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Abstract

The study of peripheral nerve repair and regeneration is particularly relevant in the light of the high clinical incidence of nerve lesions. Yet, clinical outcome after nerve lesions is often far from being satisfactory and the functional recovery is almost never complete. Therefore, a number of therapeutic approaches are being investigated, ranging from local delivery of trophic factors and other molecules to bioactive biomaterials and complex nerve prostheses. Although the translation of the new therapeutic approaches to the patients always requires a final pre-clinical step using in vivo animal models. The need to limit as much as possible animal use in biomedical research, however, makes the preliminary use of in vitro models mandatory from an ethical point of view.

In this paper, the different types of in vitro models available today for the study of peripheral nerve regeneration have been ranked adopting a *three-step stair model* based on their increasing ethical impact: 1) cell line-based models, that raise no ethical concern; 2) primary cell-based models, that have low ethical impact since animal use, though necessary, is limited; 3) organotypic ex vivo-based models, that raise moderate ethical concerns since the use of laboratory animals is required though with much lower impact on animal wellbeing in comparison to in vivo models of peripheral nerve regeneration.

This paper aims to help researchers in selecting the best experimental approach for their scientific goals being driven by the ‘‘Three Rs’’ rules (Replacement, Reduction or Refinement of animal use in research) for scientific research.

Key words: Cell line, Primary culture, Organotypic culture, Schwann cell, Neuron.

1. Introduction

Peripheral nerve regeneration after injury is a very complex phenomenon that involves a number of changes at molecular, cellular, and tissue level (Tetzlaff, Alexander et al. 1991, Navarro, Verdu et al. 1994, Fornaro, Lee et al. 2008, Geuna, Tos et al. 2009, Camara-Lemarroy, Guzman-de la Garza et al. 2010). Biological changes are even more dramatic when severe nerve lesions cause a nerve tissue defect since, in cases where autologous nerve grafting is no option, an artificial nerve scaffold (nerve prosthesis) is required to bridge the nerve gap and reestablish the connection with the distal targets (Dahlin and Lundborg 2001, Battiston, Papalia et al. 2009, Deumens, Bozkurt et al. 2010, Gonzalez-Perez, Cobianchi et al. 2014).

A number of strategies are presently being investigated with the goal of designing innovative tissue engineered devices which improve peripheral nerve repair and regeneration. These include among others: (1) the systemic administration of neuroprotective drugs; (2) the controlled local release of neurotropic factors and/or other neuroactive cytokines; (3) the production of biomimetic materials which mimic the properties of the peripheral nerve environment; (4) the surface functionalization of nerve scaffolds with bioactive molecules; (5) the local stimulation by means of pro-regenerative physical agents.

Several specific elements of the peripheral nerve regeneration processes can be effectively reproduced in vitro although the complexity of the peripheral nerve structure and the regeneration process are impossible to be entirely reproduced in vitro.. Nonetheless it is mandatory to respect the ‘‘Three Rs’’ (3Rs, Replacement, Reduction, Refinement) principle in animal use in research (Tannenbaum and Bennett 2015) and researchers have been imposed to use in vitro models of peripheral nerve regeneration as intensive and effective as possible, before they proof their concepts in in vivo animal models (European Directive 2010/63/EU).

The aim of this review is to describe and classify in vitro models for the study of peripheral nerve regeneration by ranking them on a *three-step stair model* based on the degree of ethical concern they are raising. At the bottom stand1) cell line-based models (Fig.1A), for which no animal use is needed and which thus have no ethical impact. At the middle rank 2) primary cell-based models (Fig.1B-C), that raise little ethical impact since animal use, though necessary, is usually limited. The final step build 3) organotypic ex vivo-based models (Fig.1D), for which the use of laboratory animals is unavoidable, but the impact on the animals wellbeing is much lower in comparison to in vivo models for peripheral nerve regeneration. Figure 2 provides a schematic overview of the relation between ethical concerns, costs and technical efforts, and the comparability to the complex in vivo model system.

2. Cell line-based models

The great ethical advantage of cell lines is that once created from animal tissues, they completely replace any further animal sacrifice. In many cases they are even derived from human tissues and represent a positive feature in the perspective of translational research towards the clinic.. On the other hand it has to be strengthened that biological properties of cell lines are far away from those of the corresponding mammalian cells in normal conditions since cell lines are either derived from neoplastic tissue or from normal cells that have been immortalized through genetic manipulation.

Related to these two features of cell lines, the following indications/contraindications for their use for peripheral nerve regeneration research can be specified.

Indications

Due to the lack of an ethical impact, the use of Schwann cell and nerve cell lines represent the *first research step* to acquire a body of preliminary data on several basic aspects including:

(i) the signaling pathways activated by new molecules/drugs (Armstrong, Wiberg et al. 2008, Magnaghi, Parducz et al. 2010, Gnavi, di Blasio et al. 2014, Pascal, Giovannelli et al. 2014),

(ii) the biocompatibility of new and/or modified biomaterials (Gnavi, di Blasio et al. 2014, Novajra, Tonda-Turo et al. 2014, Wrobel, Serra et al. 2014),

(iii) the cell behavior changes due to environmental alterations induced by physical agents (magnetic fields, light radiations, mechanical forces, etc.) (Gamboa, Gutierrez et al. 2007, Koppes, Nordberg et al. 2014, Liu, Huang et al. 2015).

Contraindications

Due to their profound differences compared to normal animal cells, cell lines cannot be used to predict the in vivo behavior of Schwann cells and neurons (unless they are used as models of neoplastic tissue).

Within these limitations, some examples of Schwann cell and nerve cell lines have been selected based on the following criteria: a) overall reliability assessed on the basis of literature data; b) the firsthand experience on some of them in our own research laboratories. In the next paragraphs, these examples are described and grouped according to the two major actors in peripheral nerve regeneration: Schwann cells and neurons.

2.1 Schwann cells

Several models of immortalized cell lines of peripheral glial cells, Schwann cells, serve to study basic elements of peripheral nerve regeneration.

RT4D6P2T (Imada and Sueoka 1978), JS-1 (Kimura, Fischer et al. 1990), RSC96 (Badache and De Vries 1998), R3(Ridley, Paterson et al. 1988), and S16Y (Toda, Small et al. 1994) cell lines are some of the most frequently used models of immortalized Schwann cells that are either obtained from tumors of nervous tissues or from genetic manipulation of glial precursors. In a comparative study, Hai and colleagues (Hai, Muja et al. 2002) have carefully explored that RT4-D6P2T represent the immortalized cell line with features most similar to primary Schwann cells. The RT4-D6P2T cell line maintains the expression of key genes that characterize Schwann cells, but still there are some differences to primary cells that have to be considered.

Cell proliferation is a very important functional aspect of Schwann cells to be respected when studying peripheral nerve regeneration in vitro. The neoplastic RT4-D6P2T cell line makes evaluation of cell proliferation very easy since it provides a homogeneous population, free of cellular senescence which researcher may encounter very early when using primary Schwann cells

cultures. Therefore, RT4-D6P2T cells have been found to be a useful model in assessing scaffold materials for peripheral nerve guide such as, e.g., a VEGF (vascular endothelial growth factor)-functionalized hydrogel (Gnavi, di Blasio et al. 2014), polycaprolactone/chitosan nanofibers (Prabhakaran, Venugopal et al. 2008), various types of electrospun fibrous substrates (Sangsanoh, Waleetorncheepsawat et al. 2007), or amorphous carbon substrates with varying surface texture sensitivity (Jain, Sharma et al. 2013). Moreover RT4-D6P2T cells can be easily transfected to induce over-expression of neurotrophic factors or release of other substances/drugs. As such they are particularly suitable for the evaluation of enriched and functionalized scaffolds for peripheral nerve regeneration (de Guzman, Ereifej et al. 2008).

On the other hand, the expression of the Neuregulin1/ErbB system that characterizes Schwann cell precursor growth and the interactions between Schwann cells (expressing ErbB2-ErbB3 and soluble type I/II NRG1) and axons (mainly expressing transmembrane NRG1-type III), for example, has been found to be different between primary Schwann cells and RT4-D6P2T cells, as the latter do not express NRG1 type I/II (Pascal, Giovannelli et al. 2014).

Schwann cells, however, are not the only suitable glial cell model for research on peripheral nerve regenerative processes. The neonatal olfactory bulb ensheathing cell (NOBEC) line was obtained from dissociated rat neonatal olfactory bulbs and immortalization of the primary cells by retroviral transduction of the SV40 large T antigen (Goodman, Silver et al. 1993). These versatile cells, similar to both Schwann cells and the central nervous astroglia , can be used in a wide range of experiments related to both central nervous system and peripheral nervous system regeneration . When characterizing the NOBEC cell line morphological, molecular, and migratory properties , Audisio and colleagues found homogeneity in the expression of markers typical for Schwann cells, as well as the expression of the NRG1 / ErbB system, as a key element in regeneration and myelination of peripheral nerves (Audisio, Raimondo et al. 2009, Gambarotta,

Ronchi et al. 2014). Consequently, the NOBEC line represents another useful model to determine the ability of innovative biomaterials for peripheral nerve repair to induce cell alignment (Chiono, Sartori et al. 2011, Novajra, Tonda-Turo et al. 2014).

2.2 Neuronal cells

In vitro studies using the peripheral nervous system related neuronal population are even more complex than those using glial cells. Primary neuron cultures cannot survive for considerable period of time in the absence of a permissive environment which is represented by heterogeneous populations of cells, including glial cells and fibroblasts. Therefore neuronal cell lines have to be used to screen substance and biomaterial effects on specific aspects of neuronal behavior.

The PC12 cell line, for example, which is derived from rat phaeochromocytoma cells of sympathoadrenal origin, is available to screen th pro-regenerative potential of substances and biomaterials in neurite outgrowth assays (Greene and Tischler 1976, Pittier, Sauthier et al. 2005, Morano, Wrobel et al. 2014). But it has to be strengthened, that although the PC12 cell line shows neurite extensions upon, e.g. neurotrophic factor stimulation, these cells are not closely related to sensory or motor neurons whose axons compose peripheral nerves. Especially with regard to molecular studies, where high amounts of cellular material are needed, cell lines generally provide optimal model systems. The cell lines chosen, however, have to share as much similarities with the primary cells as possible. In the following we describe two cell lines which could give more specific information in the context of peripheral nerve regeneration research than PC12 cells.

An SV40-large T antigen transfected, immortalized, cell line of sensory neurons (50B11) has been generated from rat embryonic dorsal root ganglion (DRG) neurons and provides the

properties of nociceptive DRG neurons (Chen, Mi et al. 2007, Bhattacherjee, Liao et al. 2014). These cells remain largely undifferentiated under standard culture conditions, but upon a forskolin stimulus they assume neuronal properties such as neurite extension, neuronal marker expression, and action potential generation (Chen, Mi et al. 2007). The 50B11 cells further respond to trophic factor and hormone supplementation in a manner that is largely similar to DRG neurons used in neurite outgrowth assays (Bhattacherjee, Liao et al. 2013). Once differentiated, however, the cells do only survive for additional 72 hours and this limits their use to short term assays and makes them unsuitable for e.g., in vitro myelination assays usually requiring culture times of more than 10 days (Pittier, Sauthier et al. 2005, Blugeon, Le Crom et al. 2011) .

The NSC-34 mouse motor neuron cell is a hybrid cell line derived from the fusion of mouse neuroblastoma cells and motor neuron-enriched spinal cord cells (Cashman, Durham et al. 1992). The NSC-34 cells maintained properties characteristic for motor neurons, including the generation of action potentials, expression of neurofilament triplet proteins, and acetylcholine synthesis, storage, and release. The use of NSC-34 cells is often selected for studies related to motor neuron degenerative diseases (Raimondi, Mangolini et al. 2006, Sun, Benardais et al. 2013). However, these cells offer the possibility to reveal basic mechanisms of motor neurite outgrowth (Madison, McGee et al. 2014) in an ethically uncritical, less cost intensive, and technically much easier culture system than primary or organotypic systems provide. One example is the use of NSC-34 cellsin combination with C2C12 muscle cell line to investigate the benefits derived from the use of extracellular vesicles from muscle cells for peripheral nerve regeneration (Madison, McGee et al. 2014).

3. **Primary cell-based models**

The use of cell lines in order to answer complex questions related to peripheral nerve regeneration processes has obviously a lower impact on the generality of study conclusions than the use of primary cell cultures. Unlike cell lines, primary cell cultures are composed of naive cells, i.e. Schwann cells and neurons harvested from healthy animal tissues. Consquently, in vitro experiments using primary cells are suitable to more closely reproduce what happens in vivo in the peripheral nerve. It should be noted however, that the harvest of neurons (both sensory and motor) requires the unavoidable transection of their axons and the loss of contact with their target organs, thus making it practically impossible to set-up primary cultures of uninjured neurons. The same is true for Schwann cells when they are isolated from axons. More properly, we have to admit that primary cultures mimic a condition in which the normal physiological properties of Schwann cells and neurons are subjected to an axonal trauma. In this view, these cell cultures adequately reproduce what happens to Schwann cells and neurons after peripheral nerve injury.

Therefore, the data obtained from these in vitro models are more likely to predict the phenomena occurring in vivo during nerve regeneration than cell-line based models. But, primary peripheral nerve cell cultures have also a main drawback represented by the complete destruction of the 3D tissue framework during the dissociation procedure. The 3D tissue framework , especially in the nervous system, is extremely complex and has a high impact on cellular functions. This dramatic environmental modification is thus likely to induce profound changes in the dissociated cells, and should be kept in mind when interpreting the results obtained from these in vitro models.

Related to the features of primary peripheral nerve cell cultures described above, the following indications/contraindications for their use as in vitro models for peripheral nerve regeneration research can be specified.

Indications

Due to the ethical concern that arises from the use of animals in research, although usually limited, primary cells (both of Schwann cells and neurons) should be regarded as the *second research step* to acquire information not obtainable by using cell lines; this includes data on:

(i) the signaling pathways specifically induced by various molecules/drugs on Schwann cells and/or neurons (Shin, Jang et al. 2013, Schmid, Zeis et al. 2014);

(ii) the proliferation and/or apoptosis activation induced by different types of chemical and physical stimuli (Pellitteri, Catania et al. 2014);

(iii) the biomimetic properties of biomaterials, i.e. how closely they reproduce the effects of the natural nerve environment on Schwann cells or neurons (Ribeiro-Resende, Koenig et al. 2009, Haastert-Talini, Geuna et al. 2013);

(iv) the specific changes in Schwann cell and/or neuronal behavior upon single or multiple stimuli (e.g. trophic factor administration, plus biostimulation by physical agents, plus surface functionalization of biomaterials) (Brushart, Aspalter et al. 2013, van Neerven, Pannaye et al. 2013, Morano, Wrobel et al. 2014);

(v) the neuron-glial differentiation potential of stem cells (de Luca, Faroni et al. 2015)

Contraindications

Due to the changes that might have been induced by cell dissociation, data obtained from primary cell cultures might not fully predict what happens at the whole organ level and thus,

especially prior to concluding about potential clinical implications these data should be always verified in in vivo models.

Within these limitations, in the next paragraphs we will describe the most frequently used types of primary cell cultures for peripheral nerve regeneration research. These include two types of Schwann cells (Schwann cells *strictu senso*, i.e. those dissociated from peripheral nerves, and glial cells harvested from the olfactory nerve, that are commonly referred to as olfactory ensheating cells) as well as two types of neurons (sensory neurons isolated from dorsal root ganglia (DRGs) and spinal motor neurons).

3.1 Schwann cells

Primary cultures of Schwann cells can be obtained from nerves of neonatal and adult rats, mice, dog and human (Haastert-Talini 2012, Tao 2013). They retain similar features compared to Schwann cells in vivo. Unfortunately, in culture they have a limited life span (usually at passage 10 they lose their typical bipolar shape and become multipolar as sign of senescence). The removal of contaminating fibroblasts, however, is difficult because these cells proliferate faster than Schwann cells. The first important step to minimize the amount of fibroblasts in the culture is the removal of the epineurium immediately after nerve harvest. Then different strategies can be followed to further reduce the presence of fibroblasts in the culture.

Commonly, the supplementation of Ara-C (cytosine-B-arabinoside hydrochloride), an anti-mitotic agent, to the culture medium for 24h is used to eliminate most of the fibroblasts growing among Schwann cells (Wei, Zhou et al. 2009). Antibody-mediated immunoselection is also a commonly used procedure to highly enrich Schwann cell cultures (van Neerven, Pannaye et al.

2013, Wrobel, Serra et al. 2014). Kaewkhaw and colleagues described a protocol that exploits the preferential capacity of adult rat Schwann cells to metabolize D-valine and the elimination of fibroblasts by using selective culture conditions (Kaewkhaw, Scutt et al. 2012).

Cultures of adult human Schwann cells are a precious source for pre-clinical in vitro studies. These cells can either be obtained from cauda equina donors a few hours after death (Tapinos and Rambukkana 2005) or from sural nerve specimen of patients undergoing an autologous nerve graft surgery (Haastert, Mauritz et al. 2007). Purification of adult human Schwann cell cultures can be obtained by differential detachment which washes the Schwann cells out of the initially fibroblast contaminated cultures. In combination with selective culture conditions highly purified adult human Schwann cell cultures could be maintained (Haastert, Mauritz et al. 2006, van Neerven, Pannaye et al. 2013).

Olfactory ensheating cells (OECs) share the expression of several specific markers such as S-100 and low-affinity nerve growth factor receptor (p75) with Schwann cells (Guerout, Derambure et al. 2010, Honore, Le Corre et al. 2012, Pascal, Giovannelli et al. 2014). These cells are of particular interest for their ability to promote remyelination of damaged axons both in the central nervous system (Boyd, Doucette et al. 2005) and the peripheral nervpus system (Guerout, Paviot et al. , Guerout, Paviot et al. 2014).

3.2 Neuronal cells

Neuronal survival, neurite elongation, orientation and branching, differentiation, and basic mechanisms of the neuronal physiology are parameters to be studied in order to understand the response of neurons to substance/drug administration and/or to interaction with biomaterials in vitro.

Dissociated primary neurons from adult, neonatal or embryonic DRG or spinal cord can be used to test the behavior of sensory and motor neurons, respectively, but the purification of these cultures is demanding (especially in case of spinal cord motor neurons).

A dissociated DRG neuron culture can be co-cultured with different Schwann-like derived stem cells, providing a valuable model to study nerve regeneration and myelination in vitro, mimicking the in vivo environment at the injury site (de Luca, Faroni et al. 2015).

The culture of dissociated or isolated neurons, allows the in depth study of factors that are able to modulate neuronal behavior, e.g. the involvement of aquaporin-1 in axonal growth and regeneration (Zhang and Verkman 2015).

On the other hand, the presence of Schwann cells in neuronal cultures represents an obstacle beause these cells continue to proliferate and thus limit the possibility to discriminate the selective neuronal response to any given stimulus. Recently, Zuchero described an immunopanning-based method for rapid purification in the absence of antimitotic agents and serum (Zuchero 2014), by binding a selected cell population via an antigen-antibody reaction to a solid surface.. These Schwann cell free DRG neuron cultures are useful to study the role of glia in the biology of DRG neurons or if a growth factor has a direct or indirect effect on neurons.

Embryonic spinal cord motor neurons have been also successfully cultured (Graber and Harris 2013), although they represent a minor population of developing spinal cord cells and have to be carefully purified and enriched to separate them from non-neuronal cells and other neurons (Haastert, Grosskreutz et al. 2005, Klausmeyer, Stern et al. 2015).

The culture of adult motor neurons is still difficult due to their short-term survival, and limited axonal outgrowth. To date many researchers suggest different reproducible protocols to obtain long-term cultures of healthy and functional adult motor neurons (Milligan and Gifondorwa 2011, Montoya-Gacharna, Sutachan et al. 2012). The culture of dissociated neurons allows

standardizing evaluation parameters, such as the number of cells that cannot be quantified in organotypic cell culture models.

3.3 Induced pluripotent stem cells

A final mention deserves the potential of the employment of induced pluripotent stem (iPS) cells of human origin (Robinton and Daley 2012). The possibility to convert human iPS into both neurons (Chambers, Qi et al. 2012) and Schwann cells (Liu, Swistowski et al. 2014) may open new ways in peripheral nerve regeneration related research and for the translation of research results into the clinical application. It can be foreseen that, once the differentiation technology has been optimized to reliable standards, iPS-derived human peripheral nervous system neurons and glial cells could become a powerful tool for primary cell culture in vitro models evaluating peripheral nerve regeneration both in single- and co-culture conditions.

4. Organotypic ex vivo-based models

Organotypic cultures refer to in vitro culturing conditions which mimic the 3D organization of a tissue and/or organ. Therefore, contrary to in vitro models based on dissociated cells, organotypic cultures do not completely abolish the numerous chemico-physical environmental stimuli that contribute to the regulation of Schwann cell and neuron functions in vivo.

From an ethical point of view, organotypic cell culture models still contribute to the reduction of the number of animals used for research purposes and they avoid animal discomfort occurring during the postoperative interval of in vivo experiments. The main drawback of organotypic cultures, when compared to in vivo models of peripheral nerve regeneration, is represented by the limited time window they provide for the observation of regeneration-related changes since the cultures can obviously not be maintained for months and resemble reconnection to target tissues.

These features of organotypic cultures make it possible to specify the following indications/contraindications for their use in peripheral nerve regeneration research.

Indications

Organotypic cultures represent the *third (and last) research step* in the in vitro peripheral nerve regeneration research. In particular, they can be a good alternative to in vivo models when early (up to one month) changes under given experimental conditions are sought. These include collection of data on:

(i) axon elongation, branching and orientation influenced by of different molecules, such as neurotrophic factors or biomaterials (Vyas, Li et al. 2010, Allodi, Guzman-Lenis et al. 2011, Gerardo-Nava, Hodde et al. 2014, Morano, Wrobel et al. 2014);

(ii) the onset of the myelination process which represents a key factor of nerve regeneration in the perspective of functional recovery in vivo (Levy, Garcia Segura et al. 1996, Triolo, Dina et al. 2012, Ziv-Polat, Shahar et al. 2014).

Contraindications

Although the 3D tissue environment is preserved, culture conditions cannot completely reproduce the normal tissue/organ environment (for instance the numerous circulating factors coming from the peripheral blood are lost in organotypic explants). In addition, the process of tissue explantation is itself traumatic and likely to induce biological changes in Schwann cells and neurons. Yet, as already mentioned, the observation window is limited in time to a maximum of few weeks in vitro. Finally, due to the complexity of organotypic models their set-up has a higher ethical impact than the harvest of primary cell cultures, because the number of animals that need to sacrificed is much higher .

Within these limitations, in the next paragraphs we will describe some of the commonly used organotypic culture models obtained either by 3D co-culturing of different cell components in presence of scaffolds mimicing the spatial organization of the tissue/organ, or by ex vivo explants.

4.1 3D co-cultures

While 2D co-cultures of neurons and glial cells do not reflect the 3D environment and the related complex cellular inetractions given in vivo, 3D co-cultures can be realized using complex matrices (Bozkurt, Brook et al. 2007, Gingras, Beaulieu et al. 2008) that mimic the 3D organization of a peripheral nerve during its regeneration. Bozkurt and colleagues developed a 3D, highly oriented, scaffold of cross-linked porcine collagen to promote directed axonal growth.

Furthermore, the microstructural properties of the scaffold allowed Schwann cells from explanted DRGs to migrate along the columnar structure of the scaffolds, and to thus resemble the "Bands of Büngner" (Bozkurt, Brook et al. 2007).

Gingras and colleagues developed and characterized a 3D in vitro model of motor nerve regeneration by culturing fibroblasts, Schwann cells, and spinal cord motor neurons on a collagen-chitosan sponge (Gingras, Beaulieu et al. 2008). This in vitro model allowed the long-term survival of motor neurons and the elongation and myelination of their neurites through a 3D construct.

Kraus and colleagues recently established from, the very simple hanging drop technique, a spheroidal sprouting assay (Kraus, Boyle et al. 2015). Spheroids of Schwann cells and neuronal NG108-15 cells (neuroblastoma-glioma hybrid cell line), were resuspended in a solution containing 20% FCS and a collagen mix in order to create a 3D collagen matrix. Axonal sprouts in this 3D culture significantly increased in lenght and caliber over time in comparison to the normal 2D co-culture.This simple 3D-Schwann cell-neuron spheroid model enables multiplication of cell-cell physical interactions and to study more efficiently neurite development in vitro.

4.2 Ex vivo explants

The far most used ex vivo approach for preparing peripheral nervous system organotypic cultures is the DRG explant (Morano, Wrobel et al. 2014). The explants can be either obtained from embryonic (Liu, Gao et al. 2011), postnatal (Richardson, Rementer et al. 2011), or adult DRGs (Fornaro, Lee et al. 2008). Embryonic, postnatal, and adult DRG explants provide a different growth behavior, but all of these models can be used to study substances meant to induce less branching and more elongated and oriented axons, which represent preferential conditions for efficient in vivo peripheral nerve regeneration. A quantitative evaluation of axonal elongation and

branching can be performed with different methods (Tse, Chan et al. 2007, Morano, Wrobel et al. 2014), including line intersection and point counting stereological tools (Gundersen, Bendtsen et al. 1988). DRG explants contain also glial cells that, if properly stimulated, can migrate out of the explants and start myelination of regenerating axons (Ziv-Polat, Shahar et al. 2014).

While DRG explants are a relatively simple and effective model for investigating axon regeneration and myelination in vitro, their main limitation is that they only offer insights on the behavior of sensory neurons. In order to study ex vivo the motor compartment of peripheral nerves, spinal cord explants can also be obtained, although using procedures that have a much higher degree of technical complexity (Fabbro, Villari et al. 2012, Pinkernelle, Fansa et al. 2013).

Moreover spinal cord slice cultures have a limited basal outgrowth if compared to the growth capacity of DRG explants, for that reason they require longer time of culture (Allodi, Guzman-Lenis et al. 2011)

Vyas and colleagues have developed a more advanced in vitro organotypic co-culture system (Vyas, Li et al. 2010), later also used by other authors (Gerardo-Nava, Hodde et al. 2014), that accurately models peripheral nerve repair in the adult mammal. This model is based on entire spinal cord explants with reconstructed ventral nerve roots. This organotypic culture, although technically very challenging, allows to test a full nerve scaffold (the entire nerve prosthesis) in vitro because the presence of a nerve pedicle, to which the nerve prosthesis can be sutured, reproduces the condition obtainable in vivo to a high degree.

5. Discussion

Driven by the concurring growth of interest in tissue engineering and regenerative medicine, the interest in in vitro models to study peripheral nerve repair and regeneration has continuously increased over the last years (Geuna, Gnavi et al. 2013, Salgado and Gimble 2013). In this paper we have described and classified in vitro models for the study of peripheral nerve regeneration outlining their potential as well as their limitations. Table I summarizes the pros and cons of each model described.

Besides their intrinsic scientific value for selected research purposes, in vitro models of peripheral nerve regeneration are more acceptable from an ethical point of view than in vivo models. In fact, the European Community (similar to many countries) has adopted a policy regarding the use of animals for scientific purposes that is driven by the ethically-oriented 3Rs concept of animal use in research (Tannenbaum and Bennett 2015). The three principles that underline the 3Rs concept are:

(1) *Replacement*, i.e. the preferential adoption whenever possible of non-animal methods to achieve a scientific aim.

(2) *Reduction*, i.e. the preferential adoption of methods that enable to obtain comparable information from fewer animals or more information from the same number of animals.

(3) *Refinement*, i.e. the preferential adoption of methods that alleviate or minimize the impact on animal wellbeing and/or enhance animal welfare for those animals that cannot be replaced.

Figure 2 shows how the *three-step stair model* that we have adopted to rank the in vitro models of peripheral nerve regeneration exactly copes with the three principles of the 3Rs concept that, similarly, can be ranked on three levels on the basis of the degree of ethical concern that they raise.

(1) The *Replacement* rule, which raises the lower ethical concern, is fulfilled by *cell line-based models* that completely replace animal use.

(2) The *Reduction* rule, which applies when animal use cannot be avoided completely, is fulfilled by *primary cell-based models* for which animal use is limited to sample harvest for cell isolation.

(3) The *Refinement* rules, which applies when animal use can neither be avoided completely nor be reduced, is fulfilled by *organotypic ex vivo-based models*, that have very limited impact on animal wellbeing (only the distress of anesthesia before animal sacrifice), in comparison to in vivo models, where animals necessarily experience some degree of impairment along the postoperative observation period.

From the indications/contraindications listed above for each of the described in vitro models it is obvious that the 3Rs policy can be fulfilled to a substantial degree in peripheral nerve regeneration research. Especially, when biocompatibility testing of innovative biomaterials for peripheral nerve repair is performed with selected cell lines related to the peripheral nervous system. Furthermore basic impacts of any treatment or biomaterial on Schwann cell migration and neurite outgrowth can sufficiently be tested in primary cell culture models. Organotypic culture models, although technically challenging can be used to replace short term in vivo experiments

and to finally select the most promising approaches to support peripheral nerve repair for pre-clinical in vivo studies.

Like in other fields of regenerative medicine research, however, those pre-clinical tests of innovative therapeutic approaches have to be done in specific in vivo models. Therefore, new therapeutic approaches for peripheral nerve repair and regeneration always need to be tested in animal models before their final translation to the patients. Examples from our own research attempts clearly demonstrate that approaches that proved to be very promising in vitro (e.g., a regenerative hydrogel) may completely fail to support (e.g., impair) peripheral nerve regeneration in vivo (Meyer, Wrobel et al. 2015).

As in vivo research is unavoidable it is of highest importance to design comprehensive studies able to answer as many questions as possible with the minimal number of animals dedicated that still allows statistically relevant conclusions. This can usually be achieved by combinations of in vitro studies (after careful selection of relevant cell lines and primary cells) with well-designed in vivo studies investigating especially the functional and morphological outcome of the regenerative approach (Haastert-Talini, Geuna et al. 2013).

6. Conclusions

1) The use of in vivo models is unavoidable for the final pre-clinical testing of new strategies for improving peripheral nerve repair and regeneration.

2) In vitro models, from simple and ethical concern-free models based on the use of cell lines up to technically and ethically more challenging organotypic models are a valuable tool for the preliminary screening of a number of elements before moving to long-term animal experiments.

3) The use of in vitro models enables a more limited and effective use of in vivo animal experiments, in accordance with the 3Rs policy, resulting in a much higher rate of success in the translation of basic biomedical research to the clinics.

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Figure and table legend

Figure 1: Immunofluorescent images of neuronal cell line 50B011 (A), primary cultures of adult human Schwann cells (B), primary cultures of adult rat dissociated DRG neurons (C), adult rat organotypic DRG explant (D). Scale bars: 50 μm (A,C); 200 μm (B); 500 μm (D).

Figure 2: Scheme of in vitro models for the study of peripheral nerve regeneration, by ranking them on a three-step stair model based on the degree of ethical concerns, costs and technical efforts, and the similarity to the complex in vivo model system.

Table I: Pros and Cons of the different in vitro models useful for peripheral nerve regeneration research.

References

Allodi, I., M. S. Guzman-Lenis, J. Hernandez, X. Navarro and E. Udina (2011). "In vitro comparison of motor and sensory neuron outgrowth in a 3D collagen matrix." J Neurosci Methods **198**(1): 53-61.

Armstrong, S. J., M. Wiberg, G. Terenghi and P. J. Kingham (2008). "Laminin activates NF-kappaB in Schwann cells to enhance neurite outgrowth." Neurosci Lett **439**(1): 42-46.

Audisio, C., S. Raimondo, S. Nicolino, G. Gambarotta, F. Di Scipio, L. Macri, F. Montarolo, M. G. Giacobini-Robecchi, P. Porporato, N. Filigheddu, A. Graziani, S. Geuna and I. Perroteau (2009). "Morphological and biomolecular characterization of the neonatal olfactory bulb ensheathing cell line." J Neurosci Methods **185**(1): 89-98.

Badache, A. and G. H. De Vries (1998). "Neurofibrosarcoma-derived Schwann cells overexpress platelet-derived growth factor (PDGF) receptors and are induced to proliferate by PDGF BB." J Cell Physiol **177**(2): 334-342.

Battiston, B., I. Papalia, P. Tos and S. Geuna (2009). "Chapter 1: Peripheral nerve repair and regeneration research: a historical note." Int Rev Neurobiol **87**: 1-7.

Bhattacherjee, A., Z. Liao and P. G. Smith (2013). "Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11 cells." Neurosci Lett **558**: 120-125.

Bhattacherjee, A., Z. Liao and P. G. Smith (2014). "Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11 cells." Neurosci Lett **558**: 120-125.

Blugeon, C., S. Le Crom, L. Richard, J. M. Vallat, P. Charnay and L. Decker (2011). "Dok4 is involved in Schwann cell myelination and axonal interaction in vitro." Glia **59**(3): 351-362.

Boyd, J. G., R. Doucette and M. D. Kawaja (2005). "Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord." FASEB J **19**(7): 694-703.

Bozkurt, A., G. A. Brook, S. Moellers, F. Lassner, B. Sellhaus, J. Weis, M. Woeltje, J. Tank, C. Beckmann, P. Fuchs, L. O. Damink, F. Schugner, I. Heschel and N. Pallua (2007). "In vitro assessment of axonal growth using dorsal root ganglia explants in a novel three-dimensional collagen matrix." Tissue Eng **13**(12): 2971-2979.

Brushart, T. M., M. Aspalter, J. W. Griffin, R. Redett, H. Hameed, C. Zhou, M. Wright, A. Vyas and A. Hoke (2013). "Schwann cell phenotype is regulated by axon modality and central-peripheral location, and persists in vitro." Exp Neurol **247**: 272-281.

Camara-Lemarroy, C. R., F. J. Guzman-de la Garza and N. E. Fernandez-Garza (2010). "Molecular inflammatory mediators in peripheral nerve degeneration and regeneration." Neuroimmunomodulation **17**(5): 314-324.

Cashman, N. R., H. D. Durham, J. K. Blusztajn, K. Oda, T. Tabira, I. T. Shaw, S. Dahrouge and J. P. Antel (1992). "Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons." Dev Dyn **194**(3): 209-221.

Chen, W., R. Mi, N. Haughey, M. Oz and A. Hoke (2007). "Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line." J Peripher Nerv Syst **12**(2): 121-130.

Chiono, V., S. Sartori, A. Rechichi, C. Tonda-Turo, G. Vozzi, F. Vozzi, M. D'Acunto, C. Salvadori, F. Dini, G. Barsotti, F. Carlucci, S. Burchielli, S. Nicolino, C. Audisio, I. Perroteau, P. Giusti and G. Ciardelli (2011). "Poly(ester urethane) guides for peripheral nerve regeneration." Macromol Biosci **11**(2): 245-256.

Dahlin, L. and G. Lundborg (2001). "The use of silicone tubing in the late repair of the median and ulnar nerves in the forearm." J Hand Surg Br **26**(4): 393-394.

de Guzman, R. C., E. S. Ereifej, K. M. Broadrick, R. A. Rogers and P. J. VandeVord (2008). "Alginate-matrigel microencapsulated schwann cells for inducible secretion of glial cell line derived neurotrophic factor." J Microencapsul **25**(7): 487-498.

de Luca, A. C., A. Faroni and A. J. Reid (2015). "Dorsal root ganglia neurons and differentiated adipose-derived stem cells: an in vitro co-culture model to study peripheral nerve regeneration." J Vis Exp(96).

Deumens, R., A. Bozkurt, M. F. Meek, M. A. Marcus, E. A. Joosten, J. Weis and G. A. Brook (2010). "Repairing injured peripheral nerves: Bridging the gap." Prog Neurobiol **92**(3): 245-276.

Fabbro, A., A. Villari, J. Laishram, D. Scaini, F. M. Toma, A. Turco, M. Prato and L. Ballerini (2012). "Spinal cord explants use carbon nanotube interfaces to enhance neurite outgrowth and to fortify synaptic inputs." ACS Nano **6**(3): 2041-2055.
Fornaro, M., J. M. Lee, S. Raimondo, S. Nicolino, S. Geuna and M. Giacobini-Robecchi (2008). "Neuronal intermediate filament expression in rat dorsal root ganglia sensory neurons: an in vivo and in vitro study." Neuroscience **153**(4): 1153-1163.
Gambarotta, G., G. Ronchi, S. Geuna and I. Perroteau (2014). "Neuregulin 1 isoforms could be an effective therapeutic candidate to promote peripheral nerve regeneration." Neural Regen Res **9**(12): 1183-1185.
Gamboa, O. L., P. M. Gutierrez, I. Alcalde, I. De la Fuente and M. J. Gayoso (2007). "Absence of relevant effects of 5 mT static magnetic field on morphology, orientation and growth of a rat Schwann cell line in culture." Histol Histopathol **22**(7): 777-780.
Gerardo-Nava, J., D. Hodde, I. Katona, A. Bozkurt, T. Grehl, H. W. Steinbusch, J. Weis and G. A. Brook (2014). "Spinal cord organotypic slice cultures for the study of regenerating motor axon interactions with 3D scaffolds." Biomaterials **35**(14): 4288-4296.
Geuna, S., S. Gnavi, I. Perroteau, P. Tos and B. Battiston (2013). "Tissue engineering and peripheral nerve reconstruction: an overview." Int Rev Neurobiol **108**: 35-57.
Geuna, S., P. Tos and B. Battiston (2009). "Preface: Essays on peripheral nerve repair and regeneration." Int Rev Neurobiol **87**: xxi-xxii.
Gingras, M., M. M. Beaulieu, V. Gagnon, H. D. Durham and F. Berthod (2008). "In vitro study of axonal migration and myelination of motor neurons in a three-dimensional tissue-engineered model." Glia **56**(3): 354-364.
Gnavi, S., L. di Blasio, C. Tonda-Turo, A. Mancardi, L. Primo, G. Ciardelli, G. Gambarotta, S. Geuna and I. Perroteau (2014). "Gelatin-based hydrogel for vascular endothelial growth factor release in peripheral nerve tissue engineering." J Tissue Eng Regen Med.
Gonzalez-Perez, F., S. Cobianchi, S. Geuna, C. Barwig, T. Freier, E. Udina and X. Navarro (2014). "Tubulization with chitosan guides for the repair of long gap peripheral nerve injury in the rat." Microsurgery.
Goodman, M. N., J. Silver and J. W. Jacobberger (1993). "Establishment and neurite outgrowth properties of neonatal and adult rat olfactory bulb glial cell lines." Brain Res **619**(1-2): 199-213.
Graber, D. J. and B. T. Harris (2013). "Purification and culture of spinal motor neurons from rat embryos." Cold Spring Harb Protoc **2013**(4): 319-326.
Greene, L. A. and A. S. Tischler (1976). "Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor." Proc Natl Acad Sci U S A **73**(7): 2424-2428.
Guerout, N., C. Derambure, L. Drouot, N. Bon-Mardion, C. Duclos, O. Boyer and J. P. Marie (2010). "Comparative gene expression profiling of olfactory ensheathing cells from olfactory bulb and olfactory mucosa." Glia **58**(13): 1570-1580.
Guerout, N., A. Paviot, N. Bon-Mardion, A. Honore, R. Obongo, C. Duclos and J. P. Marie "Transplantation of olfactory ensheathing cells to evaluate functional recovery after peripheral nerve injury." J Vis Exp(84): e50590.
Guerout, N., A. Paviot, N. Bon-Mardion, A. Honore, R. Obongo, C. Duclos and J. P. Marie (2014). "Transplantation of olfactory ensheathing cells to evaluate functional recovery after peripheral nerve injury." J Vis Exp(84): e50590.
Gundersen, H. J., T. F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J. R. Nyengaard, B. Pakkenberg, F. B. Sorensen, A. Vesterby and et al. (1988). "Some new, simple and efficient stereological methods and their use in pathological research and diagnosis." APMIS **96**(5): 379-394.
Haastert-Talini, K. (2012). "Culture and proliferation of highly purified adult Schwann cells from rat, dog, and man." Methods Mol Biol **846**: 189-200.
Haastert-Talini, K., S. Geuna, L. B. Dahlin, C. Meyer, L. Stenberg, T. Freier, C. Heimann, C. Barwig, L. F. Pinto, S. Raimondo, G. Gambarotta, S. R. Samy, N. Sousa, A. J. Salgado, A. Ratzka, S. Wrobel and C. Grothe (2013). "Chitosan tubes of varying degrees of acetylation for bridging peripheral nerve defects." Biomaterials **34**(38): 9886-9904.

Haastert, K., J. Grosskreutz, M. Jaeckel, C. Laderer, J. Bufler, C. Grothe and P. Claus (2005). "Rat embryonic motoneurons in long-term co-culture with Schwann cells--a system to investigate motoneuron diseases on a cellular level in vitro." J Neurosci Methods **142**(2): 275-284.
Haastert, K., C. Mauritz, S. Chaturvedi and C. Grothe (2007). "Human and rat adult Schwann cell cultures: fast and efficient enrichment and highly effective non-viral transfection protocol." Nat Protoc **2**(1): 99-104.
Haastert, K., C. Mauritz, C. Matthies and C. Grothe (2006). "Autologous adult human Schwann cells genetically modified to provide alternative cellular transplants in peripheral nerve regeneration." J Neurosurg **104**(5): 778-786.
Hai, M., N. Muja, G. H. DeVries, R. H. Quarles and P. I. Patel (2002). "Comparative analysis of Schwann cell lines as model systems for myelin gene transcription studies." J Neurosci Res **69**(4): 497-508.
Honore, A., S. Le Corre, C. Derambure, R. Normand, C. Duclos, O. Boyer, J. P. Marie and N. Guerout (2012). "Isolation, characterization, and genetic profiling of subpopulations of olfactory ensheathing cells from the olfactory bulb." Glia **60**(3): 404-413.
Imada, M. and N. Sueoka (1978). "Clonal sublines of rat neurotumor RT4 and cell differentiation. I. Isolation and characterization of cell lines and cell type conversion." Dev Biol **66**(1): 97-108.
Jain, S., A. Sharma and B. Basu (2013). "In vitro cytocompatibility assessment of amorphous carbon structures using neuroblastoma and Schwann cells." J Biomed Mater Res B Appl Biomater **101**(4): 520-531.
Kaewkhaw, R., A. M. Scutt and J. W. Haycock (2012). "Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue." Nat Protoc **7**(11): 1996-2004.
Kimura, H., W. H. Fischer and D. Schubert (1990). "Structure, expression and function of a schwannoma-derived growth factor." Nature **348**(6298): 257-260.
Klausmeyer, A., D. Stern and S. Wiese (2015). "Isolation and culture of spinal cord motor neurons." Curr Protoc Cell Biol **66**: 1 9 1-1 9 10.
Koppes, A. N., A. L. Nordberg, G. M. Paolillo, N. M. Goodsell, H. A. Darwish, L. Zhang and D. M. Thompson (2014). "Electrical stimulation of schwann cells promotes sustained increases in neurite outgrowth." Tissue Eng Part A **20**(3-4): 494-506.
Levy, A., M. Garcia Segura, Z. Nevo, Y. David, A. Shahar and F. Naftolin (1996). "Action of steroid hormones on growth and differentiation of CNS and spinal cord organotypic cultures." Cell Mol Neurobiol **16**(3): 445-450.
Liu, Z., W. Gao, Y. Wang, W. Zhang, H. Liu and Z. Li (2011). "Neuregulin-1beta regulates outgrowth of neurites and migration of neurofilament 200 neurons from dorsal root ganglial explants in vitro." Peptides **32**(6): 1244-1248.
Liu, Z., L. Huang, L. Liu, B. Luo, M. Liang, Z. Sun, S. Zhu, X. Quan, Y. Yang, T. Ma, J. Huang and Z. Luo (2015). "Activation of Schwann cells in vitro by magnetic nanocomposites via applied magnetic field." Int J Nanomedicine **10**: 43-61.
Madison, R. D., C. McGee, R. Rawson and G. A. Robinson (2014). "Extracellular vesicles from a muscle cell line (C2C12) enhance cell survival and neurite outgrowth of a motor neuron cell line (NSC-34)." J Extracell Vesicles **3**.
Magnaghi, V., A. Parducz, A. Frasca, M. Ballabio, P. Procacci, G. Racagni, G. Bonanno and F. Fumagalli (2010). "GABA synthesis in Schwann cells is induced by the neuroactive steroid allopregnanolone." J Neurochem **112**(4): 980-990.
Meyer, C., S. Wrobel, S. Raimondo, S. Rochkind, C. Heimann, A. Shahar, O. Ziv-Polat, S. Geuna, C. Grothe and K. Haastert-Talini (2015). "Peripheral nerve regeneration through hydrogel enriched chitosan conduits containing engineered Schwann cells for drug delivery." Cell Transplant.
Milligan, C. and D. Gifondorwa (2011). "Isolation and culture of postnatal spinal motoneurons." Methods Mol Biol **793**: 77-85.
Montoya-Gacharna, J. V., J. J. Sutachan, W. S. Chan, A. Sideris, T. J. Blanck and E. Recio-Pinto (2012). "Preparation of adult spinal cord motor neuron cultures under serum-free conditions." Methods Mol Biol **846**: 103-116.
Morano, M., S. Wrobel, F. Fregnan, O. Ziv-Polat, A. Shahar, A. Ratzka, C. Grothe, S. Geuna and K. Haastert-Talini (2014). "Nanotechnology versus stem cell engineering: in vitro comparison of neurite inductive potentials." Int J Nanomedicine **9**: 5289-5306.

Navarro, X., E. Verdu and M. Buti (1994). "Comparison of regenerative and reinnervating capabilities of different functional types of nerve fibers." Exp Neurol **129**(2): 217-224.
Novajra, G., C. Tonda-Turo, C. Vitale-Brovarone, G. Ciardelli, S. Geuna and S. Raimondo (2014). "Novel systems for tailored neurotrophic factor release based on hydrogel and resorbable glass hollow fibers." Mater Sci Eng C Mater Biol Appl **36**: 25-32.
Pascal, D., A. Giovannelli, S. Gnavi, S. A. Hoyng, F. de Winter, M. Morano, F. Fregnan, P. Dell'Albani, D. Zaccheo, I. Perroteau, R. Pellitteri and G. Gambarotta (2014). "Characterization of glial cell models and in vitro manipulation of the neuregulin1/ErbB system." Biomed Res Int **2014**: 310215.
Pellitteri, R., M. V. Catania, C. M. Bonaccorso, E. Ranno, P. Dell'Albani and D. Zaccheo (2014). "Viability of olfactory ensheathing cells after hypoxia and serum deprivation: Implication for therapeutic transplantation." J Neurosci Res **92**(12): 1757-1766.
Pinkernelle, J., H. Fansa, U. Ebmeyer and G. Keilhoff (2013). "Prolonged minocycline treatment impairs motor neuronal survival and glial function in organotypic rat spinal cord cultures." PLoS One **8**(8): e73422.
Pittier, R., F. Sauthier, J. A. Hubbell and H. Hall (2005). "Neurite extension and in vitro myelination within three-dimensional modified fibrin matrices." J Neurobiol **63**(1): 1-14.
Prabhakaran, M. P., J. R. Venugopal, T. T. Chyan, L. B. Hai, C. K. Chan, A. Y. Lim and S. Ramakrishna (2008). "Electrospun biocomposite nanofibrous scaffolds for neural tissue engineering." Tissue Eng Part A **14**(11): 1787-1797.
Raimondi, A., A. Mangolini, M. Rizzardini, S. Tartari, S. Massari, C. Bendotti, M. Francolini, N. Borgese, L. Cantoni and G. Pietrini (2006). "Cell culture models to investigate the selective vulnerability of motoneuronal mitochondria to familial ALS-linked G93ASOD1." Eur J Neurosci **24**(2): 387-399.
Ribeiro-Resende, V. T., B. Koenig, S. Nichterwitz, S. Oberhoffner and B. Schlosshauer (2009). "Strategies for inducing the formation of bands of Bungner in peripheral nerve regeneration." Biomaterials **30**(29): 5251-5259.
Richardson, J. A., C. W. Rementer, J. M. Bruder and D. Hoffman-Kim (2011). "Guidance of dorsal root ganglion neurites and Schwann cells by isolated Schwann cell topography on poly(dimethyl siloxane) conduits and films." J Neural Eng **8**(4): 046015.
Ridley, A. J., H. F. Paterson, M. Noble and H. Land (1988). "Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation." EMBO J **7**(6): 1635-1645.
Salgado, A. J. and J. M. Gimble (2013). "Secretome of mesenchymal stem/stromal cells in regenerative medicine." Biochimie **95**(12): 2195.
Sangsanoh, P., S. Waleetorncheepsawat, O. Suwantong, P. Wutticharoenmongkol, O. Weeranantanapan, B. Chuenjitbuntaworn, P. Cheepsunthorn, P. Pavasant and P. Supaphol (2007). "In vitro biocompatibility of schwann cells on surfaces of biocompatible polymeric electrospun fibrous and solution-cast film scaffolds." Biomacromolecules **8**(5): 1587-1594.
Schmid, D., T. Zeis and N. Schaeren-Wiemers (2014). "Transcriptional regulation induced by cAMP elevation in mouse Schwann cells." ASN Neuro **6**(3): 137-157.
Shin, Y. K., S. Y. Jang, J. Y. Park, S. Y. Park, H. J. Lee, D. J. Suh and H. T. Park (2013). "The Neuregulin-Rac-MKK7 pathway regulates antagonistic c-jun/Krox20 expression in Schwann cell dedifferentiation." Glia **61**(6): 892-904.
Sun, H., K. Benardais, N. Stanslowsky, N. Thau-Habermann, N. Hensel, D. Huang, P. Claus, R. Dengler, M. Stangel and S. Petri (2013). "Therapeutic potential of mesenchymal stromal cells and MSC conditioned medium in Amyotrophic Lateral Sclerosis (ALS)--in vitro evidence from primary motor neuron cultures, NSC-34 cells, astrocytes and microglia." PLoS One **8**(9): e72926.
Tannenbaum, J. and B. T. Bennett (2015). "Russell and Burch's 3Rs Then and Now: The Need for Clarity in Definition and Purpose." J Am Assoc Lab Anim Sci **54**(2): 120-132.
Tao, Y. (2013). "Isolation and culture of Schwann cells." Methods Mol Biol **1018**: 93-104.
Tapinos, N. and A. Rambukkana (2005). "Insights into regulation of human Schwann cell proliferation by Erk1/2 via a MEK-independent and p56Lck-dependent pathway from leprosy bacilli." Proc Natl Acad Sci U S A **102**(26): 9188-9193.

Tetzlaff, W., S. W. Alexander, F. D. Miller and M. A. Bisby (1991). "Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43." J Neurosci **11**(8): 2528-2544.
Toda, K., J. A. Small, S. Goda and R. H. Quarles (1994). "Biochemical and cellular properties of three immortalized Schwann cell lines expressing different levels of the myelin-associated glycoprotein." J Neurochem **63**(5): 1646-1657.
Triolo, D., G. Dina, C. Taveggia, I. Vaccari, E. Porrello, C. Rivellini, T. Domi, R. La Marca, F. Cerri, A. Bolino, A. Quattrini and S. C. Previtali (2012). "Vimentin regulates peripheral nerve myelination." Development **139**(7): 1359-1367.
Tse, T. H., B. P. Chan, C. M. Chan and J. Lam (2007). "Mathematical modeling of guided neurite extension in an engineered conduit with multiple concentration gradients of nerve growth factor (NGF)." Ann Biomed Eng **35**(9): 1561-1572.
van Neerven, S. G., P. Pannaye, A. Bozkurt, F. Van Nieuwenhoven, E. Joosten, E. Hermans, G. Taccola and R. Deumens (2013). "Schwann cell migration and neurite outgrowth are influenced by media conditioned by epineurial fibroblasts." Neuroscience **252**: 144-153.
Vyas, A., Z. Li, M. Aspalter, J. Feiner, A. Hoke, C. Zhou, A. O'Daly, M. Abdullah, C. Rohde and T. M. Brushart (2010). "An in vitro model of adult mammalian nerve repair." Exp Neurol **223**(1): 112-118.
Wei, Y., J. Zhou, Z. Zheng, A. Wang, Q. Ao, Y. Gong and X. Zhang (2009). "An improved method for isolating Schwann cells from postnatal rat sciatic nerves." Cell Tissue Res **337**(3): 361-369.
Wrobel, S., S. C. Serra, S. Ribeiro-Samy, N. Sousa, C. Heimann, C. Barwig, C. Grothe, A. J. Salgado and K. Haastert-Talini (2014). "In vitro evaluation of cell-seeded chitosan films for peripheral nerve tissue engineering." Tissue Eng Part A **20**(17-18): 2339-2349.
Zhang, H. and A. S. Verkman (2015). "Aquaporin-1 water permeability as a novel determinant of axonal regeneration in dorsal root ganglion neurons." Exp Neurol **265**: 152-159.
Ziv-Polat, O., A. Shahar, I. Levy, H. Skaat, S. Neuman, F. Fregnan, S. Geuna, C. Grothe, K. Haastert-Talini and S. Margel (2014). "The role of neurotrophic factors conjugated to iron oxide nanoparticles in peripheral nerve regeneration: in vitro studies." Biomed Res Int **2014**: 267808.
Zuchero, J. B. (2014). "Purification of dorsal root ganglion neurons from rat by immunopanning." Cold Spring Harb Protoc **2014**(8): 826-838.