Clin Exp Med (2016) 16:451–461
DOI 10.1007/s10238-015-0364-3

ORIGINAL ARTICLE

Human adipose-derived stem cells stimulate neuroregeneration

Ruslan F. Masgutov[1,2] · Galina A. Masgutova[1] · Margarita N. Zhuravleva[1] · Ilnur I. Salafutdinov[1,2] · Regina T. Mukhametshina[1] · Yana O. Mukhamedshina[1] · Luciana M. Lima[3] · Helton J. Reis[4] · Andrey P. Kiyasov[1] · András Palotás[1,5] · Albert A. Rizvanov[1]

Received: 4 April 2015 / Accepted: 26 May 2015 / Published online: 6 June 2015

Abstract Traumatic brain injuries and degenerative neurological disorders such as Alzheimer's dementia, Parkinson's disease, amyotrophic lateral sclerosis and many others are characterized by loss of brain cells and supporting structures. Restoring microanatomy and function using stem cells is a promising therapeutic approach. Among the many various sources, adipose-derived stem cells (ADSCs) are one of the most easily harvested alternatives, they multiply rapidly, and they demonstrate low immunogenicity with an ability to differentiate into several cell types. The objective of this study was to evaluate the effect of xenotransplanted human ADSCs on post-traumatic regeneration of rat sciatic nerve. Peripheral reconstruction following complete sciatic transection and autonerve grafting was complemented by intra-operative injection of hADSCs into the proximal and distal stumps. The injury caused gliosis and apoptosis of sensory neurons in the lumbar 5 (L5) ganglia in the control rodents; however, animals treated with hADSCs demonstrated a smaller amount of cellular loss. Formation of amputation neuroma, which hinders axonal repair, was less prominent in the experimental group, and immunohistochemical analysis of myelin basic protein showed good myelination 65 days after surgery. At this point, control groups still exhibited high levels of microglia/macrophage-specific marker Iba-1 and proliferating cell nuclear antigen, the mark of an ongoing inflammation and incomplete axonal growth 2 months after the injury. This report demonstrates that hADSCs promote neuronal survival in the spinal ganglion, fuel axonal repair and stimulate the regeneration of peripheral nerves.

Keywords Autonerve graft · Human adipose-derived stem cell (hADSC) · Xenotransplantation · Peripheral nerve injury · Regenerative medicine · Repair

Introduction

Despite rapid development of neurosciences and major advances in innovative pharmacological approaches, slowly progressive degenerative brain diseases such as Alzheimer's dementia, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, among others, remain incurable. These conditions are accompanied by a varying degree of altered structure and function, in particular loss of neurons or glia, demyelination, degeneration of nerve fibers, dysfunction of the innervated target tissues and a plethora of clinically relevant or more subtle cellular changes. Current treatments are largely supportive and in many cases only palliative in nature. To date, clinical recovery following neurotrauma is also unsatisfactory and requires the development and implementation of new approaches.

Despite innovative methods in reconstructive neurosurgery, peripheral nerve injury still has a high risk of

✉ András Palotás
palotas@asklepios-med.eu

✉ Albert A. Rizvanov
albert.rizvanov@kpfu.ru

[1] Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan, Russia

[2] Republic Clinical Hospital, Kazan, Russia

[3] Universidade Federal de Viçosa, Viçosa, Brazil

[4] Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

[5] Asklepios-Med (private medical practice and research center), Kossuth Lajos sgt. 23, Szeged 6722, Hungary

permanently losing functions in the affected extremities. The gap between the proximal and distal nerve fragments is often insufficient for end-to-end anastomosis, causing significant tension. As such, autologous nerve grafts are often used in clinical settings. This approach is also aimed at activating the proliferation of Schwann cells and locally producing neurotrophic factors and cytokines leading to cell adhesion and axon regeneration [1]. Even though the use of autologous nerve grafts for compensating the defects in nerve injury requires an additional traumatization of healthy nerve trunks, autonerve grafting is still the most effective option in the daily surgical routine. However, even high-tech microsurgical manipulations fail to offer full functional restoration of damaged nerves, creating an unmet need to explore new avenues in the treatment of nerve injury. These include transplantation of Schwann cells, mesenchymal adult precursor cells [2], olfactory ensheathing cells [3] and adipose-derived stem cells [4], and several others are also extensively studied.

Traumatic injuries of peripheral nerves are accompanied by structural and functional disorders. Experimentally induced chronic injury of the sciatic nerve causes a significant level of cell death in the spinal ganglion and usually results in incomplete functional recovery [6–8]. Transection of sciatic nerve leads to sequential loss of spinal ganglion neurons in adult rats [9]. Initially, small neurons with dark perikaryotic and unmyelinated appendage (B cells) succumb in a large volume, whereas large neurons with light perikaryotic and myelinated processes (A cells) die in a lesser degree [10]. Approximately 40 % of sensory neurons diminish within 2 months after peripheral axotomy. Similar phenomena are also observed clinically following peripheral nerve injury [11]. Swelling occurs following nerve damage in the proximal segment, while the distal stump undergoes degeneration; however, regenerating axons can be detected as early as 24 h following the injury. Initially, the regenerating units contain only unmyelinated fibers, even if the axon originally was a myelinated fiber, because myelin formation is slower. Over time these unmyelinated fibers become myelinated. Regrowth and myelination of axons per se are not signs of successful regeneration, since the functional recovery is only possible if the fiber reaches the distal nerve segment and the respective distal receptors. If regenerating fibers grow into the perineurium, neuroma will be formed, which could potentially cause loss of function of the regenerating units and may act as a barrier to nearby regenerating axons.

Schwann cells are involved in the formation of endo- and perineurium. Fibroblasts take part in the formation of the myelin sheath during axonal growth. Both of these cell types play important roles in the regeneration of the peripheral nervous system [12], but formation of blood vessels is also key to a successful recovery [13]. As such, stimulating Schwann cells and blood vessels is essential for the regeneration of the peripheral nerve.

Microglia play important roles in re-establishing homeostasis during intra-thecal injury and various degenerative processes. They also bridge the immune system with the brain [14]. However, their impact on traumatized peripheral nerve is yet to be characterized. Mesenchymal stem cells (MSCs) have the ability to differentiate into various cell types and therefore might yield significant regenerative capacity [15]. Several studies demonstrate their positive effects in degenerative neuropsychiatric conditions, but only limited data exist on their regenerative properties in the peripheral nervous system. Among the various MSC sources, adipose tissue has been recognized as one of the most promising in regenerative medicine: Adipose-derived stem cells (ADSCs) are easily harvested, multiply rapidly, demonstrate low immunogenicity and have the ability to differentiate into various cell types [5]. This work, therefore, evaluates the influence of xeno-transplanted human ADSCs (hADSCs) on the regenerative processes after sciatic nerve autografting, in particular post-traumatic proliferation of Schwann cells, axonal growth, myelination, microglial response, regeneration of blood vessels, and the response of neurons and satellite cells in the L5 spinal ganglia.

Materials and methods

Adipose tissue was obtained from healthy human donors, testing negative to hepatitis B and C viruses, and human immunodeficiency virus. The collection of adipose tissue was performed under sterile conditions in a hospital during the planned liposuction. The cell isolation was performed within 16 h after collecting the samples.

Isolation of stem cells from human adipose tissue was performed according to previously published protocols [16]. The adipose tissue was washed three times in DPBS (Biolot, Russia) at 500 g for 5 min. After removal of the lower aqueous phase, 0.4 % collagenase solution was added in the equal volume. The incubation of the tissue was carried out at 37 °C on a shaker, at 120 rpm/min for 1 h. Following the enzymatic digestion of the tissue, the cells were washed three times by centrifugation at 500*g* for 5 min. The pellet was re-suspended in αMEM medium [alpha minimum essential medium, Invitrogen) + 10 % FBS (fetal bovine serum, FBS) (Sigma, USA)] and then filtered (100 μmØ) to remove the uncleaved fragments of adipose tissue. The received cells were maintained in αMEM medium, with the addition of 10 % FBS, 2 mM L-glutamine (Sigma, USA), a mixture of antibiotics of penicillin and streptomycin (100 U/ml, 100 μg/ml) (Sigma, USA).

Previous studies using the phase-contrast microscopy have shown that obtained cells have a fibroblast-like morphology, with highly proliferative properties and antigenic characteristics, typical for MSCs [16].

The experiment was performed on 29 white outbred rats (weight 200–250 g each) obtained from Pushchino Laboratory, Russia. The tests on animals were approved by the physiological section of the Russian national committee on bioethics and were carried out in accordance with the international bioethical norms.

Animals were housed and bred pathogen-free under controlled temperature and lighting (12:12-h light/dark cycle) and fed with commercial animal feed and water. The animals under chloralhydrate anesthesia (400 mg/kg in 6.4 % solution of NaCl) had the defect with the length of 10 mm formed in their left sciatic nerve and immediately grafted the autonerve into the place. The nerve ends were sutured without tension with 10.0 Prolene thread. In the experimental group, during surgery the animals were injected by Hamilton syringe intra-neurally at three points (in the nerve autograft area, in the central and the distal segments of the nerve) with 10 µl of solution (total of 30 µl) containing 1 million MSCs suspended in 0.09 % NaCl. Similarly, the control group was injected with 10 µl of sterile 0.09 % NaCl (total of 30 µl; see Fig. 1). In both groups, after the grafting, the sciatic nerve was coated with fibrin glue "Tissucol" (Baxter, Austria). The wound was sutured in layers. On day 65 after the operation, in both the experimental and control groups, the L5 spinal ganglion was obtained on the operated side, as well as the peripheral fragment of the sciatic nerve with the nerve autograft. The material was fixed in 10 % neutral formalin and was frozen at −80 °C.

Routine methylene blue staining on 7-µm-thick serial sections was used for histological evaluation of the spinal ganglion L5 and glial cells. Images were obtained with microscope (Olympus BX51WI) using a 20× objective equipped with a digital camera (AxioCam MRm, Carl Zeiss). Areas of the spinal ganglion were outlined and measured with ImageJ 1.43.

On 7-µm-thick methylene-blue-stained serial sections of the L5 spinal ganglion, the efficiency of regeneration estimated using caspase-9 goat polyclonal IgG (Santa Cruz) and the number of caspase-9^{+} cells were assessed, as well as the number of neurons, of a large and, partly, of the average diameter was evaluated using NF-m (H60) rabbit polyclonal IgG (Santa Cruz).

In the longitudinal sections of the rat sciatic nerve, the axonal sprouting was evaluated through two seam lines via NF-m (H60) of rabbit polyclonal IgG (Santa Cruz), myelination of fibers was assessed by MBP of goat polyclonal IgG (Santa Cruz), and migration and proliferation of microglia/macrophages were assessed by double-fluorescent immunohistochemical markers Iba-1/PCNA [Iba-1 goat polyclonal IgG (AbCam)/PCNA rabbit polyclonal IgG (Santa Cruz)]. The results were then processed using the Student's *t* test method.

Results

The morphometric analysis of the spinal ganglion L5 indicates a significant visual reduction in size of the spinal ganglion compared with that of the contralateral side in the experimental and control groups. The distal fragment of the sciatic nerve is thickened due to the formation of the connective tissue on its surface. Furthermore, 65 days after the surgery, the swelling of the central nerve segment was

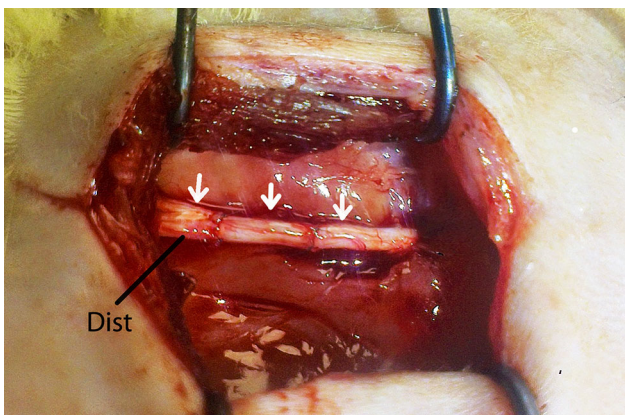

Fig. 1 Sciatic nerve of the rat on 65th day after the autonerve grafting. Dist—the distal segment of the nerve. *White arrows*—insertion site of hADSCs

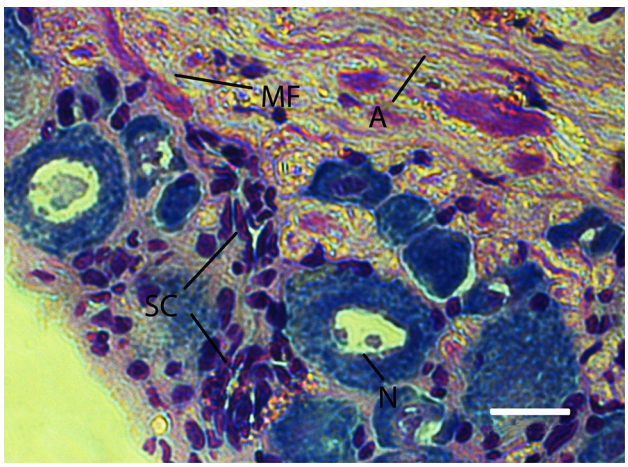

Fig. 2 Serial section of the spinal ganglion L5 in the experimental group on 65th day after the autonerve grafting and hADSC xenotransplantation. The picture shows the cluster of satellite cells (*SC*). We observe the presence of neurons containing two nucleoluses in the nucleus (*N*) in the large-diameter neurons. The presence of axons (*A*) and myelinated fibers (*MF*). *Scale bar*—50 µm

observed. The central part of the spinal ganglion contains well-expressed layers of the connective tissue. In neurons, an uneven and sometimes total central chromate lysis is developed. Part of the neurons appears as hyper-chromic neurons with perikaryons of irregular angular shapes, as well as the neurons containing two nucleoli are observed (see Fig. 2).

Due to the death of a large number of small-diameter neurons, using the method of immunohistochemistry we have evaluated the number of the proprioceptive neurons of large and partly tactile neurons of average diameter using neurofilament-m (NF-m) staining. We counted the neurons with double-fluorescent marking containing NF-m^+ marker in cytoplasm and the blue marker DAPI in the nucleus (see Fig. 3). The number of NF-m^+ neurons in the experimental group has an increase of 35.8 % ($P < 0.05$) compared with the similar cells in the control group (autonerve grafting without hADSCs injection) (Fig. 4). In comparison with the intact animals, the number of NF-m^+ neurons in the spinal ganglions has reduced 65 days after the surgery in the experimental group for 30.75 % ($P < 0.05$) and 55.5 % ($P < 0.05$) in the control group.

In the light microscopy of the spinal ganglion L5, we discovered high activity of the glial cells, the accumulation of macrophages and the formation of glial scars. The number of glial cells was determined by counting DAPI-stained nuclei which morphologically does not belong to neural cells (see Fig. 5). Thus, in L5 spinal ganglion the amount of glial cells in the experimental group increase by 37.61 % ($P < 0.05$) compared with the control group. The number of glial cells in the control group has decreased by 30.9 % ($P < 0.05$) compared with the intact animals.

Along with the proliferation of the glial cells after the peripheral nerve injury in the spinal ganglions, the sensory

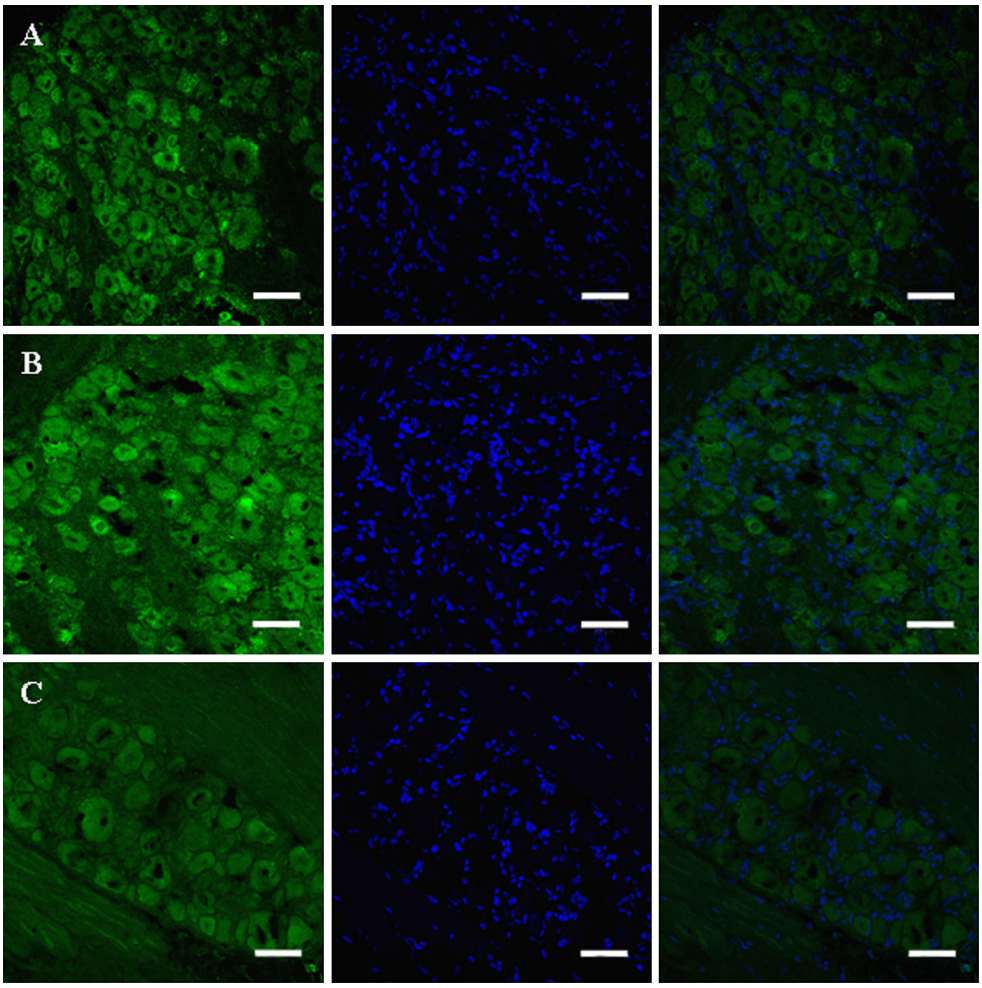

Fig. 3 Serial section of the spinal ganglion L5 in the experimental group on 65th day after the autonerve grafting. NF-m^+ cells and the glial cells in the cross section of the spinal ganglion L5. Sections of adult rats of wild type (WT) were analyzed by confocal microscopy after immunostaining using specific antibodies. *Row* **a**—the group of the intact animals. *Row* **b**—the group of animals with autonerve grafting and hADSC xenotransplantation. *Row* **c**—the group of animals with the autonerve grafting. *Green* fluorescence corresponds to the immunopositive reaction with NF-m. *Blue* fluorescence is a nuclear marker DAPI. *Scale bars*—50 μm (color figure online)

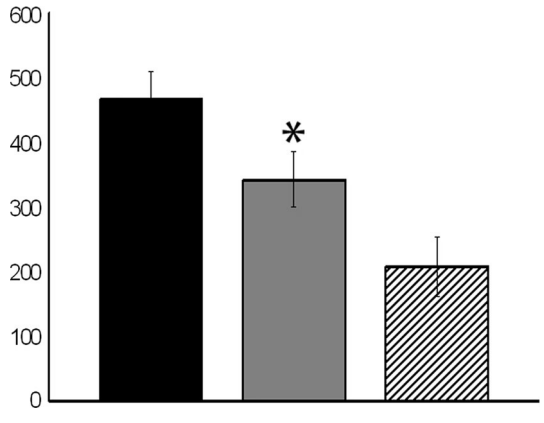

Fig. 4 Number of NF-m$^+$ cells (*Y*-axis) in the test groups on 65th day after the operation. The *black column* represents the intact animals, the *gray column* corresponds to the group of animals with the autonerve grafting and hADSC xenotransplantation, the *striped column* represents the group of animals with the autonerve grafting, and *asterisk* represents significant difference between the groups nerve autografting and administration of MSCs and the group of animals with the nerve autografting and the group of intact animals

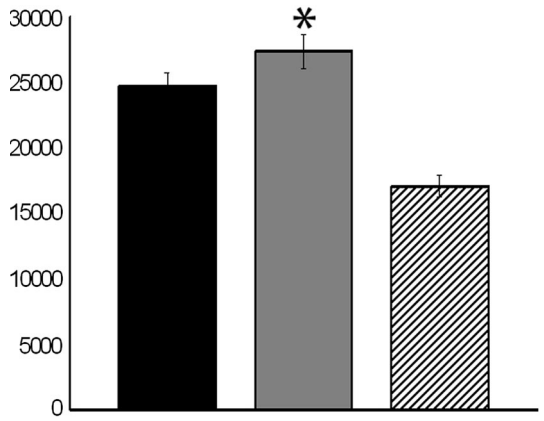

Fig. 5 Number of the glial cells (*Y*-axis) in the test groups on 65th day after the operation. The *black column* represents the intact animals, the *gray column* represents the group of animals with autonerve grafting and hADSC xenotransplantation, the *striped column* represents the group of animals with the autonerve grafting, and *asterisk* represents significant difference between the groups nerve autografting and administration of MSCs and the group of animals with the nerve autografting

neurons undergo apoptosis [17], in which the spinal ganglions were analyzed for tendency of the cells to apoptosis after the autonerve grafting (Fig. 5).

In spinal ganglia, the number of caspase-9$^+$ neurons was evaluated, and it was shown that the number of positive-stained neurons, the neurons prone to apoptosis, in the group with hADSCs transplantation is 41.6 % ($P < 0.05$) lower than in the group of animals with the autonerve graft without the injection of the cells (Figs. 6, 7). It was also, discovered that caspase-9 is localized in the cell cytoplasm only.

The analysis of the longitudinal sections of the sciatic nerve shows the presence of a large number of small- and medium-sized intra-trunk neuromas in the area of the autonerve graft in the sections of the proximal and distal sutures. At randomly selected areas of the longitudinal sections of the sciatic nerve, we evaluated the number of the neuromas. In the group with the cell stimulation, the regenerating fibers are arranged more directly. In the areas of scar formation as well as in the nerve-suture area, the scar tissue is less pronounced. Comparatively, in the control group the scar appears with overgrown nerve fibers and fibrous tissue. The number of neuromas in the experimental group was significantly lower compared with the number of neuromas in the control group. On the one hand, the presence of such structures represents the inability of axons to regenerate; however, it gave us an opportunity to determine the structure of the myelin fibers in both longitudinal and transverse sections. In the experimental group, in the area of the second (distal) suture line, the immunohistochemical marker MBP, we discovered the intra-trunk neuroma, in which the morphology of the myelinated fibers is fully consistent with the normal structure of the nervous tissue (see Fig. 8).

In the distal segment of the nerve, the neuromas were not found in any of the groups, indicating the exceptional obstacle for regenerating fibers in the form of scar tissue. In the control group, the amputation neuromas occur more frequently and in most cases their presence is accompanied by cysts of the adjacent tissue (see Fig. 9). The peripheral nerve fragment in the control group is presented by separately located fibers, and the areas containing large longitudinal cavities are discovered.

In the group of animals with cell stimulation the peripheral fibers of the sciatic nerve are located more specifically. In case of neuroma in the distal segment of the autonerve graft, the axon growth takes place due to the collateral axons, possibly via the regenerative sprouting. With the double-staining immunohistochemical reaction using a specific microglia/macrophage marker (ionized calcium-binding adapter molecule 1, or Iba-1) and the proliferating cell nuclear antigen (PCNA), we showed that in the sciatic nerve of rats in the experimental group, there were no Iba-1-positive cells, while the PCNA fluorescence is not pronounced compared with the one of the control group (Fig. 10). In the control group, we observed a large number of proliferating microglia/macrophages, the axons and myelin fibers (Fig. 11). These results suggest that hADSCs implanted intra-operatively in the injured area of the sciatic nerve prevented the appearance of microglia/macrophages 2 months after the injury. As for the results with the application of the fluorescent reaction with PCNA, we can conclude that in the control group, on 65th day following the surgery there are ongoing processes of growth and regeneration, whereas in the experimental

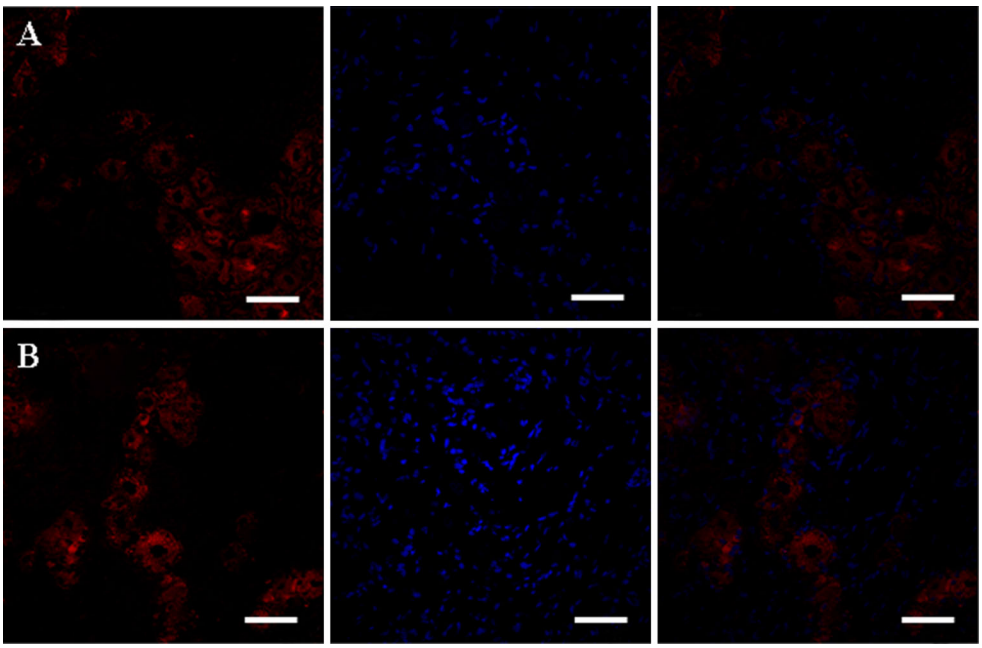

Fig. 6 Serial section of the spinal ganglion L5 in the experimental group on 65th day after the operation. Caspase-9^{+} cells in the spinal ganglion L5. *Row* **a**—the group of animals with autonerve graft. *Row* **b**—the groups autonerve grafting and hADSC xenotransplantation. *Red* fluorescence corresponds to the positive reaction with caspase-9. *Blue* fluorescence represents the nuclear marker DAPI. *Scale bars*—50 μm (color figure online)

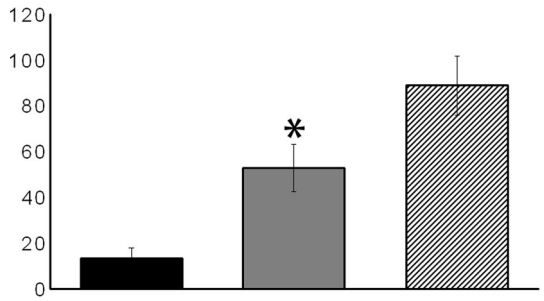

Fig. 7 Number of caspase-9^{+} cells (*Y*-axis) in the experimental group on 65th day after the operation. The *black column* represents the intact animals, the *gray column* is the group of animals with autonerve graft and hADSC xenotransplantation, the *striped column* represents the group of animals with the autonerve graft, and *asterisk* represents significant difference between the groups nerve autografting and administration of MSCs and the group of animals with the nerve autografting

group, the bulk of the axons reached the distal nerve segment and the respective distal receptors, the process of fibers myelination took place, and the processes of neuroma formation discontinued.

Discussion

Within the serious medical and social problem, a large number of scientific papers were dedicated for search of methods to reduce post-traumatic degeneration in the central and peripheral nervous system, and neurodegenerative diseases research (ALS and Alzheimer's). Since the first description of MSCs as a single cell type, a large number of studies were carried out in both preclinical and clinical animal models, for their application in various fields of medicine, and a whole literature data on the prospects for clinical application of MSCs look quite optimistic. For example, in their review Chen et al. [18] discuss the possibility of clinical application of MSCs in neurodegenerative diseases and claim that transplanted MSCs produce neurotrophic factors and promote the survival and regeneration of neurons, leading to functional recovery: This was confirmed in our work.

The nerve autografting continues to remain the golden standard for restoring the integrity of the nerve in diastase. The explanation can be given by the presence of the autologous Schwann cells and the structural proteins of the extracellular matrix supporting neuronal survival and the stimulation of the axonal regeneration [19].

The recorded high rate of neuronal death in our experimental groups is undoubtedly associated with serious traumatization of the sciatic nerve and the two suture lines create a serious obstacle for the regenerating axons [11]; however, the transplantation of the hADSCs considerably contributes to the neuronal survival of the L5 spinal ganglion after the autonerve grafting. We have found that it is only in the group with the cell stimulation, and exclusively in the neurons of a large diameter, that doubling of nucleoli

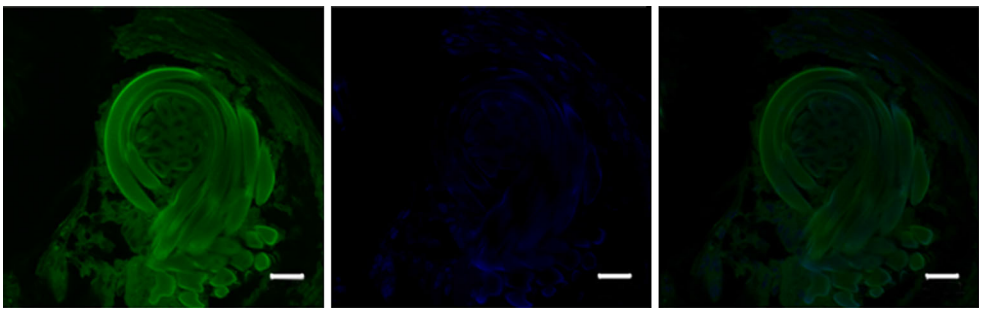

Fig. 8 Sciatic nerve fragment on 65th day after operation in the control group. The neuroma in the distal suture line in the group of animals with autonerve grafting and hADSC xenotransplantation. *Green* fluorescence corresponds to positive reaction with MBP. The *blue* fluorescence is the nuclear marker DAPI. *Scale bars*—20 μm (color figure online)

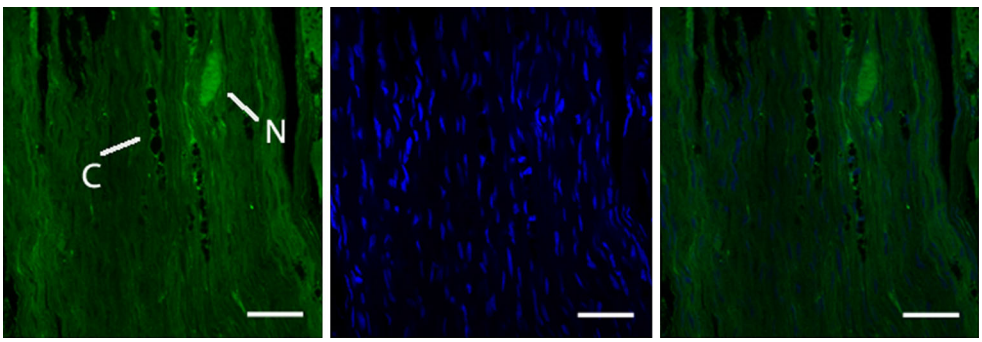

Fig. 9 Sciatic nerve fragment on 65th day after operation in the control group. On the picture, cysts are labeled as (*C*) of the sciatic nerve in nerve autograft area and intra-trunk neuroma is labeled as (*N*). *Green* fluorescence corresponds to the immunopositive reaction with NF-m. *Blue* fluorescence is a nuclear marker DAPI. *Scale bars*—50 μm (color figure online)

in cells takes place. It has been shown that nucleoli are the most elastic components of the cell nucleus, the organization and the functional activity of which change in response to many external influences [20]. Morphologically, the pathology of the cell nucleus is shown by changes in the structure and changes in the size, shape and number of nuclei and nucleoli, in the presence of a variety of nuclear inclusions as well as in the changes of the nuclear membrane. The changes of the nucleoli are essential in morphological and functional assessment of the cell condition, since the processes of transformation and transcription of ribosomal RNA (rRNA) are associated with the nucleoli. The size and the structure of the nucleoli, in most cases, correlate with the amount of the cellular protein synthesis, detectable by biochemical methods. Increasing number of nucleoli indicates an increase in their functional activity. The newly formed ribosomal RNA in the nucleolus is transported to the cytoplasm, and, possibly, through the pores of the inner nuclear membrane. The intensive protein synthesis in these cases is confirmed by the increased amount of the endoplasmic reticulum ribosomes.

It has been observed that the neuronal death, at least partially, is caused by the deficiency of the neurotrophic factors [21]. The survival of the peripheral neurons, unlike the central neurons, is less dependent on the neurotrophic factors. Many types of the peripheral neurons, including sensory and sympathetic neurons of the spinal ganglion, can survive in the absence of the neurotrophic support of the Schwann cells, their analogues or the innervated target cells [22]. This is consistent with the data stating that the peripheral neurons of a mature organism are capable to produce neurotrophic factors that may be involved in their autocrine stimulation [19].

The proliferation of glial cells, including the satellite cells surrounding the perikaryons of the sensory neurons, is phenotypically regarded as the Schwann cells analogues, as often described in the literature. However, recently there have been the results showing that the early satellite cell proliferation improves the regeneration in the peripheral nerve system [23]. Besides the recovery of the motor function, the activation of microglia/macrophages plays an important role in the pathogenesis of the neuropathic pain [24].

In turn, apoptosis is genetically controlled and morphologically defined by the type of the cell death, which can be triggered by a variety of physiological and pathological aspects. This important intracellular process is often initiated during the normal life cycle of a cell to maintain

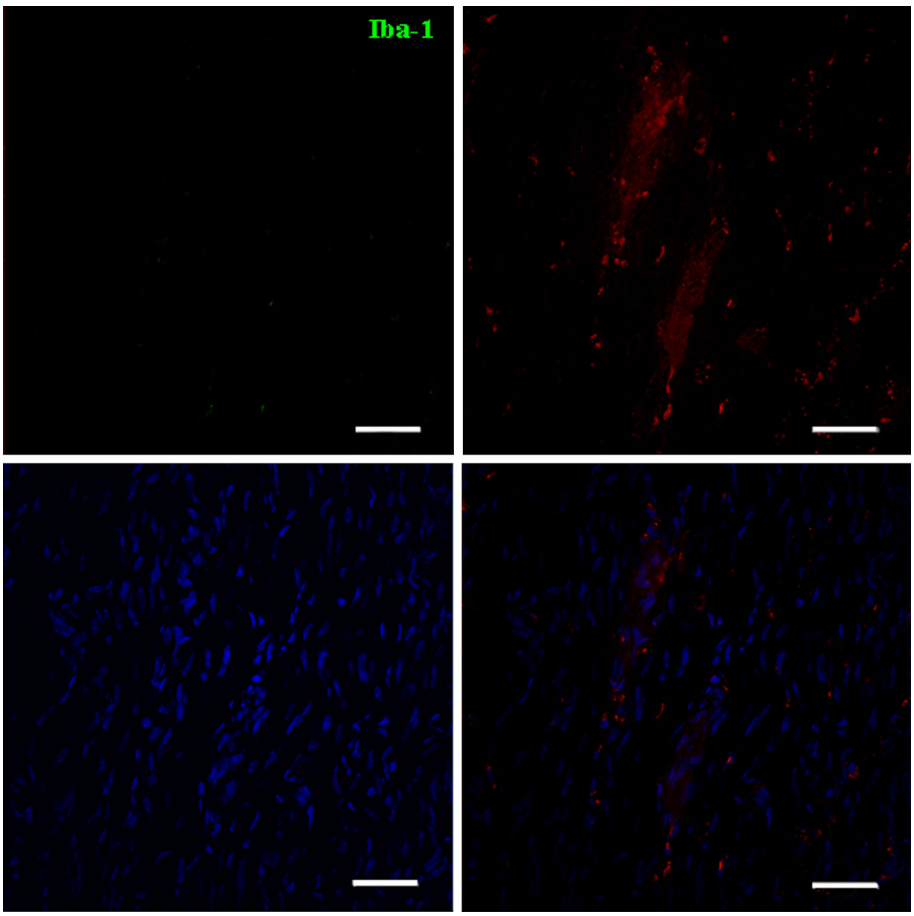

Fig. 10 Sciatic nerve fragment on 65th day after the operation in the control group. Iba-1/PCNA in the group with the autonerve graft and hADSC xenotransplantation (the experimental group). *Upper left* field is in accordance with negative expression with Iba-1. *Red* fluorescence (*right top*) corresponds to the positive reaction with PCNA. *Blue* fluorescence (*left bottom*) stands for the nuclear marker DAPI. Double immunostaining using PCNA- and Iba-1-specific antibodies (*right bottom*). Confocal microscopy of adult rat sections after double immunostaining using Iba-1- and PCNA-specific antibodies. *Scale bars*—50 μm (color figure online)

homeostasis in the body. However, apoptosis plays an important role in various pathological conditions, particularly in the neurodegenerative diseases.

The direct "performers" of apoptosis in a cell are proteins, a special family of proteases, called caspases. Caspases belong to the family of evolutionarily conserved cysteine proteases that are specifically activated in apoptotic cells and play a key role in the mechanisms of the programmed cell death. Currently, several subfamilies of caspases are being differentiated based on their structural similarity, amino acid sequence and substrate specificity.

Apoptotic protease activating factor 1 (Apaf-1) and cytochrome C are playing an important role in holoenzyme formation (caspase-9). Apaf-1 is a protein with molecular weight of 130 kDa, containing caspase activation N-end and recruitment domain (CARD) and as well as 12 repeated amino acid WD-40 sequences (WD—dipeptide consisting of tryptophan and aspartic acid). Out of these conformations, symmetrical structures may occur and molecules of dATP can join their specific areas, which favors the creation of a stable Y-shaped Apaf-1.

The released CARD domains of monomeric molecules Apaf-1 associated with each other to form heptameric structure (>700 kDa) are called apoptosome [25]. The formation of this complex leads to the conversion of procaspase-9 to caspase-9 that triggers the apoptotic cascade.

We have also discovered that caspase 9 is localized in the cell cytoplasm only, which correlates with Cheng and Zochodne's [26] data. In their paper, published in 2003 authors suggested that the increased level of caspase-9 in the nucleus cannot be regarded as a sign of irreversible cell entry into apoptosis.

This observation is consistent with our registered decrease in the total number of neurons on 65th day after the nerve injury. These data along with the dynamics of caspase-9^{+} expression prove that nerve transection is induced by apoptosis of the sensory neurons in the spinal ganglion [11] and allow to suggest that the hADSCs due to their ability to release neurotrophic factors and their retrograde transportation from the area of their administration to the target cells support the survival of the sensory neurons in the spinal ganglion, which is one of the factors determining the efficiency of the peripheral axon regeneration and restoration of the sensory function.

Although the molecular mechanisms that are in the basis of the neuronal cell death of the spinal ganglion in response to the nerve axotomy are not fully clear, the role of the exogenous trophic factors has been proved as well as the

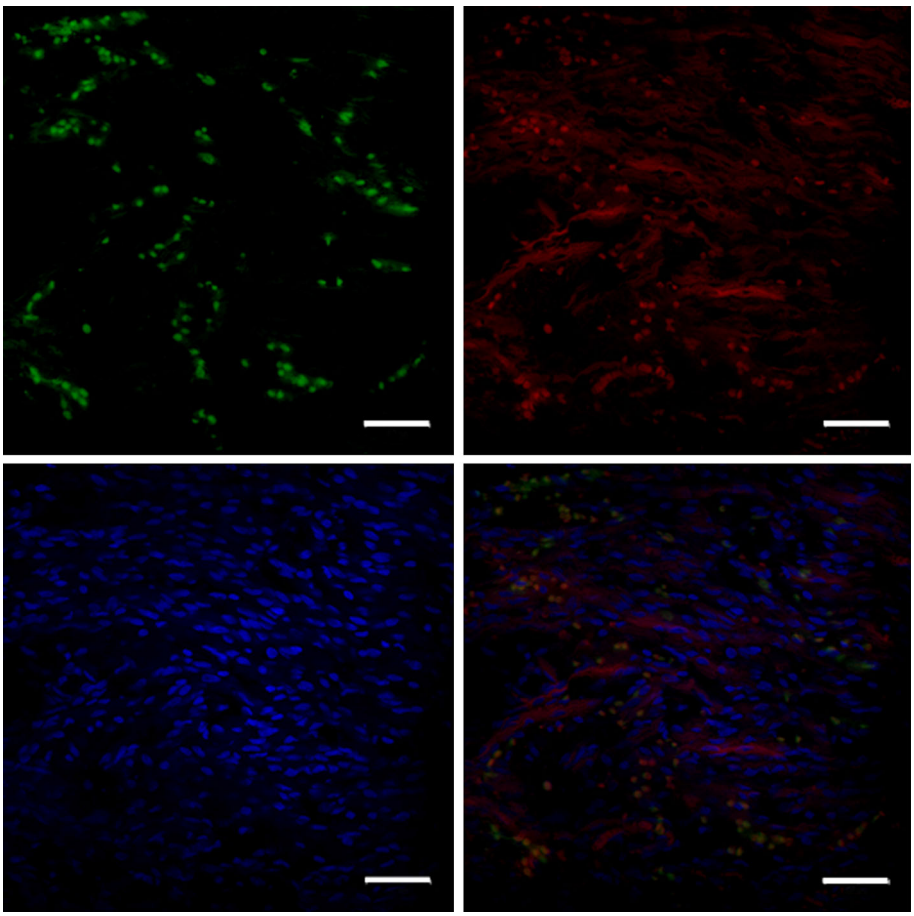

Fig. 11 Sciatic nerve fragment on 65th day after the operation in the control group. Iba-1/PCNA in the control group. *Green* fluorescence corresponds to the positive reaction with Iba-1 (*upper left*). *Red* fluorescence corresponds to the positive reaction with PCNA (*upper right*). *Blue* fluorescence (*left bottom*) stands for the nuclear marker DAPI. Double immunostaining using PCNA- and Iba-1-specific antibodies (*right bottom*). Confocal microscopy of adult rat sections after double immunostaining using Iba-1- and PCNA-specific antibodies. *Scale bars*—50 μm (color figure online)

severity of the trauma in the neuronal apoptosis and the possibility for axonal sprouting to the corresponding distal receptors [27].

Presently, scientists pay a close attention to the study of the immune system response, particularly the reaction of microglia/macrophages to the traumatic peripheral nerve injury. In early studies, it was shown that the sciatic nerve is relatively impermeable for microglia/macrophages; however, the access of the immune cells enhances after the nerve injury [28]. In the last few years, it has been shown that in response to an injury, it is not only microglia which gets activated, but also in the periphery, in the injured area, the reactive Schwann cells provide trophic support and directional growth of the regenerating axons. In response to injury, the reactive astrocytes and microglia play the key role in synaptic cleaning as well as in the phagocytosis processes of the destroyed myelin [29].

The microglial cells are resident macrophages in the central nervous system. These are the cells of mesodermal/mesenchymal origin, in trauma migrating to all areas of the central nervous system through the spinal cord parenchyma and acquiring their inherent branched morphological phenotype [30]. The recent studies have shown that even in intact spinal cord, microglia is able to migrate, thus scanning the territorial domains. It can interact with macroglia, neurons and cells of the immune system. Furthermore, the microglial cells have neurotransmitters and cytokines receptors. The microglial cells are considered to be the most sensitive sensors of the nervous system pathology. At the first signs of nervous system dysfunction, the microglial cells go through a complex, multistep activation process, which converts them into the "activated microglia". This cell form has the ability to release a large number of substances influencing the surrounding cells, either in a pathological or in a stimulating way. The activated microglia is able to migrate to the traumatized area, proliferate and phagocytize cells and cellular debris [31]. It has been observed that the activated microglia migrates to the area of an injured facial nerve already in 15 h after the injury, with the phagocytosis activity peak observed during the 2–3 weeks and lasting for 8 weeks [31].

The MSC application for regeneration and neuroprotection has been extensively studied. The recent work describes the biological properties as well as the evaluation of the MSCs quality and safety for clinical use [32] which, in accordance with our received data, allows distinguishing the regenerative potential of MSCs in enhancing post-traumatic nerve regeneration.

In conclusion, we can infer that the xenotransplantation of hADSCs in the injured rat's sciatic nerve supports the neuronal survival in the spinal ganglion L5 and stimulates the axonal growth and myelination.

One of the possible mechanisms for the neuroprotective antiapoptotic properties of the xenotransplanted cells is the production of growth factors, retrogradely transported to neurons and stimulating their survival [33]. These data allow us to suggest that ADSCs due to their ability to release neurotrophic factors and their retrograde transportation from the area of their administration to the target cells support the survival of the sensory neurons in the spinal ganglion, being a prerequisite for the successful regeneration.

Our results as well previous studies show that ADSCs represent a promising therapeutic approach in nerve tissue engineering regeneration and that one of their ability to migrate and release neurotrophic factors [34] can be considered as a promising cell type for treatment of neurodegenerative diseases.

Acknowledgments The study was supported by Grant 13-04-12035 from Russian Foundation for Basic Research. This work was performed in accordance with Program of Competitive Growth of Kazan Federal University and subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities. Yana O. Mukhamedshina was supported by Presidential grant for government support of young scientists (PhD) from the Russian Federation (4020.2015.7). Some of the experiments were conducted using equipment at the Interdisciplinary center for collective use of Kazan Federal University supported by Ministry of Education of Russia (ID RFMEFI59414X0003), Interdisciplinary center for analytical microscopy, and Pharmaceutical Research and Education Center, Kazan (Volga Region) Federal University, Kazan, Russia. The authors are also indebted to Drs. Gallyamov and Bogov for their assistance in animal handling.

Conflict of interest Authors declare no conflict of interest.

References

1. Dubovy P. Schwann cells and endoneurial extracellular matrix molecules as potential cues for sorting of regenerated axons: a review. Anat Sci Int. 2004;79(4):198–208. doi:10.1111/j.1447-073x.2004.00090.x.
2. Dezawa M, Ishikawa H, Hoshino M, Itokazu Y, Nabeshima Y. Potential of bone marrow stromal cells in applications for neurodegenerative, neuro-traumatic and muscle degenerative diseases. Curr Neuropharmacol. 2005;3(4):257–66.
3. Radtke C, Wewetzer K, Reimers K, Vogt PM. Transplantation of olfactory ensheathing cells as adjunct cell therapy for peripheral nerve injury. Cell Transplant. 2011;20(2):145–52. doi:10.3727/096368910X522081.
4. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol. 2007;207(2):267–74. doi:10.1016/j.expneurol.2007.06.029.
5. Arkhipova SS, Raginov IS, Mukhitov AR, Chelyshev YA. Satellite cells of sensory neurons after various types of sciatic nerve trauma in the rat. Neurosci Behav Physiol. 2010;40(6):609–14. doi:10.1007/s11055-010-9303-7.
6. Gordon T, Tyreman N, Raji MA. The basis for diminished functional recovery after delayed peripheral nerve repair. J Neurosci. 2011;31(14):5325–34. doi:10.1523/JNEUROSCI.6156-10.2011.
7. McKay Hart A, Brannstrom T, Wiberg M, Terenghi G. Primary sensory neurons and satellite cells after peripheral axotomy in the adult rat: timecourse of cell death and elimination. Exp Brain Res. 2002;142(3):308–18. doi:10.1007/s00221-001-0929-0.
8. Raginov IS, Chelyshev IuA. Post-traumatic survival in different subpopulations of sensory neurons. Morfologiia. 2003;124(4):47–50.
9. Tandrup T, Woolf CJ, Coggeshall RE. Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. J Comp Neurol. 2000;422(2):172–80. doi:10.1002/(SICI)1096-9861(20000626)422:2<172:AID-CNE2>3.0.CO.
10. Vigneswara V, Berry M, Logan A, Ahmed Z. Caspase-2 is upregulated after sciatic nerve transection and its inhibition protects dorsal root ganglion neurons from apoptosis after serum withdrawal. PLoS One. 2013;8(2):e57861. doi:10.1371/journal.pone.0057861.PONE-D-12-35011.
11. Weerasuriya A, Mizisin AP. The blood-nerve barrier: structure and functional significance. Methods Mol Biol. 2011;686:149–73. doi:10.1007/978-1-60761-938-3_6.
12. Tamaki T, Hirata M, Soeda S, Nakajima N, Saito K, Nakazato K, et al. Preferential and comprehensive reconstitution of severely damaged sciatic nerve using murine skeletal muscle-derived multipotent stem cells. PLoS One. 2014;9(3):e91257. doi:10.1371/journal.pone.0091257.PONE-D-13-38421.
13. Perry VH, Teeling J. Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. Semin Immunopathol. 2013;35(5):601–12. doi:10.1007/s00281-013-0382-8.
14. Kalinina NI, Sysoeva VY, Rubina KA, Parfenova YV, Tkachuk VA. Mesenchymal stem cells in tissue growth and repair. Acta Naturae. 2011;3(4):30–7.
15. Faroni A, Terenghi G, Reid AJ. Adipose-derived stem cells and nerve regeneration: promises and pitfalls. Int Rev Neurobiol. 2013;108:121–36. doi:10.1016/B978-0-12-410499-0.00005-8.
16. Solovyeva VV, Salafutdinov II, Martynove EV. Human adipose derived stem cells do not alter cytokine secretion in response to the genetic modification with pEGFP-N2 Plasmid DNA. World Appl Sci J. 2013;26(7):968–72.
17. Momeni HR, Soleimani Mehranjani M, Shariatzadeh MA, Haddadi M. Caspase-mediated apoptosis in sensory neurons of cultured dorsal root ganglia in adult mouse. Cell J. 2013;15(3):212–7.
18. Chen L, Qiu R, Xu Q. Mesenchymal stem cell therapy for neurodegenerative diseases. J Nanosci Nanotechnol. 2014;14(1):969–75.
19. Masgutov RF, Salafutdinov II, Bogov AA. The stimulation of posttraumatic regeneration of the sciatic nerve of a rat with a plasmid expressing a vascular endothelial growth factor and the basic fibroblast growth factor. Cell Transplant Tissue Eng. 2011;6(3):67–70.
20. Cheutin T, Gorski SA, May KM, Singh PB, Misteli T. In vivo dynamics of Swi6 in yeast: evidence for a stochastic model of heterochromatin. Mol Cell Biol. 2004;24(8):3157–67.
21. Lo DC. Neurotrophic factors and synaptic plasticity. Neuron. 1995;15(5):979–81.
22. Goldberg JL, Barres BA. Nogo in nerve regeneration. Nature. 2000;403(6768):369–70. doi:10.1038/35000309.

23. Ribeiro-Resende VT, Carrier-Ruiz A, Lemes RM, Reis RA, Mendez-Otero R. Bone marrow-derived fibroblast growth factor-2 induces glial cell proliferation in the regenerating peripheral nervous system. Mol Neurodegener. 2012;7:34. doi:10.1186/1750-1326-7-34.
24. Otoshi K, Kikuchi S, Konno S, Sekiguchi M. The reactions of glial cells and endoneurial macrophages in the dorsal root ganglion and their contribution to pain-related behavior after application of nucleus pulposus onto the nerve root in rats. Spine (Phila Pa 1976). 2010;35(3):264–71. doi:10.1097/BRS.0b013e3181b8b04f.
25. Rodriguez J, Lazebnik Y. Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev. 1999;13(24):3179–84.
26. Cheng C, Zochodne DW. Sensory neurons with activated caspase-3 survive long-term experimental diabetes. Diabetes. 2003;52(9):2363–71.
27. Wood MD, Kemp SW, Weber C, Borschel GH, Gordon T. Outcome measures of peripheral nerve regeneration. Ann Anat. 2011;193(4):321–33. doi:10.1016/j.aanat.2011.04.008.
28. Abram SE, Yi J, Fuchs A, Hogan QH. Permeability of injured and intact peripheral nerves and dorsal root ganglia. Anesthesiology. 2006;105(1):146–53.
29. Berg A, Zelano J, Pekna M, Wilhelmsson U, Pekny M, Cullheim S. Axonal regeneration after sciatic nerve lesion is delayed but complete in GFAP- and vimentin-deficient mice. PLoS One. 2013;8(11):e79395. doi:10.1371/journal.pone.0079395.PONE-D-13-32448.
30. Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. Origin and differentiation of microglia. Front Cell Neurosci. 2013;7:45. doi:10.3389/fncel.2013.00045.
31. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev. 2011;91(2):461–553. doi:10.1152/physrev.00011.2010.
32. Shachpazyan NR, Astrelina TA, Yakovleva MV. Mesenchymal stem cells derived from various human tissues: biological properties, evaluation of their quality and safety for clinical use. Cell Transplant Tissue Eng. 2012;7(1):23–33.
33. Reid AJ, Sun M, Wiberg M, Downes S, Terenghi G, Kingham PJ. Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis. Neuroscience. 2011;199:515–22. doi:10.1016/j.neuroscience.2011.09.064.
34. Marconi S, Castiglione G, Turano E, Bissolotti G, Angiari S, Farinazzo A, et al. Human adipose-derived mesenchymal stem cells systemically injected promote peripheral nerve regeneration in the mouse model of sciatic crush. Tissue Eng Part A. 2012;18(11–12):1264–72. doi:10.1089/ten.TEA.2011.0491.