In a novel paradigm, the insertion of specific functional genes into a BM-MSC genomic location could make the transduced cell continuously express the proteins of interest. The combination of both cell and gene-based therapies is a novel tool that has attracted attention owing to the potential of this great complementary strategy. Among several features, BM-MSCs are suitable for stable transfection and expression of exogenous genes, which make them a good candidate for delivery of therapeutic factors by genetic engineering. Recently, BM-MSCs were transduced to express NGF using an adenoviral vector. The genetically modified BM-MSCs were capable of expressing physiologically relevant levels of these growth factors for a period of greater than 1 week (Rooney et al., 2008). Despite great advances in the use of vector

biotechnology systems for genetic engineering, some important biosafety issues, including the risk of insertional mutagenesis and disruption of tumor suppression remain to be clarified and refined.

One of the most interesting features of the BM-MSCs for tissue transplants, besides their great plasticity and capability of acting as growth-factor releasing cells, is the unique ability to modulate the immune system. BM-MSCs can induce immunosuppressive effects and markedly decrease tissue inflammation, acting directly or indirectly on almost all immune cell populations. The BM-MSCs present low expression of major histocompatibility complex (MHC) class I and are negative for MHC class II markers (Uccelli, Moretta, & Pistoia, 2006). By cell-to-cell contact and release of soluble factors, the BM-MSCs exert their complex immunomodulatory effects and also play a role in migratory behavior. The interplay between BM-MSCs and the cells of the immune system is broad and complex and not yet completely clarified. BM-MSCs can suppress T-cell proliferation (Di Nicola et al., 2002) and also reactivity, by inhibiting the development of proinflammatory T helper cell 1 and natural killer cell signaling, and by promoting anti-inflammatory T helper cell 2 and/or suppressive regulatory T-cell signaling (Aggarwal & Pittenger, 2005). BM-MSCs can also indirectly reduce T-cell activation by suppressing the differentiation of monocytes into dendritic cells, which are the most potent antigen-presenting cells (Jiang et al., 2005). BM-MSCs administered intraventricularly or intravenously in the mouse model of chronic progressive experimental autoimmune encephalomyelitis, exhibit substantial immunomodulatory activity, by strongly reducing tissue inflammation and suppressing the proliferation of myelin-sensitized lymphocytes *in vitro* and *in vivo* (Kassis et al., 2008). Following a traumatic spinal cord injury (SCI) in rats, BM-MSCs transplantation favored the development of a population of alternatively activated macrophages (M2 phenotype), which have anti-inflammatory activity and enhanced phagocytic capacity, while preventing the development of a population of classically activated macrophages (M1 phenotype), which possess deleterious effects on injured tissues (Nakajima et al., 2012). Despite no direct evidence demonstrating the immunoregulatory mechanisms exerted by the BM-MSCs following a peripheral nerve injury, there is a body of literature showing that this adult stem cell auto/allotransplantation approach decreases the risk of tissue rejection (Frattini et al., 2012; Oliveira et al., 2010). The underlying mechanisms of BM-MSC immunomodulatory properties are complex and are still under investigation, but the immunosuppressive effects exerted by

these cells have been widely described, potentially contributing to the understanding of the therapeutic merits of a BM-MSC-based approach for safely treating disorders of the nervous system.

Besides the properties already mentioned, the BM-MSCs also have direct or indirect neuroprotective activity. Researchers have paid attention to this feature of these cells and the results are encouraging, supporting that BM-MSCs have the ability to improve neuronal survival following tissue damage (Frattini et al., 2012).

It is true that most peripheral neuropathies result in distal axonal degeneration rather than significant loss of neuronal cell bodies, thus protective therapeutic strategies should be directed at the axon, seeking to prevent axonal degeneration. Hoke and Keswani (2005) showed that SC-derived erythropoietin (EPO) in response to axonal injury acts as an effective endogenous neuroprotectant, preventing distal axonal degeneration. They hypothesized that following axonal injury, nitric oxide (NO) produced within neurons can stimulate the SCs to produce EPO, which in turn mediates an intrinsic axon-protective pathway. It has been demonstrated that the BM-MSCs are able to produce the potent survival cytokine EPO (Mo et al., 2012), as well as NO (Sato et al., 2007). Thus, it is possible that BM-MSCs can also possess direct or even indirect axon-protective properties.

In addition to the models previously discussed as putative mechanisms responsible for the BM-MSC-mediated nerve regeneration, there is the proposed alternative model of spontaneous cellular fusion. It is likely that the great plasticity of the BM-MSCs is not restricted to their ability to transdifferentiate, but also to their putative cellular fusion capacity. It has been demonstrated that the BM-MSCs can fuse spontaneously with other cells, adopting the phenotype of recipient cells. In addition to embryonic stem cells (Terada et al., 2002), it has been demonstrated that the BM-MSCs are able to fuse with neuronal cell types, including Purkinje neurons (Weimann, Charlton, Brazelton, Hackman, & Blau, 2003). However, it is important to notice that the BM-MSC cellular fusion envisioned mechanism took place with a very low frequency and since it was reported, there has not been much progress on its understanding and efficacy. Even more intriguingly, it has been demonstrated that not only can DNA be "transferred" from BM-MSCs to another cell type, but also the mitochondria and the mitochondrial DNA. It was suggested that this BM-MSC active transfer property might rescue aerobic respiration in somatic cells with nonfunctional mitochondria (Spees, Olson, Whitney, & Prockop, 2006). To date, there is no evidence of BM-MSCs fused with SCs or mitochondrial

DNA or mitochondria transfer, but these could possibly represent, even with low frequency, alternative underlying mechanisms by which these cells exert neuroregenerative or axon-protective activities following peripheral nerve injury.

6. BM-MSCs AND CLINICAL TRIALS

A great number of studies have reported the use of BM-MSCs in different types of neurological diseases and trauma (Harrop et al., 2012). SCI affects millions of people around the world causing functional impairment and the spontaneous neurological recovery is very limited. Cell-mediated therapy is one of the therapeutic strategies that has been used on patients with SCI and some studies report the use of cell therapy combined with other strategies, such as physical therapy (Cristante et al., 2009; Yoon et al., 2007) and pharmacological treatment (Kishk et al., 2010). BM-MSCs can improve motor and sensory outcomes after SCI, and the best results come when it is applied in the acute and subacute temporal window after injury (Harrop et al., 2012). Although results from different studies have shown that there is an improvement in neurological responses, the level of functional recovery is insignificant to cause any impact on the individuals' quality of life. Very few side effects related to cell therapy are reported, but possible complications include headache (Mehta et al., 2008) and neuropathic pain (Kishk et al., 2010) after parenchymal injection of BM-MSCs. Also, one of the limitations of this therapy is that the number of cells reaching the injury site is very low. The most commonly used route of cell delivery is intrathecal, intravenous, or direct parenchymal injection, with intrathecal injection being the most frequently used. Complications tend to be related to the application route chosen (Harrop et al., 2012). With respect to peripheral nerve injuries, the most common methods by which cells are delivered in basic and preclinical trials include microinjection direct to the injury site, cell suspension inside conduits (Chen et al., 2007; Oliveira et al., 2010), and seeding within acellular muscle and nerve grafts (Keilhoff et al., 2006). Despite important advances regarding the combined cellular and tubulization approaches, they still need to be optimized by providing transplanted cells with a favorable environment for survival and integration in host tissue, allowing appropriate nerve regeneration and full functional recovery, thus encouraging the implementation of BM-MSC-based clinical multitranslational studies for treating peripheral nerve injuries.

Finally, it is difficult to make conclusions on the use of BM-MSCs in the clinic because clinical trials usually have different variables, for instance, the methods of isolation and expansion, the approaches used to characterize BM-MSCs, the type of injury (partial or complete), time window (acute, subacute, or chronic) and the route of cell delivery (intrathecal, intravenous, or parenchymal), making it hard to compare the studies outcomes. To establish uniformity on these variables, it is necessary to control them in preclinical studies. In an attempt to address the cellular issue (characterization, methods of expansion, and isolation) the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have suggested specific criteria to define human BM-MSCs (Fernández Vallone et al., 2013). Decreasing the number of variables and developing specific protocols to be used in multicenter studies may be an important step toward the discovery of the optimal use of BM-MSCs in clinical trials.

7. CONCLUSION AND FUTURE PERSPECTIVES

Despite continuous advancements in the knowledge and development of techniques for treating peripheral nerve disorders to date, the highly expected outcome of full functional recovery has never been achieved. Among several therapeutic strategies for improving nerve regeneration, cell-based therapies are considered feasible candidates as they represent a source of tissue replacement or growth-promoting proteins delivery to the damaged tissue. In this review, we focused on the promising BM-MSC-based therapy since it owns great potential for translation into the clinical setting of nerve repair. In addition to having several advantages over embryonic stem cells, BM-MSCs have unique features, such as great plasticity, and pleiotropic and multifunctional actions, which make them the subject of study of many neuroscientists worldwide.

The majority of studies credit the neuroregenerative positive effects exerted by BM-MSCs to their great paracrine properties. However, the underlying mechanisms of the BM-MSC nerve regeneration–promotion potential, described so far in the literature are very broad, sometimes controversial, and not completely clarified.

In summary, more efforts should seek for advancement in our understanding of how BM-MSCs exert their tissue repair effects, which will facilitate their development as therapeutic agents and a clinically viable tool for encouraging peripheral nerve regeneration.

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CHAPTER FOUR

Perspectives of Employing Mesenchymal Stem Cells from the Wharton's Jelly of the Umbilical Cord for Peripheral Nerve Repair

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Contents

Abstract

Mesenchymal stem cells (MSCs) from Wharton's jelly present high plasticity and low immunogenicity, turning them into a desirable form of cell therapy for the injured nervous system. Their isolation, expansion, and characterization have been performed from cryopreserved umbilical cord tissue. Great concern has been dedicated to the collection, preservation, and transport protocols of the umbilical cord after the parturition to the laboratory in order to obtain samples with higher number of viable MSCs without microbiological contamination. Different biomaterials like chitosan-silicate hybrid,

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collagen, PLGA90:10, poly(DL-lactide-ε-caprolactone), and poly(vinyl alcohol) loaded with electrical conductive materials, associated to MSCs have also been tested in the rat sciatic nerve in axonotmesis and neurotmesis lesions. The *in vitro* studies of the scaffolds included citocompatibility evaluation of the biomaterials used and cell characterization by imunocytochemistry, karyotype analysis, differentiation capacity into neuroglial-like cells, and flow cytometry. The regeneration process follow-up has been performed by functional analysis and the repaired nerves processed for stereological studies permitted the morphologic regeneration evaluation. The MSCs from Wharton's jelly delivered through tested biomaterials should be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves. In addition, these cells represent a noncontroversial source of primitive mesenchymal progenitor cells, which can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses. The importance of a longitudinal study concerning tissue engineering of the peripheral nerve, which includes a multidisciplinary team able to develop biomaterials associated to cell therapies, to perform preclinical trials concerning animal welfare and the appropriate animal model is here enhanced.

1. INTRODUCTION

Tissue engineering focusing on the *in vitro* fabrication of autologous, living tissues with the potential of regeneration is a promising scientific and clinical field. Peripheral nerve regeneration should include a multidisciplinary team able to develop biomaterials, to develop cell therapies, and to elaborate *in vitro* analysis and preclinical trials concerning animal welfare and the most appropriate animal model before the clinical trials and clinical application approval (Hermann et al., 2004). A full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remains the principal goal of regenerative medicine. The reliability of animal models is crucial for peripheral nerve research. Because of its peripheral nerve size, the rat sciatic nerve has been the most commonly used experimental model in studies concerning the peripheral nerve regeneration and possible therapeutic approaches (Kerns, Braverman, Mathew, Lucchinetti, & Ivankovich, 1991). Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Amado et al., 2008; Sporel-Ozakat, Edwards, Hepgul, Savas, & Gispen, 1991). Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After this injury,

regeneration is usually successful since axons regenerate at a steady rate along the distal nerve supported by the reactive Schwann cells (SCs) and by the preserved endoneural tubules, which enhance axonal elongation and facilitate adequate reinnervation (Nichols et al., 2005). Crush injuries are appropriate to investigate the cellular and molecular mechanisms of peripheral nerve regeneration and to assess the role of different factors in the regeneration process (Mackinnon, Hudson, & Hunter, 1985). Nerve crush injury is also a well-established model in experimental regeneration studies to investigate the impact of various pharmacological treatments (Amado et al., 2008; Chang, Auyang, Scholz, & Nichols, 2009; Pereira et al., 2006). This injury does not imply surgical reconstruction but due to the regeneration period needed, the neurogenic atrophy of the innervated muscles may occur, so, therapeutic approaches to successfully decrease this recovery time are also important. Among various types of peripheral nerve injuries, transection injuries where the nerve trunk is completely interrupted, especially those resulting in large neural gaps, may have a devastating impact on patients' quality of life, and in these cases reconstructive surgery is required as a therapeutic management to achieve nerve regeneration and function restoration (Gu, Ding, Yang, & Liu, 2011). Unlike the adult central nervous system that fails to spontaneously regenerate after injury, the peripheral nervous system (PNS) has an intrinsic regenerative ability to a certain extent. In response to small injuries, peripheral nerves can regenerate on their own over relatively short distances under appropriate conditions. After peripheral nerves are transected, a series of molecular and cellular events, collectively called Wallerian degeneration, are triggered throughout the distal stump of transected nerves and within a small zone distal to the proximal stump, resulting in the disintegration of axoplasmic microtubules and neurofilaments (Sabatier, To, Nicolini, & English, 2011). Within 24 h most axons along the distal stump of transected nerves are reduced to granular and amorphous debris; by 48 h the myelin sheath begins to get transformed toward the short segment (Joao, Amado, Veloso, Armada-da-Silva, & Mauricio, 2010). Then macrophages and monocytes migrate into the degenerating nerve stumps to remove myelin and axon debris, while SCs proliferate to form longitudinal cell columns, known as Bands of Bü̈ngner (Luís, 2008). Under the influences of neurotrophic factors and extracellular matrix (ECM) molecules produced by SCs, the proximal portion of transected nerves sprouts new daughter axons to generate a "regenerating unit" that is surrounded by a common basal lamina (Dahlin, Johansson, Lindwall, & Kanje, 2009; Evans, 2001). New axonal sprouts usually emanate from the nodes of

Ranvier, and undergo remyelination by SCs. Functional reinnervation requires that the regenerating axons elongate under the mediation of growth cones until they reach their synaptic target, and in humans, axon regeneration occurs at a rate of about 2–5 mm/day; thus significant injuries may take many months to heal, so this delay is the cause of neurogenic muscular atrophy conducting to a poor functional recovery (Jiang, Lim, Mao, & Chew, 2010).

Tissue engineering of peripheral nerves associates biomaterials, like chitosan, poly(DL-lactide-ε-caprolactone) (PLC) copolyester, collagen, and other biomaterials, some of them, previously studied by our group (Amado et al., 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Luria, Panasyuk, & Friedenstein, 1971) to cellular systems, able to differentiate into neuroglial-like cells or even by modulating the inflammatory process, which might improve nerve regeneration, in terms of motor and sensory recovery, and also, by shortening the healing period avoiding regional muscular atrophy. Cell transplantation has been proposed as a method of improving peripheral nerve regeneration (Chen et al., 2007). SCs, mesenchymal stem cells (MSCs), embryonic stem cells, and bone marrow stromal cells are the most studied support cell candidates. To implant cultured cells into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected into the neural scaffold, which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). It can also be performed by preadding the cells to the neural scaffold via injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve (Luria et al., 1971).

2. REGENERATIVE MEDICINE AND PERIPHERAL NERVE INJURIES

2.1. Nerve reconstruction

Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery. Direct repair with end-to-end suture, should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a nerve gap, are considered for a nerve graft procedure (Luis et al., 2007). In these cases, the donor nerves used for grafting

are commonly expendable sensory nerves (Matsuyama, Mackay, & Midha, 2000). This technique, however, has some disadvantages, with the most prominent being donor site morbidity, which may lead to a secondary sensory deficit and occasionally neuroma and intensive pain. In addition, the donor and the recipient nerve diameters often do not match, which might be the basis for poor functional recovery (Matsuyama et al., 2000). Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy, and entubulation by means of autologous non-nervous tissues, such as vein and muscles (Walsh & Midha, 2009a, 2009b; Zheng & Cui, 2012) or natural and synthetic biomaterials recently revised by Gu et al. (2011). Just like most of tissue-engineered products, tissue-engineered nerve grafts are typically composed of a physical scaffold with the introduction of support cells and/or growth factors or other biomolecular components, which might improve the functional recovery after axonotmesis and neurotmesis injuries (Cheng et al., 2011; Luria et al., 1971; Shen et al., 2010; Zheng & Cui, 2010). So, entubulation for peripheral nerve repair is used for nerve defects that cannot be bridged without tension (Battiston, Geuna, Ferrero, & Tos, 2005). Nerves will regenerate from the proximal nerve stump toward the distal one, whereas neuroma formation and ingrowth of fibrous tissue into the nerve gap are prevented. Consequently, guidance of regenerating axons is not only achieved by a mechanical effect but also by a chemical effect (such as accumulation of neurotrophic factors) (Maurício et al., 2011). Nerve guides can be made of biological or synthetic materials and, among the latter, both nonabsorbable and biodegradable tubes have been developed and preclinically tested. Some of them are nowadays used in patients and are available in the national and international market (Schmidt & Leach, 2003). The aim of our research group for the past 10 years has been exploring the therapeutic value of human umbilical cord (UC) Wharton's jelly derived from MSCs both *in vitro* and *in vivo*, associated to several tube-guides of natural or absorbable synthetic biomaterials on a rat sciatic nerve axonotmesis experimental model.

2.1.1 Biomaterials

Biomaterials used in biomedical sciences are developing continuously, bringing indisputable benefits to the clinical and research field of knowledge. The use of three-dimensional materials in tissue regeneration is now an increasingly used approach that takes the name of guided tissue regeneration, in which the regeneration of peripheral nerve is a strong candidate. The biomaterials can be biological and synthetic and, among the latter, both

nonabsorbable and biodegradable have been used (Schmidt & Leach, 2003). The concept behind the use of biodegradable biomaterials is that no foreign body material will be left in the host after the device has accomplished its task. For nerve lesion applications, these biomaterials may be used in the form of membranes or tubes, which function not only as a vehicle for the cellular system and/or therapeutic molecules, but also promotes a mechanical protection at the site of the injury. These scaffolds can be mounted in the nerve injury site as tube-guides by the surgeon, facilitating the microsurgical technique in case of neurotmesis injuries (Maurício et al., 2011). An ideal scaffold for peripheral nerve reconstruction has to satisfy many biological and physicochemical requirements, among which biocompatibility, biodegradability, permeability, biochemical properties, and surface properties are the most important concerns during the development of these scaffolds (Gu et al., 2011). On the other hand, the perfect biomaterial used for the construction of these membranes and tube-guides should follow the production requirements in terms of length and wall width concerning the nerve defect, be capable of supporting the suture, and if possible, be transparent. These properties are not mandatory, but can substantially improve the material handling during the surgical procedure and the microsurgery technique (Seckel, 1990). Sufficient mechanical strength must be considered when designing the tube-guides, in order to avoid the collapse during implantation and the healing period. The tube-guide at the same time should also be flexible to allow bending without breaking, so, the balance between flexibility and hardness is an important issue to be considered during its fabrication (Harley, Hastings, Yannas, & Sannino, 2006). Another important factor is the biomaterial resorption process and its degradation rate. The resorption of a biomaterial should be adjusted to the regeneration process, which depends on its molecular weight, composition, crystal structure, and thermal history. When associating a biomaterial to a cellular system it is also important to determine properties such as hydrophobicity, surface charge, and surface rugosity to establish its ability to support adhesion and cell growth (Harley et al., 2006). Among several biomaterials, our research group focused its attention on some biodegradable ones like those made of poly(lactic-co-glycolic) acid (PLGA), made of a novel proportion (90:10) of the two polymers, poly(L-lactide): poly(glycolide) (PLGA90:10), of PLC copolyester (Vivosorb® and Neurolac®), hybrid chitosan, collagen type III (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Maurício et al., 2011; Simoes et al., 2010; Zheng & Cui, 2010) and more recently, the poly(vinyl alcohol)

(PVA) loaded with electrical conductive materials, such as carbon nanotubes (CNTs) and polypyrrole (PPy) to produce a conductive biomaterial with higher electrical conductivity than the polymer matrix (data unpublished, under analysis).

2.1.1.1 Natural biomaterials

The natural biomaterials are characterized by their biocompatibility, biodegradability, low toxicity, and low cost. In peripheral nerve lesions, our research group has been testing mainly two natural biomaterials for the past 5 years—the collagen and the chitosan (Amado et al., 2010, 2008; Maurício et al., 2011; Simoes et al., 2010; Zheng & Cui, 2010). Collagen is an integral component of the ECM of the nerve and several studies have used the collagen in peripheral nerve reconstruction (Gartner et al., 2012). In our studies, the equine collagen type III (GentaFleece®, Baxter, Nuremberg, Germany) was used with positive results, in axonotmesis and in directly sutured neurotmesis lesions (Amado et al., 2010; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). The equine type III collagen membranes wrapped around the nerve lesion (crush injury) were used in order to support an experimental cellular system (N1E-115 cells *in vitro* differentiated for 48 h in the presence of dimethyl sulfoxide (DMSO). These cells were able to secrete neurotrophic factors in the injury site without being in direct contact with regenerating axons (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). There are many studies published where collagen has been used for manufacturing tube-guides for nerve regeneration. Some of them, such as Neuragen®, are commercially available for clinical application in patients (Tyner et al., 2007).

Chitin is a biopolymer of *N*-acetyl-D-glucosamine monomeric units and it has been used in a wide range of biomedical devices. It is the second most abundant polysaccharide found in nature, right after cellulose. Chitosan is a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine and its molecular structure is very similar to laminin, fibronectin, and collagen. Therefore, like collagen, chitosan has favorable biological properties for the nerve regeneration and it is easier to process than chitin. Chitosan is quite fragile in its dry form, so it has to undergo chemical cross-linking or has to be used with other biomaterials before scaffold fabrication. The chitosan (high molecular weight, Aldrich®, USA) tested by our research group was dissolved in 0.25 M acetic acid aqueous solution to a concentration of 2% (w/v). To obtain chitosan-silicate hybrid type III membranes, GPTMS (Aldrich®,

USA) was added to the chitosan solution and stirred at room temperature (RT) for 1 h. The drying process for type III chitosan membrane was as follows: (i) the solutions were frozen for 24 h at −20 °C and then transferred to the freeze-dryer, where they were left for 12 h to become complete dry; (ii) the chitosan type III membranes were soaked in 0.25 N sodium hydroxide aqueous solution to neutralize the remaining acetic acid, washed well with distilled water, and freeze dried (Amado et al., 2008; Simoes et al., 2010). All membranes used for *in vivo* testing were sterilized with ethylene oxide gas, considered by some authors the most suitable method of sterilization for chitosan membranes (Gärtner et al., 2012). Prior to their use *in vivo*, membranes were kept for 1 week at RT to clear any ethylene oxide gas remnants (Amado et al., 2008; Simoes et al., 2010). Results of the *in vivo* study showed that type III chitosan improved both nerve fiber regeneration and functional recovery in axonotmesis and neurotmesis injuries. In fact, the main morphological predictors of nerve fiber regeneration (number of fibers, axon and fiber size, and myelin thickness) were significantly improved in the experimental group of rats where the sciatic nerve was reconstructed with chitosan III in both types of lesions (Amado et al., 2008; Simoes et al., 2010). The same positive results with chitosan III were also obtained with respect to the main predictors of functional recovery, namely withdrawal reflex latency (WRL), extensor postural thrust (EPT), sciatic functional index (SFI) and static evaluation (SSI) tests, and motion analysis of ankle joint (Amado et al., 2008; Simoes et al., 2010). Chitosan III was developed as a hybrid of chitosan by the addition of GPTMS. The addition of GPTMS improves the wettability of chitosan surfaces (Amado et al., 2008; Shirosaki et al., 2005), and therefore chitosan type III is expected to be more hydrophilic than the original chitosan (Amado et al., 2008; Shirosaki et al., 2005). Chitosan type III was developed to be more porous, with a larger surface-to-volume ratio but preserving mechanical strength and the ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported as a consequence of the difference in the ability of the matrix to hold water (Amado et al., 2008). In fact, hybrid chitosan-based membranes may retain about two times as much biological fluid as chitosan (Amado et al., 2008). A synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III and the presence of silicon ions may be responsible for the good results in promoting posttraumatic nerve regeneration (Amado et al., 2008). The significant improvement of axonal regeneration obtained

in crushed sciatic nerves surrounded by chitosan type III membranes suggests that this material may not just work as a simple mechanical scaffold but may work instead as an inducer of nerve regeneration. The neuroregenerative property of chitosan type III might be explained by the action on SC proliferation, axon elongation, and myelination (Shirosaki et al., 2005; Yuan, Zhang, Yang, Wang, & Gu, 2004). We have also tested both *in vitro* and *in vivo*, two types of hybrid chitosan membranes with the addition of an experimental cellular system of N1E-115 cells *in vitro* differentiated (Amado et al., 2008; Simoes et al., 2010). The N1E-115 cells derived from mouse neuroblastomas (Amado et al., 2008; Simoes et al., 2010) can *in vitro* differentiate into neuroglial-like cells (Amano, Richelson, & Nirenberg, 1972; Meek & Coert, 2002) and were used as a cellular model for MSCs. Previous results obtained by our research group using these N1E-115 cells *in vitro* differentiated showed that there was no significant effect in promoting axon regeneration, and they can even exert negative effects on nerve fiber regeneration specially in the case of neurotmesis injuries (Maurício et al., 2011; Simoes et al., 2010). The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by their space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading to a negative outcome on nerve regeneration, even in the presence of hybrid chitosan membranes, with previously proved positive effect in promoting the nerve regeneration (Amado et al., 2008; Maurício et al., 2011; Simoes et al., 2010).

2.1.1.2 Synthetic biomaterials

They are already available in the market tube-guides (Neurolac®) constituted by poly(DL-lactide-ε-caprolactone) for clinical application. They are 16 mm long, with an internal diameter of 2 mm and a 1.5-mm thick wall (purchased from Polyganics BV, Groningen, The Netherlands) (Luis et al., 2007; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). Neurolac® is stiffer than other biomaterials like PLGA, PVA, and hybrid chitosan (type II and type III) (Amado et al., 2008; Simoes et al., 2010) due to the structural reinforcement of the ester bonds. Neurolac® is the only transparent device approved by FDA, which is an important characteristic for the surgeon as it facilitates the insertion of the nerve stumps across the nerve gap. However, on the other hand, it is not flexible, which might make the microsurgery technique difficult during its implantation (Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes,

et al., 2008). The biodegradation rate of PLGA, which is controlled by the monomer ratio, molecular weight, and crystallinity, can range from weeks to months (Battiston et al., 2005; Luis et al., 2007). PLGA 90:10 was developed by our research group and intensively studied; it was obtained from their cyclic dimers, DL-lactide, and glycolide. Nonwoven constructs were used to prepare 16 mm long tube-guides, with an internal diameter of 2.0 and 1.5 mm thick wall, in order to be applied in a 10-mm sciatic nerve gap. These fully synthetic nonwoven materials are extremely flexible and biologically safe, and after implantation, they are able to sustain the compressive forces related to body movement. They have also some degree of porosity to allow the influx of low molecular nutrients required for nerve regeneration. The nonwoven structure allowed the tube-guide to hold the suture without difficulties; however, greater care had to be taken in order to ensure its integrity. These tube-guides of PLGA are expected to degrade to lactic and glycolic acids through hydrolysis of the ester bonds (Luis et al., 2007; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008).

It was tested *in vivo* in the PLC polymer (Vivosorb®) membranes (purchased from Polyganics BV, Groningen, Netherlands), in rat sciatic nerve axonotmesis and neurotmesis injuries (Gartner et al., 2012; Rodrigues et al., 2005) PLC membranes are hydrophilic, thereby allowing the water uptake; this is essential for the nutrient control and other metabolite transportation to the surrounding healing tissue. A few weeks after implantation, the mechanical strength gradually decreases and there is a loss of molecular weight as a result of the hydrolysis process. Approximately in 24 months, PLC degrades into lactic acid and hydroxyl-caproic acid, both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PLC has the advantage over PLGA of not creating an acidic and potentially disturbing microenvironment, which is favorable to the surrounding tissue (Luis et al., 2007). Our PLC studies (Gartner et al., 2012) demonstrated that this biomaterial does not interfere negatively with the nerve regeneration process. In fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already attested in previous published clinical trials (Jones & McGonagle, 2008).

The functional unit of the nervous system, the neuron, is an electrically excitable cell that processes and transmits information by electrical and chemical signaling, so electrical conductivity is one of the crucial characteristics for an ideal nerve guidance channel (Guillot, Gotherstrom, Chan,

Kurata, & Fisk, 2007). One of the biomaterials that has been recently tested by our research team is a polymer named poly(vinyl alcohol) (PVA), loaded with electrical conductive materials, such as CNTs and PPy to produce a conductive biomaterial with higher electrical conductivity than the polymer matrix. The PVA is a water-soluble synthetic polymer and is prepared via the hydrolysis (alcoholysis) of poly(vinyl acetate) (PVAc), in which the acetate groups are replaced by hydroxyls (Gotherstrom, Ringden, Westgren, Tammik, & Le Blanc, 2003). The catalyst is sodium hydroxide, the nature of the distribution of the residual acetyl groups in the partially hydrolysed PVA is determined by the choice of catalyst and, where solvents are used, by the nature of those solvents (Gotherstrom et al., 2003). PVA has many applications due to its resistance against organic solvents and aqueous solubility. It is used in textile industries, in the food packaging industry, and in medical devices. US Food and Drug Administration (FDA) approved PVA to be in close contact with food products. In medical devices, PVA is used as a biomaterial due to its biocompatibility, swelling properties, bioadhesive characteristics, and for being nontoxic and noncarcinogenic (Secco et al., 2008; Soland et al., 2012). It is used in contact lenses, in the coating of artificial hearts and drug administration devices. For instance, it is used in drug delivery vehicles due to the permeability and hydrophilic interface fostering (Soland et al., 2012). This material is also suitable for the production of catheters, haemodialysis, membranes, artificial skin, vascular prostheses, and wound dressing (Porada & Almeida-Porada, 2012). PVA may mimic the regulatory characteristics of natural ECMs and ECM-bound growth factors, both in clinical applications and in basic biology studies. For nerve guides, the polymers with electrical conductivity have attracted interest because they simultaneously display the physical and chemical properties of organic polymers and the electrical characteristics of metals. Conductive polymers show great promise in biomedicine, namely for regenerative medicine of peripheral nerve. The importance of those polymers is based on the hypothesis that such biomaterials can be used to host the growth of cells, and electrical stimulation can be applied directly to the cells, which proved to be beneficial in many regenerative strategies (Wood et al., 2012). PPy and CNTs are two of the most used agents to produce electrical conductive polymers for tissue engineering. Besides, the introduction of metal ions into a polymer, particularly when the metal is linked chemically into a polymer chain, often imparts new or improved properties to the polymer (Porada et al., 2011).

Our research group has been testing the application of PVA in producing a tube-guide for peripheral nerve reconstruction associated to PPy and

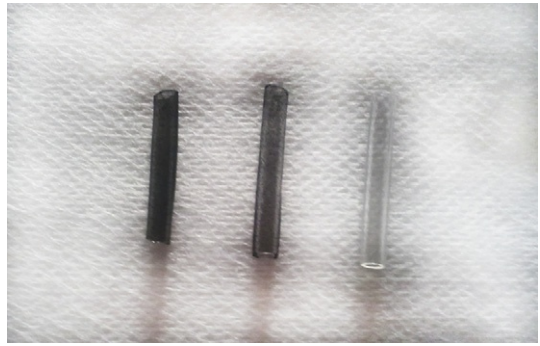

Figure 4.1 Tube-guides used to reconstruct the rat sciatic nerve after axonotmesis and neurotmesis injuries. From the left to the right panel: PVA tube-guide loaded with CNTs, PVA tube-guide loaded with PPy and PVA tube-guide.

CNTs (Fig. 4.1). These tube-guides were already fabricated and implanted in the rat sciatic nerve after axonotmesis and neurotmesis injuries, and the nerve regeneration has been evaluated through functional tests described further ahead.

Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10-98) and PVA loaded with $COOH^-$-functionalized CNTs (Nanothinx, NTX5, MWCNTs 97% –COOH), and with PPy (Aldrich, 10^{-40} S/cm of conductivity), were prepared using a casting technique to a silicone mold. A 15% (%w/v) aqueous solution of PVA was prepared. Then the solution of PVA was mixed with 0.05% (%w/v) of $COOH^-$ functionalized and 0.05% (%w/v) of PPy. The tube-guides were produced with the following dimensions: length = 16 mm, $\text{diameter}_{in} = 2$ mm, $\text{diameter}_{out} = 5$ mm. The tube-guides were produced by a freezing/thawing process consisting in three cycles of freezer (−30 °C)/incubator (25 °C), and an annealing treatment started with a stage of 14 h on an incubator (25 °C) followed by a ramp rate of 0.1 °C/min until 80 °C, and then a stage of 20 h at 80 °C. Afterwards, the tube-guides were hydrated during 2 h before use. The electrical conductivity analysis of these prepared tube-guides showed a value of 1.5×10^{-6} S/m for simple PVA tubes, which is according to bibliography (Park et al., 2001). The tubes with 0.05% (%w/v) of $COOH^-$-functionalized CNTs showed a value of 5.79×10^{-4} S/m, whereas for PVA loaded with PPy they presented a value of 1.8375×10^{-3} S/m. It is important to have in mind that PPy by itself has some conductivity (Fig. 4.1).

After obtaining these promising electrical conductivity values, the above two PVA nerve tube-guide compositions were chosen for further

characterizations and were applied in six groups of adult male Sasco Sprague–Dawley rats (Charles River Laboratories, Barcelona, Spain) with a standardized crush injury (axonotmesis) or a neurotmesis injury with a 10-mm gap. During the healing period, the regenerative process was evaluated by functional tests including kinematic analysis described further in this chapter. After the sacrifice of the experimental animals, 12 or 20 weeks postsurgery, with axonotmesis and neurotmesis injuries, respectively, the harvested regenerated nerves will be analyzed by histomorphometry (unpublished data).

Simultaneously, the biocompatibility of the PVA membranes was tested in subcutaneous implants in ovine (18 adult female white Merino sheep weighing approximately 60 kg) that were randomly divided in three groups of six animals each (one with PVA discs, one with PVA discs with a surface adsorbed with human MSCs derived from Wharton's jelly, and one control group). These PVA discs with 15.5 mm diameter were implanted subcutaneously. At 1, 2, 4, 8, 16, and 32 weeks postsurgery, the implants and the surrounding tissue were collected after a peripheral infiltration of 2% lidocaine and fixed with 10% formalin. Paraffin-embedded cuts of 2 μm were stained with hematoxylin and eosin (HE) for histological evaluation. The biological response parameters were assessed in the implant/tissue interface with three high power fields (×400) by at least two different pathologists for each sample and recorded in an appropriated formulary. Among the biological response parameters, all were evaluated according to the ISO standard 10993-6 (annex E) and included: the extent of fibrosis/fibrous capsule (layer in μm) and inflammation; the degeneration as determined by changes in tissue morphology; the number and distribution from the material/tissue interface of the inflammatory cell types, namely polymorphonuclear neutrophilic leucocytes (PMN), lymphocytes, plasma cells, eosinophils, macrophages, and multinucleated cells; the presence, extent, and type of necrosis; other tissue alterations such as vascularization, fatty infiltration, and granuloma formation; the material parameters such as fragmentation and/or debris presence, form, and location of remnants of degraded material and classified as nonirritant, slight irritant, moderate irritant, and severe irritant. According to this analysis, the PVA membranes alone or associated to the cellular system were considered slightly irritant ($N = 24$ samples analyzed) (unpublished data).

2.1.2 Fetal-derived cells for tissue engineering

Perinatal-derived cells are an ideal setting for tissue-engineering constructs as they can be harvested without harming intact donor structures and causing high risks for the child. The advantages for using these cellular systems are

the following: autologous cells source, processing with high quality standards and cryopreservation are possible, easy obtainable at various stages (pre and perinatal), present low immunogenic properties, these cell source often provides different types of cells including progenitor cells, the isolated cells have excellent cell growth capacities and they present a tumorogenic potential much lower when compared to omnipotent cells (Almeida-Porada, 2010; Cheng et al., 2011; Gartner et al., 2012; Hatlapatka et al., 2011; Maurício et al., 2011). In contrast to the highly standardized and industrially fabricated scaffolds, the quality of cells varies from patient to patient, depending on the individual tissue characteristics, on the transport conditions and time of the samples from the hospital/clinic to the laboratory, on the processing and cryopreservation techniques. These aspects are focused more ahead of this chapter concerning the collection of umbilical cord tissue (UCT) in order for isolation of MSCs from Wharton´s jelly (Cheng et al., 2011).

The MSCs isolated from extra-fetal tissues like the Wharton's jelly, the adipose tissue, and the bone marrow have a common morphologic description that includes a spindle shape, resembling fibroblasts, which are plastic adherent (Porada et al., 2010). The International Society of Cellular Therapy (ISCT) recognized the need to define the MSCs. This effort was made in order to distinguish between mesenchymal stromal cells and MSCs. The ISCT provides a clear and resumed definition: MSCs are progenitor cells for the mesenchymal lineages. These MSCs are adherent to tissue culture plastic (adherent cells), have a particular surface marker phenotype (express SH2, SH3, SH4, CD44, and HLA-class I, and do not express markers of the hematopoietic lineages CD34 and CD45 or HLA-class II), and have the capacity to differentiate into three mesenchymal lineages *in vitro* (bone, cartilage, adipocytes) (Dominici et al., 2006). The MSCs derived from the bone marrow and from the adipose tissue are able of self-renew and differentiate into specialized cells *in vitro*, there are reports of differentiation into neural cells (neurons, glial cells) (Cheng et al., 2011; da Silva et al., 2009; Gartner et al., 2012; Maurício et al., 2011).

UC-derived stem cells can be used for differentiation into all three layers, such as ekto-, endo-, and mesoderm (Cheng et al., 2011; Guillot et al., 2007; Hatlapatka et al., 2011). Concerning the nervous system, the UC-derived stem cells have been used for induction of neurons and glial cells (Cheng et al., 2011; da Silva et al., 2009; Gartner et al., 2012; Maurício et al., 2011). There are clear advantages of UC MSCs over the bone marrow MSCs. Obtaining bone marrow MSCs involves a painful bone marrow

aspirate, whereas the UC constitutes a waste product and a relatively high number of cells can be obtained by simple preparation of the cord. Extra-embryonic tissues are a good alternative to adult donor. These tissues, such as, amnion, microvillus, Wharton's jelly, and UC perivascular cells, are routinely discarded at childbirth, so slight ethical controversy attends the harvesting of the resident stem cell populations. The UC MSCs also represent "early" MSCs, which are considered superior cells obtained from more mature tissue like bone marrow, as they can undergo a significantly higher number of cell division before senescence (Cheng et al., 2011; Maurício et al., 2011). The reason for this higher proliferative capacity of fetal MSCs is because these cells have longer telomeres than adult MSCs (Galotto et al., 1999; Guillot et al., 2007). The fetal MSCs appear to lack some of the immune suppression properties observed in adult MSCs (Almeida-Porada, Zanjani, & Porada, 2010) and lack the human leukocyte antigen (HLA) class II, in contrast to adult MSCs and synthesize HLA-G, which is not present in the adult ones (Gotherstrom et al., 2003). Also, the cytokine profile of the fetal MSCs is different from that of the adult MSCs. So, the fetal MSCs like the ones isolated from the Wharton's jelly and from the UC blood, are primitive MSCs with greater ability to expand in culture due to their youth and naive status. MSCs have been isolated from several compartments of the UC, but our research group has been focused in the MSCs isolated from the Wharton's jelly and in preclinical studies regarding animal models of the application of this cellular system to the regeneration of the PNS after axonotmesis and neurotmesis injuries. Other important experimental work has been performed concerning the regeneration of bone, muscle, and vascular system concerning these MSCs but not referred since it is not included in the scope of this chapter (Shen et al., 2010).

2.2. MSCs from the Wharton jelly of the UC

2.2.1 Validation of transport of the UC from the hospital to the laboratory

The comparatively large volume of extra-embryonic tissues increases the chance of isolating suitable amounts of MSCs, despite the complex and expensive procedures needed for their isolation. Some protocols use enzymatic digestion while others use enzyme-free tissue explant methods that require longer culture time (Gartner et al., 2013). Wharton's jelly is a mature mucous tissue and the main component of the UC, connecting the umbilical vessels to the amniotic epithelium. UC derives from extra-embryonic or embryonic mesoderm; at birth it weighs about 40 g and measures

approximately 30–65 cm in length and 1.5 cm in width (Gartner et al., 2013). Cord blood (CB) and more recently, UCT have been stored cryopreserved in private and public CB and tissue banks worldwide in order to obtain hematopoietic and MSCs and, although guidelines exist (Netcord—Foundation for the Accreditation of Cellular Therapy), standardized procedures for CB and UCT transport from the hospital/clinic to the laboratory, storage, processing, cryopreservation, and thawing are still awaited. These may be critical in order to obtain higher viable stem cells number after thawing and limit microbiological contamination (Cheng et al., 2011).

Our research group focused on determining whether UCT storage and transport from the hospital/clinic to the laboratory at RT or refrigerated (0.5–20 °C) and immersed in several sterile saline solutions affects the UCT integrity in order to be cryopreserved. Twelve UCs ($N=12$) were collected from healthy donors after written informed consent and following validated procedures according to the clinical and technical guidelines of the Portuguese Private Bank, Biosckin, Molecular and Cell Therapies, SA (authorized for processing and cryopreservation CB and UCT units by the Portuguese Authority Instituto Português de Sangue e Transplantação—IPST, IP). After collection in the hospital/clinic, these UCs were transported to the laboratory during 96 h at refrigerated temperature controlled by a datalloger. From each donor UC ($N=12$), eight fragments of 1 cm were cut, and each fragment from each different donor was immersed for 168 h in four different sterile saline solutions at RT (22–24 °C) and refrigerated (0.5–20 °C): NaCl 0.9% (Labesfal, Portugal), AOSEPT®-PLUS (Ciba Vision, Portugal), Dulbecco's phosphate-buffered saline without calcium, magnesium, and phenol red (DPBS, Gibco, Invitrogen, Portugal) and Hank's balanced salt solution (HBSS, Gibco, Invitrogen, Portugal). Two UCs ($N=2$) were immersed in 4% of paraformaldehyde and processed for light microscopy, one immediately after birth and other after 96 h at transport refrigerated temperature without being immersed in any of the tested sterile saline solutions. These two UCs served as control. After 168 h, the immersed fragments were collected in 4% of paraformaldehyde and processed for light microscopy. The samples were fixed in 4% paraformaldehyde for 4 h and then washed and conserved in phosphate buffer saline (PBS) until embedding. The specimens were dehydrated and embedded in paraffin and cut at 10 μm perpendicular to the main UC axis. For light microscope analysis, sections were stained with HE and observed with a Leica DM400 microscope equipped with a Leica DFC320 digital camera. The UCT

integrity was evaluated through the following parameters: (i) detachment of vessels and retraction of vascular structures; (ii) loss of detail and integrity of the endothelium; (iii) connective tissue degradation; and (iv) loss of detail and integrity of the mesothelium. It was concluded that the best transport solutions were HBSS or DPBS at refrigerated temperature, since those solutions maintained the histological structure of UC evaluated through those five parameters previously referred (Fig. 4.2). As a matter of fact, the UC immersed for 168 h in DPBS and HBSS at refrigerated temperature presented integrity of the histological structure comparable to UCs collected and processed for histological analysis immediately after birth and transported for 96 h after collection at refrigerated temperatures of transport. With DPBS, a slight retraction of the vessels was noted, which is advantageous since the vessels are stripped and discarded before cryopreservation of the UCT. It was concluded that the transport of the UC from the hospital/clinic to the cryopreservation laboratory when performed with the UC immersed in DPBS or HBSS at refrigerated temperatures, permits to extend the time of transport more than 96 h, which is the maximal time allowed according to the technical protocols of the Private Cord Blood Bank.

2.2.2 *Validation of the isolation protocol of MSCs from the Wharton's jelly*

Various methods of isolation have been described to obtain and isolate MSCs from the UC (Can & Karahuseyinoglu, 2007). Frequently, the

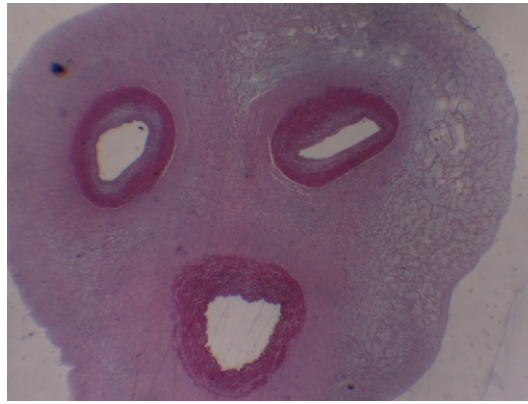

Figure 4.2 Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature 2 of 4–6 °C. Samples were stained with hematoxylin and eosin (HE). Magnification: 10 ×.

isolated MSCs although showing the properties and characteristics of MSCs, are probably different cell types. Most of the recently described methods share a common step, which is the manual separation of the tissue of interest within the UC, followed or not by enzymatic digestion of the ECM to release viable individual cells that are able to expand in culture.

Whether obtained through cesarean section or vaginal delivery, it is important to remove contaminating blood from the UC. As it was previously reported; CB itself can be a source of MSCs, but the rate of recovery is generally too low (Almeida-Porada et al., 2007). The blood vessels of the UC are another important source of MSCs (Romanov, Svintsitskaya, & Smirnov, 2003). It has been described in the isolation of MSCs from UC stripped vessels (Sarugaser, Lickorish, Baksh, Hosseini, & Davies, 2005) that these perivascular cells are isolated with collagenase incubation and present the MSCs characteristics like plastic adherence, surface markers expression, and ability to trans-differentiate in the presence of appropriate culture medium components. On the other hand, these perivascular cells express 3G5 (Sarugaser et al., 2005) and CD146 (Baksh, Yao, & Tuan, 2007), these two markers are not present in the MSCs from the Wharton's jelly. Wang and collaborators described the isolation of MSCs from the Wharton's jelly, after removing the UC vessels, by an enzymatic sequence of collagenase and trypsin (Wang et al., 2004). Weiss and collaborators also described an enzymatic method for the isolation of MSCs from the Wharton's jelly. After removing the UC vessels, the cord is cut in segments that are incubated with a cocktail of hyaluronidase, collagenase, and trypsin (Weiss et al., 2006). Other laboratories have been trying with success the isolation of MSCs from the Wharton´s jelly without enzymatic treatment like the published work by Mitchell et al. (2003). In this protocol, the cord tissue, after removal of the cord vessels, is cut in small segments that are placed directly in the culture dish (Mitchell et al., 2003).

The isolation and culture of MSCs from the Wharton's jelly was performed by our research group in order to obtain undifferentiated MSCs and *in vitro* differentiated neuroglial-like cells. Both cellular systems were tested *in vivo*, in axonotmesis and neurotmesis injuries of the rat sciatic nerve. The isolation was performed by enzyme-free tissue explant and by enzymatic isolation procedure. Despite our standard approaches, we are aware that there are still significant variations that exist between laboratories' protocols, which must be taken into account when comparing results using other methodologies. There is a wide range of individual differences among donor tissues also and our protocols usually use 15–20 cm of UC. While

most UC samples will provide a reasonable number of MSCs using the provided protocols, some samples may result in suboptimal cell isolation and expansion. The reasons behind this phenomenon still remain to be clarified, but as we have previously mentioned, the temperature and the time of transport from the hospital/clinic to the cryopreservation laboratory is crucial. Anyway, there is an important difference, described by Weiss et al., in 2006, between UC blood MSCs and Wharton's jelly MSCs: the latter can be isolated from close to 100% of the samples, even UCs that are delayed in their processing up to 48 hours (Weiss et al., 2006). These results were confirmed by our research group as it is described and discussed above.

Irrespective of the specific protocol, the washing procedure of the UC fragments is crucial in order to avoid microbiological contamination of the cultures. After obtaining the written informed consent from the parents, fresh human UCs are obtained after birth and transported from the hospital/clinic to the laboratory at refrigerated temperatures, as it was previously described. After washing the UC unit four times in rising sterile DPBS solution, disinfection is performed in 70% ethanol for 30 s. Finally, and before the dissection step, the UC unit is washed once again in sterile DPBS solution. The vessels are usually stripped with UC unit still immersed in DPBS. Once the washing step of the UC was considered essential to achieve good UCT units for cryopreservation and for MSCs isolation and culture, the washing protocol was validated. DPBS from the first washing step (used immediately after the transport of the unit to the laboratory—washing solution 1) and DPBS used in washing step after disinfection in 70% ethanol (washing solution 2) from 14 UC units ($N=14$) collected from healthy donors and transported from the hospital/clinical at refrigerated temperatures in <96 h, were tested for microbiological contamination using BacT/ALERT® (bioMérieux). Each unit was tested for aerobic and anaerobic microorganisms and fungi using 10 ml of the solution 1 and 10 ml of the solution 2, which were aseptically introduced into the BacT/ALERT® testing flasks. All procedures were performed in a laminar flow tissue culture hood under sterile conditions. All the units that presented microbial contamination in DPBS obtained from the first washing step (solution 1) presented no contamination in the analysis performed to DPBS from the last washing step immediately performed before MSCs isolation or UCT cryopreservation (solution 2). The following microorganisms were identified in the DPBS solution from solution 1: *Staphylococcus lugdunensis* ($N=2$); *Staphylococcus epidermidis* ($N=1$); *Staphylococcus coagulase* ($N=2$); *Escherichia coli* ($N=4$); *Enterococcus faecalis* ($N=1$); and *Streptococcus sanguinis* ($N=1$). The DPBS solution from

the first washing step (solution 1) from three UC units was negative for microbial contamination ($N=3$). These results permitted us to conclude that the washing protocol was 100% efficient in what concerns microbiological elimination (including aerobic and anaerobic bacteria, yeast and fungi) and appropriate for cryopreservation of UCT or for isolation and culture of MSCs obtained from the collected UCs (Cheng et al., 2011).

Once the transport and washing protocols were validated, it was important to isolate and expand *in vitro* the MSCs from the UCT units for future preclinical trials.

In the "enzymatic protocol," collagenase type I (Sigma-Aldrich) and trypsin-EDTA solution (Sigma-Aldrich) were used. With the written informed consent from the mother, fresh human UCs were obtained after birth and transported to the laboratory at refrigerated temperatures (Gibco, Invitrogen, Portugal) for 1–96 h before tissue processing to obtain MSCs. After removal of blood vessels, the Wharton's jelly is scraped off with a scalpel and centrifuged at 250 g for 5 min at RT and the pellet is washed with serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Portugal). Next, the cells are centrifuged at $250 \times g$ for 5 min at RT and then treated with collagenase (2 mg/ml) for 16 h at 37 °C, washed and treated with 2.5% trypsin-EDTA solution (Sigma-Aldrich) for 30 min at 37 °C with gentle agitation. Finally, the cells are washed and cultured in DMEM (Gibco, Invitrogen, Portugal) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/l), 1% (w/v) penicillin and streptomycin (Sigma-Aldrich), and 2.5 mg/ml amphotericin B (Sigma-Aldrich) in 5% CO_2 in a 37 °C incubator (Nuaire). Around 2×10^5 cells are plated into each T75 flask in 10 ml culture medium. Cells are allowed to attach and grow for 3 days. To remove the nonadherent cells or fragments, the flasks are gently washed using prewarmed DPBS after which 10 ml of prewarmed culture medium is added. The culture medium is changed every third day (or twice per week). Confluence (80–90%) is normally reached at day 12–16, and the cells are removed with prewarmed trypsin-EDTA solution (4 ml per flask), for 10 min at 37 °C. The cells are plated onto poly-L-lysine-coated glass coverslips (in 6- or 24-well tissue culture plates) (Sigma-Aldrich) or on biomaterials membranes used in the nerve reconstruction. Normally, 5000 cells/cm^2 are plated on the coverslips or on the membranes (Cheng et al., 2011) (Fig. 4.3).

In our "enzyme-free tissue explant protocol" for isolation of MSCs from the Wharton's jelly, enzymatic digestion is not employed. The mesenchymal tissue (Wharton's jelly) is diced into cubes of about 0.5 cm^3 and the

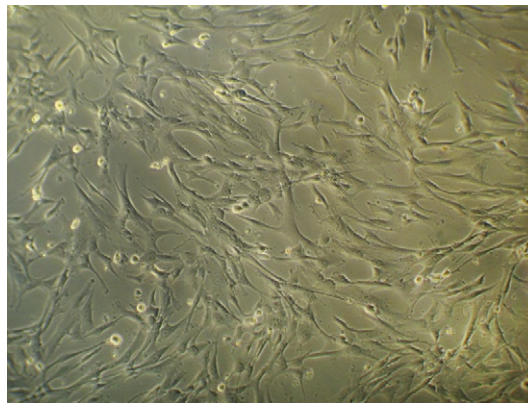

Figure 4.3 MSCs isolated from Wharton's jelly using the "enzymatic protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100 ×.

remaining vessels are removed by dissection. Using a sterile scalp, the cubes are diced in 1–2 mm fragments and transferred to a Petri dish precoated with poly-L-lysine (Sigma-Aldrich) with mesenchymal stem cell medium (PromoCell, C-28010) supplemented with 1% (w/v) penicillin and streptomycin (Sigma-Aldrich), and 2.5 mg/ml amphotericin B (Sigma-Aldrich) and cultured in 5% CO_2 in a 37 °C incubator (Nuaire). Some tissue fragments will allow cell migration from the explants in 3–4 days incubation. Confluence is normally obtained 15–21 days after (Cheng et al., 2011) (Fig. 4.4).

The laboratory's processing and cryopreservation protocols of the UCT units following the technical procedures of Biosckin, Molecular and Cell Therapies S.A. (BSK.LCV.PT.7) were validated for the ability of isolating and expanding *in vitro* MSCs after cryopreserved UCT thawing. The protocols of processing and cryopreservation of the UCT are protected by a Confidentiality Agreement between Biosckin, Molecular and Cell Therapies S.A. and all the involved researchers. Briefly, the UCT collected from healthy donors ($N = 60$), and according to Netcord guidelines and following the Portuguese law 12/2009 (Diário da República, lei 12/2009 de 26 de Março de 2009), was diced into cubes of about 0.5 cm^3 and the remaining vessels were removed by dissection. In order to ensure the viability of the UCT after parturition and limit the microbiological contamination of the samples, the UCs were transported from the hospital/clinic to the laboratory at refrigerated temperatures monitored by a datalloger in less than 96 hours. The UCT units from 15 to 20 cm long UCs and after the blood vessels

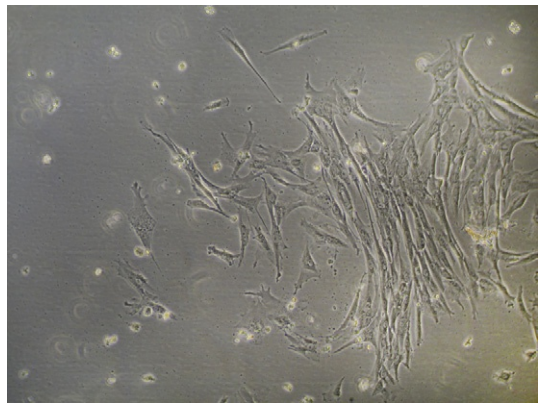

Figure 4.4 MSCs isolated from Wharton's jelly using the "enzyme-free tissue explant protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. The mesenchymal tissue (Wharton's jelly) 1–2 mm fragments transferred to a Petri dish precoated with poly-L-lysine allowed cell migration from the explants in 3–4 days incubation.

dissection were treated and processed for cryopreservation using a cryoprotective solution (freezing medium). The UCT units were transferred to a computer-controlled slow rate freezer (Sylab, Consensus, Portugal) and a nine-step freezing program was used to set up the time, temperature, and rates specifically optimized for the human UC-MSCs cooling. To thaw frozen cells, the cryovials were transferred directly to a 37 °C water bath. Upon thawing in less than a minute, the cell suspension was centrifuged at $250 \times g$ for 10 min, and the supernatant was gently removed and the cell pellet was resuspended in culture medium (Cheng et al., 2011). It was possible to obtain MSCs in culture from 52 out of 60 thawed UCT units. In some UCT cryopreserved units ($N=8$), it was not possible to isolate MSCs due to increased number of erythrocytes' lysis and/or microbiological contamination during the initial cell culture period. The MSCs morphology was observed in an inverted microscope (Zeiss, Germany) at different points of expansion. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. The MSCs obtained ($N=52$) were characterized by flow cytometry (FACSCalibur®, BD Biosciences) analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). The karyotype of undifferentiated MSCs was determined and no structural alterations were found demonstrating absence of neoplasic characteristics in these cells, as well as chromosomal stability to

the cell culture procedures. In the presence of neurogenic medium, the MSCs were able to become exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches. These results permitted to conclude that the processing and cooling protocols used for UCT units' cryopreservation were adequate to preserve the UCT viability since it was possible to isolate and expand MSCs after appropriate thaw and in presence of adequate cell culture conditions (Cheng et al., 2011).

2.2.3 MSCs cells from a cell line and differentiation into neuroglial-like cells

An established and ready-to-use human MSC cell line from the UC matrix (Wharton's jelly) was employed for promoting axonotmesis and neurotmesis lesions regeneration in the rat sciatic nerve model. Human MSCs from Wharton's jelly UC were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreserved cells were cultured and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 h. At 80–90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. MSCs at a concentration of 2500 cells/ml were cultured on poly-D-lysine coverslips (Sigma-Aldrich) or on biomaterials membranes and after 24 h cells exhibited 30–40% confluence. Differentiation into neuroglial-like cells was induced with MSC neurogenic medium (PromoCell, C-28015). Medium was normally replaced every 24 h during 3 days. The formation of neuroglial-like cells was observed after 24 h in an inverted microscope (Zeiss, Germany) (Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012; Shen et al., 2010).

This established human MSC cell line was preferred for *in vivo* testing in rats, since the number of MSCs obtained was higher in a shorter culture time, it was not dependent on donors' availability and ethic committee authorization, and the protocol was much less time-consuming, which was advantageous for preclinical trials with a large number of experimental animals. As a matter of fact, there was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure. The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are normally performed for each lot of PromoCell MSCs isolated from Wharton's jelly of UC. MSCs are tested for cell morphology, adherence rate, and viability. Furthermore, each cell lot is characterized by flow cytometry analysis for a comprehensive panel of markers (Gartner et al., 2012).

The MSCs established PromoCell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion, the cells became long spindle shaped and colonized the whole culturing surface. After 96 h of culture in neurogenic medium, cells changed in morphology. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches (Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012). Giemsa-stained cells of *in vitro* differentiated MSC cell line at passage 5 were analyzed for cytogenetic characterization. The karyotype of undifferentiated MSCs obtained from the UCT cryopreserved and isolated by the enzyme-free tissue explants ($N=52$) (described in Section 2.2) and from PromoCell was determined and no structural alterations were found demonstrating absence of neoplasic characteristics in the MSCs cells, as well as chromosomal stability to the cell culture procedures (Gartner et al., 2013). The *in vitro* differentiated MSCs karyotype could not be established, since no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated MSCs previously determined, supported the suitability of our cell culture and differentiation protocols. This concern resulted from previous negative *in vivo* results obtained with *in vitro* differentiated N1E-115 cells in axonotmesis and neurotmesis injuries of the rat sciatic nerve (Gartner et al., 2013). The differentiation into neuroglial-like cells of the MSCs from Wharton's jelly was tested based on the expression of typical neuronal markers such as growth-associated protein-43 (GAP-43), glial fibrillary acidic protein (GFAP), and neuronal nuclei (NeuN). Undifferentiated MSCs were negatively labeled GFAP, GAP-43, and NeuN. After 96 hours of differentiation, the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nuclei of neuroglial-like cells were also labeled with the neuron-specific nuclear protein called NeuN showing that differentiation of MSCs into neuroglial-like cells was successfully achieved for MSCs obtained from UCT cryopreserved ($N=52$) and for the PromoCell MSC cell line (Gartner et al., 2013).

3. *IN VIVO* TESTING IN THE RAT SCIATIC NERVE MODEL

3.1. Surgery technique and the importance of a standardized injury

The most frequently used animal model for studying the peripheral nerve regeneration is the rat because of the widespread availability of these animals

as well as the distribution of their nerve trunks, which is similar to humans (Mackinnon et al., 1985). The rat sciatic nerve is still by far the most employed experimental model as it also provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and/or introduction of grafts or tube-guides (van Neerven et al., 2012) (Fig. 4.5). And at same time, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Mackinnon et al., 1985). Peripheral nerve injuries are classified in two major groups, which are the crushing injuries (axonotmesis) and the complete nerve section (neurotmesis) with or without loss of nerve tissue (Almeida-Porada et al., 2005; Chaudhry, Glass, & Griffin, 1992; Stoll, Griffin, Li, & Trapp, 1989). The axonotmesis injury is less severe and is often used for studies concerning the physiology mechanisms of regeneration, while the neurotmesis injury is mostly used in surgical strategy and implementation of biomaterials associated with cellular systems studies (Luís, 2008). The choice of an appropriate animal model is fundamental for preclinical experiments and should be performed before the clinical trials and compassive treatments in humans (Maurício et al., 2011). Nowadays, animal welfare is crucial and not negotiable in terms of *in vivo* experiments. Some alternatives to animal experimentation exist, but what concerns cell therapies and regenerative medicine using stem cells and different biomaterials is always the last step of the research line before the clinical application in humans. Our research group has been using the rat sciatic nerve for *in vivo* experiments of peripheral nerve regeneration (Amado et al., 2010, 2008;

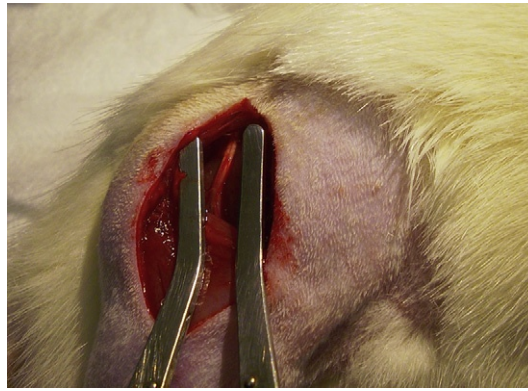

Figure 4.5 Rat sciatic nerve.

Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Maurício et al., 2011; Simoes et al., 2010), concerning different therapeutic strategies in axonotmesis (Brohlin et al., 2009) and neurotmesis (Luis et al., 2007) injuries. So, for these *in vivo* testing, Sasco Sprague adult male rats (Charles River Laboratories, Barcelona, Spain) have been used and divided in groups of 6–7 animals each. All procedures had been performed with the approval of the Veterinary Authorities of Portugal, and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The animals placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. Under deep anesthesia (ketamine 9 mg/100 g; xylazine 1.25 mg/100 g, atropine 0.025 mg/100 g body weight, intramuscular), the sciatic nerve is exposed unilaterally through a skin incision extending from the greater trochanter to the mid-thigh followed by a muscle-splitting incision. The standard crush injury (axonotmesis lesion) is performed by a nonserrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3-mm axonotmesis lesion, since the published experimental work from Luis and collaborators in 2007 (Luís et al., 2007) (Fig 4.6A). To test the scaffold (biomaterial associated or not to the cellular system) therapeutic effect in the nerve regeneration after crush, the axonotmesis lesion of 3 mm performed was enwrapped. Figure 4.6B shows the crush injury enwrapped with a PVA tube-guide loaded with CNTs. For the neurotmesis lesion, a transaction injury is usually performed using straight microsurgical scissors and the surgical procedure is performed with the aid of an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). In both models, the nerve injury is performed at a level as low as possible, in general, immediately above the terminal nerve ramification, considering always individual anatomical differences. The reconstruction of the injured nerve after neurotmesis by an end-to-end suture, implies cooptation with 7/0 monofilament nylon sutures of the two injured nerve endings performed under magnification. In the rats in which tube-guides were used for the neurotmesis injured sciatic nerve, the proximal and distal nerve stumps were inserted 3 mm into the tube-guide and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon, respectively (Fig. 4.7A–C). For the

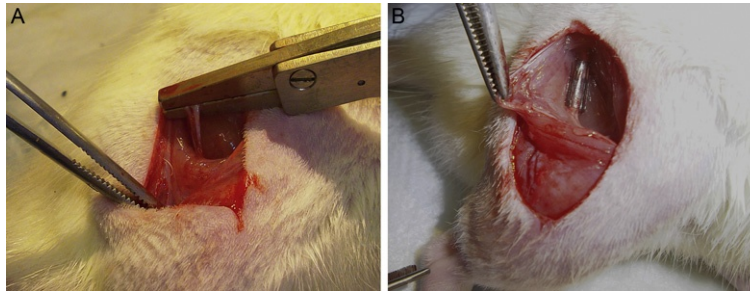

Figure 4.6 The standard crush injury (axonotmesis lesion) is performed by a nonserrated clamp exerting a constant force of 54 N for a period of 30 s, inducing a 3-mm axonotmesis lesion (panel A). Crush injury enwrapped with a PVA tube-guide loaded with CNTs (panel B).

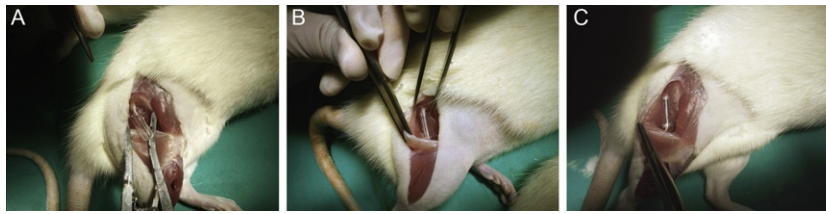

Figure 4.7 In the rats in which a PVA tube-guide was used for the neurotmesis injured sciatic nerve, the proximal (panel A) and distal nerve stumps (panel B) were inserted 3 mm into the tube-guide and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon (panel C).

group of animals in which an autologous graft procedure was tested, the sciatic nerve was transected immediately above the terminal nerve ramification and at a 10 mm distal point. The nerve graft obtained, with a length of 10 mm, was inverted at 180° and sutured with 7/0 monofilament nylon. Normally the muscle and skin are closed with 4/0 resorbable sutures. An antibiotic (enrofloxacin, Alsir® 2.5%, 5 mg/kg b.w., subcutaneously) is always administered to prevent any infections. To prevent autotomy, a deterrent substance must be applied to the rats' right foot (Sanada et al., 2012; Wood et al., 2012). There was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure.

3.2. Functional assessment

Experiments on peripheral nerve regeneration are often performed on the rat sciatic nerve model (Dellon & Mackinnon, 1989). Research on

peripheral nerve injury needs to combine both functional and morphological assessment. It is not generally agreed which type of evaluation tool is the most useful descriptor of functional recovery; for this reason, the use of different methods for an overall assessment of nerve function has been recommended by several investigators (Morris, Hudson, & Weddell, 1972). In our lab, we perform a variety of independent evaluation tools in order to understand and estimate the potential therapeutic benefit of a nerve repair strategy.

After injury and treatment of animals, follow-up results are very important for analysis of functional recovery. Animals have been tested preoperatively (week 0), and every week during 12 and 20 weeks, for axonotmesis and neurotmesis of the rat sciatic nerve, respectively.

For SFI, animals are usually tested in a confined walkway that they cross, measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end. Several measurements must be taken from the footprints: (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). In the SSI, only the parameters TS and ITS, are measured. For SFI and SSI, all measurements are taken from the experimental (E) and normal (N) sides. Prints for measurements are chosen at the time of walking based on preciseness, clarity, and completeness of footprints. The mean distances of three measurements are used to calculate the following factors (dynamic and static):

$$\text{Toe spread factor (TSF)} = \frac{(\text{ETS} - \text{NTS})}{\text{NTS}} \tag{4.1}$$

$$\text{Intermediate toe spread factor (ITSF)} = \frac{(\text{EITS} - \text{NITS})}{\text{NITS}} \tag{4.2}$$

$$\text{Print length factor (PLF)} = \frac{(\text{EPL} - \text{NPL})}{\text{NPL}} \tag{4.3}$$

SFI is calculated as described by Bain et al. (1989) according to the following equation:

$$\begin{aligned} \text{SFI} &= \frac{-38.3}{\text{NPL}} + \frac{109.5(\text{ETS} - \text{NTS})}{\text{NTS}} + \frac{13.3(\text{EIT} - \text{NIT})}{\text{NIT}} - 8.8 \\ &= (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \end{aligned} \tag{4.4}$$

For SFI and SSI, an index score of 0 is considered normal and an index of −100 indicates total impairment. When no footprints are measurable,

the index score of −100 is given. In each walking track, three footprints should be analyzed by a single observer, and the average of the measurements is used in SFI calculations.

Among the large variety of available motor and nociceptive tests, the EPT and the WRL, respectively, have been proven to be reliable, valid and highly efficient methods to determine functional recovery following sciatic nerve injury (Gartner et al., 2013). For EPT test, the affected and normal limbs should be tested at least three times, with an interval of 2 min between consecutive tests, and the three values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (Eq. 4.5) to derive the percentage of functional deficit, as described in the literature (Koka & Hadlock, 2001):

$$\%\ \text{Motor deficit} = \left[\frac{(\text{NEPT} - \text{EEPT})}{\text{NEPT}}\right] \times 100 \tag{4.5}$$

The nociceptive WRL was adapted from the hotplate test developed by Masters et al. (1993). Normal rats withdraw their paws from the hotplate within 4 s or less. The cutoff time for heat stimulation is set at 12 s to avoid skin damage to the foot.

The restoration of locomotor activity following damage of the nervous system has emerged as one of the most pressing and challenging problem in clinical neuroscience. Many patients with neurological injuries, like peripheral nerve or spinal cord injuries suffer from muscle weakness and loss of independent joint control, often resulting in gait disorders. During the past 10–15 years, exciting work is being carried out on rat gait analysis that may significantly alter the future of peripheral nerve research (Costa, Simoes, Mauricio, & Varejao, 2009). Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles, which is very useful and accurate to evaluate different therapeutic approaches (Varejao, Cabrita, Geuna, et al., 2003; Varejao, Cabrita, Meek, et al., 2003; Varejao, Melo-Pinto, Meek, Filipe, & Bulas-Cruz, 2004). It is important to realize that the number of kinematic variables (positions, velocities, and accelerations) required to describe one-step cycle is very high. Therefore, it is only through high-speed digital cameras that we can achieve a full kinematic description during gait (Costa et al., 2009).

Ankle kinematics analysis has been carried out prior nerve injury, at week 2 and every 4 weeks during the 12 or the 20-week follow-up time,

for axonotmesis and neurotmesis lesions, respectively. The motion capture is performed with two digital high speed cameras (Oqus, Qualysis®) at a rate of 200 images per second, and Qualysis Track Manager software (QTM, Qualysis®). The cameras operate on an infra-red light frequency ensuring a high level of accuracy on the determination of reflective marker position and a position residual of less than 2.7 mm was obtained. Cameras are usually positioned to not recorder significant signal deflection during the test and three reflective markers are placed at the skin of the rat right hindlimb at the proximal edge of the tibia, the lateral malleolus and the fifth metatarsal head. Advanced analysis of the 2D movement (sagittal plan) data is usually performed with Visual3D software (C-Motion®, Inc.). The rats' ankle angle is determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (Θ°) indicate dorsiflexion and plantarflexion, respectively. For each step cycle, the following time points are identified: midswing, midstance, initial contact (IC) and toe-off (TO) (Gartner et al., 2013) and are time normalized for 100% of step cycle. The normalized temporal parameters are averaged over all recorded trials. Angular velocity of the ankle joint ($\Omega\,^{\circ}$/s) is also determined where negative values correspond to dorsiflexion. A total of at least six walking trials for each animal with stance phases lasting between 150 and 400 ms are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) (Gartner et al., 2013). The motion capture is performed when the animals walk on a Perspex track with length, width, and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments and the rats are daily trained for 2 weeks before the surgery, in order to walk on the Perspex corridor.

Individual joint kinematics either in control or nerve-injured animals is characterized by high variability, with notable differences between different animals and even from step to step (Jacobson & Guth, 1965). Such high level of variability, which seems to be an intrinsic property of normal quadruped walking, seriously affects the precision of joint kinematic measures of functional recovery after nerve injury. Reducing this variability is a challenge for efficient use of walking analysis to assess functional recovery. Attempts to overcome this limitation include constraining walking velocity by using treadmill walking instead of self-paced locomotion (Gartner et al., 2013). This, of course, is likely to reduce step-by-step variability in joint kinematics but has the disadvantage of requiring expensive equipment and limits the

possibility of combining kinematic analysis with other data, such as ground reaction forces. Other possibilities look at a global, limb-level movement analysis as an alternative to individual joints kinematics (Jacobson & Guth, 1965; Pereira et al., 2013). To better assess hindlimb joint kinematics during walking, we recently analyzed hip, knee, and ankle joint kinematics during recovery of less severe sciatic nerve crush injury, using a more sophisticated motion capture system to track the motion of reflective markers attached to the rat hindlimb (unpublished data). Recently a segmental kinematic analysis using both planar angles computation and a tridimensional (3D) reconstruction of the rat hindlimb was also performed, regarding the morphology and the movement of each segment. Seven rats without any peripheral injury were evaluated for natural overground walking, and motion capture of the right hindlimb was collected with an optoelectronic system while the animals walked in the track. 3D biomechanical analyses were carried out and hip, knee, ankle, and metatarsophalangeal joint angular displacements were calculated. A comparison between planar and 3D segmental kinematic analysis using a tridimensional reconstruction of the rat hindlimb demonstrated that different joints have different motion patterns within motion planes, probably related with physiological constraints and muscle actions. A major indication of the need for an anatomical reference frame kinematic analysis is supported by the knowledge that neuromuscular diseases are related to important clinical signs or motor deficits that should be observed, qualified, and quantified (Harley et al., 2006). On the other hand, systematic changes in the biomechanical and movement control constraints of the locomotor task, such as using up- and down-slope walking might also increase the accuracy of walking analysis within the context of peripheral nerve research (Pereira et al., 2013). Walking analysis is a promising method to assess functional recovery after hindlimb nerve injury. However, in order to provide accurate measures of functional recovery, walking analysis after hindlimb peripheral nerve injury will have to evolve from simply analyzing ankle kinematics to reach a full biomechanical description of hindlimb motion including analysis of hip, knee, and ankle joints. Further refinements of walking analysis in the field of peripheral nerve research using the rat model will probably include the combined use of joint kinematics, ground reaction forces, and electromyographical data of muscle activity (Harley et al., 2006; Maurício et al., 2011).

3.3. Morphologic assessment

It has been recently pointed out that morphological analysis is the far most common method for the study of peripheral nerve regeneration (Raimondo

et al., 2009). Actually, the investigation of nerve morphology can give us important information on various aspects of the regeneration processes which relates with nerve function (Geuna et al., 2009). Although different types of fixatives can be used for peripheral nerve histology, the nerve samples are fixed in a solution of 2.5% purified glutaraldehyde (Histo-line Laboratories S.R.L., Milano, Italy) and 0.5% saccarose (Merck, Darmstadt, Germany) in 0.1 M Sörensen phosphate buffer, pH 7.4, for 6–8 h. Nerves are then washed and stored in 0.1 M Sörensen phosphate buffer added with 1.5% saccarose at 4–6 °C prior to embedding. Sörensen phosphate buffer is made with 56 g di-potassium hydrogen phosphate 3-hydrate (K_2HPO_4-$3H_2O$) (Fluka, Buchs, Switzerland) and 10.6 g sodium di-hydrogen phosphate 1-hydrate (NaH_2PO_4-H_2O) (Merck, Darmstadt, Germany) in 1 liter of doubly-distilled water. Just before the embedding, nerves are washed for few minutes in the storage solution and then immersed for 2 h in 2% osmium tetroxide (Sigma, St. Louis, MO) in the same buffer solution. The specimens are then carefully dehydrated in passages in ethanol and embedded in Glauerts' mixture of resins, which is made of equal parts of Araldite M and the Araldite Härter, HY 964 (Merck, Darmstad, Germany). At the resin mixture, 2% of accelerator 964, DY 064 is added (Merck, Darmstad, Germany). Finally, the plasticizer (0.5% of dibutylphthalate) is added to the resin. In Stefano Geuna laboratory (Department of Clinical and Biological Sciences, University of Turin, Italy), histomorphometry (stereology) is carried out on toludine-blue-stained semi-thin sections (2.5 micron-thick) of nerve samples using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). We adopt a final magnification of 6600 × in order to enable accurate identification of myelinated nerve fibers. A 2D-disector method, (Raimondo et al., 2009) is finally used for estimating the total number of myelinated fibers (N), the mean diameter of fiber (D) and axon (d) as well as mean $[(D-d)/2]$ and g-ratio (D/d).

4. DISCUSSION AND FINAL REMARKS

Despite great progress in the fields of tissue engineering and stem cell therapy, translational and preclinical studies are required to accelerate the clinical application of scaffolds as an alternative to autologous nerve grafts for peripheral nerve repair. The PNS is able to regenerate after traumatic injury, but most frequently, the functional outcomes following damage are limited and poor. Nowadays, most tissue-engineered nerve grafts are

composed of a neural scaffold prepared with a variety of synthetic or natural biomaterials through well-defined fabrication techniques. Introduction of support cells, an important biochemical cue, represents an optimal way for constructing tissue-engineered nerve grafts with enhanced ability to repair extended peripheral nerve defects. MSCs are multipotent cells that have been used in studies of peripheral nerve regeneration and have yielded promising results. The aim of our research group for the past 10 years has been exploring the therapeutic value of human UC Wharton's jelly-derived MSCs both *in vitro* and *in vivo*, associated to several tube-guides of natural or absorbable synthetic biomaterials on a rat sciatic nerve axonotmesis experimental model. Undifferentiated MSCs from human UC Wharton's jelly have been expanded and have exhibited a normal star-like shape with a flat morphology in culture. To prevent the possibility of eventual mutations due to expansion artifacts, Giemsa-stained metaphases of these cells were analyzed for numerical aberrations. The karyotype was determined in a completely analyzed G-banding metaphase and no structural alterations were found. The karyotype analysis to the MSCs cell line derived from human Wharton jelly demonstrated that this cell line has not neoplasic characteristics and was stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes (Gartner et al., 2012). The MSCs from Wharton's Jelly were differentiated into neuroglial-like cells in the presence of neurogenic culture medium during 96 h. The MSCs became exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches. The differentiation was also tested based on the expression of typical neuronal markers such as GFAP, GAP-43, and NeuN by neural-like cells attained from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43, and NeuN and after 96 h of differentiation the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nucleus of neuroglial-like cells were also labeled with the neuron-specific nuclear protein called NeuN showing that differentiation was successfully achieved (Gartner et al., 2012). The *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization. Although preclinical and clinical data demonstrated the safety and effectiveness of MSCs therapy in some pathologies such as neurological, there are still questions surrounding the mechanism of action. MSCs' maintenance and differentiation to neuroglial-like cells depends on metabolic modulation. *In vitro*, glucose is the most widely used substrate for the generation of ATP, which is

essential for cell growth and maintenance. It has been proposed that cells undergoing high proliferation rates depend on glycolysis to generate ATP, known as the Warburg effect (Gartner et al., 2012, 2013). Our results showed that during expansion, the undifferentiated MSCs consume glucose and produce high concentration of lactate as a metabolic sub product, which is consistent with the Warburg effect and glycolysis stimulation. MSCs do not require oxidative phosphorylation to survive as alternative, hypoxia extends the lifespan, increases their proliferative ability, and reduces differentiation (Gartner et al., 2012, 2013). We recently *in vivo* tested using the rat model, the efficacy of several natural and synthetic biomaterials associated with cellular systems including the MSCs isolated from the UC Wharton's jelly in the treatment of sciatic nerve axonotmesis and neurotmesis injuries (Amado et al., 2008; Gartner et al., 2012; Maurício et al., 2011). Following transection, axons show staggered regeneration and may take substantial time to cross the injured site and enter the distal nerve stump (Gartner et al., 2012, 2013). However, delayed axonal elongation might be caused by growth inhibition originated from the distal nerve itself, growth-stimulating influences may overcome axons stagger. As a potential source of growth-promoting signals, MSCs transplantation is expected to give a positive outcome. Our results showed that the use of either undifferentiated or differentiated MSCs in axonotmesis and neurotmesis lesions boosted the recovery of sensory and motor function. In both cell-enriched experimental groups, we observed that the myelin sheath was thicker; this suggests that MSCs might apply their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Gartner et al., 2012, 2013). Also results from *in vivo* testing previously performed showed that infiltration of MSCs from the Wharton's jelly or the combination of chitosan type III membrane enwrapment and MSCs enrichment after nerve crush injury provide an advantage to post-traumatic nerve regeneration (Amado et al., 2008; Costa et al., 2009; Gartner et al., 2012; Maurício et al., 2011). Chitosan type III was developed as a hybrid of chitosan by adding GPTMS. A synergistic effect of an extra permeability and physicochemical properties of chitosan type III and the presence of silica ions may be responsible for the good results in post-traumatic nerve regeneration promotion observed in the sciatic nerve after axonotmesis and neurotmesis suggesting that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration (Amado et al., 2008; Simoes et al., 2010). The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation,

and myelinization (Amado et al., 2008; Gärtner et al., 2012; Simoes et al., 2010). Our data also showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients (Maurício et al., 2011). The MSCs from the Wharton's jelly may be a valuable source in the repair of the PNS with capacity to differentiate into neuroglial-like cells. The transplanted MSCs are also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Gartner et al., 2012; Gärtner et al., 2012). Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplasic origin (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Simoes et al., 2010). The MSCs isolated from the Wharton's jelly and delivered through PLC and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. Results demonstrated that the use of either undifferentiated or neuroglial-like differentiated MSCs enhanced the recovery of sensory and motor function of the rat sciatic nerve in axonotmesis and neurotmesis injuries (Gartner et al., 2012; Gärtner et al., 2012). The myelin sheath was thicker in the regenerated nerves, suggesting that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Gartner et al., 2013). Many researchers believe that the implanted MSCs exert neurotrophic functions by producing an array of soluble factors or via a direct cell-to-cell contact rather than replacement of damaged nerve

tissues and cells. We have been using undifferentiated MSCs with positive results concerning the functional and morphologic recovery of the nerve after axonotmesis and neurotmesis injuries, in an attempt to diminish the possible adverse effects related to an *in vitro* induced differentiation of the MSCs. Moreover, direct use of MSCs without additional treatments is more favorable to clinical applications owing to simplify the treatments, which implies a less financial burden. In addition, the MSCs isolate from the Wharton's jelly represent a noncontroversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used for that transport) of the UC units from the hospital/clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved tissue. It is highly recommended that the transport from the clinic to the hospital should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

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CHAPTER FIVE

Adipose-Derived Stem Cells and Nerve Regeneration: Promises and Pitfalls

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Contents

Abstract

In order to improve the outcome of nerve regeneration following peripheral trauma injuries, the development of bioengineered nerve grafts has attracted great attention in the field of tissue engineering. Adult stem cells constitute the ideal alternative to Schwann cells (SCs) as transplantable cells in bioartificial nerve grafts. Among the various sources of stem cells with potential applications for regenerative medicine, the adipose tissue has been proven to be one of the most promising. Adipose-derived stem cells (ASCs) are easily obtained, rapidly expanded, show low immunogenicity, and can be differentiated into SCs *in vitro*. This chapter will focus on recent advances in the use of differentiated and undifferentiated ASCs for peripheral nerve regeneration, with a critical attention for the clinical exploitability of ASC in nerve repair strategies.

1. INTRODUCTION

Injuries to peripheral nerves constitute a substantial clinical problem, which affects 1:1000 people, constituting up to 5% of total trauma patients and representing a major economical burden for society (Adams, Arruda, & Larkin, 2012; Zochodne, 2012). The peripheral nervous system (PNS)

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possesses an intrinsic regeneration capability, which allows the nerve to spontaneously regenerate when there is nerve continuity after the damage (Chen, Yu, & Strickland, 2007). Nevertheless, the functional outcome of this spontaneous regeneration is often not satisfactory, leaving patients with physical morbidity that decreases their quality of life (Terenghi, 1999). Moreover, the delay occurring between injury and repair causes peripheral atrophy in the tissues that are lacking innervation, together with neuronal cell death of a portion of the motor and sensory neurons that have lost contact with the periphery (Reid et al., 2011; Terenghi, Hart, & Wiberg, 2011). This is of particular relevance especially for severe transection injuries, in which the regenerating nerves need to grow across large gaps (Sulaiman & Gordon, 2009).

Current techniques for the treatment of peripheral nerve injuries consist in surgical intervention to join the proximal and distal stumps of the damaged nerves. However, when the gap is not repairable without creating tension on the nerve fibers, a nerve autograft is performed (Wiberg & Terenghi, 2003). This requires the discomfort of a second surgery to source the donor nerve tissue, which is limited in quantity and can cause neuroma formation and donor site morbidity (Bell & Haycock, 2012; Keilhoff & Fansa, 2011). Allografts and xenografts represent a viable alternative to autografts; nevertheless, they require systemic immunosuppression to avoid rejection (Bell & Haycock, 2012; Wiberg & Terenghi, 2003). More recently, great efforts in the field of tissue engineering have been focused on the development of bioengineered nerve grafts (Fig. 5.1). This approach consists in the creation of a construct composed of a nerve guide, or conduit, enriched with elements aimed to improve the outcome of the regeneration, such as transplanted supporting cells, extracellular matrix (ECM) molecules, and growth factors (Chalfoun, Wirth, & Evans, 2006).

The obvious choice of transplantable cells to be used in engineered nerve grafts would be Schwann cells (SCs), the glial cells of the PNS (Tohill & Terenghi, 2004). SCs play extensive key roles in the response of the PNS to axonal injury. They participate in the immune response that recruits macrophages and they actively help in removing myelin debris in the distal stump, a process known as Wallerian degeneration (Bhatheja & Field, 2006; Chen et al., 2007). They also form structures called bands of Büngner, which are essential to guide the sprouting axons toward the target organs (Ide, 1996). Finally, they produce several cytokines and growth factors that are known to help and facilitate nerve regrowth, such as nerve growth

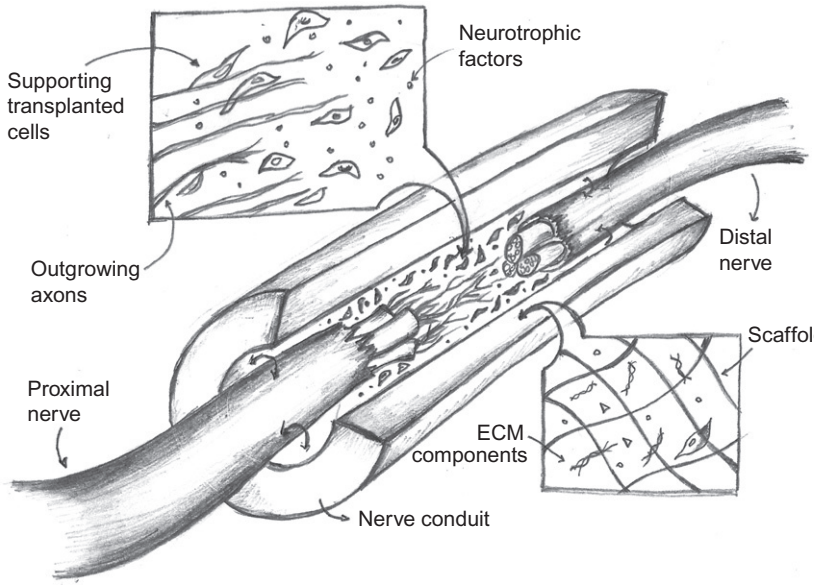

Figure 5.1 Schematic model of a bioengineered nerve graft. As alternative for the autologous graft, which remains the "gold standard" for nerve repair, several alternatives are under investigation. The development of ideal bioengineered nerve conduits requires the optimization of critical components: (i) scaffold biomaterials, (ii) transplanted cells supporting axonal regeneration, (iii) neurotrophic factors (opportunely delivered or released by transplanted cells), and (iv) extracellular matrix (ECM) molecules.

factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor as well as basic fibroblast growth factor and neurotrophin-3 (Chen et al., 2007; Ide, 1996; Jessen & Mirsky, 2008). As a proof of these neurotrophic effects, cultured SCs have been successfully transplanted in animal models in order to promote regeneration following peripheral nerve injuries (Guenard, Kleitman, Morrissey, Bunge, & Aebischer, 1992; Kalbermatten et al., 2008; Mosahebi, Fuller, Wiberg, & Terenghi, 2002; Mosahebi, Woodward, Wiberg, Martin, & Terenghi, 2001). Nevertheless, SCs lack some of the features that characterize the ideal transplantable cells for tissue engineering, such as the ease of harvest, the rapid expansion in culture, and a low immunogenicity (Tohill & Terenghi, 2004). For this reason, the attention of the researchers has moved toward the use of stem cells, which present all the advantages of an ideal candidate cell for regenerative medicine.

2. STEM CELLS FOR PERIPHERAL NERVE REPAIR

Stem cells are undifferentiated precursors that can divide into daughter cells with identical potential in a process called self-renewal, or that can commit to a pathway leading to differentiation along a variety of cell lineages (Bajada, Mazakova, Richardson, & Ashammakhi, 2008; Raff, 2003). Stem cells are defined *pluripotent* if they can generate all the three classical germ layers of the embryo (i.e., ectoderm, mesoderm, and endoderm); this is the case of embryonic stem cells (ESCs), which are derived from the inner cell mass of the blastocyst (Bajada et al., 2008). Although they have shown huge potential, ESCs have not yet been clinically used in regenerative medicine because of moral and ethical issues as well as for the known risks of solid tumor formation (*teratomas*) and possible differentiation into undesirable cell types (Bajada et al., 2008; Choumerianou, Dimitriou, & Kalmanti, 2008). Most, if not all, adult organs contain small subpopulations of adult stem cells, or at least can produce stem cells in culture (Raff, 2003). Adult stem cells are considered *unipotent* or *multipotent* if they can generate only one cell type or all the lineage of a specific germ layer, respectively. The primary role of this pool of undifferentiated cells is to maintain and repair the functional specialized cells of the tissue to which they belong (Tohill & Terenghi, 2004). In the past decades, increasing interest in the field of regenerative medicine has focused on adult stem cell plasticity, which is the ability of somatic stem cells to transdifferentiate into cells of another lineage under proper stimulation (Bhatheja & Field, 2006; Forbes, Vig, Poulsom, Wright, & Alison, 2002; Raff, 2003; Tohill & Terenghi, 2004). More recently, many studies have been focused on the genetic reprogramming of somatic cell in order to produce the so-called induced pluripotent stem cells (Chambers & Studer, 2011). These new findings on plasticity overturn the dogma of organ-specific differentiation of adult stem cells and move the interest of regenerative medicine toward adult stem cells as a potential ESC alternative for therapeutic use, due to fewer moral and ethical implications related to the use of adult tissues (Tohill & Terenghi, 2004).

Adult stem cell niches are distributed throughout the body including the brain, bone marrow, fat, skeletal muscle, liver, retina, and skin (Raff, 2003). Hematopoietic stem cells and mesenchymal stem cells derived from the bone marrow (BM-MSC) were the first stem cell populations to be extensively characterized and to be used in clinical applications (Raff, 2003; Tropel et al., 2004). BM-MSC can differentiate both *in vitro* (under the

influence of appropriate signals) and *in vivo* (after transplantation) into mesenchymal cell types, which lead to formation of bone, cartilage, and fat (Bajada et al., 2008; Barry & Murphy, 2004; Garcia-Castro et al., 2008). Moreover, cultured BM-MSCs have also been reported to transdifferentiate into nonmesodermal lineages—such as myocyte, endothelial, and hepatic cells as well as smooth muscle, neurons, and notably glial cells (Caddick, Kingham, Gardiner, Wiberg, & Terenghi, 2006; Garcia-Castro et al., 2008; Tohill & Terenghi, 2004). In particular, under appropriate stimuli, BM-MSC can originate SC-like cells with similar molecular markers, morphology, and secretion of growth factors (Caddick et al., 2006; Dezawa, Takahashi, Esaki, Takano, & Sawada, 2001; Mahay, Terenghi, & Shawcross, 2008), which were shown to promote nerve regeneration after injury (Tohill, Mantovani, Wiberg, & Terenghi, 2004).

The adipose tissue contains, besides adipocytes and pre-adipocytes, a heterogeneous stromal population of cells called stromal vascular fraction (SVF), which consists of microvascular endothelial cells, blood cells, fibroblasts, smooth muscle cells, and stem cells (Gimble, Katz, & Bunnell, 2007; Strem et al., 2005). Like BM-MSC, adipose-derived stem cells (ASCs) present important characteristics typical of stem cells such as extensive self-renewal capacity and ability to differentiate into multiple lineages (bone, fat, cartilage, and muscle) (Zuk et al., 2002). Furthermore, ASC and BM-MSC share more than 90% of phenotype markers; however, differences in surface protein expression have been reported (Gimble et al., 2007). ASCs express general mesenchymal stem cell markers (i.e., CD29, CD44, CD54, CD90, CD105, and stro-1) and lack expression of the hematopoietic antigens CD14 and CD45 (Faroni, Terenghi, & Magnaghi, 2012; Kalbermatten, Schaakxs, Kingham, & Wiberg, 2011; Kingham et al., 2007; Locke, Windsor, & Dunbar, 2009; Reid et al., 2011). The harvest of BM-MSC from bone marrow is a highly invasive procedure that presents practical constraints such as pain at the harvest site and limited volume of harvested fluid (Locke et al., 2009). By contrast, humans have abundant subcutaneous fat deposits and ASCs can easily be isolated by conventional liposuction procedures under local anesthesia, thus overcoming the problems related to painful bone marrow harvesting procedures (Kingham et al., 2007). Furthermore, the reported number of MSCs among the whole bone marrow cell population is between 1 in 25,000 and 1 in 100,000, whereas the average frequency of ASCs in processed lipoaspirate cells is ~2% (Strem et al., 2005). In addition, compared to BM-MSC, ASCs are also easier to culture for longer periods and show faster growth rates (Locke et al.,

2009). Regarding their immunological properties, it is important to note that less than 1% of ASCs expresses HLA-DR proteins, whereas the majority of ASC population expresses MHC class I molecules, making them optimal candidates for allogeneic transplantation (Strem et al., 2005). Furthermore, it also appears that ASCs may provide angiogeneic (Miranville et al., 2004; Rehman et al., 2004) and, possibly, hematopoietic support (Gimble & Guilak, 2003a, 2003b).

Besides the ability to differentiate into classical mesenchymal lineages, ASCs have also shown the capacity of commitment to differentiation following nonmesenchymal pathways, generating cells expressing markers of cardiac myocytes, hepatocytes, pancreatic cells, skeletal muscle, blood vessels, and neurons (Gimble & Guilak, 2003b; Gimble et al., 2007; Locke et al., 2009; Strem et al., 2005). Recently, different groups have also demonstrated that ASC can be differentiated *in vitro* into an SC-like phenotype using a protocol analogous to BM-MSC glial differentiation (Jiang et al., 2008; Kingham et al., 2007; Xu et al., 2008). Nevertheless, other groups have used different protocols to achieve similar results (Radtke, Schmitz, Spies, Kocsis, & Vogt, 2009; Razavi, Ahmadi, Kazemi, Mardani, & Esfandiari, 2012). In the following sections of this chapter, we will summarize recent progress in the study of ASC for the treatment of peripheral nerve injuries. In particular, *in vitro* experiments as well as *in vivo* animal models employing undifferentiated and differentiated stem cells will be discussed with a view to future clinical application of ASC for nerve repair.

3. UNDIFFERENTIATED ADIPOSE-DERIVED STEM CELL (UASC)

Many *in vitro* studies have been addressed to the regenerative potential of undifferentiated ASC (uASC). One of the main concerns related to the use of uASC in cell-based therapies is arguably the potential differentiation into nondesirable phenotypes, such as adipocytes (Santiago, Clavijo-Alvarez, Brayfield, Rubin, & Marra, 2009), or the risk of tumor formation. Nevertheless, the lack of an *in vitro* differentiation step may constitute an advantage when there is a time-urgency in treating peripheral nerve injuries to avoid chronic denervation of peripheral targets.

Depending on the anatomical site of harvesting, uASCs have shown different properties in cell culture models. Human uASCs obtained from superficial fat deposits are obtained with higher yields; they proliferate faster and promote increased neurite outgrowth when compared with ASCs

obtained from deep layers (Kalbermatten et al., 2011). Moreover, the harvest site and the age of the donor influence the growth rate and the neurotrophic potential of mouse and rat uASC (Engels et al., 2013; Sowa, Imura, Numajiri, Nishino, & Fushiki, 2012). If lipoaspirates are directly used for nerve repair, without the isolation of ASC from the SVF, nerve regeneration is hindered, probably due to the fat occluding the nerve conduits (Papalia et al., 2012). Interestingly, uASC conditioned media were shown to promote neurite growth (Sowa et al., 2012) and prevent neuronal death (Zhao et al., 2009), effects that have been associated with the release of neurotrophic factors such as NGF and BDNF.

Several *in vivo* models have been employed to demonstrate the neurotrophic potential of uASC for the treatment of peripheral nerve damage. Santiago et al. reported that human uASCs enhanced peripheral nerve regeneration and decreased muscular atrophy when transplanted into poly-caprolactone nerve guides to repair a 6-mm nerve gap in athymic rats; cells survived up to 12 weeks after transplantation but did not differentiate into SCs (Santiago et al., 2009). Similarly, uncultured and fluorescent-labeled ASCs were found 2 weeks after surgery in repaired nerve gaps, where they improved regeneration, although they did not colocalize with endogenous SCs nor differentiated (Suganuma et al., 2013). Nonetheless, another study reported a significant loss of cell number only 2 weeks after transplantation, although the initial trophic boost due to uASC, together with the purported recruitment of endogenous SCs, was still able to improve nerve regeneration (Erba et al., 2010). A similar trophic support was shown following transplantation of ASC in laminin-deficient mice; ASCs were able to rescue the neuropathic phenotype and induce endogenous SCs to facilitate axon sorting, myelination, and functional recovery (Carlson et al., 2011). The uASCs were used to populate acellular nerve allografts which improved nerve regeneration across a 15 mm nerve gap; walking behavior, muscle mass, nerve conduction velocity, and number of myelinated axons were all improved in the ASC-populated allografts (Liu et al., 2011). Similarly, porcine de-cellularized nerves enriched with uASC successfully improved nerve regeneration across 10-mm rat sciatic nerve gaps, through a mechanism involving a transforming growth factor β-dependent release of vascular endothelial growth factor (VEGF) (Luo, Zhang, Zhang, & Jin, 2012). VEGF is known to be produced by ASC and might be responsible to neo-angiogenesis and improved regeneration in the injury site (Luo et al., 2012; Marconi et al., 2012; Sowa et al., 2012). Interestingly, a restricted number of human uASCs were able to migrate to the site of a sciatic nerve crush injury following systemic injection;

this was associated with increased expression of neurotrophic factors in the injured milieu, with reduced inflammation and with improved regeneration (Marconi et al., 2012). This could represent an alternative approach for stem cell delivery in cell-based therapies.

4. DIFFERENTIATED ADIPOSE-DERIVED STEM CELL (SC-LIKE ASC)

Although uASCs have been proved to be beneficial for nerve repair, most likely through trophic support rather than direct transdifferentiation into SCs, many studies have attempted to predifferentiate ASC *in vitro*, prior to transplantation, into SC-like differentiated ASC (dASC). This approach brings two important benefits: (i) Since the transplanted cells are already committed, there is a reduced risk of precursor differentiation into undesired cell phenotypes or teratoma formation; (ii) Besides providing trophic support, dASC could actively participate, together with endogenous SCs, in the processes related to regeneration and myelination.

Different approaches have been employed to obtain glial differentiation of ASC. Kingham et al. obtained dASC from rat uASC using a 2-week protocol involving the combination of several growth factors aimed to mimic the developmental environment of native SCs (Kingham et al., 2007). dASC obtained by this means expresses SC markers (such as glial fibrillary acidic protein (GFAP), S100, and the low affinity receptor for NGF p75), and they were shown to promote neurite outgrowth *in vitro* (Kingham et al., 2007). The mechanism of this differentiation is poorly understood, nevertheless it has been shown that it is independent from notch signaling pathways (Kingham, Mantovani, & Terenghi, 2009). Shortly after the first report, other groups used the same protocol to obtain and further characterize dASC with *in vitro* and *in vivo* models. Moreover, dASCs with similar phenotypes were also obtained with different protocols involving the formation of neurospheres (Radtke et al., 2009; Razavi et al., 2012, 2013) or by indirect cocultures with native SCs (Wei et al., 2010). Kaewkhaw et al. showed that the anatomical origin of the fat from which ASCs are derived can influence the glial differentiation and suggested subcutaneous and perinephrium fat as the optimal source for deriving dASCs (Kaewkhaw, Scutt, & Haycock, 2011). The age of the donor did not seem to affect the neurotrophic potential of dASC, however, markers of senescence were detected in dASC from old rat, but not in cells obtained from young or new-born animals (Mantovani et al., 2012). Using *in vitro* coculture models with dorsal root ganglia (DRG)

neurons or PC12 cells, dASCs have been shown to express myelin protein (Mantovani et al., 2010) and form myelin structures (Xu et al., 2008).

Different strategies have been attempted to improve further the neurotrophic potential of dASC. Experiments using direct and indirect cocultures of dASC and DRG neurons showed that ECM molecules influence cell viability, adhesion, and neurotrophic behavior of dASCs (di Summa, Kalbermatten, Raffoul, Terenghi, & Kingham, 2013). This is of particular relevance for the engineering of bioartificial construct in which the cells will be delivered; coating nerve conduits with ECM molecules could enhance the regeneration rate and improve the final outcome. Another approach consists in the treatment of the cells with growth factor and other drugs able to improve the regenerative potential. Treatment of dASCs with leukemia inhibitory factor increased the expression levels of the glial markers S100, GFAP, and of the myelin basic protein, proving that the myelinating potential can be improved with the treatment with cytokines (Razavi et al., 2013). Interestingly, dASCs have been shown to express $GABA_A$ and $GABA_B$ receptors, which can be targeted with specific agonists to modulate important physiological parameters such as cell proliferation and neurotrophin expression and release (Faroni, Calabrese, Riva, Terenghi, & Magnaghi, 2013; Faroni et al., 2011, 2012). This opens new opportunities for a pharmacological modulation of the regenerative potential of dASC for the treatment of nerve damage.

Many *in vivo* studies have confirmed the increased ability of dASC to promote nerve regeneration. Fibrin conduits seeded with dASC were shown to promote regeneration toward a 1-cm nerve gap after 2 weeks (di Summa et al., 2010), and to reduce muscle atrophy while increasing the size and the number of myelinated fibers after 16 weeks (di Summa et al., 2011). A similar increase in myelinated axons and improvements in sciatic function index and nerve conduction velocity were also observed 6 months after surgery in nerve repaired with silicon tubes seeded with dASC in a collagen gel (Orbay, Uysal, Hyakusoku, & Mizuno, 2011). In addition, a novel synthetic nerve guide made of poly ε-caprolactone seeded with dASC was effective in preventing DRG neuronal loss after axotomy (Reid et al., 2011). Nerve regeneration and remyelination were shown to improve after repair with allogeneic grafts from de-cellularized nerves (Wang et al., 2012) and arteries conduits (Sun, Zhou, Mi, & Qiu, 2011) repopulated with rat dASCs. Recently, human ASCs were also successfully differentiated into dASCs and transplanted into crushed tibial nerves of athymic rats where they remyelinated regenerated axons with a higher extent

compared to human uASC (Tomita et al., 2013). This suggests that human ASC might be a clinically viable option for the cell-based therapies for peripheral nerve disorders, and that dASC might be a preferable option rather than the undifferentiated counterparts.

5. CONCLUSIONS

There is an increasing body of literature supporting the evidence that ASC could constitute a potential tool for the treatment of injuries to the peripheral nerves. We envisage a clinical scenario in which fat could be harvested from patients suffering from nerve injuries to derive and expand quickly adipose stem cells *in vitro*. ASC could then be directly transplanted into bioengineered nerve guides for the repair of the nerve damage, or trans-differentiated into dASC prior to reimplantation (Fig. 5.2). Great debate is

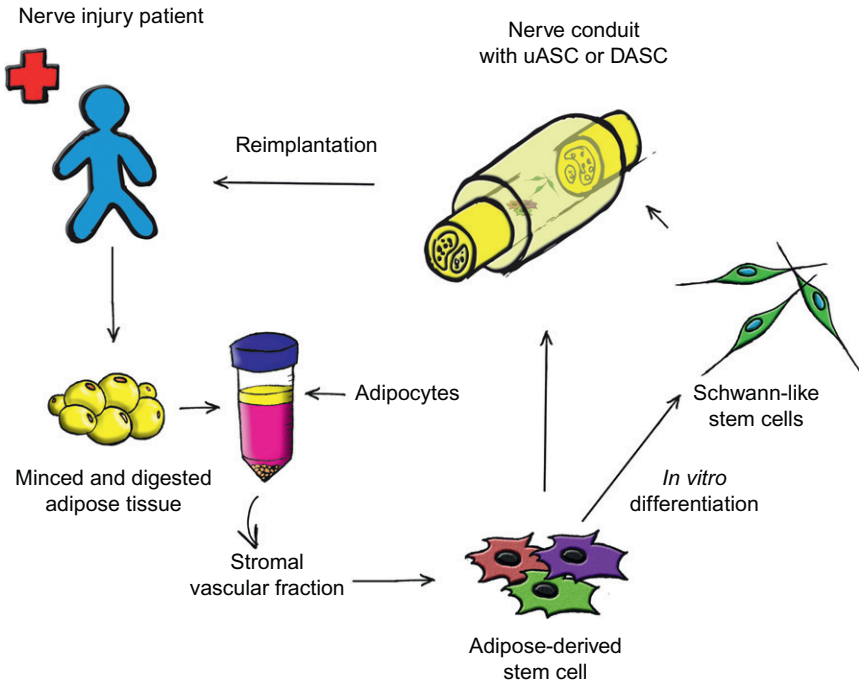

Figure 5.2 Clinical scenario for adipose stem cell-based therapy for peripheral nerve injuries. Adipose stem cells could be derived from patients suffering from peripheral nerve trauma and transplanted into nerve conduits with or without prior differentiation in SC-like cells.

focused on whether undifferentiated or differentiated ASCs represent the most clinically viable option. Whereas, uASCs have the advantage of being readily available without requiring an *in vitro* predifferentiation step, their use could be associated with differentiation in unwanted cell types or risks of teratoma formation. Moreover, uASCs only provide trophic support without *in vivo* commitment to SC phenotype or myelin formation. By contrast, dASCs are generally believed to provide a better regeneration outcome and, in combination with endogenous SCs, they actively participate in the remyelination of the regenerated axons. For this reason, they seem to be the best option for cell-based therapies for peripheral nerve regeneration. However, a better understanding of the differentiation mechanisms and a deeper characterization of the final phenotype are key requirements before dASC could be employed in a clinical setup. Finally, other factors such as the engineering of the graft microenvironment with ECM molecules or the pharmacological modulation of dASC should be considered in order to improve cell survival, neurotrophic potential and, by consequence, the final outcome of nerve regeneration.

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CHAPTER SIX

The Pros and Cons of Growth Factors and Cytokines in Peripheral Axon Regeneration

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Contents

Abstract

Injury to a peripheral nerve induces a complex cellular and molecular response required for successful axon regeneration. Proliferating Schwann cells organize into chains of cells bridging the lesion site, which is invaded by macrophages. Approximately half of the

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injured neuron population sends out axons that enter the glial guidance channels in response to secreted neurotrophic factors and neuropoietic cytokines. These lesion-associated polypeptides create an environment that is highly supportive for axon regrowth, particularly after acute injury, and ensure that the vast majority of regenerating axons are directed toward the distal nerve stump. Unfortunately, most neurotrophic factors and neuropoietic cytokines are also strong stimulators of axonal sprouting. Although some of the axonal branches will withdraw at later stages, the sprouting effect contributes to the misdirection of reinnervation that results in the lack of functional recovery observed in many patients with peripheral nerve injuries. Here, we critically review the role of neuronal growth factors and cytokines during axon regeneration in the peripheral nervous system. Their differential effects on axon elongation and sprouting were elucidated in various studies on intraneuronal signaling mechanisms following nerve lesion. The present data define a goal for future therapeutic strategies, namely, to selectively stimulate a Ras/Raf/ERK-mediated axon elongation program over an intrinsic PI3K-dependent axonal sprouting program in lesioned motor and sensory neurons. Instead of modulating growth factor or cytokine levels at the lesion site, targeting specific intraneuronal molecules, such as the negative feedback inhibitors of ERK signaling, has been shown to promote long-distance regeneration while avoiding sprouting of regenerating axons until they have reached their target areas.

ABBREVIATIONS

ART artemin
BDNF brain-derived neurotrophic factor
Cdc42 cell division control protein 42 homolog
CNS central nervous system
CNTF ciliary neurotrophic factor
CT-1 cardiotrophin 1
DRG dorsal root ganglion
ERK extracellular signal-regulated kinase
ESCRT endosomal sorting complex required for transport
FGF fibroblast growth factor
FGFR fibroblast growth factor receptor
GDNF glial cell-derived neurotrophic factor
GFR GDNF receptor
gp130 glycoprotein 130
Grb2 growth factor receptor-bound protein 2
GSK-3β glykogen synthase kinase 3 beta
IGF insulin-like growth factor
IL interleukin
LIF leukemia inhibitory factor
MVB multivesicular body
NGF nerve growth factor
NT-3/4/5 neurotrophin-3/4/5
NTN neurturin
OSM oncostatin-M

PACAP pituitary adenylate cyclase-activating peptide
PI3K phosphatidylinositol-3 kinase
PKC protein kinase C
PLC phospholipase C
PNS peripheral nervous system
PNT-1 pan-neurotrophin-1
PSP persephin
RET "rearranged during transfection" (tyrosine kinase receptor)
RTK receptor tyrosine kinase
SCG superior cervical ganglion
siRNA small interfering RNA
STAT signal transducer and activator of transcription
Trk tropomyosin receptor kinase
TSC terminal Schwann cells
VIP vasoactive intestinal peptide

1. KEY PROBLEMS IN PERIPHERAL NERVE REGENERATION: SPROUTING AND MISGUIDANCE OF REGROWING AXONS

1.1. Sprouting of axons at the lesion site

Restoration of function after transection of peripheral nerves is poor (Lundborg, 2003), and the occurrence of a postparalytic syndrome, such as paresis, synkinesis, and dysreflexia, is inevitable (Kerrebijn & Freeman, 1998). As demonstrated in the facial nerve lesion model (Fig. 6.1), insufficient recovery is due to the misdirected reinnervation of muscles (Moran & Graeber, 2004). Thousands of regrowing axons are misguided and fail to rejoin their original nerve fascicles (Baker et al., 1994). Each transected axon can sprout up to 25 branches (Mackinnon, Dellon, & O'Brien, 1991). Excessive sprouting leads to reinnervation of several muscle groups by a single motoneuron, often with antagonizing action. Such axonal misdirection during regeneration causes abnormally associated movements (Ito & Kudo, 1994). Furthermore, upon reaching a target, axons can undergo additional intramuscular sprouting and reinnervate several muscle fibers (Son & Thompson, 1995).

Axonal sprouting has been regarded as an adaptive mechanism to compensate for reduced functional capacity after injury. However, it will inevitably result in the enlargement of motor units (Gordon, Hegedus, & Tam, 2004) and reinnervation of motor endplates by more than one axon, a state known as "polyinnervation" (Rich & Lichtman, 1989; Vleggeert-Lankamp

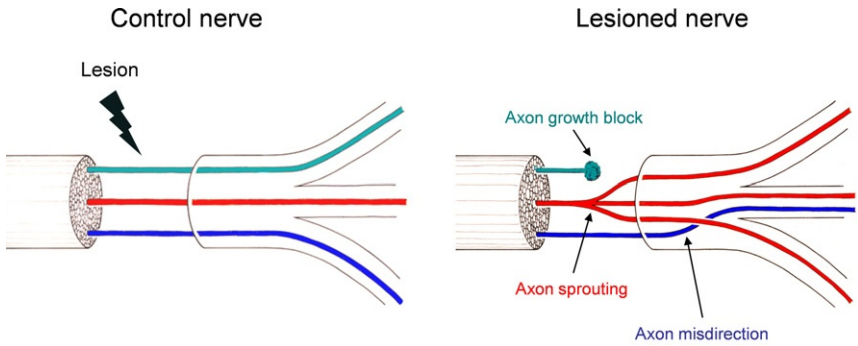

Figure 6.1 After peripheral nerve injuries, only ~60% of patients regain useful function. Three main problems have been identified. First, axons stop elongating and form local sprouts, resulting in neuroma formation. Second, axons elongate but form sprouts that innervate more than one peripheral nerve branch. Third, false regeneration into the wrong nerve can occur, leading to unsuccessful regeneration, for example, if motor axons grow into sensory nerves or vice versa. Current research efforts attempt to identify the relevant neuronal signaling mechanisms and specifically enhance axon elongation without promoting axonal sprouting at the lesion site.

et al., 2005), with muscle fibers being controlled by two or more asynchronously firing motoneurons. Attempts to inhibit the first step of aberrant reinnervation, that is, achieving a "fascicular specificity" (Evans, Bain, Mackinnon, Makino, & Hunter, 1991; Mackinnon, Dellon, Lundborg, Hudson, & Hunter, 1986), have failed to date. Though claimed to be transient (Hennig & Dietrichs, 1994), misguided axons may persist for extended periods of time with deleterious effects on synchronized function (Mackinnon et al., 1991; Madison, Archibald, Lacin, & Krarup, 1999). Thus, postlesional axonal sprouting results in failure to solely reinnervate the original muscle (Angelov et al., 1999).

Significant problems arise in the sensory system as well. For example, injury-induced sprouting of A-, C- or sympathetic fibers is likely to be involved in the generation of intractable pain at the level of the peripheral nociceptor or within affected dorsal root ganglia (for a review see Ramer, Thompson, & McMahon, 1999). Since neuropathic pain is often exacerbated by increased activity of the sympathetic nervous system, coupling of sympathetic and sensory axons may be involved in the development of neuropathic pain.

1.2. Cellular mechanisms of axonal regeneration and sprouting

Injury to peripheral nerves initiates a complex series of changes distal to the site of injury, collectively known as Wallerian degeneration. Within 24 h after lesion, the axonal content begins to necrotize and axonal debris is phagocytosed by blood-borne macrophages and proliferating Schwann cells (Hirata, Mitoma, Ueno, He, & Kawabuchi, 1999; Perry & Brown, 1992). The process of Wallerian degeneration creates an environment that is highly supportive for axonal growth. When resorption is complete, the Schwann cells form long chains (called bands of Buengner) that bridge the inter-fragmentary gap and form guidance channels for the regenerating axons on their way to target organs. The preference for axonal growth into a degenerating nerve ensures that the vast majority of axons will regrow into the distal stump if it remains in continuity with the proximal stump (Bisby, Tetzlaff, & Brown, 1995). As stated above, the regenerating axons do not merely elongate toward the distal stump, but respond with axonal sprouting by lateral budding mainly at the nodes of Ranvier up to 6 mm proximal to the injury site. As regeneration proceeds, some of these supernumerary branches are pruned off over a period of up to 12 months (Bray & Aguayo, 1974).

Axonal sprouting begins from the end bulb within 3 h after injury (Sjoberg & Kanje, 1990). The regenerating branches initially lie on the surface of the Schwann cells. Later, these branches increase in diameter and become surrounded by Schwann cell processes. The guidance of these immature axons to their final destination can be considered as a series of short-range projections to intermediate targets under the influence of local guidance cues. Neurons respond to these cues by means of a motile sensory apparatus at the tip of the advancing growth cone, which often does not emerge from the axon at the precise site of injury, but proximal to it (Ziv & Spira, 1997). The initial formation of growth cones occurs before newly synthesized axonal proteins arrive at the site of axon injury, which is too rapid to be dependent on metabolic changes in the cell body (Smith & Skene, 1997). Actin-rich filopodia are responsible for the recognition of specific guidance cues by detecting gradients of trophic or adhesive factors (Lin & Forscher, 1993). Individual filopodia respond to alterations in the environment by changing internal calcium concentration, and filopodia on different parts of the growth cone respond independently via local changes in actin metabolism (Bixby & Harris, 1991; Letourneau, 2009).

1.3. Terminal sprouting of axons in denervated muscles

Regarding the cellular correlates of muscle polyinnervation, it is well known that following nerve injury, denervated terminal Schwann cells (TSCs) enlarge and sprout processes that reach adjacent innervated motor endplates (O'Malley, Waran, & Balice-Gordon, 1999; Reynolds & Woolf, 1992). Using these bridges, TSCs reach, attract, and direct intramuscular axonal sprouts toward denervated endplates (Love & Thompson, 1999; Reddy, Koirala, Sugiura, Herrera, & Ko, 2003). Interestingly, it has been shown that the outgrowth of TSC processes precedes the outgrowth of sprouts from intact intramuscular axons. In other words, TSC are able to initiate intramuscular axonal sprouting (Son & Thompson, 1995). Thus, the beneficial effect of stimulation on muscle reinnervation may be mediated by interfering with the extension of TSC processes and their ability to bridge between the endplates. TSC bridges may be modulated by running exercises (Tam & Gordon, 2003) or electrical stimulation (Love, Son, & Thompson, 2003). Hence, any form of artificially excited muscular activity may inhibit the bridge formation by TSC and reduce postlesional intramuscular sprouting. Considering the molecular correlates of muscle polyinnervation, it should be noted that denervated muscles have been shown to produce short-range diffusible sprouting stimuli (Slack & Pockett, 1982; Zhao, Veltri, Li, Bain, & Fahnestock, 2004). Various neurotrophic factors have been identified as possible candidate stimuli (Raivich & Makwana, 2007; Sendtner, 1998).

2. INDUCTION OF NEURONAL GROWTH FACTORS AND NEUROPOIETIC CYTOKINES FOLLOWING NERVE INJURY

2.1. Polypeptide families involved in peripheral axon regeneration

Neuronal growth factors were originally advocated to be novel treatments to overcome growth arrest by enhancing axon outgrowth during long-distance regeneration, presumably leading to faster recovery of function. They were initially described as target-derived polypeptides synthesized in limited amounts then retrogradely transported along the axon to, ultimately, induce transcriptional events that promote survival and maintenance of neuronal subpopulations required for correct organ innervation (Purves, Snider, & Voyvodic, 1988; Reichardt, 2006). In response to a peripheral nerve injury, a number of neurotrophic factors are upregulated, mainly in glial cells and also in resident or invading macrophages and affected neurons. These

molecules fall into two major classes: the neurotrophic factors (primary roles in neuronal development and adult plasticity) and the neuropoietic cytokines (key functions in various pathological situations, such as trauma, immune responses, and inflammation) (Hopkins & Rothwell, 1995; Lewin & Barde, 1996). They are subgrouped into peptide families according to sequence similarity and homology of receptors to which they bind.

Several comprehensive reviews have described the localization, regulation, and function of neurotrophins (NGF, BDNF, NT-3/4/5), glial cell-derived neurotrophic factor (GDNF) family members (GDNF, NTN, PSP, ART), fibroblast growth factors (FGF-1, FGF-2), insulin-like growth factors (IGF-I, IGF-II), neuregulins (GGF), neuropeptides (galanin, VIP, PACAP), and neuropoietic cytokines belonging to the gp130 family (CNTF, LIF, CT-1, OSM, IL-6, IL-11) in the lesioned peripheral nervous system (PNS) (Boyd & Gordon, 2003; Fawcett & Keynes, 1990; Fu & Gordon, 1997; Gordon, 2009; Grothe, Haastert, & Jungnickel, 2006; Hökfelt, Zhang, & Wiesenfeld-Hallin, 1994; Klimaschewski, Kummer, & Heym, 1996; Terenghi, 1999; Zigmond, 2012). Here, we focus our discussion on the ability of these molecules to promote neuronal survival and regeneration in the lesioned PNS, with particular emphasis on their role in axonal sprouting.

2.2. Requirement of growth factors for neuronal survival after lesion in adults

Neurotrophic factors are important for neuronal survival and neurite outgrowth during development. However, during the perinatal and early postnatal period, neurons gradually lose their dependence on trophic support. In fact, the vast majority of axotomized sensory and motor neurons can survive a peripheral nerve injury in adults, provided that the lesion is not located too close to the neuronal cell body (Vanden Noven, Wallace, Muccio, Turtz, & Pinter, 1993; Xu, Forden, Walsh, Gordon, & Midha, 2010). This is confirmed by earlier observations that adult sensory neurons do not depend on trophic factors if dissociated (axotomized) and placed into culture in defined medium (Lindsay, 1996). It should be noted that significant neuron death (up to 30%) following peripheral nerve injury has been described in sensory ganglia of adult animals (Schmalbruch, 1987). Obviously, this cell death occurs in the presence of endogenous upregulation of growth factors and cytokines. It is possible that growth factors themselves actively participate in the induction of neuronal apoptosis in dorsal root ganglia (DRG). For example, increased FGF levels may lead to DRG neuron death via

activation of FGFR3 (Jungnickel, Gransalke, Timmer, & Grothe, 2004). Alternatively, BDNF has been proposed to negatively influence survival and regeneration via activation of p75 (Boyd & Gordon, 2001). On the other hand, there is evidence that exogenous treatment with FGF-2 (via FGFR1), BDNF (via TrkB), GDNF (via RET), or CNTF (via gp130) protects some of the lesioned motor or sensory neurons after axotomy in adult animal models (Boyd & Gordon, 2003; Terenghi, 1999; Verge, Gratto, Karchewski, & Richardson, 1996).

The true significance of lesion-associated growth factors and cytokines for neuronal survival or axon regeneration requires investigation of the appropriate conditional knockout mice lacking these molecules (or their receptors) in Schwann cells and/or axotomized neurons at postnatal age. Unfortunately, these studies are yet to be performed. Until now, the involvement of growth factors and cytokines in peripheral axon regeneration has been demonstrated mainly by classical pharmacologic approaches. As described below, studies applying neutralizing antibodies rule out an absolute requirement for any of the discussed polypeptides during regeneration in the adult PNS.

2.3. Induction of growth factors and cytokines after nerve lesion is cell-type dependent

The neurotrophins NGF, BDNF, NT-4/5, but not NT-3, are induced in Schwann cells in the distal nerve stump after transection (Funakoshi et al., 1993; Heumann, Korsching, Bandtlow, & Thoenen, 1987; Meyer, Matsuoka, Wetmore, Olson, & Thoenen, 1992). The primary neuronal receptor for nerve growth factor (NGF), TrkA, is neither present nor upregulated in axotomized motoneurons, but decreases in a small neuron population of lesioned DRG (Krekoski, Parhad, & Clark, 1996). In contrast, the pan-neurotrophin receptor p75 is upregulated in denervated Schwann cells and motoneurons (Ernfors, Henschen, Olson, & Persson, 1989; Raivich & Kreutzberg, 1993), but diminished in DRG neurons after sciatic nerve lesion (Verge et al., 1996). Furthermore, BDNF, but not NGF, is rapidly upregulated in motoneurons for a few days after lesion (Kobayashi, Bedard, Hincke, & Tetzlaff, 1996). Synthesis of the BDNF receptor, TrkB, is elevated, too. NT-3 is downregulated in axotomized motoneurons (Funakoshi et al., 1993). Following transection of the buccal branch of a rat facial nerve, rapid upregulation of NGF and BDNF is observed during the first few days postlesion (Streppel et al., 2002). GDNF receptors, GFR-α1 and Ret, are upregulated in motoneurons (Burazin & Gundlach,

1998b; Trupp, Belluardo, Funakoshi, & Ibanez, 1997), and synthesis of GFR-α1 as well as GDNF is increased in the distal nerve following nerve lesion (Naveilhan, Elshamy, & Ernfors, 1997).

FGF-2 isoforms are strongly and rapidly upregulated in response to peripheral nerve lesions. In sympathetic ganglia, expression of low and high molecular weight FGF-2 isoforms is induced at the mRNA and protein level within 24 h after axotomy (Klimaschewski, Meisinger, & Grothe, 1999). In lesioned sciatic nerves, FGF-2 isoforms and FGF receptors (type-1, -2, and -3) are induced in Schwann cells 1 week after lesion. Moreover, FGF-2 is detected in DRG neurons (Grothe et al., 2006; Grothe, Meisinger, Hertenstein, Kurz, & Wewetzer, 1997; Meisinger & Grothe, 1997). Following transection of the facial nerve, upregulation of FGF-2 and IGF-I is observed in Schwann cells of the distal nerve stump, invading macrophages and target muscles of the whisker pad (Glazner, Morrison, & Ishii, 1994; Streppel et al., 2002).

Among neuropeptides with growth factor-like activity (Tatemoto, Rökaeus, Jörnvall, McDonald, & Mutt, 1983), galanin is dramatically upregulated in response to axotomy of sympathetic (Klimaschewski, Grohmann, & Heym, 1996; Klimaschewski, Tran, Nobiling, & Heym, 1994; Mohney, Siegel, & Zigmond, 1994; Schreiber, Hyatt-Sachs, Bennett, & Zigmond, 1994), sensory (Dahlin, Stenberg, & Kanje, 2003; Holmes et al., 2000; Ma & Bisby, 1997; Villar et al., 1989), and motor neurons (Burazin & Gundlach, 1998a; Rutherfurd, Widdop, Louis, & Gundlach, 1992; Saika et al., 1991). Previous studies showed elevated galanin peptide and mRNA in lesioned peripheral ganglia in a majority of axotomized neurons for at least 1 month after nerve injury. Similarly, the proportion of galanin mRNA-expressing neurons in human sensory ganglia increases from approximately 13% of cervicothoracic neurons to ~33% after brachial plexus lesion (Landry et al., 2003). Nerve transection also influences the level of another neuropeptide, pituitary adenylate cyclase-activating peptide (PACAP), that belongs to the secretin/vasoactive intestinal peptide (VIP) family (Arimura et al., 1994). PACAP is upregulated within 24 h after nerve lesion in sensory ganglia (Pettersson, Dahlin, & Danielsen, 2004; Zhang et al., 1996), the sympathetic superior cervical ganglion (Klimaschewski, Hauser, & Heym, 1996; Moller et al., 1997), and the adult rat facial motor nucleus (Zhou et al., 1999). VIP is a 28-amino acid peptide (Said & Mutt, 1970) that is also upregulated after axotomy. VIP expression increases in a subpopulation of mainly intermediate to large-sized neurons of the axotomized rat superior cervical ganglion (Klimaschewski

et al., 1994; Mohney et al., 1994), which is partially due to the release of LIF after axotomy (Sun & Zigmond, 1996).

LIF is an important neuropoietic cytokine rapidly induced at the site of injury, along with CNTFRα, IL6-R, and gp130 (Ito et al., 1998). IL-6 is transiently upregulated in axotomized motoneurons (Kiefer, Lindholm, & Kreutzberg, 1993) and Schwann cells after sciatic nerve injury (Ito et al., 1998). Expression of the signal transduction subunit for all of these cytokines, gp130, is unchanged in axotomized sympathetic (Banner & Patterson, 1994), sensory (Brazda, Klusakova, Svizenska, Veselkova, & Dubovy, 2009), and facial motor neurons (Haas, Hofmann, & Kirsch, 1999), but downregulated in motoneurons after sciatic nerve lesion, whereas specific LIF receptors are induced (Hammarberg, Piehl, Risling, & Cullheim, 2000). CNTF is strongly expressed in Schwann cells of the intact nerve, but its expression decreases in response to injury (Friedman et al., 1992; Sendtner, Stöckli, & Thoenen, 1992). Recently, IL-1β was shown to be increased in Schwann cells within 1 day after sciatic nerve lesion (Temporin et al., 2008).

3. EXOGENOUS MODULATION OF GROWTH FACTORS AND CYTOKINES AT THE LESION SITE

3.1. Functional significance of neuronal growth factors as putative treatments for PNS lesions

Over the years, the possibility of enhancing the regenerative potential of lesioned neurons by exogenous application of growth-promoting molecules has been addressed in a number of studies. Local application of growth factors to the lesioned nerve resulted in enhanced axon outgrowth, which was often accompanied by stimulation of axon sprouting. Neurotrophins act as potent promoters of axon outgrowth *in vitro*, even in the presence of CNS inhibitors (Cai, Shen, De Bellard, Tang, & Filbin, 1999). NGF, BDNF, and NT-3 have all been shown to reverse various changes induced by axotomy of neonatal and adult sensory neurons (Eriksson, Lindsay, & Aldskogius, 1994; Verge et al., 1992; Verge, Richardson, Wiesenfeld-Hallin, & Hökfelt, 1995). On the other hand, the decreased availability of NGF after axotomy may be the appropriate stimulus to induce the conditioning lesion effect (Shoemaker, Sachs, Vaccariello, & Zigmond, 2006), that is, enhanced axon outgrowth following axotomy if neurons have been injured some days before (preconditioned). Peripheral axon regeneration *in vivo* is enhanced by administration of NGF to the lesion site (Terenghi, 1999). However,

antibody-mediated neutralization of NGF does not interfere with regeneration (Diamond, Foerster, Holmes, & Coughlin, 1992; Lankford et al., 2013; Rich, Yip, Osborne, Schmidt, & Johnson, 1984). Rather, it reduces the maladaptive axonal sprouting (Krenz, Meakin, Krassioukov, & Weaver, 1999). NGF and BDNF also play a major role in facial nerve (Streppel et al., 2002) and sympathetic sprouting (Ramer et al., 1999) since treatment with neutralizing antibodies significantly reduce sprouting of facial axons and basket formation in DRGs.

BDNF promotes local axon regeneration of motoneurons after ventral root avulsion (Kishino, Ishige, Tatsuno, Nakayama, & Noguchi, 1997; Novikov, Novikova, & Kellerth, 1997a, 1997b) and increases size and myelin thickness of regenerating sciatic nerve axons, particularly in combination with CNTF (Lewin, Utley, Cheng, Verity, & Terris, 1997). Exogenous BDNF or GDNF, however, do not increase the proportion of regenerating neurons or functional recovery, but contribute significantly to axonal sprouting (Gordon, 2009). Antibodies against BDNF interfere with axon regeneration, reduce the density of myelinated axons, and impair sensory reinnervation (Zhang, Luo, Xian, Liu, & Zhou, 2000). When applied continuously to the lesion site, BDNF and GDNF both increase the number of regenerating neurons in a *chronic* lesion model, where nerve transection is followed by delayed nerve repair after 1 month (for a review see Gordon, 2009). BDNF and TrkB are induced by brief electrical stimulation (20 Hz) and, thereby, promote axon regeneration across the surgical lesion site at which axons grow in a much slower and staggered fashion (in contrast to the more continuous growth rate of 1–3 mm/day observed during distal stump regeneration). Sustained delivery of NGF, GDNF, or NT-3 to the injury site via synthetic nerve guidance channels promotes regeneration of both sensory and motor axons over long gaps (Barras, Pasche, Bouche, Aebischer, & Zurn, 2002; Fine, Decosterd, Papaloizos, Zurn, & Aebischer, 2002).

Neurotrophin (NT)-3 and NT-4/5 also contribute to regeneration in the PNS. Lesioned nerves treated with NT-4 have more regenerated axons with increased diameter and myelin (Yin, Kemp, Yu, Wagstaff, & Frostick, 2001). This is supported by studies in mice with deletion of NT-4/5 that exhibit a decreased axon regeneration rate and reduced response to electrical stimulation (English, Meador, & Carrasco, 2005; English, Schwartz, Meador, Sabatier, & Mulligan, 2007). NT-3 has been shown to support functional regeneration of large diameter afferent axons into the spinal cord after rhizotomy (Ramer et al., 2002) and improve muscle mass and

reinnervation of fast muscle fibers (Sterne, Coulton, Brown, Green, & Terenghi, 1997). Furthermore, delivery of NT-3 restores motor and sensory conduction velocities similar to GDNF treatment (Munson, Shelton, & McMahon, 1997). Nerve lesion in transgenic mice expressing a chimeric neurotrophin, pan-neurotrophin-1 (PNT-1), increases axonal sprouting, but apparently improves motor and sensory innervation of the paw following sciatic nerve crush (Funakoshi et al., 1998).

Insulin-like growth factors (IGFs) control Schwann cell viability (Meier, Parmantier, Brennan, Mirsky, & Jessen, 1999), promote neurite outgrowth of motoneurons (Neff et al., 1993), increase regeneration rate *in vivo* after lesions of the sciatic nerve (Pu, Zhuang, Marsh, & Ishii, 1999), and enhance axonal regeneration in chronically injured neurons (Houle, Ye, & Kane, 1996). The neutralization of IGF causes a sustained reduction in regeneration rate (Glazner, Lupien, Miller, & Ishii, 1993; Near, Whalen, Miller, & Ishii, 1992).

FGFs promote axonal regeneration across a collagen-filled nerve conduit (Aebischer, Salessiotis, & Winn, 1989) and related regeneration models (Danielsen, Pettmann, Vahlsing, Manthorpe, & Varon, 1988; Fujimoto, Mizoguchi, Hanada, Yajima, & Ide, 1997). Conversely, neutralization of FGF-2 causes a significant decrease in the number of regenerating axons (Chen et al., 1999). Channels containing Schwann cells overexpressing the high molecular weight isoform of FGF-2 are particularly useful for promoting regeneration, since axon sprouting appears to be reduced and recovery of thermoception is faster (Haastert, Lipokatic, Fischer, Timmer, & Grothe, 2006; Timmer, Robben, Muller-Ostermeyer, Nikkhah, & Grothe, 2003). This is consistent with our own observations that adult sensory neurons taken from prelesioned animals exhibit longer axons with a reduced number of branches if treated with FGF-2 versus NGF (Klimaschewski, Nindl, Feurle, Kavakebi, & Kostron, 2004). Due to their effects on mitogenesis of mesoderm- and neuroectoderm-derived cells, it is assumed that FGFs not only directly support axonal regeneration but also increase proliferation of Schwann cells and enhance angiogenesis (Aebischer et al., 1989). However, conflicting data come from knockout studies. FGF-2-deficient mice were found to have five times more regenerated myelinated axons with increased myelin and axon diameters compared to wild type if analyzed close to the injury site (Jungnickel, Claus, Gransalke, Timmer, & Grothe, 2004). In FGF-2-overexpressing mice, the number of regenerated axons doubled, but myelin thickness was significantly less 1 week after sciatic nerve crush (Jungnickel, Haase,

Konitzer, Timmer, & Grothe, 2006). Morphometric analysis and functional tests revealed no differences in recovery of sensory and motor nerve fibers in these animals. The number of proliferating cells was significantly increased distal to the crush site compared to wild type, suggesting that endogenously synthesized FGF-2 influences early peripheral nerve regeneration mainly by regulating Schwann cell proliferation and remyelination (reviewed in Grothe et al., 2006).

3.2. Reduction of axonal sprouting by application of neutralizing antibodies to trophic factors

The excessive axonal sprouting could, at least in part, be due to increased expression of trophic molecules at the lesion site. Accordingly, inhibition or blockade of these factors would reduce sprouting and improve the accuracy of reinnervation. Therefore, neutralizing antibodies to several neurotrophic agents were tested in the facial nerve lesion model. Following analysis of local protein expression, the facial nerve trunk of adult rats was transected and both ends were inserted into a silicon tube containing collagen gel with neutralizing concentrations of antibodies to NGF, BDNF, FGF-2, IGF-I, CNTF, and GDNF alone or in combination. Two months later, retrograde labeling was used to estimate the proportion of motoneurons with axons present in all of the three major branches of the facial nerve. Anti-NGF, anti-BDNF, and anti-IGF-I significantly reduced axon sprouting. The most pronounced effect was achieved after application of anti-BDNF, which reduced the portion of branched neurons to 18%. All effects after single application of antibodies were concentration-dependent and superior to those observed after combined treatment. Thus, treatment with antibodies against NGF, BDNF, FGF-2, IGF-I, CNTF, or GDNF nearly doubles the precision of reinnervation, as evaluated by multiple retrograde labeling of motoneurons, compared to control animals (Streppel et al., 2002).

3.3. Effects of exogenous neuropeptides and cytokines on axon regeneration

Galanin and PACAP38 are utilized as modulators of synaptic transmission under normal conditions (Klimaschewski, Kummer, et al., 1996), but act as potent inducers of peripheral axon sprouting in motor and sensory neurons after axotomy. Treatment of DRG neurons with either peptide stimulates axonal branching *in vitro*, and application of galanin or PACAP38 to transected facial nerve dramatically increases the number of facial neurons

with axon collaterals in different branches (Suarez et al., 2006). Functional analysis of these animals revealed that the peptides negatively affect the reinnervation of the whisker pad. Endogenous galanin or PACAP38 may be released to directly activate intracellular signaling pathways involved in axon sprouting after nerve lesion. This hypothesis is confirmed by nerve lesion studies in mice lacking PACAP (Armstrong et al., 2008), galanin (Holmes et al., 2000), or galanin receptors (Hobson, Holmes, Kerr, Pope, & Wynick, 2006).

Cytokines involved in neuropeptide regulation, such as LIF and IL-6, enhance axonal growth in lesioned DRGs (Thompson & Majithia, 1998). Based on partial denervation studies in knockout mice, CNTF also contributes to axonal sprouting (Siegel, Patton, & English, 2000). IL-6 improves axon regeneration in the lesioned hypoglossal (Hirota, Kiyama, Kishimoto, & Taga, 1996) and sciatic nerve (Zhong, Dietzel, Wahle, Kopf, & Heumann, 1999), and IL-6 knockout mice show impairment in sensory, but not motor, reinnervation after sciatic nerve crush (Yao, Moir, Wang, To, & Terris, 1999). These mice exhibit a prominent decrease in inflammatory response around facial neurons after axotomy, accompanied by a moderate reduction in peripheral axon regeneration (Galiano et al., 2001). Interestingly, IL-6 appears to act as a key mediator for the conditioning of lesion-induced enhanced regeneration in injured dorsal column afferents (Cafferty et al., 2004).

4. INTRANEURONAL MECHANISMS UNDERLYING AXON ELONGATION VERSUS SPROUTING

To understand the cellular and molecular biology of the different modes of axon regeneration (axon elongation, axon sprouting/branching, and axon collateralization), it is important to first consider the growth cone forming at the tip of lesioned axons in more detail. Flattened processes (lamellipodia) and numerous stiff fine processes (filopodia) extend from a central core, giving the growth cone a shape similar to a webbed foot. Three major intracellular cytoskeletal components are responsible for the cytomechanical forces at the leading edge of elongating axons: actin microfilaments, myosin, and microtubules (Challacombe, Snow, & Letourneau, 1996). Growth cone formation begins with restructuring of neurofilaments and microtubules to form a region proximal to the tip of the transected axon in which vesicles accumulate. This rearrangement of the cytoskeleton forms a transient cellular compartment that traps the transported vesicles and serves as a locus for

microtubule polymerization. Microtubuli, in turn, facilitate the fusion of vesicles with the plasma membrane, promoting the extension of growth cone lamellipodia (Spira, Oren, Dormann, & Gitler, 2003).

Axonal branching can occur via two mechanisms. During terminal branching, the bifurcation of the growth cone gives rise to two or more axon branches. In contrast, interstitial branching results in *de novo* initiation of axon branches from previously quiescent regions of the axon (Bastmeyer & O'Leary, 1996; Schmidt & Rathjen, 2010). Terminal branching is believed to occur only during early axonal outgrowth, whereas interstitial branching is observed during development and regeneration. During transition from a dynamic filopodium to a stable branch, microtubule bundles splay apart and form loops. Microtubules are then fragmented into shorter pieces, which invade actin-rich structures in the newly formed branch (Gallo, 1998; Kalil, Szebenyi, & Dent, 2000). Subsequently, microtubules become bundled again, thereby stabilizing the new branch (Fig. 6.2). Collateral axon branches are formed by neighboring *uninjured* axons, a process which is distinct from sprouting of lesioned axons. Axons often stop elongating before they start to develop branches (Dent & Gertler, 2003). Sprouting and elongation are regulated by different signaling mechanisms, and inhibitors of axon elongation have almost no effect on collateral branch formation (Gallo & Letourneau, 1998). Similarly, inhibition of branching rarely affects axonal elongation (Szebenyi et al., 2001).

4.1. Neuronal signaling pathways activated by growth factors and cytokines

As outlined above, the growth of axons is strongly influenced by neurotrophic factors and cytokines during development and regeneration of the nervous system (Boyd & Gordon, 2003; Chen, Yu, & Strickland, 2007). While neuropeptides act via specific G-protein-coupled receptors, cytokines utilize the gp130 transmembrane receptor, which leads to activation of JAK/Tyk tyrosine kinases and STAT transcription factors. Growth factors elicit a response via receptor tyrosine kinases (RTKs) at the plasma membrane of neurons. Ligand-induced activation and autophosphorylation of RTKs result in the recruitment of adapter molecules, followed by activation of several intracellular signaling pathways (Zhou & Snider, 2006). The main signaling cascades required for axon growth are the Ras/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3 kinase (PI3K)/Akt pathways (Fig. 6.3). Although signaling pathways overlap for receptors of neuropeptides, cytokines, and growth factors, the duration and strength

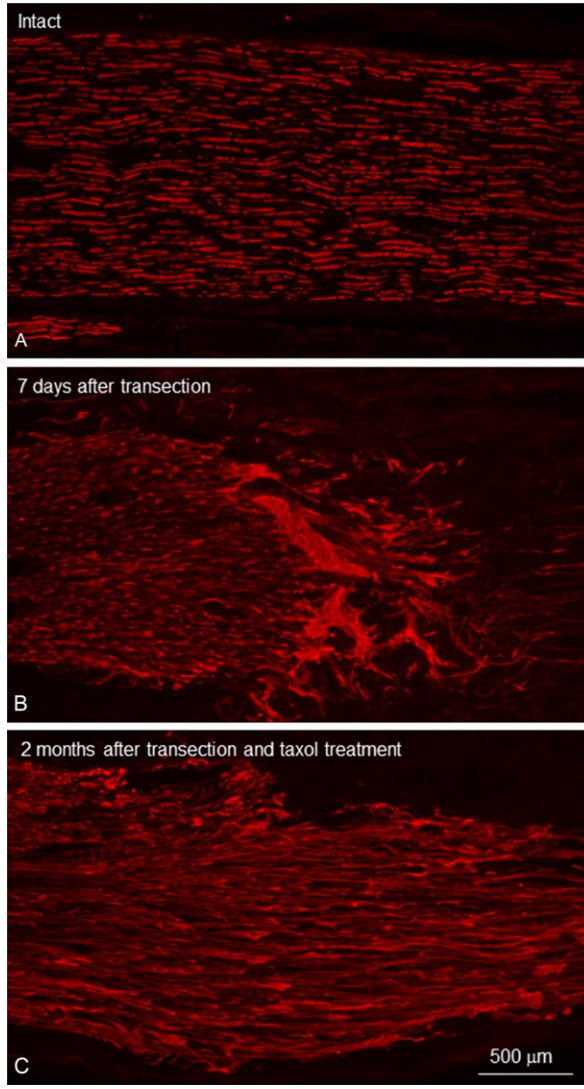

Figure 6.2 Immunocytochemical staining of axons for neuronal class III β-tubulin in the rat buccal branch of the facial nerve. Representative longitudinal sections of an intact nerve with no axonal branching (A), of the proximal stump of the transected nerve inserted into an empty silicon tube displaying vigorous axonal branching (B), and of the proximal fragment, which had been inserted into a silicon tube filled with 10 µg/ml taxol in collagen (C). Please note the reduced amount of axonal branches by taxol which acts via stabilizing microtubules.

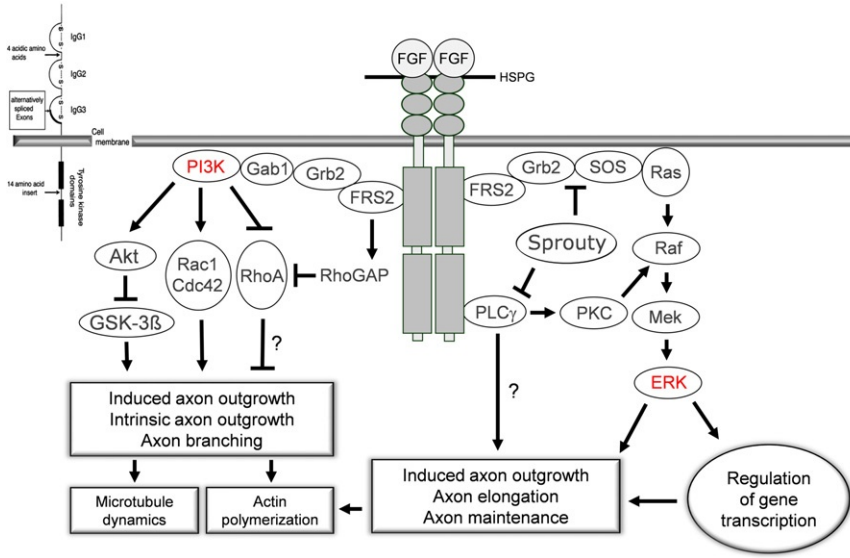

Figure 6.3 The PI3K/Akt- and Ras/Raf/ERK-signaling pathways are activated by receptor tyrosine kinases upon growth factor binding which is here exemplified by FGF-dependent activation of the FGF receptor (FGFR1). Both pathways are crucial for axon outgrowth during development and regeneration. The activation of the ERK machinery is implicated in neuronal survival and elongative axon growth following injury. PI3K/Akt signaling is central to the regulation of cytoskeletal proteins and is linked to neuronal survival and axonal branching. Controlled by PI3K signaling, small GTPases are key regulators of the growth factor-dependent effects on microtubule dynamics and actin polymerization.

of their activation differ with respect to receptor isoforms between families and even within the same family. Here, we will focus our discussion on growth factor-dependent RTKs.

Upon ligand-induced activation of RTKs, the ligand–receptor complex is internalized by endocytosis and transported through the cytoplasm in early and late endosomes/multivesicular bodies (MVBs) and finally lysosomes (Fig. 6.4; Romanelli & Wood, 2008). The signaling endosomes are transported to considerable distances by retrograde axonal transport (Grimes et al., 1996). The whole complex, including the transport vesicle, ligand, receptor, and eventually downstream signaling molecules, is carried by the motor protein dynein along microtubules. Upon arrival at the cell body, some of the signaling molecules are released, pass through the nuclear pore, and regulate neuronal gene expression (Wu et al., 2007). Unlike RTKs

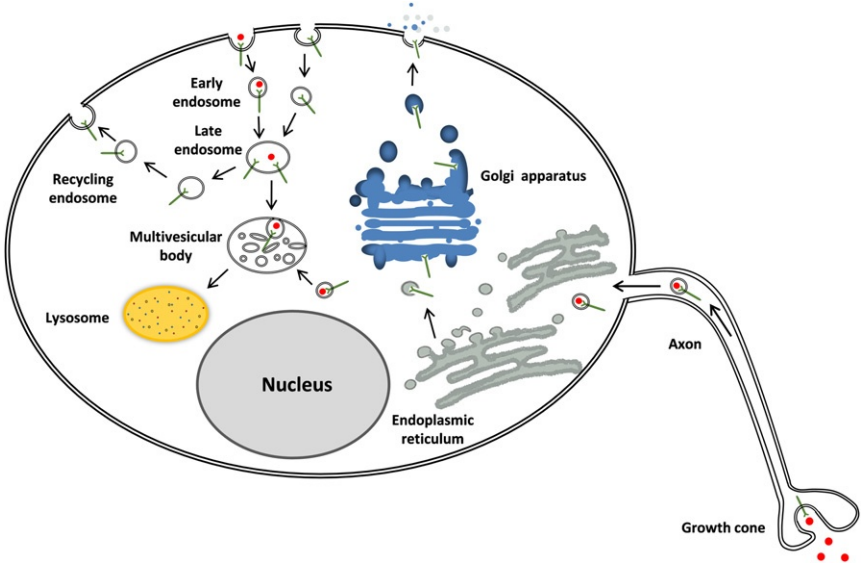

Figure 6.4 Intraneuronal transport of growth factors and their receptors. Receptors (green) are synthesized at the rough endoplasmic reticulum (gray), shuttled to the Golgi apparatus (blue) for posttranslational modification and incorporated into the plasma membrane via vesicle exocytosis. Upon stimulation with the appropriate ligand (red) various signaling cascades are activated. The receptors are internalized by endocytosis at the injury site (growth cones) and at the plasma membrane of the perikaryon, since growth factors and cytokines are also released from glial cells located around neuronal cell bodies. At the level of the early endosome, it is then decided whether the receptor is shuttled back to the cell surface via recycling endosomes or whether the early endosomes fuse with multivesicular bodies targeting the receptors for degradation inside lysosomes (yellow).

located at the plasma membrane, receptors within endosomal membranes can continue to stimulate activation of distinct signaling pathways (Sorkin & Von, 2002; Zhang, Moheban, Conway, Bhattacharyya, & Segal, 2000). Signaling of the receptor stops upon transfer to the lysosome as a consequence of endosomal sorting complex required for transport (ESCRT)-mediated endocytosis into the inner membranes of the MVB. Alternatively, receptors located in early endosomes may be recycled back to the plasma membrane either directly or via the endocytic recycling compartment (Platta & Stenmark, 2011). The decision of whether a receptor signals from the outer plasma membrane or from recycling endosomes is critical for axon regeneration (Fig. 6.5). Enhanced recycling of RTKs has recently been demonstrated to promote elongative axon growth *in vitro* (Ascano,

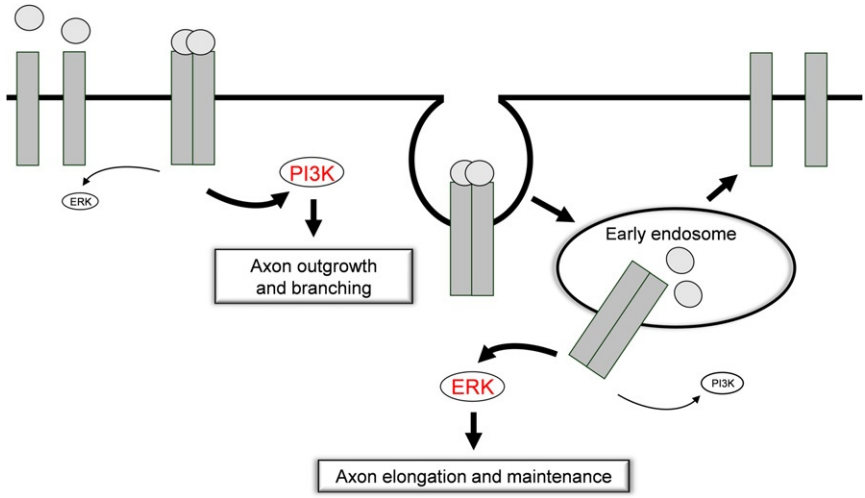

Figure 6.5 Plasma membrane-bound RTKs (e.g., FGFR1) primarily stimulate PI3K to promote axonal branching, whereas endocytosis of FGFR1 into early endosomes is strongly activating the ERK pathway to induce axon elongation. The balance of activation of ERK over PI3K appears to be the decisive parameter for long-distance regeneration of adult peripheral neurons.

Richmond, Borden, & Kuruvilla, 2009; Hausott, Schlick, Vallant, Dorn, & Klimaschewski, 2008; Hausott, Vallant, Hochfilzer, Mangger, Irschick, Haugsten et al., 2012).

Both the Ras/ERK and the PI3K/Akt pathways are required for growth factor-dependent axon regeneration, and each pathway induces distinct axonal morphologies (Atwal, Massie, Miller, & Kaplan, 2000; Markus, Zhong, & Snider, 2002). Furthermore, activation of PI3K/Akt and downstream targets represents the primary survival pathway in neurons (Crowder & Freeman, 1998; Dudek et al., 1997). The Ras/ERK pathway plays a role in neuronal survival following cell injury as well (Hetman, Kanning, Cavanaugh, & Xia, 1999).

4.1.1 The ERK pathway

The Ras/ERK pathway includes the kinases Raf and MEK, which activate the serine/threonine kinase ERK (Fig. 6.3). This intracellular cascade is mainly responsible for the regulation of gene expression required for axon growth (Zhou & Snider, 2006). Furthermore, ERK is required for local axon assembly and polymerization of microtubules and actin filaments

(Goold & Gordon-Weeks, 2005). Accordingly, inhibition of ERK induces actin depolymerization and growth cone collapse (Atwal, Singh, Tessier-Lavigne, Miller, & Kaplan, 2003). Phosphorylation of ERK is enhanced in regenerating nerves in response to sciatic nerve crush (Agthong, Kaewsema, Tanomsridejchai, & Chentanez, 2006; Yamazaki et al., 2009) or transection (Sheu, Kulhanek, & Eckenstein, 2000). In addition, ERK is necessary for retrograde signaling (Perlson et al., 2005). Cell culture studies with embryonic DRG and SCG neurons revealed that ERK is mainly involved in elongative axon growth (Atwal et al., 2000; Markus et al., 2002). The duration and/or strength of the ERK signal influence the rate of neurite outgrowth. Different tyrosine kinase receptors vary in their ability to generate long-lived signaling endosomes, which elicit long-term ERK activation (Wiley & Burke, 2001). Sustained ERK activation is required for neurite outgrowth in PC12 pheochromocytoma cells, whereas transient ERK activation leads to proliferation in this cell line (Traverse, Gomez, Paterson, Marshall, & Cohen, 1992).

Inhibition of ERK has no effect on spontaneous axon outgrowth of adult DRG explants, while growth factor-induced outgrowth is significantly impaired (Sjogreen, Wiklund, & Ekstrom, 2000; Sondell, Lundborg, & Kanje, 1999). Furthermore, ERK is required for axotomy-induced growth cone formation after *in vitro* axon lesions (Chierzi, Ratto, Verma, & Fawcett, 2005) and axon growth of preaxotomized DRG explants, which model *in vivo* conditions controlled by growth factors (Wiklund, Ekstrom, & Edstrom, 2002). In dissociated adult DRG neuron cultures, conflicting reports on inhibition of ERK have demonstrated both no effect on intrinsic axon outgrowth (Kimpinski & Mearow, 2001; Tucker, Rahimtula, & Mearow, 2008) and possible stimulation of outgrowth (Jones, Tucker, Rahimtula, & Mearow, 2003). Furthermore, regenerative axon growth in response to a preconditioning lesion is not affected by ERK inhibition in dissociated DRG cultures (Liu & Snider, 2001). Although some studies are controversial, probably due to different culture conditions, ERK appears to have an important role in growth factor-induced axon elongation and axon maintenance.

4.1.2 The PI3K pathway

The PI3K pathway is involved in the reorganization of actin filaments of the axonal cytoskeleton through Rac and Cdc42, as well as microtubules via inhibition of GSK-3β (Fig. 6.3; Zhou & Snider, 2006). Thus, inactivation of GSK-3β leads to enhanced axon growth in adult DRG neurons (Jones

et al., 2003). PI3K is activated at the leading edge of the growth cone (Zhou, Zhou, Dedhar, Wu, & Snider, 2004). Phosphorylation of Akt downstream of PI3K is enhanced in regenerating nerves after sciatic nerve crush (Yamazaki et al., 2009). It is clear that PI3K activity induces branching and turning of axons (Gallo & Letourneau, 1998; Ming et al., 1999). Consistent with these results, enhanced phosphorylation of Akt increases axonal branching in both developing and adult DRG neurons (Jones et al., 2003; Markus et al., 2002). PI3K also mediates branching of sensory axons induced by NGF (Gallo & Letourneau, 1998). NT-3 is a stronger activator of Akt than NGF in embryonic DRG cultures (Markus et al., 2002). Accordingly, NT-3 induces more highly branched axon morphologies than NGF (Lentz, Knudson, Korsmeyer, & Snider, 1999; Ulupinar, Jacquin, & Erzurumlu, 2000).

Inhibition of PI3K decreases spontaneous and growth factor-induced axon outgrowth in adult DRG explants (Edström & Ekström, 2003) and in dissociated adult DRG neuron cultures (Jones et al., 2003; Kimpinski & Mearow, 2001). In contrast, as with ERK inhibition, regenerative axon growth in response to a preconditioning lesion is not affected by PI3K inhibition in dissociated DRG cultures (Liu & Snider, 2001). Furthermore, PI3K inhibition has no effect on growth cone initiation after axotomy of adult DRG explants (Chierzi et al., 2005). Thus, in contrast to the ERK pathway, PI3K signaling is involved in spontaneous and growth factor-induced axon outgrowth and branching.

4.2. Sprouty negative feedback inhibitors of RTK signaling

RTK signaling is tightly regulated by negative and positive feedback mechanisms (Lemmon & Schlessinger, 2010). As discussed above, activation of the ERK pathway promotes axonal elongation. Sprouty proteins belong to a group of intracellular negative feedback inhibitors of the ERK pathway (Hanafusa, Torii, Yasunaga, & Nishida, 2002; Tefft et al., 2002; Yusoff et al., 2002). Four functionally conserved Sprouty proteins (Sprouty1–4) exist in mammals, of which Sprouty-1, -2, and -4 represent the major isoforms (Minowada et al., 1999; Ozaki, Miyazaki, Tanimura, & Kohno, 2005). In response to growth factor stimulation, Sprouty-1 and -2 bind Grb2, thereby preventing downstream Ras/ERK activation (Fig. 6.3). Furthermore, Sprouty-2 and -4 bind Raf to interfere with ERK activation downstream of Ras (Sasaki et al., 2003). Additional inhibition of the PLC/PKC pathway (Akbulut et al., 2010; Ayada et al., 2009) and Rac1 GTPase has been

described (Alsina et al., 2012; Poppleton et al., 2004). In contrast, activation of Akt or p38 is not inhibited by Sprouty-2 (Hausott et al., 2009).

Sprouty proteins were analyzed in great detail over the last few years. Knockdown of Sprouty-2 or -4 strongly promotes axonal elongation of hippocampal pyramidal neurons, and this effect is enhanced by FGF-2 (Hausott, Vallant, Hochfilzer, et al., 2012; Hausott, Vallant, Schlick, et al., 2012). In the PNS, Sprouty-2 is highly expressed in the adult DRG. Downregulation of Sprouty-2 induces elongative axon growth of adult DRG neurons without stimulating additional branching (Fig. 6.6). Conversely, overexpression of Sprouty-2 inhibits axon growth of adult DRG neurons. Enhanced activation of ERK was observed in sensory neuron cultures in response to inhibition of Sprouty-2 (Hausott et al., 2009). Although Sprouty-2 mRNA is not regulated *in vivo* in response to a sciatic nerve lesion (Hausott et al., 2009), Sprouty-2 protein levels are reduced posttranscriptionally by microRNA miR-21 (Strickland et al., 2011). Interestingly, upregulation of miRNA-21 is observed in the DRG 2 days after axotomy. This increase is sustained up to 28 days after injury, indicating an important role for Sprouty-2 during peripheral axon regeneration. Recent results from our laboratory suggest that a 50% reduction of Sprouty-2 *in vivo* in heterozygous Sprouty-2 knockout mice is sufficient to significantly enhance axon elongation and accelerate functional recovery (Marvaldi et al., unpublished). Thus, accumulating evidence suggests that targeting endogenous inhibitors of ERK signaling promotes rapid and specific axon regeneration via promotion of axon elongation without stimulation of axon sprouting.

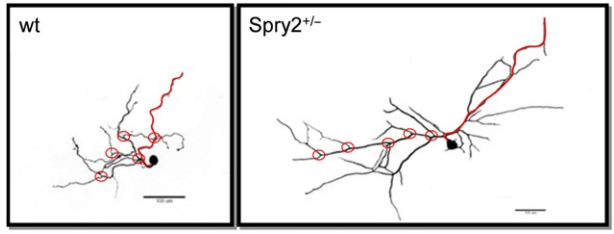

Figure 6.6 Sensory neurons extend axons over 24 h in culture. Neurons dissociated from heterozygous Sprouty2 knockout DRG exhibit longer axons (longest axon indicated in red) as compared to neurons obtained from wild-type littermates (Marvaldi et al., unpublished). The extent of axonal branching is not affected (examples of axonal branch points indicated by circles). Sprouty2 acts as a negative feedback inhibitor of the ERK, but not of the PI3K-signaling pathway. Bar = 100 μm.

5. CONCLUSIONS

Considering recent technical advances in the life sciences, it is now feasible to apply drugs intrathecally or via mini osmotic pumps and bioresorbable substances in patients with severe nerve lesions. As discussed in this review, polypeptidergic growth factors and neuropoietic cytokines can improve the extent, but not the specificity, of peripheral axon regeneration due to their stimulation of axon branching. Results from spinal cord injury models reveal that combinatorial treatment strategies may be required, including cAMP-elevating agents, electrical stimulation, and inhibitors of axonal sprouting, such as Sprouty siRNAs. Interfering with intraneuronal RTK degradation and increasing ERK signaling from early endosomes represent novel strategies to promote long-distance axon regeneration. Guidance factors (adhesion molecules or gradients of extracellular factors) may also be necessary to ensure that axons find their way to their original target. Recovery of specific motor patterns in patients with proximal lesions of the facial nerve, for example, will be particularly difficult to achieve. Correct reinnervation of the facial muscles may also require extensive training after axon regeneration is completed. The complexities of growth factors and cytokines in CNS lesions, such as development of spasticity in response to BDNF treatment (Lu et al., 2012), appear to be reduced in the PNS. Hence, the PNS will remain the primary research field to study neurotrophic factors and cytokines, their receptors and signaling pathways with the aim to ultimately develop new therapeutic approaches that promote fast and specific axon regeneration, leading to fully functional recovery following peripheral nerve injury.

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CHAPTER SEVEN

Role of Inflammation and Cytokines in Peripheral Nerve Regeneration

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Contents

Abstract

This chapter provides a review of immune reactions involved in classic as well as alternative methods of peripheral nerve regeneration, and mainly with a view to understanding their beneficial effects. Axonal degeneration distal to nerve damage triggers a cascade of inflammatory events alongside injured nerve fibers known as Wallerian degeneration (WD). The early inflammatory reactions of WD comprise the complement system,

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arachidonic acid metabolites, and inflammatory mediators that are related to myelin fragmentation and activation of Schwann cells. Fine-tuned upregulation of the cytokine/chemokine network by Schwann cells activates resident and hematogenous macrophages to complete the clearance of axonal and myelin debris and stimulate regrowth of axonal sprouts. In addition to local effects, immune reactions of neuronal bodies and glial cells are also implicated in the survival and conditioning of neurons to regenerate severed nerves. Understanding of the cellular and molecular interactions between the immune system and peripheral nerve injury opens new possibilities for targeting inflammatory mediators to improve functional reinnervation.

1. INTRODUCTION—AN OVERVIEW OF IMMUNE REACTION FUNCTIONS IN NORMAL AND INJURED NERVOUS SYSTEMS

The integrity of animal tissues is maintained by the nervous and endocrine systems as well as by mechanisms that involve immune cells and production of a variety of cellular mediators, such as cytokines.

Traumatic injury to peripheral nerve results in a loss of axons' functional connection to peripheral tissue. Renewed functional connection is achieved through regeneration of severed axons and their correct navigation to target tissues. Peripheral nerve trauma generally triggers an inflammatory response, which is required for nerve repair and functional reinnervation. The innate and adaptive immune systems are activated by endogenous signals that originate from injured or necrotic cells distal to the damaged nerve without septic or exogenous stimuli (i.e., aseptic inflammation). As in other tissues, inflammatory reaction after nerve lesion is a dynamic process driven by numerous inflammatory mediators. However, the role of inflammation in the peripheral nervous system is more controversial. On the one hand, inflammatory reaction and its mediators in damaged nerve participate significantly in the processes of nerve regeneration (Camara-Lemarroy, Guzman-de la Garza, & Fernandez-Garza, 2010). On the other hand, these also develop conditions for neuropathic pain (NPP) induction (Austin & Moalem-Taylor, 2010). Therefore, detailed knowledge of inflammation after traumatic nerve injury and during nerve regeneration is important for improving functional recovery after peripheral nerve reconnection/reconstruction as well as while pursuing tissue engineering. Moreover, early suppression of inflammatory reactions after nerve injury in an attempt to reduce NPP induction may result in decelerating or halting axon regeneration.

2. WALLERIAN DEGENERATION AFTER TRAUMATIC NERVE INJURY AS ASEPTIC INFLAMMATION

Although Wallerian degeneration (WD) was originally described after complete disconnection of peripheral nerve, more recently the term is used also to depict similar cellular and molecular processes that occur after various types of nerve injury (Zochodne, 2012). As used in this review, the term "Wallerian degeneration" refers only to WD induced by traumatic (mechanical) peripheral nerve injury. WD is a cascade of stereotypical cellular and molecular events distal to injury of nerve fibers and considered to be a sort of innate immune reaction or neuroinflammation (Gaudet, Popovich, & Ramer, 2011). The cellular and molecular events develop throughout nerve fibers distal from lesion sites in the direction of denervated target tissues.

The cellular events comprise degeneration of the distal parts of severed axons (Coleman, 2005), activation of Schwann cells, breakdown of the blood–nerve barrier (Mizisin & Weerasuriya, 2011), and recruitment of hematogenous macrophages and other types of immune cells that produce cytokines and chemokines (Rotshenker, 2011; Taskinen & Roytta, 1997). In addition, axon injury triggers molecular changes mostly related to inflammation and includes modulation of the endoneurial extracellular matrix (ECM) by metalloproteinases (Gantus, Nasciutti, Cruz, Persechini, & Martinez, 2006; Tona, Perides, Rahemtulla, & Dahl, 1993) and elevation of neurotrophin and cytokine production (Camara-Lemarroy et al., 2010; Fu & Gordon, 1997; Terenghi, 1999). Both cellular and molecular events distal to nerve injury create conditions to induce axon regrowth from proximal stumps (Fu & Gordon, 1997; Raivich & Makwana, 2007; Webber & Zochodne, 2010).

2.1. Early phase of WD and consequent axon regeneration

The early phase of WD (to 48 h) begins with prompt degradation of such axon components as axoplasm, axolemma, and axonal mitochondria distal to the site of nerve fiber lesion. Axonal degeneration is an active, intrinsic process of self-destruction that proceeds independently of non-neuronal cells surrounding the axon (Saxena & Caroni, 2007). Calcium influx and activation of calpains are early events implicated in a proteolytic degradation of axonal cytoskeletal elements and axonal fragmentation (Beirowski et al., 2005; Glass, Culver, Levey, & Nash, 2002). Activation of calpains also causes

an early peak of cytokine upregulation in Schwann cells (Uceyler, Tscharke, & Sommer, 2007).

The inflammatory response initiated by axonal disintegration of injured nerve concerns both humoral (e.g., involving the complement system, cyclooxygenase–lipoxygenase pathways, and cytokines/chemokines) and cellular (blood–nerve barrier permeabilization, activation of Schwann cells and resident macrophages and recruitment of hematogenous macrophages) components. These early reactions of WD are probably triggered via receptors sensitive to tissue damage, such as toll-like receptors (TLRs).

2.1.1 Toll-like receptor signaling, activation of complement system, and their critical roles for early phase of WD and consequent axon regeneration

2.1.1.1 Toll-like receptors

Endogenous danger signals released from necrotic or stressed cells triggering the inflammatory response after tissue trauma have been termed alarmins or danger-associated molecular patterns (DAMPs) (Hirsiger, Simmen, Werner, Wanner, & Rittirsch, 2012).

Disintegration of axons in distal nerve stump results in the production of such various DAMPs as galectins, adenosine, HMBG-1, hyaluronan, heparan sulfate proteoglycan, fibrin, and fibronectin. Some DAMPs are endogenous ligands for TLRs, the signaling of which plays a critical role in WD and axon regeneration after peripheral nerve injury (Boivin et al., 2007). For example, disintegrated axolemma and degraded endoneurial ECM in the distal nerve stump triggers TLR signaling of Schwann cell activation (Brunn, Bungum, Johnson, & Platt, 2005). Early activation of TLR4 distal to nerve injury is shown in Fig. 7.1A and B.

2.1.1.2 Complement system

Activation of complement components constitutes another important regulatory system in the early phase of WD. The complement (C) system is a part of the innate immune response consisting of about 30 soluble as well as membrane-embedded complement proteins. The distribution of the various complement components differs considerably among axon, Schwann cells, endoneurium, and perineurium, and this might play a role in the regeneration of peripheral nerve (Ramaglia, Daha, & Baas, 2008). It has been demonstrated that activated complement components are already present in the myelin sheath 4 h after nerve injury (De Jonge, van Schaik, Vreijling, Troost, & Baas, 2004) and that these are involved in myelin breakdown

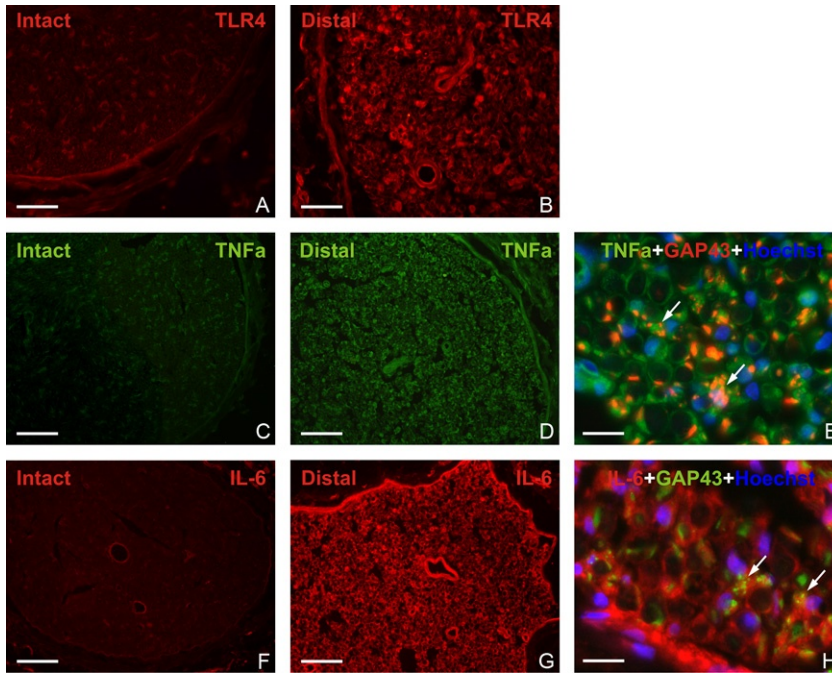

Figure 7.1 Cross sections through rat intact sciatic nerve (Intact) and distal (Distal) to ligature after 1 day (A, B) and 7 days (D, E, G, H). Immunofluorescence staining illustrates upregulation of TLR4 (A, B), TNFα (C, D), and IL-6 (F, G). Regrowing axons decorated by GAP43 immunostaining (arrows) are in close contact with Schwann cells that display high intensity of immunofluorescence for TNFα (E) or IL-6 (H). Nuclei of cells are stained with Hoechst. Scale bars for A–D, F, G = 50 μm, for E, H = 12 μm.

and myelin phagocytosis by macrophages during WD (Ramaglia et al., 2008). These processes are based on myelin opsonization by complement components, because deficiency of C3 blocks myelin phagocytosis and delays WD (Bruck & Friede, 1991; Dailey, Avellino, Benthem, Silver, & Kliot, 1998). Moreover, macrophage recruitment and activation after nerve injury is inhibited in C5- and C6-deficient rats (Bruck & Friede, 1991; Liu et al., 1999; Ramaglia, King, et al., 2007; Ramaglia, Wolterman, de Kok, et al., 2007; Ramaglia, Wolterman, Vigar, et al., 2007).

Mechanical nerve injury induces binding of C1q to axonal and myelin epitopes, the damaged nerve structures are then opsonized by C4b, C3b, and C5b, and these are selectively phagocytized by macrophages (Ramaglia, King, et al., 2007; Ramaglia, Wolterman, de Kok, et al., 2007; Ramaglia, Wolterman, Vigar, et al., 2007). Activation of the C system during WD thus

leads to rapid and efficient clearance of the axons and their myelin sheaths without damage to other cellular components of the distal nerve stump (De Jonge et al., 2004; Ramaglia, King, et al., 2007; Ramaglia, Wolterman, de Kok, et al., 2007; Ramaglia, Wolterman, Vigar, et al., 2007).

2.1.1.3 Roles of TLRs and complement components in axon regeneration

TLRs and complement activation drive the early phase of WD in relation to the disintegration and clearance of damaged axons and myelin sheaths. During this early period of WD, however, axonal sprouts also begin immediately to grow from proximal axon segments (Stoll, Jander, & Schroeter, 2000). Therefore, most of the early processes influence the final results of nerve regeneration and functional recovery after nerve reconstruction.

The implication of TLRs in nerve regeneration is illustrated by delayed WD and axonal regeneration after sciatic nerve injury of TLR2- and TLR4-deficient mice compared to wild-type mice. The slowing of nerve regeneration in TLR2- and TLR4-deficient mice probably is due to reduced upregulation of IL-1β and MCP-1 by Schwann cells; both IL-1β and MCP-1 proteins are important for recruitment of macrophages. On the other hand, activation of TLR2 and TLR4 by injection of their agonists into the sciatic nerve has been shown to accelerate myelin debris clearance and axon regeneration while improving recovery of peripheral nerve function (Boivin et al., 2007).

In contrast to the positive effect of C during WD, it has been found that recovery of sensory and motor functions is accelerated in C6-deficient and sCR1-treated rats. This accelerated nerve regeneration might be explained by a decreased quantity of recruited macrophages and reduced production of matrix metalloproteinases with subsequent rescue of the endoneurial basal lamina tubes from degradation. In addition, it was surprising to find that delayed clearance of myelin debris in C6-deficient nerve or following sCR1 treatment does not appear to hamper axon regeneration (Ramaglia et al., 2009).

2.1.2 Cytokines, clearance of myelin debris from the distal nerve stump, and consequent axon regeneration

Clearing of the myelin debris is essential for nerve reinnervation because it contains myelin associated glycoprotein (MAG) and other molecules that inhibit axon growth (Barrette, Calvo, Vallieres, & Lacroix, 2010; Schafer, Fruttiger, Montag, Schachner, & Martini, 1996; Shen, DeBellard, Salzer, Roder, & Filbin, 1998). Schwann cells of the distal stump first respond to

the nerve injury by their detachment from axons, dedifferentiation, and activation. The activated Schwann cells acquire a phagocytic capacity and begin to remove myelin and degenerating axons. Then, the degraded myelin is phagocytized by resident endoneurial and recruited hematogenous macrophages to effect full myelin clearance (Mueller et al., 2003; Stoll, Jander, & Myers, 2002).

Recruitment of macrophages is regulated by immune activation of Schwann cells that had lost contact with their axons. The activated Schwann cells upregulate a cascade of cytokines and chemokines that are stimuli for invasion of macrophages and other immune cells. Predominantly, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and neuropoietic cytokines like interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) are produced by Schwann cells during the early phase of WD and contribute to a selective accumulation of macrophages in the distal stump of the injured peripheral nerve (Liefner et al., 2000; Perrin, Lacroix, Aviles-Trigueros, & David, 2005; Shamash, Reichert, & Rotshenker, 2002; Tofaris, Patterson, Jessen, & Mirsky, 2002).

The molecular mechanisms of myelin phagocytosis by Schwann cells and macrophages are thought to be different. The first step of myelin phagocytosis by Schwann cells is lectin mediated (i.e., opsonin independent), whereas that of macrophages is mainly opsonin dependent (Hirata & Kawabuchi, 2002).

Cytokine relationships, delayed removal of axon and myelin debris, and axon regeneration are well studied in injured sciatic nerves of C57/BL/6NHSD (C57/BL) and C57/BL/6-WLD/OLA/NHSD (Wld) mice. Such cellular and molecular events of WD as myelin removal, Schwann cell activation, and NGF production are efficient and rapid in C57/BL mice (rapid WD) but deficient and slow in Wld mice (slow WD) (Be'eri, Reichert, Saada, & Rotshenker, 1998; Reichert, Saada, & Rotshenker, 1994; Stoll & Muller, 1999).

Cytokine mRNAs and proteins are constitutively expressed and synthesized at no or low levels in Schwann cells of intact sciatic nerve and they are significantly induced after nerve injury in C57/BL mice. However, no protein upregulation of cytokines (TNFα, IL-1α, and IL-1β, GM-CSF, IL-6, and IL-10) has been found during slow WD in the injured sciatic nerve of Wld mice (Be'eri et al., 1998; Reichert, Levitzky, & Rotshenker, 1996; Saada, Reichert, & Rotshenker, 1996; Shamash et al., 2002).

These results of experiments achieved from Wld mice illustrate the relevance of a fine-tuned spatiotemporal expression of cytokines/chemokines

for myelin clearance in the context of peripheral nerve regeneration (Brown, Booth, Lunn, & Perry, 1991; Carroll & Frohnert, 1998; Schafer et al., 1996).

3. CYTOKINES AND PERIPHERAL NERVE REGENERATION

Cytokines comprise a heterogeneous group of polypeptide mediators that have been associated classically with activation of the immune system and inflammatory responses. Recently, there is a growing body of evidence that cytokines and their group of chemokines have important roles in maintaining normal physiological conditions in the nervous system and their expression is upregulated rapidly following various types of stress to restore nervous tissue homeostasis. General features of cytokines are the extremely broad range of their activities, which may overlap or act synergistically, and the wide range of cells that are able to produce several cytokines. Furthermore, cytokines are frequently regulated in cascades where induction of the early cytokines serves to influence the synthesis of later ones (Fregnan, Muratori, Simoes, Giacobini-Robecchi, & Raimondo, 2012). Cytokines of the peripheral nervous system are produced by peripheral neurons and their glial cells (e.g., Schwann cells, satellite glial cells (SGCs) of the sensory ganglia), as well as by resident and recruited immune cells.

Published results regarding cytokine effects upon axon regeneration are frequently controversial, because cytokines, like inflammation itself, constitute a "double-edged sword" with respect to nerve regeneration. These opposing effects are related to the degree and timing of inflammatory reactions in severed nerves. A beneficial effect of local inflammation upon axon regeneration probably depends on orchestrated production of cytokines and its very precise control to keep cytokines at optimal levels during critical periods. It is known that chronic overproduction of cytokines after a traumatic nerve injury or other nerve diseases is implicated in conditions inducing NPP (Austin & Moalem-Taylor, 2010). However, optimal levels of cytokines and the mechanisms of balancing these to achieve their beneficial effects in lesioned peripheral nerve are still unknown.

Most cytokines are upregulated in the distal stump of the injured nerve in two or three waves. The upregulation of cytokines/chemokines by activated Schwann cells during the early phase of WD is implicated predominantly in myelin destruction and recruitment of hematogenous macrophages. This

early peak is followed by a second, later phase in cytokine/chemokine expression that includes termination of inflammatory response (Shamash et al., 2002; Taskinen & Roytta, 2000).

Schwann cells proliferate and dedifferentiate to immature stage distal to nerve injury. The immature Schwann cells aligned in bands of Büngner of the distal nerve stump comprise the main source of cytokines (Dubovy, 2011; Taskinen & Roytta, 2000), neurotrophic factors (Gordon, 2010; Terenghi, 1999), and cell adhesion molecules (Gardiner, 2011; Kirsch, Friz, Vougioukas, & Hofmann, 2009) that stimulate and guide axonal regeneration. After the early phase of WD, an inflammatory reaction of Schwann cells relates mainly to their multiplication, survival, and axon growth promotion. It has been proven under *in vitro* conditions that low levels of proinflammatory cytokines (e.g., TNFα and IL-6) can stimulate neurotrophin-dependent neurite outgrowth of dorsal root ganglion (DRG) neurons. The cytokine–neurotrophin interaction can be suggested as one of the mechanisms by which inflammatory response modulates axonal regeneration (Golz et al., 2006; Marz, Heese, Dimitriades-Schmutz, Rose-John, & Otten, 1999).

It has been demonstrated over the past two decades that cytokines/chemokines produced by Schwann cells of the distal nerve stump have direct or indirect effects on axonal growth. In addition to this local effect, cytokines/chemokines and their signaling molecules contribute significantly to axotomy-induced events in the neuronal bodies and surrounding glial cells and may affect neuron survival and axon regeneration. In the present review, we select only representative cytokines that are frequently studied in relation to nerve regeneration.

3.1. Interleukin-1β

IL-1β is a proinflammatory cytokine, the expression of which is increased only to a limited extent in the immature Schwann cells of the distal nerve stump in the early stage of WD and which disappears thereafter when Schwann cells begin their remyelination (Shamash et al., 2002).

It is well known that this proinflammatory cytokine promotes inflammation and cell death, including neurodegeneration (Basu, Krady, & Levison, 2004). On the other hand, IL-1β regulates synthesis of nerve growth factor (NGF) by Schwann cells and fibroblasts (Lindholm, Heumann, & Thoenen, 1987; Rotshenker, Aamar, & Barak, 1992), and, together with neurotrophin-3, it synergistically promotes neurite growth (Boato et al.,

2011). Moreover, this cytokine is able to overcome MAG-induced RhoA activation and axon growth inhibition to promote sensory axon outgrowth under *in vitro* and *in vivo* conditions (Temporin et al., 2008a, 2008b). In summary, a locally regulated elevation of IL-1β during the early phase of WD contributes to macrophage recruitment (Perrin et al., 2005; Shamash et al., 2002), Schwann cell proliferation (Conti et al., 2002), and initial axonal elongation (Temporin et al., 2008a, 2008b).

3.2. Tumor necrosis factor α

TNFα expression is highly upregulated in the peripheral nerves early (within hours) after injury (George, Buehl, & Sommer, 2004; Shamash et al., 2002; Temporin et al., 2008a). Thereafter, two distinct peaks of TNFα mRNA have been seen in the endoneurium after 5 days and, in the more distal segments of transacted nerve, also after 2 weeks (Taskinen et al., 2000). Later upregulation of TNFα distal to nerve ligature predominantly in Schwann cells and their close contact with growing axons are illustrated in Fig. 7.1C–E. During the early phase of WD, TNFα has beneficial effects on myelin degradation and phagocytosis, Schwann cell activation, and macrophage recruitment, as demonstrated in experiments under *in vitro* and *in vivo* conditions (Liefner, Maruschak, & Bruck, 1998; Schafers, Schmidt, Vogel, Toyka, & Sommer, 2002; Shubayev et al., 2006).

Published findings as to TNFα's direct effects on peripheral nerve regeneration are controversial. While TNFα may promote motor functional recovery after crushing of peripheral nerve (Chen, Seaber, Wong, & Urbaniak, 1996), it has also been found that TNFα stimulates fibroblast proliferation to induce neuroma formation that results in impairment of functional nerve regeneration (Lu et al., 1997). There is further evidence of TNFα's having a detrimental effect upon nerve regeneration from experiments with systemic and local administration of the TNFα antagonist etanercept that enhanced the rate of axonal regeneration (Kato, Liu, Kikuchi, Myers, & Shubayev, 2010). Even *in vitro* experiments do not provide unequivocal findings. While there are results showing that TNFα reduces neurite sprouting (Lu et al., 1997), a recent paper by Saleh et al. (2011) proved an increased branching of DRG neurons after application of TNFα through an NF-κB-dependent pathway. These controversial results illustrate that the effects of TNFα upon peripheral nerve regeneration are complicated and call for further experiments.

3.3. Neuropoietic cytokine family

The neuropoietic cytokine family includes ciliary neurotrophic factor (CNTF), LIF, oncostatin M, cardiotrophin-1, cardiotrophin-like cytokine (CLC), neuropoietin (NPN), IL-27, and IL-31 (Bauer, Kerr, & Patterson, 2007). All of these cytokines cause homo- or hetero-dimerization of the glycoprotein 130 (gp130) receptor in target cells and subsequent tyrosine phosphorylation of Janus kinases (JAKs) and of signal transducers and activators of transcription (STATs). For example, phosphorylated STAT3 is then dimerized and translocated to the neuron nucleus, where it activates transcription of many genes (Heinrich et al., 2003). Several lines of evidence implicate mainly CNTF, LIF, and IL-6 in the regenerative conditioning of peripheral nerves and their neurons (Murphy et al., 2000).

Myelinating Schwann cells of intact peripheral nerve display high levels of CNTF protein and mRNA, but these are dramatically reduced in the distal segment of the injured sciatic nerve (Rabinovsky, Smith, Browder, Shine, & McManaman, 1992; Sendtner, Stockli, & Thoenen, 1992). It has been demonstrated that administration of recombinant CNTF promotes axonal regeneration and sprouting of motor axons (Dubovy et al., 2011; Sendtner et al., 1992; Siegel, Patton, & English, 2000). For example, intrathecal CNTF administration results in improved functional motor reinnervation in the model of end-to-side neurorrhaphy by promoting direct reinnervation and collateral sprouting and those sprouts' final maturation (Dubovy et al., 2011).

When the sciatic nerve is transected, LIF mRNA is increased significantly in those regions immediately proximal and distal to the lesion site while LIF receptor mRNA somewhat decreases (Banner & Patterson, 1994; Curtis et al., 1994). Peripheral nerve injury initiates increased retrograde transport of LIF to both primary sensory and motor neurons in order to induce their regeneration program (Curtis et al., 1994). Moreover, LIF promotes elongating but not arborizing axon outgrowth *in vitro* and is required for normal regeneration of injured adult primary sensory neurons *in vivo* (Cafferty et al., 2001).

Of special interest regarding nerve regeneration is IL-6, which exerts pleiotropic effects and is considered to be a key component in the neuronal and immune responses to nerve injury. IL-6 first binds to a membrane-bound, specific IL-6 receptor (IL-6R), which is then associated with the ubiquitously distributed gp130. This receptor complex activates a signal transduction cascade through transcription factors JAKs and STAT3.

Phosphorylated STAT3 dimers then translocate to the nucleus and initiate transcription of target genes (for review, see Eulenfeld et al., 2012).

Proteins and mRNAs of IL-6, IL-6R, and IL-6's signaling molecules are constitutively expressed at low levels in the structures of intact peripheral nerve but at higher levels in non-myelinating Schwann cells (Bolin, Verity, Silver, Shooter, & Abrams, 1995; Grothe et al., 2000; Kurek, Austin, Cheema, Bartlett, & Murphy, 1996; Lara-Ramirez, Segura-Anaya, Martinez-Gomez, & Dent, 2008). Schwann cells of the distal nerve stump constitute the main source of increased levels of IL-6 (Fig. 7.1F and G), its receptors, and signaling molecules. While IL-6 is increased distal to a sciatic nerve lesion (Bolin et al., 1995), retrograde transport of this cytokine to the neuronal bodies does not seem to occur (Kurek et al., 1996). Double immunostaining revealed regrowing axons in close contact with activated Schwann cells that expressed high level of IL-6 protein (Fig. 7.1H).

During the early phase of WD, IL-6 is involved in an autocrine-signaling inflammatory cascade also involving LIF and MCP-1, which gradually amplifies the Schwann cell-derived chemotactic signals for macrophage recruitment (Tofaris et al., 2002). In addition, IL-6 in DRG neurons overcomes axon outgrowth inhibition by MAG (Cao et al., 2006). Implication of IL-6 biological activity in nerve regeneration has been studied in various nerve injury models applied in genetically modified animals. The increase of GAP-43 normally observed in DRG neurons of wild type mice after axotomy is abolished in IL-$6^{-/-}$ mice (Cafferty et al., 2004). Other experiments with genetic deletion of IL-6 have also revealed retardation of regeneration speed in sciatic (Cafferty et al., 2004; Zhong, Dietzel, Wahle, Kopf, & Heumann, 1999) and facial nerves (Galiano et al., 2001).

However, no significant differences were found in functional recovery between wild-type and IL-6 knockout mice after sciatic nerve transection with epineurial suture repair (Inserra, Yao, Murray, & Terris, 2000), while sensory impairments were observed after crush lesion of that nerve (Zhong et al., 1999). In addition, the regeneration of axotomized hypoglossal nerve was delayed when IL6-R was inhibited with specific antibodies while accelerated nerve regeneration was achieved in transgenic mice with upregulated expression of IL-6 and IL-6R (Hirota, Kiyama, Kishimoto, & Taga, 1996). It was also demonstrated that IL-6, by activating STAT3, can enhance synthesis of myelin proteins in Schwann cells (Haggiag et al., 2001; Ito, Ikeda, Tomita, & Yokoyama, 2010).

Gp130-deficient mice die in early embryonic stages, and conditional gp130 gene deletion after birth has been shown to cause Schwann cell

degradation and degeneration of both myelinated and non-myelinated peripheral nerves (Betz et al., 1998).

The actions of neuropoietic cytokines are mediated by the gp130 receptor, which activates the phosphorylation and dimerization of STAT3. Local STAT3 activation after sciatic nerve injury occurs in axons within 15 min of lesioning, leading to retrograde signaling to the nucleus (Lee, Neitzel, Devlin, & MacLennan, 2004), where it is involved in the program promoting axon growth. Activation of STAT3 is subject to feedback inhibition by suppressor of cytokine signaling 3 (SOCS3) (Croker et al., 2003). It has been found that SOCS3 protein is restricted mainly to Schwann cells distal to nerve injury and is negatively correlated with the expression of IL-6. This suggests that the increased expression of SOCS3 may reduce expression of IL-6 in order to eliminate its detrimental inflammatory effects. In addition, a higher level of SOCS3 was detected in cut/ligated nerves as compared to crushed nerves (Girolami, Bouhy, Haber, Johnson, & David, 2010), but any relationship between these findings and differences of axon regrowth following various types of nerve injury are not yet clear. More important, STAT3/SOCS3 regulation linked with nerve regeneration occurs in injured neurons. Further information on this subject is provided in the next subchapter.

3.4. Prostanoids

Arachidonic acid is a polyunsaturated fatty acid that is found in the cell membrane. Once it has been freed from the cell membrane by phospholipase A2, it is metabolized by cyclooxygenase enzymes in the arachidonate cascade to compounds that include prostaglandins, thromboxanes, and leukotrienes. Cyclooxygenase-1 (COX-1) is expressed constitutively in most tissues and has a homeostatic or housekeeping role, while cyclooxygenase-2 (COX-2) expression is usually low but can be induced by proinflammatory and downregulated by anti-inflammatory cytokines (Flower, 2003). Prostaglandin E2 (PGE2) is a well-known mediator of inflammation, and COX2-dependent PGE2 (COX2/PGE2) is one of the important mediators abundantly produced in injured nerves (Ma & Quirion, 2008). A biphasic increase of COX-2 expression was found in Schwann cells at day 1, while in ED1+ macrophages within 7–14 days, after nerve injury (Takahashi et al., 2004), and this upregulation can last for months to years (Durrenberger et al., 2006). Application of nonsteroidal anti-inflammatory drugs or inhibition of COX-2 by the selective inhibitor celecoxib promotes

axon regeneration (Camara-Lemarroy et al., 2008; Fu, Hue, & Li, 2007) and indicates a direct implication of COX2/PGE2 in nerve regeneration.

4. IMMUNE REACTIONS OF NEURONAL BODIES AND THEIR GLIAL CELLS, AND NERVE REGENERATION

Rescue of neurons from their deaths and promotion of their axon regeneration program are important prerequisites for successful functional reinnervation after nerve injury. Molecular changes during WD constitute a source of both positive and negative signaling molecules for inflammatory reactions in the neuronal somata and their surrounding glial cells. This retrograde signaling-induced inflammation may lead to death and/or protection of the neurons as well as switching off of the neurons for regeneration of their damaged axons. The balance of nerve injury-induced inflammatory responses of the neurons may promote axon regeneration on the one hand but also induce development and maintenance of NPP on the other hand (Dubovy, 2011).

4.1. Interleukin-1β

The cytokine IL-1β binds to specific cell surface receptors IL-1RI and IL-1RII that are members of the TLR superfamily (Martin & Wesche, 2002). Whereas IL-1RI transduces the biological signal of IL-1β, and IL-1RII serves as a "decoy" receptor restricting the effect of the cytokine on its target cells. It has been shown that only IL-1RI but not IL-1RII is expressed in the DRG and motor neurons (Copray et al., 2001; Obreja, Rathee, Lips, Distler, & Kress, 2002; Sasaki, Seo-Kiryu, Kato, Kita, & Kiyama, 2001). The highly selective IL-1 receptor antagonist (IL-1ra) is a naturally occurring inhibitor of IL-1β and competes with IL-1β for binding to IL-1R1 (Arend, Palmer, & Gabay, 2008).

Nerve injury-induced elevation of IL-1β in the neurons has opposing outcomes as to neurotoxic and neuroprotective effects related to the degree and timing of inflammation (Basu et al., 2004). Moreover, it seems that IL-1β is not neurotoxic per se, but it induces activation of surrounding glial cells and their excitotoxic effect (Hailer, Vogt, Korf, & Dehghani, 2005). Although there is evidence that administration of IL-1R antagonist or IL-1β blocking antibodies reduces neuronal death (Allan, Tyrrell, & Rothwell, 2005; Touzani, Boutin, Chuquet, & Rothwell, 1999), other experiments under *in vitro* and *in vivo* conditions have yielded controversial results about direct IL-1β neurotoxic or neuroprotective effects. Therefore,

IL-1ra has been used extensively in experiments to clarify the role of IL-1 in neuronal injury. Exogenous administration of IL-1ra or overexpression of endogenous IL-1ra markedly inhibits neuronal injury in experimental animals (Allan et al., 2005).

The cytokine IL-1β is synthesized as a 35-kDa precursor that is proteolytically processed to the 17 kDa mature form by the IL-1β-converting enzyme (ICE) (Thornberry & Molineaux, 1995). ICE's deletion does not alter neuron death induced by a facial nerve injury in the neonatal periods, but adult ICE knockout mice exhibit greater facial motoneuron loss following axotomy. These data suggest that ICE may contribute to an environment for neuronal survival after nerve injury (De Bilbao, Giannakopoulos, Srinivasan, & Dubois-Dauphin, 2000) and provide indirect evidence that natural regulation of IL-1β levels may protect neurons from their death after nerve injury.

4.2. Tumor necrosis factor α

The pleiotropic activities of TNFα are mediated via two distinct receptor subtypes, constitutively expressed receptor-1 (TNF-R1, p55) and inducible receptor-2 (TNF-R2, p75). Several studies now point to an important contribution of TNF-R2 to immune cell activation based on a cooperative action with TNF-R1 (Chadwick et al., 2008).

As its local effects in nerve, TNFα provides diverse bioactivities in neurons suffering from axon injury. At present, it is not entirely clear under which conditions TNFα promotes beneficial or deleterious effects on injured neurons.

The TNFα produced at nerve lesion sites is retrogradely transported to DRG neurons and the spinal cord dorsal horn, where it correlates with the expression of TNF-R1 and TNF-R2 (Shubayev & Myers, 2001, 2002). In addition to this retrogradely transported TNFα, the DRG neurons and their SGCs synthesize TNFα after sciatic nerve injury (Dubovy, Jancalek, Klusakova, Svizenska, & Pejchalova, 2006).

Several lines of evidence demonstrate that neurotoxic effects of TNFα are mediated through TNF-R1 (Chadwick et al., 2008). The endogenous TNFα does not exhibit neurotoxic effects in the presence of NGF. When NGF is withdrawn, the endogenous TNFα has neurotoxic effects on neurons through TNF-R1. Contrary to these neurotoxic effects, there is evidence that TNFα can promote neuron survival through TNF-R2. However, TNFα is upregulated in both dying and surviving neurons

(Barker, Middleton, Davey, & Davies, 2001) and definite involvement of TNFα in both processes has not yet been clarified.

According to findings as to the immunohistochemical distribution of TNFα and its receptors in DRG after nerve injury, we can speculate about autocrine and paracrine action of TNFα. As shown in Fig. 7.2, after nerve injury, TNF-R1 was upregulated only in the neuronal bodies while TNF-R2 was enhanced in their SGCs. TNFα produced by both neurons and their SGCs binds to TNFR1 present only in the neurons and produces a destructive effect by means of autocrine and paracrine mechanisms. However, TNFα binding to TNFR2 of SGCs and some small-sized DRG neurons—again by autocrine and paracrine fashions—protect these glial and neuronal cells from the destructive effect of TNFα (Fig. 7.3).

Combined deletion of both TNFα receptors (TNF-R1 and -R2$^{-/-}$ mice) led to almost complete disappearance of motoneuron loss in the first 29 days after facial nerve axotomy. However, there is a second phase of motoneuron loss independent as to the presence of TNFα receptors that begins in the 4th week after injury. This suggests that the destructive effect of TNFα on neurons is probably limited to the early periods after nerve injury (Raivich et al., 2002).

4.3. Neuropoietic cytokine family

Several lines of evidence indicate that neuropoietic cytokines and their signaling pathways are activated in the primary sensory neurons by nerve injury and are necessary for regenerative responses of these neurons. Axotomy of adult peripheral neurons stimulates synthesis of IL-6 in the DRG neurons and their SGCs (Dubovy, Klusakova, Svizenska, & Brazda, 2010; Murphy, Grondin, Altares, & Richardson, 1995), as well as LIF synthesis in the DRG neurons (Banner & Patterson, 1994). The nerve injury upregulation of IL-6 in the neurons is linked with increased expression of growth-associated genes and reactivation of their intrinsic growth program to promote axonal regrowth (Cafferty et al., 2004; Pieraut et al., 2011; Yang, Wen, Ou, Cui, & Fan, 2012). Injured DRG neurons also display increased IL-6R levels, which is important for an autocrine effect of IL-6 on DRG neuron survival (Dubovy, Klusakova, Svizenska, & Brazda, 2010; Thier, Marz, Otten, Weis, & Rose-John, 1999).

It has been demonstrated that the presence of LIF is critical for motoneuron survival (Holtmann et al., 2005). In addition, LIF is retrogradely transported to injured neuronal bodies, where it is involved in processes that regulate their intrinsic growth status (Cafferty et al., 2001).

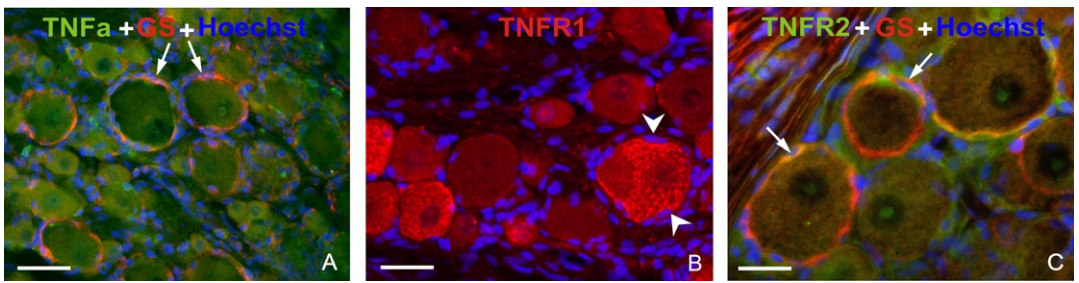

Figure 7.2 Sections through L4-DRG ipsilateral to sciatic nerve ligature after 7 days. Increased immunofluorescence staining of TNFα protein is seen in neuronal bodies (arrowheads) and satellite glial cells (arrows), with simultaneously stained glutamine synthetase (GS) used as a marker (A). Nuclei of cells are stained with Hoechst. Immunostaining for TNFR1 is elevated only in the neuronal bodies, while satellite glial cells are free of the signals (arrowheads, B). By contrast, TNFR2 immunostaining is increased mainly in satellite glial cells that were simultaneously stained for GS (arrows, C). Scale bar for A, B = 30 μm; for C = 20 μm.

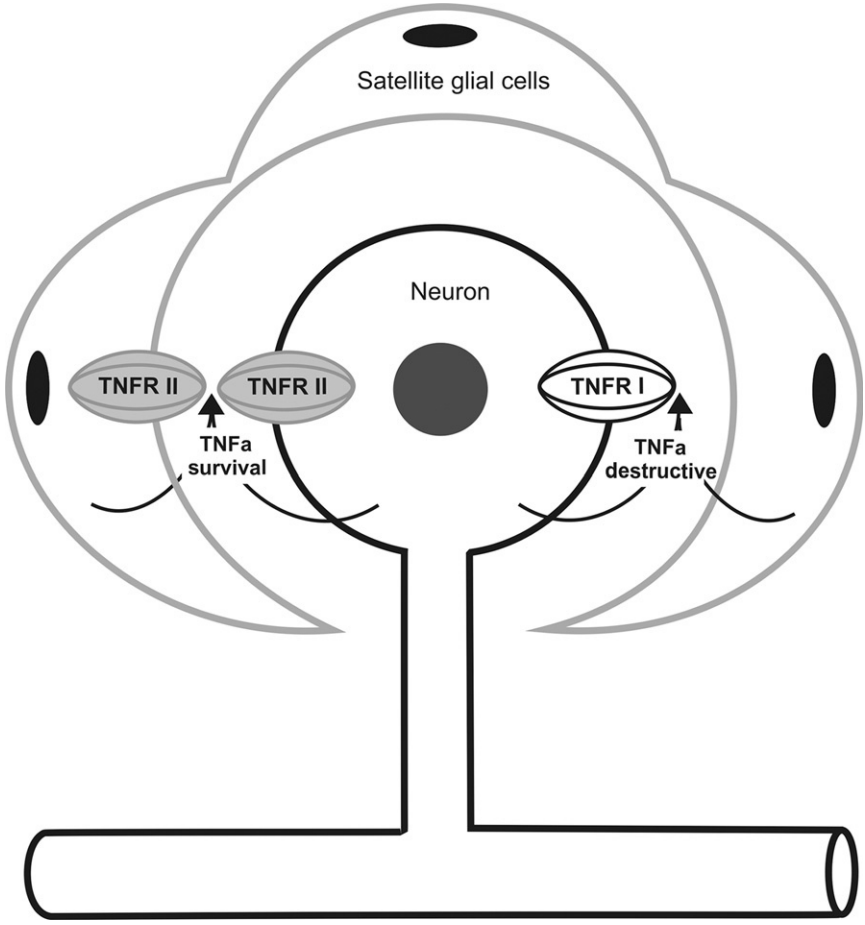

Figure 7.3 Schematic presentation of TNFα and its receptors (TNFR1, TNFR2) distribution in DRG according to the results of immunohistochemical staining after nerve injury. TNFα is upregulated in the neurons and their satellite glial cells while TNFR1 is upregulated only in the neuronal bodies. Immunostaining for TNFR2 was observed in satellite glial cells and small- and medium-sized neuronal bodies (Fig. 7.2, Dubovy, Jancalek, Klusakova, Svizenska, & Pejchalova, 2006). Based on published conclusions (Chadwick et al., 2008), we can speculate on autocrine and paracrine actions of TNFα upon neuronal bodies via TNFR1 to induce a destructive effect. In contrast, autocrine and paracrine actions of TNFα via TNF-R2 probably have protective effects upon satellite glial cells and a subpopulation of the DRG neurons.

As members of the neuropoietic cytokine family, LIF and CNTF utilize common signaling pathways and have overlapping effects on neurons. Although CNTF is dramatically reduced in the distal segment of injured sciatic nerve (Rabinovsky et al., 1992), experiments with CNTF-deficient

mice point to CNTF as an early retrograde signal in axotomized motoneurons to support their survival (Kirsch, Terheggen, & Hofmann, 2003; Sendtner, Gotz, Holtmann, & Thoenen, 1997). However, the later conclusions based on experiments with CNTF knockout mice do not take into account the effects of other neuropoietic cytokines that are also able to activate STAT3 and mediate neuron survival.

The gp130/JAK/STAT3 signaling pathway is activated by neuropoietic cytokines in axotomized DRG neurons, as demonstrated by immunohistochemical evidence of STAT3 phosphorylation (Dubovy, Klusakova, Svizenska, & Brazda, 2010; Qiu, Cafferty, McMahon, & Thompson, 2005). STAT3 is locally translated and activated at the nerve injury site, then transported retrogradely to modulate survival of peripheral sensory neurons, and stimulate axon outgrowth by modifying the transcription of genes (Ben-Yaakov et al., 2012). Moreover, local origin STAT3 is activated by neuropoietic cytokines directly in the DRG neurons, as has been demonstrated immunohistochemically in DRG neurons contralateral to nerve injury (Dubovy, Klusakova, Svizenska, & Brazda, 2010). This neuronal activation of STAT3 after axotomy may prevent the neurons from excitotoxic death (Park, Nozell, & Benveniste, 2012) as the most important prerequisite for successful nerve reinnervation.

5. INFLAMMATORY REACTIONS IN NERVE GRAFTING

To overcome more extensive defects of peripheral nerves, autologous grafts prepared from cutaneous nerves are usually used (Millesi, 1984). Although autologous grafts (autografts) are considered as the gold standard for peripheral nerve grafting, they have several limitations that include limited donor source and secondary site morbidity (loss of function in the distribution of the donor peripheral nerve, scarring, or painful neuroma formation) (Colen, Choi, & Chiu, 2009). Despite the autologous antigenic content, an autograft from donor peripheral nerve undergoes a neuroinflammatory reaction during WD that includes activation of Schwann cells, recruitment of macrophages, and clearance of axon and myelin debris (see Section 2). It has been found that the prevention of macrophage invasion impairs reinnervation of autografts (Dahlin, 1995).

The tissue for a nerve graft can also be taken from another individual of the same species (allogeneic grafts, allografts) and/or from another species (xenogeneic grafts or xenografts).

5.1. Biological and synthetic nerve conduits

Generally, any tissue containing the basal lamina tubes—such as freeze-dried muscle or vein packed with muscle fibers—can be used as a possible alternative biological conduit to bridge the peripheral nerve gap. Allogeneic and xenogeneic grafts are alternatives only for peripheral nerve repair, because immunological rejection due to foreign antigens poses a major obstacle to their common clinical use.

Concerns as to the need for an amount of graft material sufficient to bridge a long defect, ensuring only an acceptable neurological deficit after harvesting of a nerve autologous graft, and the side effects of immunosuppressive medication in the case of allo- and xenotransplantation have prompted the development of nerve conduits. The conduits can be divided into biological conduits of autologous or non-autologous origin and non-biological (synthetic) conduits. Experimental and clinical studies have verified axonal regeneration through the autologous venous and arterial conduits for a short distance with restoration of appropriate nerve function (Chiu, 1999). Skeletal muscle can also be used as a nerve conduit because of its suitable ECM components and similarities between the longitudinally oriented basal lamina of muscles and the endoneurial tubes of degenerating nerves (Meek, Varejao, & Geuna, 2004). Because collagen is a major component of the nerve ECM, conduits with collagen type I/III tubes are also generally useful for nerve repair (Keilhoff, Stang, Wolf, & Fansa, 2003).

Also available are various synthetic conduits, but most of these remain experimental (Wang & Cai, 2010). Although the main features of synthetic materials are their inertness and biocompatibility, some of them use immune reaction with invasion of macrophages for biodegradation (Hong et al., 2011). Currently, tissue engineering procedures are used to form conduits enriched with Schwann cells that release many bioactive factors—such as growth factors—promoting axonal regeneration (e.g., NGF, BDNF, IGF-1, IGF-2, PDGF, FGF, and CNTF), permissive ECM molecules (laminin and fibronectin), and some forms of collagen that promote axonal extension (Daly, Yao, Zeugolis, Windebank, & Pandit, 2012). Moreover, other sorts of synthetic conduits and scaffolds are prepared to fix or continuously release neurotrophic factors or neuropoietic cytokines (Cao et al., 2011; Uebersax et al., 2007). These engineered conduits could represent the future of peripheral nerve repair, because they can lead to better functional results than do autologous nerve grafts (Jiang, Lim, Mao, & Chew, 2010).

5.2. Immunobiology of non-autologous nerve grafts

While non-autologous nerve grafts provide unlimited material for use in peripheral nerve reconstruction, the presence of foreign antigens triggers an immune reaction leading to rejection of the graft. Although recipient Schwann cells migrate into the graft and support axon regeneration, donor Schwann cells in the graft are required for regeneration through a long nerve graft and the loss of regenerated host axons contributes to an immune reaction against these non-autologous Schwann cells (Hall, 2002). Schwann cells and fibroblasts of the peripheral nerve express major histocompatibility complex class I (MHC-I) molecules, which play an important role in eliciting immunological rejection after non-autologous nerve transplantation (Rovak et al., 2005). These cell surface molecules present intracellular proteins to cytotoxic CD8+ T cells, resulting in their specific activation and destruction of recognized foreign cells (Barry & Bleackley, 2002). The time course of MHC-I expression in neurons correlates with axonal regeneration after peripheral nerve lesion and proves that this cell surface molecule plays a role in nerve regeneration (Zanon et al., 2010).

MHC class II molecules (MHC-II) present antigens from genetically different individuals to helper CD4+ T cells, resulting in the late rejection of non-autologous tissue transplants. MHC-II is expressed on professional antigen-presenting cells (APCs), such as macrophages. Although Schwann cells express low levels of MHC-I and not MHC-II, significant elevation of MHC-II molecules has been detected on their surface in the presence of activated T cells upon stimulation by interferon-γ (IFN-γ) with synergistic effect of TNFα (Meyer zu Hörste, Hu, Hartung, Lehmann, & Kieseier, 2008). Thus, Schwann cells have the ability to process and present antigens in some specific conditions to be facultative APCs.

Due to MHC incompatibility of recipient and nerve allografts or xenografts, regenerative success of nerve transplantation depends upon immune reaction by immunocompetent cells of hematopoietic origin and/or neural resident cells. Although humoral and cellular immunities participate in the general creation of an obstacle to non-autologous transplantation, the main critical role in nerve graft rejection is played by the cellular immune response (Lu et al., 2009). Neovascularization with restoration of blood flow in avascular nerve grafts is an important condition for successful regeneration, because the Schwann cells survive only 7 days while depending purely on diffusion (Prpa, Huddleston, An, & Wood, 2002). Thus, the use of vascularized nerve for long nerve defects or those within a hypovascular and

scarred recipient bed results in outcomes superior to those achieved using conventional nerve grafts (Vargel et al., 2009). On the other hand, the restoration of blood supply allows donor nerve invasion by macrophages and T cells with predominance of the helper subtype that initiates the rejection response via donor-specific antigen recognition (Caballero et al., 2006).

The direct pathway of allorecognition is based on donor APCs that present donor antigens to the host T cells in the context of MHC-II molecules. In addition to donor professional APCs, like endoneurial resident macrophages, Schwann cells act as facultative APCs that are primary targets of the immune response to the nerve allograft (Lilje, 2002). This immune response to donor MHC molecules is directed toward a variety of antigens and plays a critical role during the early phase of acute graft rejection by sensitizing the host to graft antigens.

T cells become activated when coming into contact with APCs. In addition to direct killing activity by cytotoxic T cells that recognize their targets by binding to antigens associated with MHC-I, graft rejection can also occur through T-cell-mediated mechanisms based on cytokine production (e.g., of IFN-γ, TNFα, IL-2, IL-4, IL-6, IL-12, and IL-17), as well as recruitment and activation of other cytotoxic cells (Yang & Sykes, 2007). For example, graft rejection is significantly suppressed after application of IL-17 and IFN-γ neutralizing antibodies (Yu et al., 2012). TNFα is involved in the rejection process by its capacity to kill cells as well as its ability to activate macrophages (Gulati, 1998). IFN-γ, produced mainly by helper CD4+ T cells, contributes significantly to the acute rejection process by driving macrophages and endothelial cells into a proinflammatory status with T-cell sensitization and rapid development of graft rejection. However, the limiting of IFN-γ in IFN-γ-deficient mice leads to the development of a CD8-mediated mechanism of allograft rejection, which is resistant to experimental immunosuppression (Bishop, Wood, Eichwald, & Orosz, 2001).

5.3. Strategies for attenuation of host immune response

5.3.1 Graft pretreatment

Graft pretreatment is a method for reducing the host immune response to nerve allografts, including both its cellular and structural elements. The pretreatment methods include freeze-drying techniques, cryopreservation, lyophilization, freeze thawing, predegeneration, irradiation, and cold storage (Siemionow & Sonmez, 2007). Promising results were obtained with cold storage in the University of Wisconsin Storage Solution because the technique does not decrease the number of viable donor Schwann cells

but reduces the expression of MHC-II molecules that trigger activation of T cells (Evans et al., 1998, 1999). Another method that is suitable not only for graft pretreatment but also for tissue banking is cryopreservation, which does not alter the cell's viability. However, cryopreserved nerve grafts have been seen to display significantly reduced axon counts and less myelination compared to fresh conventional autografts (Fansa, Lassner, Kook, Keilhoff, & Schneider, 2000). Treatment with the cryoprotectant 10% dimethyl sulfoxide before using liquid nitrogen does not improve morphometric parameters of regenerated nerve fibers in the nerve graft (Fansa, Lassner, et al., 2000).

Other methods keep the basal lamina intact but destroy cellular elements of the graft in an attempt to decrease antigenicity. Although the nerve grafts are gradually repopulated by migrating recipient Schwann cells, the reinnervation of initial acellular graft is delayed and is poorer in comparison with cellular graft (Haninec, Dubový, Houšt'ava, & Stejskal, 2000). Local delivery of soluble neurotrophic factors is a method for reducing the disadvantage of acellular nerve grafts. Grafts pretreated with vascular endothelial growth factor have been shown to stimulate the migration of Schwann cells and outgrowth of blood vessels but not of axons. Neither pretreatment with laminin nor NGF affected the migration of Schwann cells, but NGF treatment increased the number of axons in the graft (Sondell, Lundborg, & Kanje, 1999).

5.3.2 Host immunosuppression

Successful suppression of the immune response and regeneration across nonautologous peripheral nerve grafts using cyclosporine A have been demonstrated in some experimental models, but long-term immunosuppression for non-vital organ transplantation is controversial because of the potential risks. Although withdrawal of immunosuppression after successful reinnervation through nerve grafts results in graft rejection, the rejection is only short term in nature, and the recovery of graft histological and functional parameters is comparable with that of continuous immunosuppression (Midha et al., 1993). Although T cells are the primary target of cyclosporine A's effect, the reduced number of recruited macrophages during WD results in retarded axonal degeneration and subsequent reinnervation (Taskinen & Roytta, 2000). Therefore, cyclosporine A has been replaced by tacrolimus (FK-506) in several nerve grafting protocols because its application resulted in accelerated WD and increased axon sprouting into the graft (Mackinnon, Doolabh, Novak, & Trulock, 2001). The neurotrophic effect of FK-506 is

mediated through binding to FKBP-12 that increases the level of intracellular calcium via calmodulin and thus induces Schwann cell proliferation (Fansa, Keilhoff, et al., 2000; Toll, Seifalian, & Birchall, 2011). Although neurotoxic complications have been noted after clinical application of FK-506, these side effects are reversible and usually occur in association with an underlying predisposing condition (Eidelman et al., 1991). Some new strategies in peripheral nerve bioengineering combined with cyclosporine A have been published that are shown to induce tolerance while enhancing the viability of transplanted cells (Scharpf, Strome, & Siemionow, 2006).

6. CONCLUSION

Endogenous signals from injured or necrotic cells distal to nerve lesion or nerve graft activate inflammatory reaction without septic or exogenous stimuli. The so-called aseptic inflammatory reaction and its mediators under physiological control participate significantly in the process of nerve regeneration, but chronic overproduction of inflammatory mediators develops into NPP induction. Activation of the complement system during WD leads to rapid clearance of injured axons and their myelin sheaths without attack to the other cellular components of the distal nerve stump. Cytokines upregulated in the distal nerve stump are involved in clearance of axon and myelin debris as well as axon regeneration. Similarly to the distal nerve stump, inflammatory responses in and around damaged neurons are implicated not only in their conditioning to regenerate severed axons, but also in NPP induction. Precise understanding of inflammatory reactions during nerve regeneration is needed for correct application of anti-inflammatory treatment of NPP without prevention of axon growth.

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CHAPTER EIGHT

Ghrelin: A Novel Neuromuscular Recovery Promoting Factor?

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Contents

Abstract

Promoting neuromuscular recovery after neural injury is a major clinical issue. While techniques for nerve reconstruction are continuously improving and most peripheral nerve lesions can be repaired today, recovery of the lost function is usually unsatisfactory. This evidence claims for innovative nonsurgical therapeutic strategies that can implement the outcome after neural repair.

Although no pharmacological approach for improving posttraumatic neuromuscular recovery has still entered clinical practice, various molecules are explored in experimental models of neural repair. One of such molecules is the circulating peptide hormone ghrelin. This hormone has proved to have a positive effect on neural repair after central nervous system lesion, and very recently its effectiveness has also been demonstrated in preventing posttraumatic skeletal muscle atrophy. By contrast, no information is still available about its effectiveness on peripheral nerve regeneration although preliminary data from our laboratory suggest that this molecule can have an effect also in promoting axonal regeneration after nerve injury and repair.

Should this be confirmed, ghrelin might represent an ideal candidate as a therapeutic agent for improving posttraumatic neuromuscular recovery because of its putative effects at all the various structural levels involved in this regeneration process, namely, the central nervous system, the peripheral nerve, and the target skeletal muscle.

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1. INTRODUCTION

One of the most frequent causes of movement impairment are lesions of peripheral nerves that induce dramatic muscle atrophy and can occur as a consequence of a variety of traumas (e.g., work accidents) and diseases (e.g., diabetes) with high social costs (de Putter et al., 2012).

The consequences of nerve injuries may be disastrous and can result in substantial functional loss. The increasing number of patients undergoing nerve surgery represents an enormous stimulus for more research in peripheral nerve regeneration and, most of all, for defining innovative strategies for improving functional recovery of repaired nerves.

Peripheral nerve regeneration is usually far from satisfactory (Navarro, Vivo, & Valero-Cabre, 2007; Sun et al., 2009) and there is no technique to guarantee total recovery and normalization of functional sensibility following repair of an injured nerve.

The significant improvements made in understanding the basic biological and molecular mechanisms underlying the progression of nerve regeneration have resulted in the identification of a number of key molecules involved in the process. Among these molecules, there are environmental factors (laminin, integrin, dystroglycan, L-periaxin, and fibrin), neurotrophic factors (such as glial cell-derived neurotrophic factor, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, Neuregulin1, transforming growth factor-β), cytokines (interleukin-6 (IL-6), leukemia inhibitory factor), transcription factors (signal transducer and activator of transcription 3), plasmalemma-associated PKC substrates (GAP-43, myristoylated alanine-rich c kinase substrate, and cytoskeleton-associated protein 23) (Chen, Yu, & Strickland, 2007).

A number of hormones have also been shown to play a regenerative-promoting role after a peripheral nerve injury. Progesterone has been demonstrated to be involved in remyelination since blockage of its action leads to the decrease of myelin sheath after injury and regeneration. In contrast, administration of either exogenous progesterone or its precursor pregnenolone to the injured site promotes myelin sheath formation (Koenig et al., 1995). Also, thyroid hormone (T3) plays an important role in neuronal maturation and myelination. Administration of T3 to an injured rat sciatic nerve results in an increased number of remyelinated fibers, along with the increment of their diameter and myelin thickness compared with control nerves (Mercier, Turque, & Schumacher, 2001). Parathyroid

hormone-related peptide (PTHrP) is widely expressed in the PNS and is upregulated in Schwann cells following sciatic nerve crush (Macica, Liang, Lankford, & Broadus, 2006). Addition of PTHrP to dorsal root ganglion explants stimulates Schwann cell migration, but does not affect their proliferation and survival (Macica et al., 2006). After sciatic nerve crush, the expressions of erythropoietin (Epo) and Epo receptors are increased in Schwann cells. Addition of exogenous Epo to injured sciatic nerves stimulates Schwann cell proliferation (Li, Gonias, & Campana, 2005) and reduces the expression of tumor necrosis factor alpha (TNF-α) in Schwann cells at the injured site, improving peripheral nerve regeneration (Campana et al., 2006). Growth hormone (GH)-treated rats showed improved functional recovery and axonal remyelination after rat sciatic nerve injury (Devesa et al., 2012).

Alpha-melanocyte stimulating hormone (alphaMSH) and corticotropin (ACTH) are known to improve the postlesion repair of injured peripheral nerves by accelerating and enhancing nerve regeneration and muscle reinnervation (Strand et al., 1993). Moreover, alphaMSH has been shown to stimulate the sprouting and neuritogenesis from spinal and sensory neurons *in vitro* (van der Neut, Hol, Gispen, & Bar, 1992). In addition, stimulation with alphaMSH of spinal cord trauma showed a profound and significant stimulation of neurite outgrowth (Joosten, Majewska, Houweling, Bar, & Gispen, 1999).

The ability of gonadal steroids to enhance functional recovery and outgrowth rates after nerve injury was also demonstrated. Indeed, rats subjected to crush injury of the sciatic nerve and treated with testosterone propionate showed a faster regeneration of axonal regrowth. Accelerating regeneration rate was also obtained after exposure with the nonaromatizable androgen, dihydrotestosterone, and E2 (Jones, Alexander, Brown, & Tanzer, 2000).

2. GHRELIN: AN OVERVIEW OF ITS ROLE IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Ghrelin is a circulating peptide hormone, acylated on Ser 3, that, through binding and activation of its receptor GHSR-1a in hypothalamus and pituitary, promotes a potent release of GH (Kojima et al., 1999). Ghrelin is now widely acknowledged as the "hunger hormone" because, alongside the GH-releasing effect, its secretion, regulated by fasting, stimulates food intake, promotes adiposity, and controls energy homeostasis (Tschop, Smiley, & Heiman, 2000; Wren et al., 2001).

Plasma ghrelin levels, normally high during fasting and falling to basal values after the assumption of food, are altered in pathological conditions affecting body mass and/or body energy metabolism. Circulating ghrelin concentrations negatively correlate with body mass index and are lower in overweight or obese subjects compared with normal subject (Tschop et al., 2001). On the other hand, plasma ghrelin levels increase in conditions characterized by energy deficiency such as anorexia nervosa (Ariyasu et al., 2001) or anorexia/cachexia associated to cancer (Garcia et al., 2005; Shimizu et al., 2003), chronic heart failure (Nagaya et al., 2001), chronic kidney disease (Yoshimoto et al., 2002), and chronic obstructive pulmonary disease (Itoh et al., 2004).

Besides the regulation of feeding, ghrelin also exhibits a broad array of other biological activities on the cardiovascular systems, where it decreases blood pressure (Lambert et al., 2011) and improves cardiac function after heart damage (Nagaya et al., 2004, 2001). Moreover, it inhibits the apoptosis of cardiomyocytes and endothelial cells by activating PI3K/Akt and ERK-1/2 pathways *in vitro* (Baldanzi et al., 2002). Ghrelin has also an anti-inflammatory action, inhibiting the activity of the transcription factor NF-kB and suppressing the production of proinflammatory cytokines (Balasubramaniam et al., 2009; Dixit et al., 2004; Lee, Kim, Li, & Park, 2012; Li et al., 2004).

Acylation is essential for ghrelin activity through GHSR-1a: the most abundant unacylated circulating form of ghrelin does not bind to and activate GHSR-1a (Porporato et al., 2013). Far from being the inactive by-product of acylated ghrelin metabolism, a constantly increasing number of evidences proves that unacylated ghrelin is a biologically active peptide, sharing most of acylated ghrelin peripheral activities and participating in the regulation of food intake and adipogenesis through mechanisms not fully elucidated, but very likely independent from GHSR-1a (Asakawa et al., 2005; Toshinai et al., 2006).

Among the wide range of common biological functions, acylated and unacylated ghrelin exert a protective activity on several cell lines including cardiac and endothelial cells, β-pancreatic cells, human pancreatic islets, and cortical neurons (Baldanzi et al., 2002; Chung, Seo, Moon, & Park, 2008; Granata et al., 2007). Moreover, both peptides promote differentiation of skeletal myoblasts, Leydig cells, preadipocytes, and human cardiac embryonic stem cells (Barreiro et al., 2004; Filigheddu et al., 2007; Gao et al., 2012; Liu, Chen, Xu, Vicaut, & Sercombe, 2009).

The findings that unacylated ghrelin shares with ghrelin common binding sites in cell lines, including cells lacking GHSR-1a (Baldanzi et al., 2002;

Cassoni et al., 2001; Filigheddu et al., 2007; Granata et al., 2007; Jeffery, Herington, & Chopin, 2002), and that both peptides have common biological activities *in vivo*, also in *Ghsr* null mice (Porporato et al., 2013), strongly suggest that both peptides act through a common, although yet unidentified, receptor.

In both human patients and experimental models, ghrelin administration ameliorates the cachectic state associated with several pathological conditions such as chronic heart failure (Nagaya et al., 2004), chronic kidney disease (Deboer et al., 2008), arthritis, cancer (Argiles & Stemmler, 2013; DeBoer et al., 2007; Neary et al., 2004), burn injuries (Balasubramaniam et al., 2006), and COPD (Nagaya et al., 2005).

Ghrelin treatment for cancer cachexia is a good candidate for muscle wasting treatment because ghrelin levels are elevated in cancer cachexia—suggesting a compensatory effect—and it controls mediators involved in the cachectic process (Argiles & Stemmler, 2013).

Since ghrelin induces GH release—and therefore the activation of GH/IGF-1 axis—promotes food intake, exhibits anti-inflammatory activities, and stimulates positive energy balance, it has been obviously assumed that the beneficial effect of ghrelin on the cachectic state was a consequence of its activity mediated by GHSR-1a. Although ghrelin may undeniably inhibit cachexia through these GHSR-1a-mediated activities, several evidences prove that both acylated and unacylated ghrelin have a direct antiatrophic activity in skeletal muscles: unacylated ghrelin, which does not bind GHSR-1a and does not activate the GH/IGF-1 axis, reduces burn-induced skeletal muscle proteolysis and local TNF-α upregulation in rats (Sheriff et al., 2012); the cardiac overexpression of the ghrelin gene, resulting in the upregulation of circulating unacylated ghrelin, counteracts muscle atrophy induced by either fasting or denervation; and, finally, both acylated and unacylated peptides impair fasting-induced atrophy in *Ghsr* null mice (Porporato et al., 2013).

Ghrelin antiatrophy effect in muscle was also demonstrated using a mouse model of hindlimb suspension, where ghrelin administration was shown to diminish the reduction of hindlimb muscle mass, thus facilitating the recovery from muscle atrophy (Koshinaka et al., 2011).

3. GHRELIN AND THE NERVOUS SYSTEM

Ghrelin receptor GHSR-1a is detected in various hypothalamic nuclei (anteroventral preoptic nucleus, anterior hypothalamic area, suprachiasmatic

nucleus, lateroanterior hypothalamic nucleus, supraoptic nucleus, ventromedial hypothalamic nucleus, arcuate nucleus, paraventricular nucleus, and tuberomammillary nucleus) and in other areas of the rat brain such as the dentate gyrus, CA2 and CA3 regions of the hippocampal formation, thalamic regions, and several nuclei within the brain stem. Moreover, the finding that GHSR-1a is also observed in the anterior lobe of the pituitary gland is consistent with its role in regulating GH-releasing activity (Guan et al., 1997).

Since circulating ghrelin deriving from the stomach may bind to brain target neurons, it has been demonstrated that it is able to cross the blood–brain barrier in the brain-to-blood direction (Banks, Tschop, Robinson, & Heiman, 2002). Recent findings also reveal that vagotomy prevents peripheral ghrelin's effect on the hypothalamus (Date et al., 2002), suggesting ghrelin's direct effect on the brain (Nakazato et al., 2001; Tschop et al., 2000) may be of intrinsic origin.

The localization of ghrelin in specific brain area, in particular in hypothalamic nuclei, suggests an interaction between this hormone and the pain modulation system. In fact, it was found that ghrelin directly induces neuropeptide Y (NPY) neurons to release NPY (Cowley et al., 2003; Korbonits, Goldstone, Gueorguiev, & Grossman, 2004), a neuropeptide highly expressed throughout the central and peripheral nervous systems that mediates several physiologic activities including the nociceptive process at the level of the spinal cord (Gibbs, Flores, & Hargreaves, 2004). It has also been observed that ghrelin hypothalamic neurons innervate other peptidergic systems such as proopiomelanocortin (POMC) neurons (Cowley et al., 2003). β-Endorphin resulting from POMC gene plays an important role in the descending antinociceptive pathway (Sibilia et al., 2006; Sun, Lundeberg, & Yu, 2003). Moreover, ghrelin increases the levels of hypothalamic nitric oxide (NO) synthase (Gaskin, Farr, Banks, Kumar, & Morley, 2003). It is well known that neuronal NO modulates the antinociceptive effect of endogenous opioids by activating μ-opioid receptors; it is therefore possible that ghrelin enhances the antinociceptive effects of endogenous opioids via NO pathway. In addition, it has been observed that ghrelin decreases serotonin release, a modulator of pain and analgesia (Andersen & Dafny, 1983) in hypothalamus (Brunetti et al., 2002) and raphe nucleus (Carlini et al., 2004).

Several studies showed that ghrelin has an anti-inflammatory activity by decreasing the expression level of the proinflammatory cytokines IL-1β, IL-6, and TNF-α (Dixit et al., 2004) by lymphocytes and monocytes. It is known that these cytokines contribute to central and peripheral inflammatory pain hypersensitivity (Samad et al., 2001). The antihyperalgesic

and neuroprotective effects of ghrelin may therefore be due to the prevention of the production of these proinflammatory cytokines (Dixit et al., 2004; Guneli, Kazikdas, & Kolatan, 2007; Moon, Kim, Hwang, & Park, 2009; Theil et al., 2009; Wang, Bansal, Falk, Ljubanovic, & Schrier, 2009). Finally, ghrelin may exert antinociceptive effects also by directly increasing inhibitory (GABAergic/glycinergic) neurotransmission in a subset of deep dorsal horn neurons, mainly localized in the medial aspect of laminae IV–VI (Vergnano et al., 2008). All these observations suggest the role of ghrelin as a peptide participating in the inhibitory control of pain in pathological states.

Ghrelin has also been shown to promote neurogenesis in several areas of the brain. Indeed, it has been demonstrated that ghrelin increases neural cell proliferation in cultured neuronal precursor cells from the fetal spinal cord (Sato et al., 2006), but this effect is greater when the cells are taken from E17 embryos instead of P2 pups (Inoue, Nakahara, Kangawa, & Murakami, 2010). This effect is promoted by both acylated and unacylated ghrelin, suggesting that ghrelin acts through both the GHSR-dependent and GHSR-independent mechanisms to mediate neurogenesis of the embryonic spinal cord.

Moreover, systemic administration of ghrelin increases neurogenesis in the dorsal motor nucleus of the vagus (DMNV) and in the nucleus of the solitary tract (NTS) following vagotomy: ghrelin produced a significant increase in BrdU incorporation both *in vivo* (increases vagotomy-induced BrdU incorporation in the DMNV and NTS in adult rats) and *in vitro* (cultured DMNV and NTS neurons respond to ghrelin with increased BrdU incorporation), suggesting its potential to promote neuronal development and regeneration (Zhang, Hu, Lin, Fan, & Mulholland, 2005; Zhang et al., 2004).

Finally, recent studies demonstrate that ghrelin is also involved in hippocampal neurogenesis. Indeed, mice treated with ghrelin showed increased BrdU incorporation and doublecortin-positive neuroblasts in the subgranular zone (SGZ) of the dentate gyrus, and this number was significantly reduced after anti-ghrelin antibody treatment (Moon, Kim, Hwang, & Park, 2009). Moreover, ghrelin knockout mice resulted in reduced numbers of BrdU-positive cells, immature neurons, and newly generated neurons in the SGZ, while ghrelin treatment restored these cell numbers to those of wild type (Li et al., 2013). Finally, ghrelin stimulates increased incorporation of ^{3}H-thymidine in adult rat hippocampal progenitor cells, indicating an increased cell proliferation (Johansson et al., 2008).

Intracerebroventricular (icv) administration of ghrelin in the central nervous system induces anxiogenic effects (elevated plus maze test) and

increases memory retention in rats (step down test) (Carlini et al., 2002, 2004), suggesting that ghrelin influences several biochemical processes in the hippocampus. In addition, in a neonatal rat model with experimental unilateral hypoxic-ischemic injury, icv injections of the synthetic peptidic GH-secretagogue hexarelin significantly reduced the area of injury in the cerebral cortex, hippocampus and thalamus, demonstrating a neuroprotective effect of the hormone *in vivo* (Brywe et al., 2005).

More recent studies indicate that ghrelin exerts neuroprotective effects also against chronic glutamate toxicity. Indeed, in an *in vitro* study with a model of excitotoxic motoneuron degeneration of organotypic spinal cord cultures, it has been observed that treatment with ghrelin significantly decreases motoneuron loss by preventing microglial activation in the spinal cord (Lee et al., 2012). Moreover, ghrelin protects spinal cord motoneurons after glutamate excitotoxicity also through the activation of extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-3-kinase (PI3K)/Akt/glycogen synthase kinase (GSK)-3β pathways (Lim, Lee, Li, Kim, & Park, 2011). Finally, ghrelin has a neuroprotective effect in neurodegenerative diseases such as Parkinson's disease, where it functions as a microglia-deactivating factor (Moon, Kim, Hwang, Seo, et al., 2009).

Zhang et al. (2012) demonstrated the acute effect of ghrelin on ischemia/reperfusion (I/R) injury in the rat spinal cord. Their results suggest that ghrelin administration may inhibit spinal I/R injury, thanks to the anti-apoptotic properties of the ghrelin mechanism that inhibits the apoptosis molecules in the mitochondrial pathway and activates endogenous protective molecules.

Little is known about ghrelin activities in peripheral nervous system. Erriquez et al. (2009) showed that DRG cells express GHSR-1a and that ghrelin induces a change in cytosolic calcium concentration in both glia and neurons of embryonic chick DRG.

Daily administration of ghrelin to rats subjected to chronic constriction injury (CCI) of the sciatic nerve improved the histological appearance of the nerve. Moreover, ghrelin prevented mechanical hyperalgesia in CCI rats in a dose-related manner (Guneli et al., 2010).

4. OVEREXPRESSION OF GHRELIN PROMOTES MOTOR NERVE FUNCTION RECOVERY AFTER TRAUMATIC INJURY

So far, no information are available about the effects of GHR on peripheral nerve regeneration. Therefore, in order to evaluate if ghrelin can act on

neuromuscular recovery, a pilot study was performed in our laboratory on four adult male FVB1 WT and four FVB1 *Myh6/Ghrl* transgenic mice.

Transgenic FVB1 animals were obtained by cloning the murine ghrelin gene (*Ghrl*) under the control of the cardiac promoter sequences of the βMHC 3′ UTR and the first three exons of the αMyHC isoform (De Acetis et al., 2005; Porporato et al., 2013). Phenotypical characterization and experiments were carried out on hemizygote animals.

Under deep anesthesia, the median nerve of the left forelimb was approached from the axillary region to the elbow with a longitudinal skin approach and, under operative microscope, was carefully exposed and cut. Transected median nerve was immediately repaired by means of a termino-terminal suture. In order to prevent interferences with the grasping test, the contralateral median nerve was transected at the middle third of the brachium and its proximal stump was sutured in the pectoralis major muscle to avoid spontaneous reinnervation.

Functional recovery was assessed by grasping test every 5 days for 70 days after surgery. For the control value, the grasping test was performed the day before the operation. Preliminary results on functional recovery showed that neuromuscular recovery was significantly faster in *Myh6/Ghrl* mice compared to WT mice. Moreover, after 40 days from the injury, WT value was still statistically different compared to the preinjury value, whereas

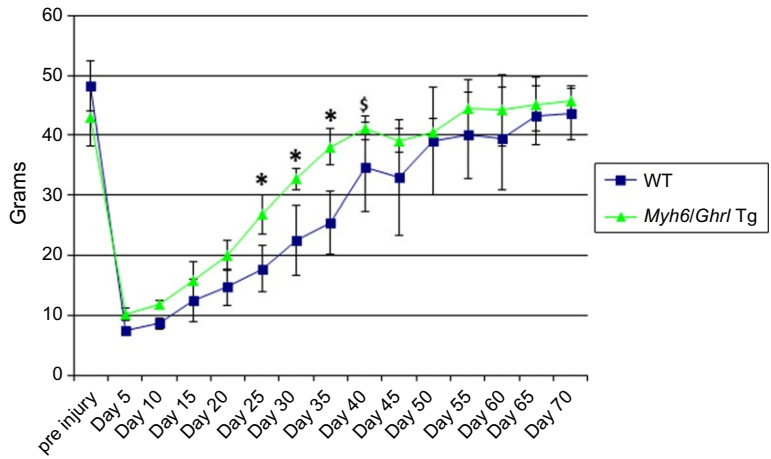

Figure 8.1 Functional recovery assessment by grasping test after termino-terminal median nerve repair in wild type (WT) and *Myh6/Ghrl* transgenic (Tg) mice. Functional recovery is faster in Tg mice ($^{*}p \leq 0.05$ WT vs. Tg). After 40 days, WT value was still statistically different compared to the preinjury value, whereas Tg value was similar to the preinjury value ($^{\$}p \leq 0.05$ WT vs. preinjury).

Myh6/Ghrl value was similar to the preinjury value, showing that the overexpression of *Ghrl*, and the resulting higher levels of circulating unacylated ghrelin, sped up motor recovery after injury. Finally, at the end of the experiment (day 70), the two experimental groups reached values similar to the preinjury values (Fig. 8.1).

These preliminary data suggest that this molecule can have an effect also in promoting axonal regeneration after nerve injury and repair. Further studies are needed to understand where and how ghrelin acts.

5. CONCLUSIONS

Today, no pharmacological approach has still been introduced in the clinics with the goal of improving posttraumatic neuromuscular recovery. Among the various molecules that are currently being explored in experimental models with such a goal, the circulating peptide hormone ghrelin is receiving increasing interest.

In fact, literature review provided in this chapter shows that ghrelin has both a regeneration promoting effect in the central nervous system and a preventive effect against skeletal muscle atrophy. Preliminary data from our laboratory also suggested that this hormone can have an effect in promoting regeneration after nerve injury and repair.

The putative positive effects of ghrelin at all the various structural levels involved in posttraumatic neuromuscular recovery, namely—the central nervous system, the peripheral nerve, and the target skeletal muscle—make this molecule an ideal candidate as a therapeutic agent for improving this complex and multilevel regenerative process.

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CHAPTER NINE

Neuregulin 1 Role in Schwann Cell Regulation and Potential Applications to Promote Peripheral Nerve Regeneration

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Contents

Abstract

Neuregulin 1 (NRG1) is a multifunctional and versatile protein: its numerous isoforms can signal in a paracrine, autocrine, or juxtacrine manner, playing a fundamental role during the development of the peripheral nervous system and during the process of nerve repair, suggesting that the treatment with NRG1 could improve functional outcome following injury. Accordingly, the use of NRG1 *in vivo* has already yielded encouraging results.

The aim of this review is to focus on the role played by the different NRG1 isoforms during peripheral nerve regeneration and remyelination and to identify good candidates to be used for the development of tissue engineered medical devices delivering NRG1, with the objective of promoting better nerve repair.

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1. INTRODUCTION TO NEUREGULIN 1

Neuregulins are a family of soluble and transmembrane growth factors encoded by four different genes. Among them, the most studied is Neuregulin 1 (NRG1), which is involved in the development of heart, mammary gland, brain, and nerve through the activation of different combinations of the tyrosine kinase receptors ErbB2, ErbB3, and ErbB4 (Mei & Xiong, 2008; Yarden & Sliwkowski, 2001). NRG1 plays an important role in myelination and peripheral nerve regeneration and, for this reason, was the subject of recent comprehensive reviews (Fricker & Bennett, 2011; Nave & Salzer, 2006; Pereira, Lebrun-Julien, & Suter, 2012; Salzer, 2012Syed & Kim, 2010; Taveggia, Feltri, & Wrabetz, 2010) and also other reviews that for space limits have not been cited here.

This review aims to give an overview of what is known about the role of NRG1 in axonal growth, myelination, and remyelination, focusing the attention on its prospective clinical application to promote peripheral nerve regeneration, taking into consideration the different possible delivery systems.

1.1. NRG1 gene products

Over time, different NRG1 isoforms were identified and named according to the context in which they were found (neu differentiation factor/NDF, heregulin/HRG, acetylcholine receptor-inducing activity/ARIA, glial growth factor/GGF, and sensory and motor neuron-derived factor/SMDF), until all of them were recognized to belong to the same gene, and named neuregulin1/NRG1 (Falls, 2003; Marchionni et al., 1993).

In this review, the different NRG1 isoforms will be named according to the scheme proposed and shared by different authors (Falls, 2003; Mei & Xiong, 2008), based on exon composition (Fig. 9.1). NRG1 is encoded by a gene spanning 2,6 mbp in humans and rats, 2,4 mbp in mice (Chou & Ozaki, 2010). All NRG1 isoforms contain an epidermal growth factor (EGF)-like domain that is located in the extracellular portion of the protein and is necessary and sufficient for the receptor activation.

Six types of NRG1 were described (I–VI), which differ for N-terminal exons, arising from alternative splicing and use of alternative promoters. Types I (NDF/HRG/ARIA), II (GGF), and III (SMDF) are encoded in a wide range of vertebrate genomes, type IV appears to be restricted to

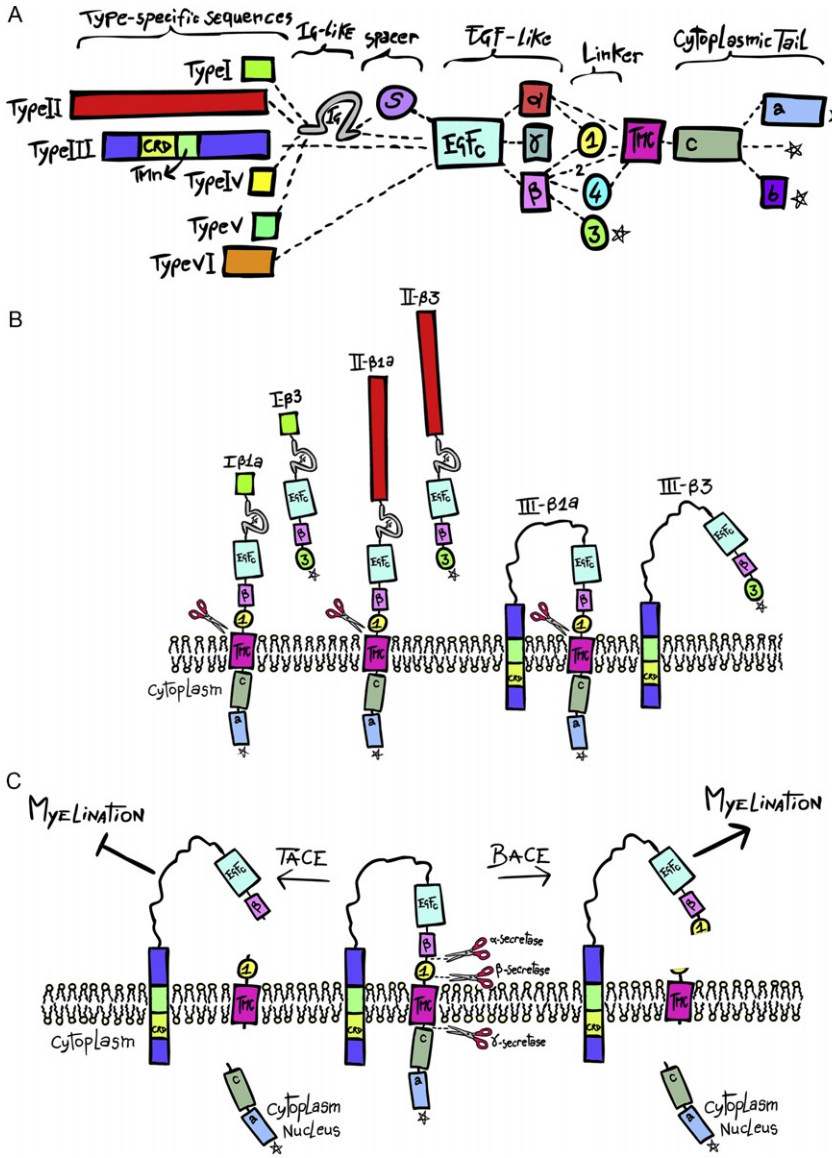

Figure 9.1 NRG1 isoforms. Panel A: Six types of NRG1 are described (I–VI), which differ for N-terminal exons. NRG1-types I, II, IV, and V (with or without a spacer region) are characterized by an immunoglobulin (Ig)-like domain, located between the N-terminal sequence and the EGF-like domain; NRG1-types III and VI present an N-terminal region connected directly to the EGF-like domain. The type III N-terminal sequence contains a cystein-rich domain (CRD) with an additional transmembrane domain (TMn). Variants derive also by splicing in the linker and in the cytoplasmic tail;

(*Continued*)

mammalian, types V and VI appear to be restricted to primates (Chou & Ozaki, 2010).

An immunoglobulin (Ig)-like domain-located between the N-terminal sequence and the EGF-like domain-characterizes NRG1 types I, II, IV, and V (with or without a spacer region), whereas NRG1 types III and VI present an N-terminal region connected directly to the EGF-like domain. In type III NRG1, the N- and the C-terminal regions are both located inside the cell (except one isoform-NRG1-type III-β3-which presents only the N-terminal inside the cell). The type III N-terminal sequence also contains a cystein-rich domain (CRD) with an additional transmembrane domain (TMn).

The number (>30) and the nomenclature of the identified NRG1 derives also from the alternative use of different exons located downstream the EGF-like domain: the first exon can be α or β or can be missing (γ); the following exon can be 1 or 4 or 3 (followed by a stop codon) or missing (isoform 2), the next exon is a transmembrane domain (TMc), followed

Figure 9.1—Cont'd the C-terminal transmembrane domain (TMc) is located between these two regions. The great number of NRG1 isoforms derives also from the alternative use of different exons located downstream the EGF-like domain: the first exon can be α or β or can be missing (isoform γ); the second exon can be 1 or 4 or 3 (followed by a stop codon) or can be missing (isoform 2); the third exon can be a transmembrane domain (TMc), followed by a cytoplasmic exon c, that can be followed by a stop codon (isoform c), by exon a or exon b. Panel B: In this scheme is reported the structure of those isoforms (I, II, and III, β1a and β3) cited in this review which is focused on peripheral nerve regeneration (see also Table 9.1). Many soluble and mature NRG1 proteins are produced in the form of transmembrane precursors and are generated by the cleavage by different secretases, except in the case of NRG1-β3 isoforms, which are already released as soluble mature proteins for autocrine/paracrine interactions (type I/II) or as transmembrane mature proteins for juxtacruine interactions (type III). Panel C: NRG1-type III can be cleaved by α-secretases belonging to tumor necrosis factor-α-converting enzyme (TACE) or a disintegrin and metalloprotease (ADAM) family (e.g., TACE/ADAM17) or by β-secretases (BACE-1/β-site of amyloid precursor protein-cleaving enzyme). BACE-1 cleavage stimulates myelination (Hu et al., 2006; Willem et al., 2006) and remyelination (Hu et al., 2008); TACE cleavage inhibits myelination (La Marca et al., 2011). A second cleavage by a γ-secretase-dependent protease, in those NRG1 isoforms containing the TMc, generates a cytoplasmic fragment that can translocate into the nucleus and influence gene transcription (Bao et al., 2004). For the contents of this figure, the authors got inspiration from different reviews and papers (Bao, Wolpowitz, Role, & Talmage, 2003; Falls, 2003; La Marca et al., 2011; Mei & Xiong, 2008; Velanac et al., 2011).

by a cytoplasmic exon c, which can be followed by a stop codon (isoform c), by exon a or exon b. The activity of β isoforms is 100-fold higher than that of α isoforms.

Cleavage by the β-secretase BACE-1 (β-site of amyloid precursor protein-cleaving enzyme) or by the α-secretases TACE (tumor necrosis factor-α-converting enzyme) or ADAM (members of "a disintegrin and metalloprotease" family), generates soluble NRG1, except in the case of type III NRG1 that remains transmembrane. As discussed in the following paragraph, BACE-1 cleavage of axonal NRG1-type III positively regulates myelination (Hu et al., 2006; Willem et al., 2006) and remyelination (Hu et al., 2008), while TACE cleavage has a negative effect on myelination (La Marca et al., 2011).

Types IV–VI NRG1 are less characterized, but it is likely that they are processed like types I and II (Mei & Xiong, 2008).

The expression of the different isoforms is spatially and temporally regulated, suggesting that different isoforms display specific and unique characteristics.

Different types of NRG1 I, II, and III are expressed in the PNS; they activate-in *cis* and in *trans*-the heterodimeric receptor ErbB2/ErbB3 expressed by Schwann cells (SC). After ligand binding to ErbB3, ErbB3, and ErbB2 heterodimerize, ErbB2 tyrosine kinase switches on, and phosphorylates intracellular tyrosine residues which become docking sites for adaptor proteins involved in different signal transduction pathways, which eventually regulate the transcription of genes implicated in survival, migration, differentiation, proliferation, and myelination (Fricker & Bennett, 2011; Yarden & Sliwkowski, 2001, and references therein).

NRG1 is involved in many steps of the peripheral nerve development, playing a fundamental role in the development of SC precursors and in the interactions between axons (expressing mainly transmembrane NRG1-type III) and SC (expressing ErbB2-ErbB3 and soluble type I/II NRG1). The absence of NRG1-type III (Wolpowitz et al., 2000) or of the co-receptors ErbB3 (Riethmacher et al., 1997) or ErbB2 (Morris et al., 1999; Woldeyesus et al., 1999) gives rise to animals without or with severely reduced SC precursors; the absence of NRG1-type I/II gives rise to normal SC (Meyer et al., 1997), suggesting that NRG1-type III is the most important isoform for SC development. Nevertheless, it has been shown-in the embryonic chick-that soluble NRG1 is also released by axons during the critical period of SC survival (Ma, Wang, Song, & Loeb, 2011).

2. NRG1 ISOFORMS AND THEIR ROLE IN SC MYELINATION

The existence of a signal instructing SC to deposit layers of myelin-the thickness of which is proportional to the diameter of the axon-had long been hypothesized. Transgenic and knock-out mice (Table 9.1) allowed to demonstrate that neuronal NRG1-type III plays an instructive role on myelination, determining the ensheathment fate of axons: reduced NRG1 expression causes hypo-myelination (Michailov et al., 2004; Taveggia et al., 2005), which can be rescued by lentiviral (LV)-mediated expression of NRG1-type III-β1a (Taveggia et al., 2005). Moreover, neuronal NRG1-type III has been shown to be required for remyelination and regeneration after nerve injury: single axons in which NRG1 has been ablated are hypomyelinated and regenerate more slowly (Fricker et al., 2011).

Neuronal transmembrane NRG1-type III-β1a overexpression induces hyper-myelination (Michailov et al., 2004), converts unmyelinated axons of sympathetic neurons to myelination (Taveggia et al., 2005), and improves remyelination after peripheral nerve injury (Stassart et al., 2013).

Neuronal NRG1-type III-β3 overexpression has no effect on myelin thickness (Gomez-Sanchez et al., 2009), but stimulates SC proliferation and enlarges peripheral nerve and ganglia.

Neuronal soluble NRG1-type I-β1a overexpression does not alter myelination (Michailov et al., 2004), but improves remyelination after peripheral nerve injury (Stassart et al., 2013). These authors demonstrate that NRG1 produced by SC is not necessary for myelination, but it is strongly involved in remyelination, which is highly impaired after nerve crush in mice lacking SC NRG1, resulting in severe hypo-myelination. In these mice, the ectopic neuronal expression of NRG1-type I-β1a does not completely rescue for the absence of SC NRG1, suggesting that NRG1 released by SCs is relevant for remyelination. This role is confirmed by the observation that NRG1-type I (both α and β isoforms) is strongly up-regulated following injury (Carroll, Miller, Frohnert, Kim, & Corbett, 1997; Ronchi et al., 2013; Stassart et al., 2013).

Intriguingly, Syed et al. (2010) investigated whether NRG1-type III-provided in a paracrine manner-would still promote myelination and they demonstrated that this is the case: a recombinant "soluble" (but containing all domains, including the CRD and the TMn domain) NRG1-type III-β3 is able to promote *in vitro* myelination of dissociated dorsal root ganglia

Table 9.1 Study of the role of the different NRG1 isoforms in myelination and remyelination in transgenic and knock out mouse experimental models

NRG1 isoform	Genotype	Axonal expr.	Glial expr.	*In vitro* assays	*In vivo* assays	Peripheral nerve phenotype	References
II-β3 (soluble)	WT			+		Soluble NRG1-II-β3 (GGF2) isoform blocks axon ensheathment and myelination, and leads to extensive demyelination when added to mature cocultures	Zanazzi et al. (2001)
All	$Nrg1^{+/-} \times ErbB2^{+/-}$ (KO)				+	Reduced NRG1 expression causes hypomyelination (Fig. 2a)	Michailov et al. (2004)
III-β-1a (transmembrane)	Thy1.2-NRG1-III-β-1a (transgenic mice expressing NRG1 in postnatal motoneurons and DRG neurons)	+			+	Nrg1-III-β1a overexpression causes hypermyelination (Figs. 4 and 5)	
I-β-1a (soluble)	Thy1.2-NRG1-I-β-1a (transgenic mice expressing NRG1 in postnatal motoneurons and DRG neurons)	+			+	Nrg1-I-β1a overexpression does not alter myelination (Fig. 4c)	
III (transmembrane)	NRG1-type $III^{-/-}$ (KO)	−		+	+	NRG1-type $III^{+/-}$ mice are hypomyelinated and aberrantly ensheathed (Fig. 7); Nrg1-III-β1a forced expression rescues myelination defects (Fig. 3)	Taveggia et al. (2005)

Continued

Table 9.1 Study of the role of the different NRG1 isoforms in myelination and remyelination in transgenic and knock out mouse experimental models—cont'd

NRG1 isoform	Genotype	Axonal expr.	Glial expr.	*In vitro* assays	*In vivo* assays	Peripheral nerve phenotype	References
III-β-1a (transmembrane)	WT	+		+		Nrg1-III-β1a forced expression converts unmyelinated axons of sympatetic neurons to myelination (Fig. 4)	
III-β-3 (transmembrane)	NSE-hSMDF (transgenic mice expressing NRG1-III-β-3 under the promoter of neuron specific enolase)	+			+	Nrg1-III-β3: no major effects on myelin thickness; peripheral nerves are enlarged; Remak bundles altered: small caliber axons not separated, but closely packed and ensheathed as a single unit	Gomez-Sanchez et al. (2009)
III-β-3 ("soluble," but containing all domains, including CRD and TMn; produced as a soluble factor by R&D)	WT			+		Soluble Nrg1-III-β3 promotes Schwann cell myelination (Fig. 1, cocultures of SC and dissociated DRG neurons)	Syed et al. (2010)
	WT			+		Soluble Nrg1-III-β3 induces myelination on normally nonmyelinated superior cervical ganglion (SCG) neurons (Fig. 3)	

	WT	+		Soluble Nrg1-III-β3 at high concentration inhibits Schwann cell myelination (Fig. 6)	
	NRG1 type III$^{-/-}$ (KO)	+		Soluble Nrg1-III-β3 rescues the myelination defect on Nrg1 type III$^{+/-}$ neurons, but not on Nrg1 type III$^{-/-}$ neurons (Fig. 2)	
II-β3 (soluble, produced by Acorda Therapeutics)	WT	+		Soluble Nrg1-II-β3 (GGF2) at very low concentration promotes Schwann cell myelination (Fig. 7), at medium-high concentration inhibits it	
III (transmembrane)	WT + Tace shRNA	+		TACE downregulation induces precocious myelination and hypermyelination *in vitro* (Fig. 1) that is neuron-autonomous (Fig. 2)	La Marca et al. (2011)
	Tace fl/fl × HB9-cre (transgenic line that drives motor neuron specific recombination using the promoter of Mnx1 gene)		+	TACE inactivation in motor neurons leads to precocious myelination (Fig. 3); nerves are hypermyelinated during development and in the adult, and Remak fibers are aberrantly ensheathed (Figs. 4 and 5)	

Continued

Table 9.1 Study of the role of the different NRG1 isoforms in myelination and remyelination in transgenic and knock out mouse experimental models—cont'd

NRG1 isoform	Genotype	Axonal expr.	Glial expr.	*In vitro* assays	*In vivo* assays	Peripheral nerve phenotype	References
	Tace fl/fl × Mpz-cre (transgenic line that drives Schwann cell specific recombination using the promoter of Mpz gene)				+	TACE inactivation in SC does not alter myelination (Fig. 6); myelinated fibers have more periaxonal space and an accumulation of organelles in the inner cytoplasmic collar (Fig. S5), suggesting that glial TACE process molecules implicated in myelin compaction and/or adhesion	
	NRG1 type III$^{-/-}$ (KO) + Tace shRNA			+		Ablation of Tace in type III$^{-/-}$ neurons does not rescue myelination: Tace inhibits myelination by modulating NRG1 type III	
III (transmembrane)	SLICK-A Cre; NRG$^{fl/fl}$	+			+	Axonal NRG1 ablation (single neuron labeling with inducible Cre-mediated knock-out) causes severe deficits in remyelination	Fricker et al. (2011)
III-β-1a (transmembrane)	Thy1.2-HA-NRG1-III-β1a^{FL} & Thy1.2-HA-NRG1-III-β1a^{GIEF} (transgenic mice expressing NRG1 in postnatal moto-neurons and DRG neurons)	+			+	Neuronal overexpression of NRG1-III-β1a^{GIEF}—designed to mimic BACE cleavage—induces hypermyelination *in vivo*	Velanac et al. (2012)

	NRG1-type III$^{-/-}$ (KO)		+	Neuronal overexpression of NRG1-III-β1a^{GIEF} is sufficient to restore myelination in NRG1-III$^{-/-}$ sensory neurons, suggesting that the C terminal domain and the cytoplasmic tail are not required for myelination	
	BACE1$^{-/-}$		+	Overexpression of full-length NRG1-III-β1a promotes hypermyelination in BACE1$^{-/-}$ mice. NRG1 processing is impaired but not abolished in BACE1$^{-/-}$. BACE1 is not essential for the activation of NRG1 type III to promote myelination	
III-β-1a (transmembrane)	Thy1.2-NRG1-III-β1a (transgenic mice expressing NRG1 in postnatal motoneurons and DRG neurons)	+	+	Overexpression of the axonal Nrg1-III-β1a isoform improves remyelination after peripheral nerve injury (Fig. 1S)	Stassart et al. (2013)

Continued

Table 9.1 Study of the role of the different NRG1 isoforms in myelination and remyelination in transgenic and knock out mouse experimental models—cont'd

NRG1 isoform	Genotype	Axonal expr.	Glial expr.	*In vitro* assays	*In vivo* assays	Peripheral nerve phenotype	References
I-β-1a (soluble)	Thy1.2-NRG1-I-β1a (transgenic mice expressing NRG1 in postnatal motoneurons and DRG neurons)	+			+	Overexpression of the soluble Nrg1-I-β1a isoform (which has not effect on normal myelination) improves remyelination after peripheral nerve injury (Fig. 1)	
All	Dhh-Cre x $Nrg1^{loxP/loxP}$ (Cre-lox mice missing NRG1 exons α and β in Schwann Cells)		−		+	SC $NRG1^{-/-}$: no defects in myelination; remyelination is strongly impaired after nerve crush, resulting in severe hypomyelination (Fig. 4)	
I-β-1a (soluble)	Thy1.2-NRG1-I-β-1a × Dhh-Cre X $Nrg1^{loxP/loxP}$ (transgenic mice expressing NRG1 in postnatal motoneurons and DRG neurons, and missing NRG1 in Schwann cells)	+	−		+	Remyelination after crush is inefficient: neuronal Nrg1-I-β1a does not compeletely compensate for the absence of NRG1 expression in SC (Fig. 5e)	
I (soluble)	WT		+		+	Type I NRG1 expression is induced after nerve injury (Fig. 2c)	
I/II (α/β) (soluble)	WT and BALB-neuT (ErbB2 overexpression)		+		+	Type I/II NRG1 (α and β isoforms) is upregulated after nerve injury (Fig. 12)	Ronchi et al. (2013)

(DRG) neurons and of normally nonmyelinated superior cervical ganglion (SCG) neurons, and is able to rescue the myelination defects on NRG1-type $III^{+/-}$ neurons (but not on NRG1-type $III^{-/-}$ neurons). These data suggest that NRG1-type III must be expressed-even at low levels-as a transmembrane protein in order to allow early events of axonal segregation and ensheathment but, to further promote myelination, it can be delivered as a soluble protein.

Then, they examined whether the NRG1 concentration also played a role on myelinating activity and they, amazingly, demonstrated that soluble NRG1-type II-β3-when used at very low concentration-promotes myelination, while at higher concentration inhibits it, as previously shown by others (Zanazzi et al., 2001). Similarly, they demonstrated that "soluble" NRG1-type III-when used at high concentration-inhibits myelination (while at low concentration stimulates it). These data show that the concentration of soluble NRG1 (regardless of the isoform) plays an important role in determining the myelination fate of axons. Syed and colleagues demonstrate that there is a concentration threshold beyond which the AKT pathway is activated, playing a promyelinating effect, and a higher concentration threshold beyond which the ERK pathway is activated, leading to myelination inhibition (and the thresholds are lower for NRG1-type II, higher for "soluble" NRG1-type III).

Following nerve injury, SC respond to axonal damage up-regulating NRG1-type I/II (Carroll et al., 1997; Ronchi et al., 2013; Stassart et al., 2013) and activating ERK signaling pathway (Harrisingh et al., 2004). Accordingly, it has been shown that activation of the ERK-signaling pathway in myelinating SC drives them back to a dedifferentiated state, that is reversible: as soon as ERK signal diminishes, SC respond to axonal signals and redifferentiate (Napoli et al., 2012). Indeed, when *Mycobacterium leprae*, the leprosy pathogen, binds to and activates ErbB2, it activates ERK pathway and demyelination (Tapinos, Ohnishi, & Rambukkana, 2006). On the contrary, it has been recently shown that sustained activation of ERK in SC and oligodendrocytes stimulates myelin growth and increases myelin thickness (Ishii, Furusho, & Bansal, 2013). To explain these different results, it has been suggested that ERK activation can be promyelinating or demyelinating, depending on the context, on the strength and on the duration of activation; it has been proposed that, during development, ERK activation promotes myelination, while its activation in differentiated SC promotes demyelination.

2.1. NRG1 cleavage and consequences on myelination activity

All NRG1 are synthesized with the EGF-like domain exposed to the extracellular environment (Fig. 9.1). With the exception of the β3 isoforms-lacking the transmembrane domain (TMc)-which are produced as soluble or transmembrane proteins (Falls, 2003), ready to interact with their receptors, most NRG1 isoforms are synthesized as transmembrane precursor proteins and need a proteolytic cleavage to release a soluble ligand for paracrine/autocrine signals (all type isoforms, except type III) or to expose the EGF-like domain toward the extracellular environment for juxtacrine interactions (isoform type III).

As discussed above, axonal NRG1-type III regulates myelin sheath thickness (Michailov et al., 2004), determines the ensheathment fate of axons (Taveggia et al., 2005), and is required for remyelination (Fricker et al., 2011). NRG1-type III can be cleaved by α-secretases belonging to the ADAM family (e.g., TACE/ADAM17) or by the β-secretase BACE-1 (Fig. 9.1, panel C). BACE-1 cleavage stimulates myelination (Hu et al., 2006; Willem et al., 2006) and remyelination (Hu et al., 2008), ADAM cleavage inhibits myelination (La Marca et al., 2011).

Moreover, it has been shown that in NRG1 isoforms containing the TMc and the cytoplasmic tail (all but the β3 isoforms), a second cleavage by a γ-secretase-dependent protease can occur following ligand–receptor interactions, to activate "reverse signaling" (Bao et al., 2003). A cytoplasmic fragment is released that can translocate into the nucleus and influence transcription (Bao et al., 2004).

TACE/ADAM17 cleaves NRG1 in the exon "1" a few aminoacids (3–6) upstream the cleavage site of BACE-1 (La Marca et al., 2011) and its downregulation leads to precocious- and hyper-myelination, that is neuron-autonomous (indeed, TACE inactivation in SC does not alter myelination). These results suggest that TACE inhibits myelination by limiting the amount of functional axonal NRG1-type III.

It has also been demonstrated that ADAM10 cleaves NRG1 in the same region, although its downregulation in a co-culture system was unable to affect normal myelination (Luo et al., 2011).

Conversely, BACE-1 inhibition impaires normal myelination (Luo et al., 2011) and neuronal overexpression of NRG1-type III-β1a^{GIEF}, a recombinant protein designed to mimic BACE-1 cleavage, induces hyper-myelination *in vivo*, and is sufficient to restore myelination in NRG1-type III$^{-/-}$ sensory neurons (Velanac et al., 2011), suggesting that

the C-terminal domain-that is missing in NRG1-type III-β1a^{GIEF} recombinant protein-is not required for myelination. Nevertheless, NRG1-type III-β1a overexpression promotes myelination also in BACE-1$^{-/-}$ mice, suggesting that BACE-1 promotes myelination, but is not essential for NRG1 processing and pro-myelination activity (Velanac et al., 2012).

It will be intriguing to understand how a few aminoacids in the NRG1-type III C terminus are able to guide the fate of axon myelination: NRG1-type I-β1a cleaved by TACE or BACE-1 differ for a few aminoacids; moreover, the C-terminal aminoacids that characterize β3 are just 11 and, as previously mentioned, the neuronal overexpression of NRG1-type III-β3 does not induce hyper-myelination (Gomez-Sanchez et al., 2009). As already discussed by Velanac, these 11 aminoacids can serve as an acylation-like modification site, which tightly associate this isoform to the membrane (Cabedo, Luna, Fernandez, Gallar, & Ferrer-Montiel, 2002), likely activating different signaling pathway. On the other hand, β3 exon aminoacids are present in the isoform NRG1-type II-β3 (GGF2) which is currently used for clinical trials and, at very low concentration, promotes myelination, at higher concentration inhibits it (Syed et al., 2010; Zanazzi et al., 2001). Understanding the role played by the "GIEF" aminoacids that remain following BACE-1 cleavage (Velanac et al., 2011), and by the β3 exon aminoacids, will contribute to understand the myelination process.

3. NRG1 TO PROMOTE NERVE REPAIR

3.1. Peripheral nerve injury and repair

Axons in the adult peripheral nervous system (PNS) undergo spontaneous regeneration after injury. Following a peripheral nerve injury, the type of nerve repair depends on the length of the nerve gap between the proximal and the distal stumps (Siemionow & Brzezicki, 2009), as shown in Fig. 9.2. Short gaps (few mm) can be repaired by direct suture of the proximal and the distal stumps with end-to-end coaptation and epineural suturing. The most common treatment for longer nerve gaps is surgical repair using autologous nerve grafts (autografts) (Deumens et al., 2010; Kandenwein, Kretschmer, Engelhardt, Richter, & Antoniadis, 2005). Autografts have shown high efficacy in inducing nerve regeneration because they provide to the regenerating axons a natural guidance channel with SC surrounded by their basal lamina, allowing appropriate alignment of outgrowing axons (Deumens et al., 2010). However, there are some drawbacks in using

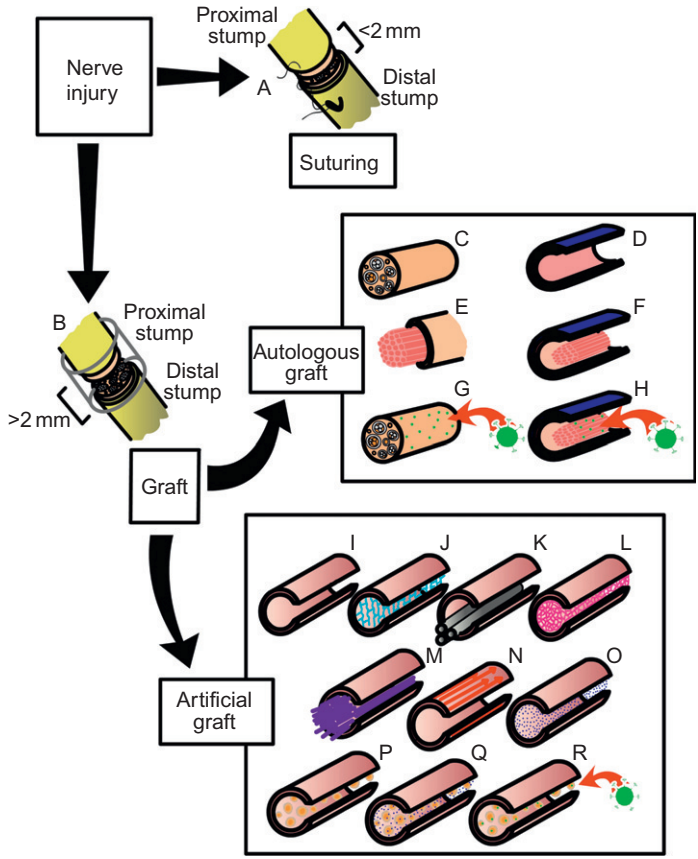

Figure 9.2 Short gap versus long gap peripheral nerve injury. (A) Short gaps (<2 mm) can be directly repaired by suturing the proximal and distal stumps one to each other without tension. (B) Longer gaps (>2 mm) can be repaired by using autologous or artificial nerve grafts, also called nerve guidance channels (NGCs). Autologous grafts are: (C) nerve autograft, (D) blood vessel, (E) muscle fiber, (F) vein filled with muscle (muscle-vein combined conduit). Modifications of autologous grafts are: (G) autograft infected with viruses, (H) vein filled with muscle infected with viruses. Artificial grafts can be: (I) empty artificial nerve graft, which can be used to repair gaps (2–10 mm); modifications to the lumen of empty artificial NGC may be helpful to repair longer gaps (>20 mm): (J) internal framework-filled artificial NGC, (K) multichannel-filled artificial NGC, (L) sponge-filled artificial NGC, (M) filament-filled artificial NGC, (N) conductive artificial NGC, (O) artificial NGC incorporated with growth factors, (P) artificial NGC incorporated with supportive cells, (Q) artificial NGC incorporated with supportive cells and (R) growth factors and artificial NGC incorporated with virus infected supportive cells.

autografts: donor site morbidity and sensory loss, scarring, neuroma formation, and limited length of available graft material encouraging the search of alternatives for nerve gap reconstruction. On the other hand, current techniques and artificial nerve conduit devices available to support regeneration across large lesion gaps have limited success (Deumens et al., 2010; Steed, Mukhatyar, Valmikinathan, & Bellamkonda, 2011). Allografts have been used, but immunosuppression is required and this technique results in poor success rates (Mackinnon & Dellon, 1990). Autologous and autogenous blood vessels (Chiu et al., 1988) and muscle fibers (Glasby, Gschmeissner, Hitchcock, & Huang, 1986) have also been used as conduits for nerve regeneration with different success rates; however, they present the same disadvantages as auto- and allografts (Doolabh, Hertl, & Mackinnon, 1996). In 1993, Brunelli et al. described a new biological conduit represented by a vein segment filled with fresh skeletal muscle (Brunelli, Battiston, Vigasio, Brunelli, & Marocolo, 1993). Both the vein and the skeletal muscle of this autologous graft are withdrawn in the site of surgery and are sutured to bridge the two stumps of a severed nerve. The clinical use of this autologous device led to good results in terms of motor and sensory recovery in 85% of patients in which it was applied (Battiston, Tos, Cushway, & Geuna, 2000).

Instead of autografts, artificial nerve guidance channels (NGCs) may be used to enhance regeneration avoiding availability and immune rejection problems (Kehoe, Zhang, & Boyd, 2012; Steed et al., 2011). The basic designs are hollow tubes in which the proximal and distal stumps of the injured nerve are inserted. More recently, research has been focused mainly on improving the single lumen nerve tube to bridge larger nerve gaps (de Ruiter, Malessy, Yaszemski, Windebank, & Spinner, 2009; de Ruiter, Spinner, Yaszemski, Windebank, & Malessy, 2009). The artificial conduit may be implanted empty, or it may be filled with collagen and laminin-containing gels (Labrador, Buti, & Navarro, 1998; Madison, Da Silva, & Dikkes, 1988; Verdu et al., 2002), internal frameworks (de Ruiter, Spinner, et al., 2009; Francel, Francel, Mackinnon, & Hertl, 1997; Lundborg & Kanje, 1996; Meek et al., 2001; Nakamura et al., 2004; Yoshii & Oka, 2001; Yoshii, Oka, Shima, Taniguchi, & Akagi, 2003), supportive cells (Ansselin, Fink, & Davey, 1997; Evans et al., 2002; Guenard, Kleitman, Morrissey, Bunge, & Aebischer, 1992; Kim et al., 1994; Rodriguez, Verdu, Ceballos, & Navarro, 2000; Sinis et al., 2005), growth factors (Derby et al., 1993; Fine, Decosterd, Papaloizos, Zurn, & Aebischer, 2002; Hollowell, Villadiego, & Rich, 1990; Lee et al., 2003; Midha, Munro, Dalton, Tator, & Shoichet, 2003; Sterne, Brown, Green, & Terenghi, 1997), and

conductive polymers, but combinations have also been used already (Fig. 9.2). An artificial graft can meet many of the needs of regenerating fibers by concentrating neurotrophic factors, reducing cellular invasion, and providing directional neuritis outgrowth to prevent neuroma formation.

3.2. NRG1 isoform expression during nerve regeneration

SC dedifferentiation and proliferation are a precondition for axonal regeneration in the lesioned PNS (Carroll et al., 1997). The different NRG1 isoforms play an important role in this process, thanks to their ability to stimulate dedifferentiation, proliferation, and differentiation of SC *in vitro* (Stassart et al., 2013).

Many studies have examined the expression of the different NRG1 isoforms at various times following peripheral nerve injury. It has been reported that expression of NRG1-type I and -type II isoforms is induced after sciatic nerve transection (3–30 days post injury) and is strictly associated with SC, strengthening the idea that NRG1 acts not only by juxtacrine interactions, but also by autocrine/paracrine communication (Carroll et al., 1997; Ronchi et al., 2013; Stassart et al., 2013). Interestingly, these authors reported that NRG1-type II-β3 expression in SC coincides with the DNA synthesis phase and this expression lasts in spite of markedly diminished SC mitogenesis, raising the question whether SC could regulate mitosis in the presence of persistent NRG1.

A rapid but transient activation of the NRG1 receptor ErbB2 in myelinating SC after sciatic nerve axotomy was detected (Guertin, Zhang, Mak, Alberta, & Kim, 2005). The authors defined the role of this activation using an ErbB2 antagonist so preventing, *in vivo*, SC response to axotomy and, *in vitro*, SC demyelination in neuron-SC co-cultures. Two days post nerve injury, NRG1 receptors are strongly down-regulated (Ronchi et al., 2013); 4–5 days post nerve injury, when Wallerian degeneration and demyelination occur, ErbB2 is expressed and activated (Carroll et al., 1997; Kwon et al., 1997). An earlier increase, at 3 days post injury, is observed for Erbin, an ErbB2 interacting protein whose expression is required for remyelination (Liang et al., 2012).

Indeed, the heterodimer receptor ErbB2–ErbB3 is coordinately induced in axotomized nerve SC, suggesting that the density of functional NRG1 receptors may modulate NRG1 activity during the process of peripheral nerve Wallerian degeneration.

These results are in accordance with a previous study (Li, Terenghi, & Hall, 1997) in which chronically denervated SC were missing ErbB2.

It was proposed that the lack of ErbB2 renders SC chronically insensitive to axonal-derived NRG1, so contributing to the failure of axonal regeneration through chronically denervated distal nerve stumps.

Neuronal NRG1-type III, as previously discussed, plays an important role in myelination and nerve regeneration. This isoform is anchored to the axonal surface and directly communicates with adjacent glial cells. Recent studies have confirmed the actual importance of the axon-derived NRG1 in nerve regeneration (Fricker et al., 2011). Through single-neuron labeling-by means of inducible Cre-mediated knock-out mice (Young et al., 2008)-it was demonstrated that juxtacrine NRG1 signaling is not essential for maintenance of the myelin sheath and neuromuscular junctions, but it is necessary for the reparative response after nerve injury, including remyelination, axon regeneration, and reinnervation of the neuromuscular junctions.

The NRG1 importance in the nerve regenerative process and in remyelination is more relevant when the functional outcome of non nervous nerve grafts is analyzed. The analysis of a successful graft represented by a fresh muscle-vein-combined conduit (Geuna et al., 2007; Nicolino et al., 2003; Tos et al., 2007) revealed that SC, in supporting axonal regeneration, very early and massively colonize the tubular graft migrating from both the proximal and the distal nerve stumps (Fornaro, Tos, Geuna, Giacobini-Robecchi, & Battiston, 2001; Raimondo et al., 2005). SC graft colonization is not only supported by migration from the nerve stumps, but also by active SC proliferation accompanied by NRG1 upregulation (Geuna et al., 2003). mRNA expression analysis of the early events occurring in this nonnervous tube placed to bridge a peripheral nerve gap has highlighted NRG1 α2 isoform-released by the muscle used to fill the tube-as suitable candidate to promote SC survival and activity in early postoperative phases, when regenerating axons are still not present. It has been proposed that this molecule may promote SC survival and differentiation rather than proliferation (Raabe, Clive, Neuberger, Wen, & DeVries, 1996). These results suggest that NRG1 α2 isoform plays a role in supporting early SC survival and activity in the absence of axons and provides a possible explanation for the observed effectiveness of the fresh muscle-vein-combined technique for nerve repair.

3.3. Biomaterials and delivery system

As discussed previously, several studies demonstrated that endogenous NRG1 is required for nerve repair, suggesting that the treatment with recombinant NRG1 could improve peripheral nerve regeneration and functional outcome following injury. Different strategies have been used

to deliver NRG1: subcutaneous injection, protein release by biomaterials, transplantation of NRG1 expressing cells, and injection of adenoviruses coding for NRG1 (Table 9.2).

3.3.1 NRG1 subcutaneous injection

Treatment with 1 mg/kg NRG1-II-β3 (recombinant human glial growth factor 2) via subcutaneous injection promotes nerve regeneration and accelerates functional recovery after rat sciatic nerve injury (Chen et al., 1998). Histological assessment shows less severe degeneration and earlier robust axon remyelination, with improvement in axon diameter and myelin thickness, in the treated group. Epinerium injection with 500 ng "GGF" (heuregulin α, Sigma Aldrich) after rabbit facial nerve anastomosis results in high SC and glial cell proliferation and in better nerve regeneration (Yildiz et al., 2011).

3.3.2 NRG1 release by biomaterials

The most promising approach for growth factor delivery to the site of nerve injury is to load growth factors directly into the conduit (Simon, Terenghi, Green, & Coulton, 2000; Sterne et al., 1997; Whitworth, Dore, Green, & Terenghi, 1995). Alginate hydrogel provides a sustained and controlled release of neurotrophic factors *in vitro* and *in vivo* (Austin, Bower, Kurek, & Muldoon, 1997; Ko, Dixit, Shaw, & Gitnick, 1995) and freeze-dried alginate supports axonal regeneration across 50-mm gap in the cat sciatic nerve (Suzuki et al., 1999). Moreover, coating of alginate hydrogel with fibronectin supports SC viability and neuronal regeneration (Mosahebi, Wiberg, & Terenghi, 2003). An ultrapure endotoxin-free low-viscosity alginate with high manuronic content was used for recombinant NRG-1-type II-β3 (human GGF2, CeNes Pharmaceutical, USA) delivery in a poly 3-hydroxybutyrate (PHB) polymer conduit to repair 20 and 40 mm gaps in rabbit common peroneal nerve (Mohanna et al., 2003). The same authors demonstrated that NRG-1-type II-β3 addition significantly increased SC quantity, sustained axonal regeneration over short and long gaps in comparison with empty and alginate conduits, and improved target muscle reinnervation (Mohanna et al., 2005).

Nerve conduits can be produced to have characteristics as similar as possible to the native nerve, by combining growth factors, aligned extracellular matrix and biomaterial filaments, improving the nerve conduit performance, and ensuring better functional recovery after *in vivo* transplantation. *In vitro* oriented collagen and a combination of differentiation factors (NGF,

Table 9.2 Different strategies to deliver NRG1

NRG1 isoform	Dose	Treatment/Nerve tube	Animal	Nerve	Injury	Gap size (mm)	Methods	Controls	Follow up	Outcome	References
NRG1 subcutaneus injection											
II-β3 soluble (GGF2, recombinant human glial growth factor 2)	1 mg kg^{-1}	Subcutaneous injection 1 day before surgery and daily for the following 4 days	Rat	Sciatic nerve	Crush injury	5	Motor functional test, muscle contractility test and histological examination	Injection equivalent volume of saline	49 days	Nerve function recovery at 11 days postsurgery, stronger isometric tetanic contractile force 11–21 days postsurgery. Less severe degeneration and earlier remyelination	Chen et al. (1998)
α (Heregulin-α, by Sigma Aldrich)	500 ng	Epineurin injection at time of surgery, 24 and 48 h postsurgery	Female New Zeland rabbit	Facial nerve	Anastomosis	1	Electron microscopy	No medication with NRG1	2 months	Increased regeneration, new axons and myelin formation, higher SC proliferation, lower number of myelin debris	Yildiz et al. (2011)
NRG1 release by biomaterials											
II-β3 soluble (recombinant human GGF2, by CeNes Pharmaceutical)	1.250 mg ml^{-1}	Poly 3-hydroxybutyrate (PHB) conduits filled with GGF suspended in alginate hydrogel	Female New Zeland rabbit	Common peroneal nerve	Nerve transection	20 and 40	Immunohistochemisty	PHB conduits filled with alginate hydrogel and empty PHB conduits	63 days	SC number and axonal regeneration increased. 20 and 40 mm gaps bridged by axons after 63 days	Mohanna, Young, Wiberg, and Terenghi (2003)
β1a soluble (EGF-like domain, human heuregulin β1, by R&D system)	800 ng	Silicone conduits filled with matrigel containing poly-L-lactic acid (PLLA) microfilaments containing heuregulin-β1	Female Sprague Dawley rats	Sciatic nerve	Nerve transection	14	Immunohistochemistry	Silicone conduits filled with matrigel containing poly-L-lactic acid (PLLA) microfilaments	10 weeks	Extensive regeneration with improvement of the number and longitudinal organization of SC and axons	Cai et al. (2004)
II-β3 soluble (recombinant human GGF2, by CeNes Pharmaceutical)	1.250 mg ml^{-1}	Poly 3-hydroxybutyrate (PHB) conduits filled with GGF suspended in alginate hydrogel	Female New Zeland rabbit	Common peroneal nerve	Nerve transection	20 and 40	Immunohistochemisty, histological examination, electron microscopy and motor reinneravtion evaluation	PHB conduits filled with alginate hydrogel and empty PHB conduits	120 days	SC number and axonal regeneration increased. Reduction of muscle mass percentage loss	Mohanna, Terenghi, and Wiberg (2005)

Continued

Table 9.2 Different strategies to deliver NRG1—cont'd

NRG1 isoform	Dose	Treatment/Nerve tube	Animal	Nerve	Injury	Gap size (mm)	Methods	Controls	Follow up	Outcome	References
Transplantation of NRG1 expressing cells											
II-β3 soluble (recombinant human GGF2, by CeNes Pharmaceutical)	15×10^7 cells/animal	Poly 3-hydroxybutyrate (PHB) conduits filled with PHB fibers and differentiated marrow stromal cell (MSC)	Sprague Dawley rats	Sciatic nerve	Nerve transection	10	Immunohistochemistry	Poly 3-hydroxybutyrate (PHB) conduits filled withPHB fibers and SC	15 days	Following transplantation, MSC mantain S100 expression and enhance nerve regeneration	Tohill, Mantovani, Wiberg, and Terenghi (2004)
?	5×10^5 cells/animal	Injection of differentiated SC trasfected with pcDNA3.1-NRG1	Male and female Wistar rats	Spinal cord (CNS)	Hemisection spinal cord injury model	20	Immunohistochemistry and Basso, Beattie Bresnahan locomotor rating score (BBB)	DMEM and DMEM containing SC injection	8 weeks	Increased spinal cord injury repair. Increased proliferation of glial cells and protection of neurons from apoptosis	Zhang, Zhao, Wu, Li, and Jin (2010)
?	3×10^5 cells/animal	Injection of differentiated SC trasfected with pcDNA3.1-NRG1	Male and female Wistar rats	Spinal cord (CNS)	Hemisection spinal cord injury model	/	Immunohistochemistry and Basso, Beattie Bresnahan locomotor rating score (BBB)	SC injection	4 weeks	Reduced size of cystic cavities; increased axonal regeneration and hind limb functional recovery	Zhang et al. (2011)
?	1×10^6 cells/tube	Silicon tube filled with a mixture of type I collagen gel and adipose-derived regenerative cells (ADRC)	Wistar rats	Sciatic nerve	Nerve transection	10	Immunohistochemistry	Silicon tube filled with saline solution or type I collagen gel	2 weeks	Increased SC migration and proliferation, and axons regrowth. ADCR express high NRG1 (Neu-1) level	Suganuma et al. (2013)
Injection of NRG1 coding adenoviruses											
β soluble (EGF-like domain)	2 μl 1×10^{11} PFU ml^{-1}	Injection of recombinant adenovirus coding for EGF-like domain of NRG1-β	Male Sprague Dawley rats	Sciatic nerve	Nerve transection	/	Immunohistochemistry and motor function test	Injection of recombinant adenovirus-LacZ or saline injection	5 weeks	Augmented expression of neurofilaments, GAP43 and S100 in the distal stump of the injury site; increased length of regenerated axons, recovery of sensory and motor function	Joung et al. (2010)

NRG-1, and TGF-β) induce SC alignment (Ribeiro-Resende, Koenig, Nichterwitz, Oberhoffner, & Schlosshauer, 2009). TGF-β stimulates α1α β1 and α6β1 integrin expression and affects cell polarity (Rogister et al., 1993; Stewart, Turner, Jessen, & Mirsky, 1997); NRG1 increases N-cadherin expression (Gess et al., 2008); at low concentration NRG1 accelerates SC migration (Meintanis, Thomaidou, Jessen, Mirsky, & Matsas, 2001), at high concentration increases proliferation via activation and phosphorylation of the ErbB2/ErbB3 receptor (Porter, Clark, Glaser, & Bunge, 1986; Rosenbaum et al., 1997). Poly-ε-caprolacton filaments induce pronounced SC alignment with a polarized expression of the cell adhesion molecule L1 similar to that seen *in vivo* in bands of Büngner after sciatic nerve crush in adult rats (Ribeiro-Resende et al., 2009). Integration of bioengineered bands of Büngner-which would guide axonal regrowth-and growth factors for the development of innovative nerve guide implants may be a promising strategy to facilitate and accelerate axonal regeneration.

Silicone implants containing matrigel, poly-L-lactic acid microfilaments, and NRG1-type I-β1 (human heuregulin-β1 by R&D systems) have been used to bridge a 14-mm gap in adult rats (Cai, Peng, Nelson, Eberhart, & Smith, 2004). Microfilaments provide organized guidance channels that direct SC migration, and enhance cable formation and axonal regeneration longitudinally across nerve stumps (Lundborg & Kanje, 1996; Ngo et al., 2003; Zhao, Lundborg, Danielsen, Bjursten, & Dahlin, 1997). NRG1 treatment caused an increase in SC number by inducing proliferation by an autocrine mechanism (Carroll et al., 1997). Application of a low dose of NRG1 promotes SC migration and trophic factor release that support neuronal survival and regeneration (Mahanthappa, Anton, & Matthew, 1996). Implants containing NRG1 and microfilaments act synergistically inducing a significant improvement in the number and longitudinal organization of both SC and axons, leading to axonal regeneration and nerve repair (Cai et al., 2004).

3.3.3 Transplantation of NRG1 expressing cells

The transplantation of cultured cells, transfected to express growth factors, into bioengineered conduits, may be used to improve nerve regeneration. Due to their role in peripheral nerve regeneration, SC may be used as a tool to deliver growth factors. Adult stem cells from adipose tissue or bone marrow can be differentiated into a SC-like phenotype and used as SC replacements (Faroni et al., 2011). Transplantation of cultured SC (Magnaghi,

Procacci, & Tata, 2009; Mosahebi, Woodward, Wiberg, Martin, & Terenghi, 2001), bone marrow mesenchymal stem cells (MSC) (Tohill et al., 2004), and adipose-derived regenerative cells (ADRC) (Suganuma et al., 2013) has been used to promote peripheral nerve regeneration.

In vitro, MSC exposed to NRG-1-type II-β3 (human GGF2, CeNes Pharmaceutical, USA) expresses s100 and glial fibrillary acidic protein (Tohill et al., 2004). Following transplantation into 10-mm nerve conduits in the rat sciatic nerve, MSC maintained s100 expression and promoted nerve regeneration (Tohill et al., 2004).

ADRC in combination with type I collagen gel have been used to bridge 10-mm sciatic nerve gaps in Wistar rats (Suganuma et al., 2013). ADRC promote peripheral nerve regeneration not by differentiating into SC, but-probably-by secreting NRG1 and VEGFA factors that promote proliferation and migration of SC (Suganuma et al., 2013).

These data suggest that NRG1 stimulates the proliferation of glial cells, inhibits apoptosis, and facilitates repair and regeneration of injured nerves in the PNS.

NRG1 has been successfully used in combination with different kinds of supportive cells, also to repair spinal cord injury. SC transfected to express high levels of NRG1 (of a nonspecified isoform) have been implanted into rats with hemisection spinal cord injury (Zhang et al., 2010). Transfected SC secreted a large amount of NRG1 both *in vitro* and *in vivo*, which results-*in vivo*-in ErbB2–ErbB4 upregulation in neurons and neuroglia cells. After transplantation, cells survived and migrated into the spinal cord injured areas. Significant recovery of hemisection spinal cord injury was observed in the group of rats implanted with transfected SC expressing NRG1. These results suggest that SC expressing NRG1 can significantly improve the repair of spinal cord injury by up-regulating ErbB receptor expression in the target cells, increasing proliferation of glial cells, and protecting neurons from apoptosis (Zhang et al., 2010).

Co-transplantation of MSC with transfected SC expressing NRG1 (of a nonspecified isoform) into a rat model of spinal cord hemisection injuries reduced the size of cystic cavities and promoted axonal regeneration and hind limb functional recovery in comparison with SC or MSC transplantation alone or together (Zhang et al., 2011). This treatment could provide important insights into potential therapies of spinal cord hemisection injuries to improve functional recovery. Delivery of NRG1 with transfected supportive cells may be a promising strategy for the repair of both spinal cord and peripheral nerve.

3.3.4 Injection of NRG1 coding viruses

Gene therapy, using adeno-associated viral vectors (Kaplitt et al., 1994) and LV vectors (Naldini et al., 1996), may be a promising strategy to promote peripheral nerve regeneration (Hoyng, Tannemaat, De Winter, Verhaagen, & Malessy, 2011).

Recombinant adenoviruses have been used to express NRG1 to improve axonal regeneration in the injured PNS (Joung et al., 2010). Rats, injected into both proximal and distal stumps of the sciatic nerve with a recombinant adenovirus expressing the β-EGF-like domain of NRG1-type I, display an augmented expression of neurofilaments, GAP43 and S100 in the distal stump of the injury site and an increased length of regenerating axons leading to sensory and motor functions improvement (Joung et al., 2010). These results suggest a therapeutic potential for β-EGF-like domain of NRG1 in the treatment of peripheral nerve injury.

4. CONCLUSIONS

When a peripheral nerve is severely injured, the use of an artificial conduit becomes necessary if the nerve gap is too long to be directly sutured, as an alternative to the autograft that gives good results but has some clinical drawbacks. It has been shown that SC of the nerve autograft and the muscle of the muscle-vein conduit release soluble NRG1 which contributes to the effectiveness of these approaches (Nicolino et al., 2003). The aim of this review is to get a clear view of the role played by NRG1 during peripheral nerve regeneration, to identify the right isoform to be delivered to the injured nerve by grafted artificial NGCs.

NRG1-type III could be the candidate with the therapeutic potential to improve nerve repair: literature data demonstrate that axonal transmembrane NRG1 is required for remyelination and regeneration after nerve injury, while it is dispensable for myelin maintenance (Fricker & Bennett, 2011; Fricker et al., 2011). However, exogenous axonal transmembrane expression implies the use of viruses that present some problems and risks (toxicity, immune and inflammatory responses, gene control, and targeting issues).

An alternative strategy to increase the amount of NRG1-type III suitable to stimulate myelination is the inhibition of those α-secretases (e.g., TACE/ADAM17 and ADAM10), whose activity negatively interferes with NRG1, impairing myelination. Several TACE inhibitors have been developed for the treatment of rheumatoid arthritis and other inflammatory disorders (Bahia & Silakari, 2010; DasGupta, Murumkar, Giridhar, & Yadav, 2009),

because tumor necrosis factor alpha is one of the most common pro-inflammatory cytokines involved in rheumatoid arthritis and in other auto-immune diseases. However, most of them have broad spectrum inhibitory activity for other matrix metalloproteases involved in other processes and are not suitable candidates for clinical trials.

Several authors demonstrated that different soluble isoforms of NRG1 (including a type III isoform provided as "soluble" recombinant protein) can improve remyelination after peripheral nerve injury (Tables 9.1 and 9.2), with an activity that is concentration- and isoform-dependent (Syed et al., 2010). They demonstrated that soluble type III isoforms have pro-myelinating activity in a broad concentration range, while type II isoforms have promyelinating activity when supplied at very low concentration, below the threshold that stimulates ERK activation. Therefore, soluble NRG1 could be the candidate with the therapeutic potential to improve nerve repair (Syed & Kim, 2010).

However, because the promyelinating activity of NRG1 seems to depend also on the exons which are downstream the EGF-like domain (α, β, γ, 1, 3, 4, a, b, c, . . .), it is important to understand which isoforms have been used by the different authors. Nevertheless, in many papers it is really difficult to find this information, because the authors call it "NRG1" or use the old nomenclature.

One of these soluble isoforms, type II-β3 (often called GGF2), has recently completed the Phase 1 of a clinical trial in patients with heart failure: Acorda Therapeutics is conducting a clinical program for GGF2 in heart failure, and preclinical development to treat peripheral nerve injury and stroke. Other groups performed a Phase II clinical trial to study the efficacy and the safety of recombinant human NRG1 in patients with chronic heart failure (Gao et al., 2010).

It has been shown that after damage to peripheral nerve, the injured nerve reacts with a peak of production of soluble NRG1-type I/II (Carroll et al., 1997; Ronchi et al., 2013; Stassart et al., 2013), ERK and AKT pathway activation (Harrisingh et al., 2004; Napoli et al., 2012; Sheu, Kulhanek, & Eckenstein, 2000)-that *in vitro* can be activated either by soluble NRG1 (Syed et al., 2010) either by other factors-SC dedifferentiation and proliferation. Subsequently, NRG1-type I/II level decreases, ERK pathway is switched off, and SC remyelinate axons expressing transmembrane NRG1-type III.

These data suggest that soluble NRG1-type I/II could be the candidate to improve nerve repair when there is a large lesion and endogenous SCs are

not enough to produce an adequate amount of NRG1. Therefore, when designing tissue engineered medical devices to bridge large gap injuries and deliver NRG1, it is essential to get an early release of highly concentrated soluble NRG1 to stimulate ERK pathways and SC dedifferentiation, followed by a late release of NRG1 at low concentration to stimulate AKT activation and remyelination.

In addition to NRG1, other molecules are certainly involved in the regeneration process (Pereira et al., 2012; Taveggia et al., 2010); it is expected that the regenerative research will go toward the concomitant use of different factors, to obtain synergistic effects and better outcome.

After the submission of this review, two papers related to NRG1 role in myelination were published.

In the first (Fricker et al., 2013) NRG1 was ablated in the adult nervous system using a tamoxifen inducible Cre ricombinase, demonstrating that axonal NRG1 promotes nerve repair after injury, but at later stages of repair it is not essential for remyelination.

In the second paper (Fleck et al., 2013) the authors demonstrated that the EGF-like domain of NRG1 type III can be proteolytically processed at both N- and C-terminal sites, which results in the secretion of the domain, suggesting that NRG1 type III dependent myelination could be not only controlled in a juxtacrine manner by membrane-bound NRG1, but also in a paracrine manner by the soluble EGF-like domain.

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CHAPTER TEN

Extracellular Matrix Components in Peripheral Nerve Regeneration

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Contents

Abstract

Injured axons of the peripheral nerve are able to regenerate and, eventually, reinnervate target organs. However, functional recovery is usually poor after severe nerve injuries. The switch of Schwann cells to a proliferative state, secretion of trophic factors, and the presence of extracellular matrix (ECM) molecules (such as collagen, laminin, or fibronectin) in the distal stump are key elements to create a permissive environment for axons to grow. In this review, we focus attention on the ECM components and their tropic role in axonal regeneration. These components can also be used as molecular cues to guide the axons through artificial nerve guides in attempts to better mimic the natural environment found in a degenerating nerve. Most used scaffolds tested are based on natural molecules that form the ECM, but use of synthetic polymers and functionalization of hydrogels are bringing new options. Progress in tissue engineering will eventually lead to the design of composite artificial nerve grafts that may replace the use of autologous nerve grafts to sustain regeneration over long gaps.

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1. INTRODUCTION

Injuries to the peripheral nervous system generally cause disconnection of the fibers distal to the site of injury from the neuronal soma. Consequently, the distal nerve undergoes degeneration, leaving the peripheral target organs denervated and, thus, functionally useless. In comparison to central axons, peripheral axons show a good capability to regenerate. This difference is partly due to not only the cellular and molecular changes occurring at the distal stump during Wallerian degeneration but also the intrinsic capabilities of the axotomized peripheral neurons to switch to a proregenerative state. Injured axons are able to elongate into the distal nerve stump if they find a permissive substrate, usually provided by tropic and trophic support from Schwann cells and connective cells (Fig. 10.1). Schwann cells that lose contact with axons switch to an immature proregenerative state, proliferating and secreting trophic factors with a temporo-spatial pattern that provides a regenerative-promoting terrain for the growing axons. The basal lamina remains in place in the endoneurium and helps to guide the axons toward distal target organs. Within the endoneurial tubules, proliferating Schwann cells form the aligned bands of Büngner. Eventually, regenerating axons will be able to reach the distal target organs and reinnervate

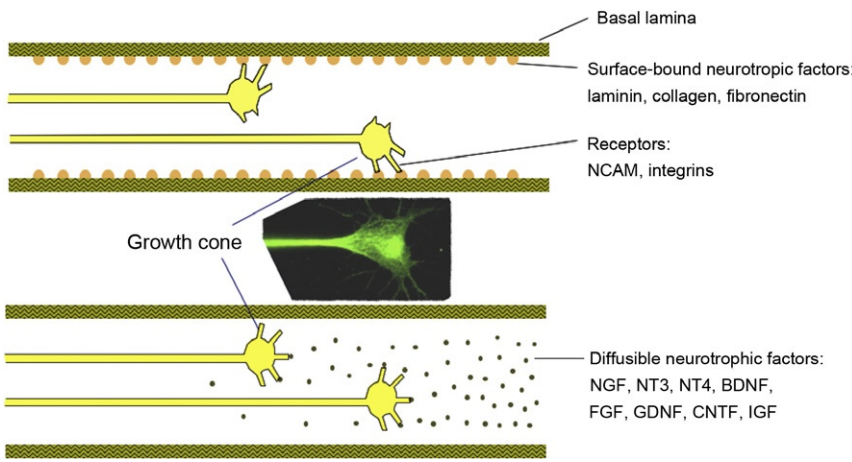

Figure 10.1 Trophic and tropic support for the axonal growth cone. The molecular cues that the regenerating axons find at the distal stump can be diffusible neurotrophic factors or membrane-bound neurotropic factors, as the ECM components laminin, fibronectin, and collagen.

them, thus allowing for the recovery of lost functions (for reviews see Allodi, Udina, & Navarro, 2012; Fu & Gordon, 1997; Navarro, Vivó, & Valero-Cabré, 2007).

After a peripheral nerve transection, surgical repair is mandatory to allow the severed axons to grow into the distal degenerating nerve. The microsurgical intervention reconnects the proximal and distal nerve stumps, trying to match individual fascicles to facilitate the appropriate navigation of growing axons to their targets. However, matching of proximal and distal endoneurial tubes is impossible and thus, misdirection of axons to a wrong distal path is common after these types of lesions. When direct suture is not possible due to loss of neural tissue, the interposition of a graft to reunite both stumps is needed. The interposition of an autologous graft is commonly used as the gold standard technique even if it causes the sacrifice of a noninjured nerve from the patient to be used as a graft. To reduce the secondary effects of autografting, alternatives to bridge the nerve gap have been investigated. Artificial nerve guides could be a good alternative to repair damaged nerves by mimicking the natural environment that the degenerating distal stump or autograft offers (Deumens et al., 2010; Doolabh, Hertl, & Mackinnon, 1996; Gu, Ding, Yang, & Liu, 2011; IJkema-Paassen, Jansen, Gramsbergen, & Meek, 2004). Tube repair has thus emerged as an alternative repair method. However, it is important to take into account that the regenerative process through tubular guides differs from that occurring in a degenerating nerve segment. Regeneration in a guide starts with the formation of a fibrin cable that bridges the gap between the transected nerve stumps. This fibrin cable provides a guiding surface for the ingrowth of fibroblasts, blood vessels, and Schwann cells that migrate from both stumps (Williams, Longo, Powell, Lundborg, & Varon, 1983). It is replaced by collagen secreted by the invading fibroblasts and laminin produced by the Schwann cells, in fibrils longitudinally oriented, and the tube lumen fluid is enriched by trophic factors. Then regenerating axons are able to grow from the proximal stump along the newly formed scaffold. Failure of the formation of the initial cable or limited supply of migrating cells into the tube due to excessive length of the gap lead to failure of regeneration (Yannas, Zhang, & Spilker, 2007). Prefilling the repair tube with components of the extracellular matrix (ECM) or with artificial scaffolds have been investigated to improve the regenerative potential of the guides, increasing the length of the gap that can be bridged by a nerve guide. Nowadays, efforts are addressed to improve the quality of artificial nerve guides to reach clinical application. To closely mimic the degenerating nerves, artificial nerve guides must offer

a proregenerative environment rich in Schwann cells, trophic factors, and components of the ECM that promote axonal regeneration.

2. EXTRACELLULAR MATRIX AND CONNECTIVE LAYERS OF THE NERVE

2.1. Peripheral nerve microstructure

In addition to bundles of axons, from spinal motor neurons, dorsal root ganglia (DRG), sensory neurons, and postganglionic autonomic neurons, the peripheral nerve is composed of three layers or compartments: epineurium, perineurium, and endoneurium. The epineurium is the outermost layer that delimits the nerve from the surrounding. It is composed of loose connective tissue and carries the blood vessels that supply the nerve. Delimiting each fascicle in the nerve there is the perineurium, a thin but dense sheath composed of flat perineurial cells and an outer layer of collagen fibers organized in bundles. The supporting connective tissue that fills each fascicle is the endoneurium, composed of fibroblasts, collagen, and reticular fibers and ECM, occupying the space between nerve fibers. The endoneurial tubes are composed of basal lamina sheets arranged in continuity around the axon–Schwann cell units and collagen fibrils.

The ECM is a physiological integrative matrix of complex molecular nature, where axons and supportive cells are immersed. The ECM is a three-dimensional network arranged in the intercellular space, which includes proteins and carbohydrates synthesized and secreted by the cells. It is present in the interstitial spaces of all tissues, playing important roles in cell migration, proliferation, and differentiation, and providing structural support and regulating intercellular communication. It contributes to mechanical tissue properties, allows the cells to form tissues, serving to cell communication, and forms paths where cells can move. In the peripheral nerve, the ECM is found in the basal lamina of Schwann cells and the endoneurium.

The basal lamina, produced by the Schwann cells, may be considered a layer of the ECM, mainly composed of collagen type IV, laminin, fibronectin, and nidogens (Bannerman, Mirsky, Jessen, Timpl, & Duance, 1986; Baron-Van Evercooren, Gansmüller, Gumpel, Baumann, & Kleinman, 1986; Bryan et al., 2012). After injury, besides the degenerative process behold in the distal stump, basal lamina tubes remain as scaffolds where proliferative Schwann cells align forming the bands of Büngner.

2.2. ECM components

The ECM is composed of a complex network of secreted proteins, glycoproteins, proteoglycans, and non-proteoglycan polysaccharides. The first group of components, the glycoproteins, can be classified into collagen and noncollagenous molecules.

Collagens are a superfamily of trimeric molecules composed of three identical triple helical α chains that define tissue structures (Brown & Phillips, 2007; Gordon & Hahn, 2011). Up to 26 different types of collagens have been described, that are divided into different groups according to the structures they form. The main subfamilies are fibril-forming collagens (types I, II, III, V, XI), collagens banded-fibrils associated (IX, XVI, XIX, XXI, XXII), networking collagens (IV, VI, VIII, X), transmembranous collagens (XIII, XXIII, XV), endostatin precursor collagens (XV, XVII), and other collagens. The collagen types most relevant for peripheral nerve regeneration are described in later sections. However, it is interesting to highlight the importance of those related to fibril formation (collagen type I) and the basement membrane (collagen type IV).

Among the ECM noncollagenous molecules of glycoprotein origin, the most important are laminins and fibronectins.

Laminins are major proteins of the ECM, participating in cell differentiation, migration, and adhesion activities. They are an active part of the natural scaffolding which structure the tissues. They are mainly found in the basal lamina. Laminins are heterotrimers of α, β, and γ chains, and 18 different types have been described to date (Durbeej, 2010). The trimers are named according to the composition of the different chain types, but usually it is the α chain that identifies the isoform. Secreted by Schwann cells, laminin-2 (α2, β1, γ1) and laminin 8 (α4, β1, γ1) are found in the peripheral nerves (Wallquist et al., 2002), whereas laminin 10 (α5, β1, γ1) can be detected in sensory end organs (Caissie, Gingras, Champigny, & Berthod, 2006). Laminin is the adhesive component that gives the regenerative-promoting capability to basal lamina scaffolds after nerve injury (Wang, Hirai, Shimada, Taji, & Zhong, 1992) and has been shown to promote neuritogenesis *in vitro* (Agius & Cochard, 1998).

Fibronectin is the other major component of noncollagen glycoproteins of the ECM (Singh, Carraher, & Schwarzbauer, 2010). It forms a fibrillar matrix similar to collagen and mediates cell-binding. Fibronectin is a dimer existing in different isoforms because of alternative splicing generation. Totally, 12 isoforms for mice and 20 for humans have been described. At

first, soluble fibronectin is produced by hepatocytes, being found in the blood plasma. The insoluble form is incorporated into the membrane of many cells. In the nervous system, it is synthesized and secreted by Schwann cells and fibroblasts (Baron-Van Evercooren et al., 1986; Chernousov & Carey, 2000). The important relations that it maintains with collagen type IV and laminins and with fibril formation make fibronectin an interesting candidate for scaffolding in nerve regeneration (Brown & Phillips, 2007).

The ability of cells to interact with both laminins and fibronectins is mainly due to the expression of the cell adhesion molecule integrins in their membrane (Hynes, 2002). Integrins are glycosilated heterodimers formed by α and β subunits. The integrin β1 subfamily is composed of integrins with a β1 subunit, which bounds to actin cytoskeleton, and the α subunit, which determines the specificity to the ECM molecule adhesion. Thus, integrin α1, α3, α6, and α7 can interact with laminin, whereas integrin α5 interacts with fibronectin. Expression of integrins on the growth cone determines the ability of the growing axon to interact with the ECM. Moreover, laminin and fibronectin have the characteristic of binding to other ECM components. For instance, laminin interacts with nidogens, agrin, perlecan, fibulin-1, heparin, and sulfatides (Holmberg & Durbeej, 2013; Rudenko, Hohenester, & Muller, 2001), whereas fibronectin binds to collagen, fibrin, and heparan sulfate proteoglycans (Hörmann, 1982).

There are other noncollagen glycoprotein molecules of the ECM although they are probably not related to axonal regeneration after nerve injury. Nidogen-1 (also called entactin) forms noncovalent unions with laminin and collagen type IV and may play its role as a promigratory factor for adult Schwann cells (Lee et al., 2007). On the other hand, vitronectin binds to collagen and glycosaminoglycans (GAGs) (heparin), acting as a regulatory molecule controlling cell adhesion (Schvartz, Seger, & Shaltiel, 1999).

The GAGs are carbohydrate polymers that are covalently bound to glycoproteins in their native state, forming *proteoglycans*. These molecules include heparan, keratin, chondroitin, dermatan, and their respective sulfates (Rutka, Apodaca, Stern, & Roseblum, 1988). Proteoglycans are formed by a GAG linked to a hydroxyl group of certain amino acids (serine and threonine) or a core molecule which is also linked to hyaluronic acid. Proteoglycans like chondroitin sulfate proteoglycans (CSPGs) create an inhibitory environment by neutralizing the growth-promoting activities of other ECM elements (McKeon, Höke, & Silver, 1995; Muir, Engvall, Varon, & Manthorpe, 1989). CSPG has been found in the peripheral nerve where it may inhibit the growth-promoting activity of endoneurial laminin

(Zuo, Ferguson, Hernandez, Stetler-Stevenson, & Muir, 1998; Zuo, Hernandez, & Muir, 1998).

Fibrin is not associated with the mature tissue structure but it is a key factor in the repair strategy of the ECM components. It will form a provisional mesh after damage that will be later replaced by the mature components of the ECM secreted by invading cells. In the presence of thrombin, fibrinogen polymerizes into fibrin to form a dense meshwork of fibers (Brown & Phillips, 2007; McKee, Mattock, & Hill, 1970). In fact, formation of a fibrin cable between the two stumps when a gap nerve is repaired by a tube is needed to guarantee successful axonal regeneration.

3. ROLE OF THE ECM COMPONENTS IN AXONAL REGENERATION

3.1. *In vitro*

To assess peripheral axon regeneration *in vitro*, the most used paradigm is the primary culture of DRG, either dissociated or as an explant. Tropic support of different molecules can be assessed by surface coating or by using 3D matrices (in which cells or explants are embedded) (Fig. 10.2).

It is well known that dissociated sensory neurons show longer neurite extension on laminin and fibronectin-coated substrates compared to poly-L-lysine-coated surfaces (Rogers, Letourneau, Palm, McCarthy, & Furcht, 1983). When comparing the ability of different ECM components, laminin-coated surfaces sustained better neurite outgrowth than vitronectin, collagen IV, fibronectin, or collagen I (Plantman et al., 2008; Wood & Willits, 2009). Laminin is also a preferred substrate for Schwann cells that extend and acquire better morphology on laminin-coated rather than fibronectin-coated substrates (Palm & Furcht, 1983). When focusing on the different isoforms of laminin,

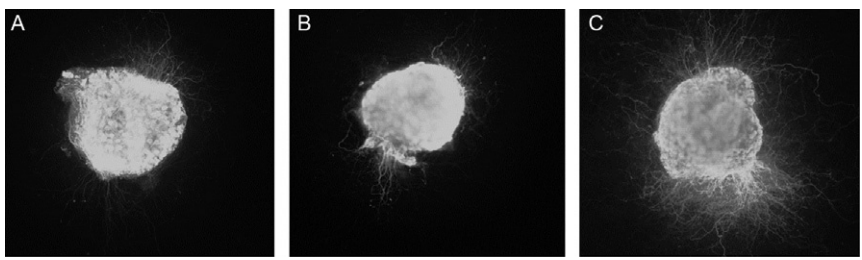

Figure 10.2 DRG explants cultured for 48 h in a 3D (A) collagen type I-containing gel, (B) fibronectin-containing gel, and (C) laminin type I-containing gel. Longer neurites can be observed in the matrix enriched with laminin type I.

DRG neurons grow better on laminin-1- and laminin-10-coated surfaces. Interestingly, when nerve growth factor was added to the culture, there was a marked increase of neurite elongation on laminin-2 and laminin-8 in comparison to laminin-1- and laminin-10-coated surfaces, which seemed to sustain a neurotrophic-independent growth (Plantman et al., 2008).

Sulfated proteoglycans have inhibitory effects when studying neurite extension on explant cultures from DRG (Ughrin, Chen, & Levine, 2003). Some authors claimed that proteoglycans inhibit neurite growth acting at the cone elongation (Snow, Smith, & Gurwell, 2002) but others relate this inhibition to the effect of sulfated proteoglycans on the neuron soma (Kuffler, Sosa, & Reyes, 2009).

When studying neurite elongation from DRG explants in 3D cultures, longer neurites were found in matrigel (a laminin-containing gel) than in collagen type I gel (Tonge et al., 1997), although the results might be affected by the additional factors present in matrigel. In another study, explants of DRG grew better on laminin gels compared to fibronectin, collagen type I, or hyaluronic gels (Deister, Aljabari, & Schmidt, 2007), supporting the findings from 2D cultures. On the other hand, different populations of sensory neurons may have different substrate preferences. Thus, embryonic proprioceptive neurons grow similarly on fibronectin and laminin, whereas cutaneous ones prefer laminin (Guan, Puthenveedu, & Condic, 2003). The inhibitory effect of the proteoglycans has also been proved in the 3D cultures (Tonge et al., 1997). When using 3D cultures, not only the components of the matrix but also the density should be taken into account. For example, when comparing different concentrations of an inert agarose gel, neurite extension was inversely correlated with the porous diameter that decreases with the concentration of the gel (Balgude, Yu, Szymanski, & Bellamkonda, 2001). Similarly, longer neurite extension was seen in lower concentration collagen gels (Willits & Skornia, 2004).

In contrast to the widely studied role of the ECM components on primary sensory neuron outgrowth *in vitro*, motoneuron outgrowth *in vitro* has been hardly analyzed. This fact is probably related to the technical difficulties of culturing primary spinal motoneurons compared to DRG neurons. Spinal cord slices embedded in a 3D matrix appear as an adequate model to investigate the role of ECM molecules on motor neurite outgrowth. Preliminary results from our laboratory indicate that motoneuron outgrowth is enhanced in matrices containing fibronectin more than in those containing laminin or collagen, suggesting that there may be a differential role of ECM molecules on different axonal populations.

3.2. *In vivo*

The role of the ECM components in nerve regeneration has been widely demonstrated in nerve graft experimental models. Even when nerve grafts are used, acellular axons are able to regenerate through the scaffolds of basal lamina (Hall, 1997). By using antilaminin antibodies, it was demonstrated that the capability of basal lamina to sustain axon growth was highly dependent on laminin. Antifibronectin antibody reduced neurite outgrowth, but did not influence the capability of axons to grow through the basal lamina (Wang et al., 1992). In similar models, the inhibitory role of proteoglycans was reduced when enzymes that degrade CSPG were applied into the distal stump or a graft, accelerating regeneration of motor and sensory axons (Krekoski, Neubauer, Zuo, & Muir, 2001; Udina et al., 2010).

Besides the studies that evaluate the role of different ECM in the distal stump of the injured nerve, the effects of these molecules have also been studied when used to fill artificial nerve guides. Moreover, addition of these substrates to a hollow tube can increase the capability of the tube to sustain regeneration and, thus, it is a first step toward engineering an artificial guide that mimics the autograft (Fig. 10.3). In fact, the sole addition of plasma, an important source of fibrin, into a silicone tube increased the gap length

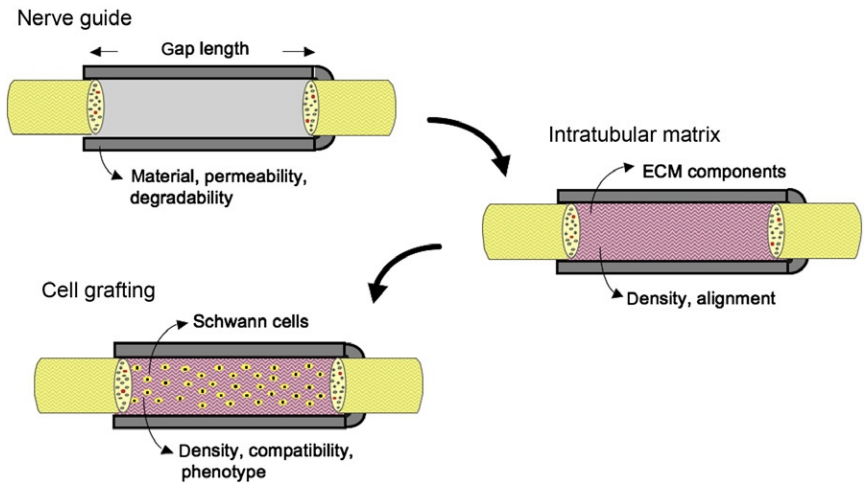

Figure 10.3 Schematic representation of the key points needed to engineer an artificial nerve graft. When designing a nerve guide, physical properties of the tube must be taken into account. For long gaps, introduction of matrices and supporting cells into the lumen improve the final outcome.

permissive for regeneration (Williams, Danielsen, Müller, & Varon, 1987). On the other hand, collagen-based (Chamberlain, Yannas, Arrizabalaga, et al., 1998; Chamberlain, Yannas, Hsu, Stritchartz, & Spector, 1998; Labrador, Butí, & Navarro, 1998; Rosen et al., 1990) and laminin-based matrices (Bailey, Eichler, Villadiego, & Rich, 1993; Labrador et al., 1998) have been successfully used to enhance axonal regeneration in nerve guides. Prefilling tubes with collagen- or laminin-containing gels improved regeneration through long gaps, whereas for short gaps, where regeneration is usually successful, addition of matrices into the tubes did not benefit the final outcome when compared to saline-filled tubes (Labrador et al., 1998; Valentini, Aebischer, Winn, & Galletti, 1987). When comparing the effects of different ECM components, laminin-containing gel performed slightly better than collagen I and hyaluronate gels on long gaps (Labrador et al., 1998). Combinations of ECM components have been tested in attempts to provide complex neurotropic support. The number of regenerated axons was higher in a gel mixture of collagen, laminin, and fibronectin compared to the control group (Chen et al., 2000). Similarly, a combination of laminin and fibronectin used to fill silicone tubes improved regeneration through long gaps in rats (Bailey et al., 1993). Fibronectin was also tested with laminin in double-coated collagen fiber bundles inserted into collagen tubes. The results were better than when using uncoated collagen fibers, revealing that laminin and fibronectin together may act positively on axonal growth (Tong et al., 1994).

As in 3D cultures, it is important to take into account the concentration of the matrix used, since the gel substrate, even if containing neurotropic agents, may impair the regenerative process by physically impeding the diffusion of factors, the migration of cells, or the elongation of axons. Lower concentrations of agarose, which forms a gel whose pore size decreases as concentration increases, were more permissive for regeneration through a silicone tube than higher ones (Labrador, Butí, & Navarro, 1995), demonstrating the importance of the density of the matrix used to guarantee axonal growth. Similar results were obtained when using collagen, laminin, and hyaluronate gels (Labrador et al., 1998; Tona & Perides, 1993).

Nerve regeneration using gel-filled tubes across long gaps usually remains inferior to that obtained with nerve autografts. A relevant difference accounts by the endoneurial tubules in nerve grafts offering a mechanical guide to the regenerating axons, while gels filling tubes do not provide local direction for axons and may even impede the ingrowth of non-neuronal cells and axons. By using a longitudinally aligned ECM gel, the rate and

the direction of axonal elongation should improve due to contact guidance with the fibrils aligned along the tube axis. This option has been reported for tubes prefilled with magnetically or gravitationally aligned collagen gel, matrigel (Ceballos et al., 1999; Dubey, Letourneau, & Tranquillo, 1999; Verdú et al., 2002), and fibrin matrix (Dubey, Letourneau, & Tranquillo, 2001), and with a collagen–GAG matrix (Chamberlain, Yannas, Arrizabalaga, et al., 1998; Chamberlain, Yannas, Hsu, et al., 1998; Chamberlain, Yannas, Hsu, Stritchartz, & Spector, 1998, 2000). Conduits containing longitudinally orientated extruded collagen microfibers and collagen scaffolds with orientated micropores, fabricated by directional ice-crystal formation, have been reported to allow regeneration over quite long gaps in the rat sciatic nerve (Bozkurt et al., 2012; Yoshii, Oka, Shima, Taniguchi, & Akagi, 2003). When fibronectin-oriented strands were introduced in nerve conduits to repair the rat sciatic nerve, axonal regeneration and Schwann cell recruitment were better than when using freeze-thawed muscle grafts (Whitworth, Brown, Doré, Green, & Terenghi, 1995). Fibronectin mats have been developed as oriented substrates that are capable of improving longitudinal migration of neurites and Schwann cells (Ahmed, Underwood, & Brown, 2003).

In conclusion, an adequate exogenous matrix designed to promote nerve regeneration within a nerve guide should have neuritotropic activity, be diluted in order to provide wide-enough pores for cellular and axonal migration, and also longitudinally oriented pathways that mimic the endoneurial tubules of the nerve.

4. ECM COMPONENTS IN THE DESIGN OF ARTIFICIAL BIOMATRICES

Further efforts are focused on the progress in tissue engineering and the improvement of artificial nerve conduits able to mimic the capabilities of autografts to sustain regeneration over long gaps. To form 3D scaffolds, besides the classical natural components of the ECM, several bioartificial polymers have been used to promote and guide axonal regeneration inside a neural guide. Such polymers can be introduced as a gel, coated directly into the internal wall of the tube or forming frameworks (like filaments or sponges used to enrich the internal architecture of the guide).

Gelatin is a natural-origin protein derived from collagen that maintains its inert properties, and can be functionalized by cross-linking techniques (Ciardelli & Chiono, 2006). Another natural polymer is chitosan,

a polysaccharide obtained from chitin. Its molecular structure is similar to GAGs in the ECM. It can be introduced into the lumen of a tube as nano/microfibers that can be functionalized with laminin and trophic factors (Patel, Mao, Wu, & Vandevord, 2007) or combined with cells (Wang et al., 2009) to enhance nerve regeneration. Alginate is a biodegradable polysaccharide with repeat units of mannuronic acid and glucuronic acid. It has been used in the form of freeze-dried sponge to promote axon regeneration across a long gap in the cat sciatic nerve (Suzuki et al., 1999). Alginate has also received attention as a slow-release hydrogel for the controlled supply of trophic factors. Other natural polymers, with potential to support nerve regeneration, are silk fibroin (Yang et al., 2007) and agarose (Labrador et al., 1995; Martin, Minner, Wiseman, Klank, & Gilbert, 2008).

Synthetic polymers can also be used to form the intratubular scaffolds that should combine the biocompatible characteristics of the natural polymers with improved mechanical and chemical performance. They also have to be surgically practicable, immunocompatible, and allow the diffusion of nutrients and growth factors to supply the regrowth of the injured nerve. Polymers can be presented as hydrogels when having high water content. Some examples of synthetic scaffolds used in peripheral nerve regeneration studies include, among others, poly(ethylene glycol) (Scott, Marquardt, & Willits, 2010; Shepard et al., 2012), poly-(L-lactic acid) (Ngo et al., 2003), poly(lactide-co-glycolide) (Subramanian, Krishnan, & Sethuraman, 2011), polycaprolactone (Daud, Pawar, Claeyssens, Ryan, & Haycock, 2012), and poly(*N*-isopropylacrylamide-co-acrylic acid) (Newman et al., 2006) in the form of hydrogels or fibers.

Recent developments of nanotechnology propose the use of fibrous scaffolds at the nanoscale level for the artificial replacement of basal lamina in tissue-engineered nerve grafts. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicon, and glass. Moreover, they can serve as an extracellular scaffold, filling a hollow nerve conduit, to guide directed axonal growth (Cao, Liu, & Chew, 2009; Olakowska, Woszczycka-Korczyńska, Jędrzejowska-Szypułka, & Lewin-Kowalik, 2010). Surfaces with nano-sized topographies, electrospinned nanofibers, or replicas of the ECM with nanoresolution were found to guide neurite outgrowth from sensory and autonomic ganglia in culture (Kanje & Johansson, 2011). *In vivo* studies showed that conduits filled with aligned polymer nanofibers resulted in better functional recovery than hollow conduits (Neal et al., 2011). These studies provide the basis for the use of nanofibers combined with molecular constituents of the ECM to enhance nerve conduits.

The performance of either synthetic tubes or scaffolds can be improved by functionalizing them with specific cues. These cues are usually motifs from ECM components with special effects on cell migration, attachment, and proliferation. Tubes can be directly functionalized by linking cues to the internal wall of the conduit. For instance, a laminin-2 motif has been reported to have a positive effect on nerve regeneration when it was used to coat a PLGA guide (Seo et al., 2012). Also, a laminin-1 motif TATVH was proposed to promote neurite outgrowth and cell attachment on coated tubes (Nickels & Schmidt, 2012).

ECM peptides are being increasingly used for functionalizing hydrogels. Based on the results of *in vitro* studies, laminin cues have been the most widely used sequences for improving hydrogel performance on nerve regeneration. For instance, a laminin-1 sequence, the IKVAV pentapeptide, has been shown to promote axonal elongation on central and peripheral neurons (Bellamkonda, Ranieri, & Aebischer, 1995; Tashiro et al., 1989). YIGSR is another pentapeptide used to graft agarose inert gels (Borkenhagen, Clémence, Sigrist, & Aebischer, 1998; Yu, Dillon, & Bellamkonda, 1999) and collagen polymers (Newman et al., 2006). The results revealed a significant increase in neuronal extension on YIGSR-coated gels in comparison to controls. Other motifs from laminin isoforms 1 and 2 have not been directly proved to enhance nerve regeneration.

Fibronectin cues have also been proposed for peripheral nerve repair. The GRGDS amino acid sequence regulates cell adhesion (Rutka et al., 1988), and it is probably the most studied short sequence from fibronectin, although the RGD sequence is also found in other ECM components such as laminin-1 and tenascin-C (Meiners & Mercado, 2003). The GRGDS sequence has been used to functionalize hydrogels. For instance, gellan gum, which is a polysaccharide from bacterial origin, functionalized with this fibronectin sequence improved axonal growth *in vitro* (Luo & Shoichet, 2004) and survival and proliferation of neural stem/progenitor cells (Silva et al., 2012).

Nowadays, it is accepted that to design an artificial nerve guide as efficient as a nerve graft, it is important to combine different approaches. After designing the best conduit, addition of neurotropic cues to the luminal space, either as a matrix or by functionalizing a gel, may improve axonal growth. Certainly, this growth can be further enhanced by introducing supportive cells or trophic factors. However, another important key point is the need to mimic the guiding endoneurial tubes found in the degenerating distal stump of the nerve. This might be achieved by longitudinally orienting

scaffolds, to provide wide-enough channels for cellular and axonal ingrowth (Brown & Phillips, 2007). Further assays are needed to improve the microgeometry of such a bioartificial matrix with added cells in order to mimic the longitudinal axis offered by endoneurial tubules and aligned Schwann cells (forming bands of Büngner) in a degenerated nerve.

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