Journal of Plastic, Reconstructive & Aesthetic Surgery (2019) 72, 1316–1325

JPRAS
An International Journal of Surgical Reconstruction
www.JPRASurg.com

Seeding decellularized nerve allografts with adipose-derived mesenchymal stromal cells: An in vitro analysis of the gene expression and growth factors produced

Nadia Rbia[a,b,1], Liselotte F. Bulstra[a,b,1], Eric A. Lewallen[a,c], Steven E.R. Hovius[b,d], Andre J. van Wijnen[a,e], Alexander Y. Shin[a,*]

[a] *Department of Orthopedic Surgery, Division of Hand and Microvascular Surgery, Mayo Clinic, 200 First Street S.W., Rochester, MN 55905, USA*
[b] *Department of Plastic, Reconstructive and Hand Surgery, Erasmus Medical Center, 's Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands*
[c] *Department of Biological Sciences, Hampton University, 100 E Queen St, Hampton, VA 23668, USA*
[d] *Xpert Clinic, Hand and Wrist Surgery, Jan Leentvaarlaan 14-24, 3065 DC Rotterdam, the Netherlands*
[e] *Department of Biochemistry and Molecular Biology, Mayo Clinic, 200 First Street S.W., Rochester, MN 55905, USA*

Received 4 May 2018; accepted 27 April 2019

KEYWORDS
Processed nerve allograft;
MSCs;
qPCR;
Peripheral nerve;
Gene expression;
Adipose-derived stem cells

Summary Mesenchymal stromal cells (MSCs) secrete many soluble growth factors and have previously been shown to stimulate nerve regeneration. MSC-seeded processed nerve allografts could potentially be a promising method for large segmental motor nerve injuries. Further progress in our understanding of how the functions of MSCs can be leveraged for peripheral nerve repair is required before making clinical translation. The present study, therefore, investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can improve gene and protein expression of growth factors that may support nerve regeneration. Human nerve allografts ($n = 30$) were decellularized and seeded with undifferentiated human adipose-derived MSCs. Subsequently, the MSCs and MSC-seeded grafts were isolated on days 3, 7, 14, and 21 in culture for RNA expression analysis by qRT-PCR. Evaluated genes

Parts of this article have been presented at the following meeting/conference: 1. International Federation of Societies for Surgery of the Hand, Buenos Aires, Argentina, October 27th, 2016. 2. Annual Meeting of the American Society for Peripheral Nerve, Hawaii, United States, January 13th, 2017.

[1] Both authors contributed equally to this work.

* Corresponding author.
E-mail addresses: shin.alexander@mayo.edu, riemersma.donna@mayo.edu (A.Y. Shin).

https://doi.org/10.1016/j.bjps.2019.04.014

included *NGF, BDNF, PTN, GAP43, MBP, PMP22, VEGF,* and *CD31*. Growth factor production was evaluated and quantified using enzyme-linked immunosorbent assay (ELISA). On day 21, semi-quantitative RT-PCR analysis showed that adherence of MSCs to nerve allografts significantly enhances mRNA expression of neurotrophic, angiogenic, endothelial, and myelination markers (e.g., *BDNF, VEGF, CD31,* and *MBP*). ELISA results revealed an upregulation of BDNF and reduction of both VEGF and NGF protein levels. This study demonstrates that seeding of undifferentiated adipose-derived MSCs onto processed nerve allografts permits the secretion of neurotrophic and angiogenic factors that can stimulate nerve regeneration. These favorable molecular changes suggest that MSC supplementation of nerve allografts may have potential in improving nerve regeneration.

Introduction

Processed nerve allografts have shown promising results in peripheral nerve reconstruction. However, for large segmental motor nerve injuries, results remain inferior to autograft reconstruction.[1-6] In autograft reconstruction, Schwann cells facilitate nerve regeneration by promoting myelination through growth factor production and interaction with the extracellular matrix (ECM).[7] The internal structure and ECM components of a nerve graft are critical for guiding cell migration and nerve fiber elongation. Therefore, in optimally decellularized allografts, the internal structure is preserved, while cellular components including Schwann cells are removed to create a nonimmunogenic graft.[8]

To improve the performance of allografts, an optimized environment of biological support must be created around the allograft. Many authors have studied the in vivo delivery of growth factors, mostly using delivery mechanisms such as micropumps and microspheres. This constant infusion has not shown to support nerve regeneration, and in some cases, even impaired nerve regeneration.[9-11]

In different stages of nerve regeneration, there is a biological demand for different growth factors.[12] With this understanding, we addressed whether cell-based production of local growth factors mimics the biological requirements in the microenvironment of the allograft. Undifferentiated mesenchymal stromal cells (MSCs) including those isolated from the stromal vascular fraction of adipose tissue have trophic functions in tissue repair. They can secrete many soluble growth factors including VEGF, NGF, BDNF, and interleukins and have previously been shown to stimulate nerve regeneration.[13-15] MSCs are preferred compared to embryonic stem cells for ethical concerns and reported teratoma formation. MSCs are preferred compared to neural stem cells owing to difficulties in harvesting and reported neuroblastoma formation.[16,17] MSCs can be obtained from multiple sources including adipose tissue, bone marrow, skin, dental pulp, and hair follicles.[14] Adipose-derived MSCs have some important advantages including the ease of accessibility with low donor morbidity, rapid proliferation with high yield, and capability to produce growth factors.[13,18,19]

Although MSCs have proven to be beneficial for nerve regeneration, the actual mechanism is yet unclear. MSCs are thought to support peripheral nerve regeneration through local production of growth factors rather than as active participants in the regeneration process, and it is postulated that the remaining nerve allograft ECM still has biological activity that influences the MSCs and their differentiation.[20,21]

The combination of a patient's own adipose-derived stem cells and the high availability of processed nerve allograft is an attractive method for individualized peripheral nerve repair, which provides results equal to the patient's own harvested nerves. However, further understanding of how the capacities of MSCs can be leveraged for peripheral nerve repair is required before clinical translation can be considered. The present study, therefore, investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can increase mRNA and protein expression of growth factors that may support nerve regeneration.

Materials and methods

General design

This study was approved by our Institutional Review Board (IRB 13-008081). To determine the interaction between the optimized decellularized nerve allograft and MSCs, a total of 30 human cadaver nerve segments were decellularized and seeded with clinical-grade human MSCs. Subsequently, the MSCs and MSC-seeded grafts were cultured for 21 days and sampled at various time points (3, 7, 14, and 21 days). Changes in gene expression profiles (phenotype) of the MSCs were quantified, and production of growth factors was measured (Figure 1).

Allograft preparation

Human motor nerves (thoracodorsal and long thoracic nerves) were obtained from one fresh human cadaver within 12 h post mortem (male, Caucasian, age 62 years). The subject had no known history of conditions that affect the peripheral nervous system. A total of 30 3-cm nerve segments were aseptically removed, cleared of peripheral fat and connective tissue, and were decellularized using the protocol described by Hundepool et al.[22] Briefly, the samples

A

Group	Description	Time points (days)	N	Outcome measurements
I	Seeded nerve graft (nerve graft + MSCs)	T= 0 T= 3 T= 7 T= 14 T= 21	n=5 per experimental group for each time point	**qPCR** analysis for: experimental genes: NGF, BDNF, GDNF, PTN, GAP43, VEGFA, CD31, MPZ, PMP22, MBP, COL1A1, COL3A1, LAMB2, FBLN1, CASP3, EGR1, CD96 reference genes: GAPDH, AKT1 **ELISA** for: NGF, BDNF, GDNF, VEGFA
II	Only MSCs (in static culture)			
III	Only nerve graft (negative control)	T= 0		

B

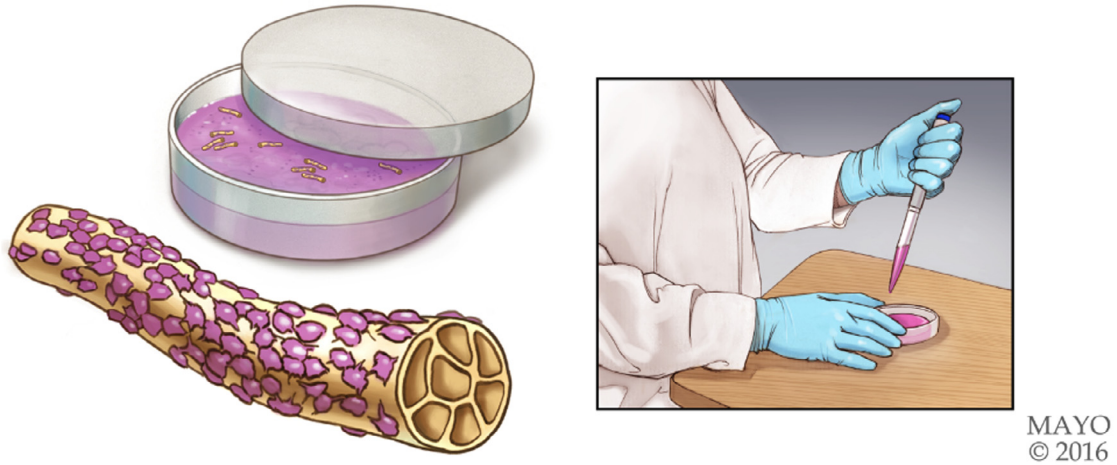

Figure 1 Experimental design. (**A**) A total of 25 human nerve allografts were decellularized and seeded in vitro with clinical-grade human MSCs. Subsequently, the MSC-seeded grafts and MSCs cultured in media only were cultured for 21 days and sampled at various time points (days 3, 7, 14, and 21); total RNA was extracted, then reverse transcribed into cDNA, and qRT-PCR was performed for genes essential for nerve regeneration and additional genes to map MSC characteristics including proliferation, apoptosis, myelination, and ECM molecules. Growth factor production was evaluated and quantified using enzyme-linked immunosorbent assay (ELISA). (B) Shows an image of MSCs seeded onto a decellularized nerve graft.

were treated with a series of detergents including Triton X-200, sulfobetaine-16, sulfobetaine-10, chondroitinase, and elastase. The nerves were sterilized using γ-radiation and stored at 4 °C for 7 days before seeding. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all solutions were autoclaved or filter-sterilized before use.

Isolation of mesenchymal stromal cells

Human MSCs were isolated from lipo-aspirates from a representative 41-year-old male donor with written individual consent and institutional approval. These cells have been routinely used at our institution for clinical trial applications and have been extensively tested for the following cell surface markers: CD73, CD90, CD105, CD44, CD14, and CD45, RNA-sequence transcriptome profiles, and multilineage potential.[23,24] Cell isolation and culture conditions have previously been described.[23,25,26] Cell culture expansion for this experiment started with passage 4 cells and were passaged 2 additional times to obtain 35 million cells (1 million per nerve graft). All the MSCs used in the experiment were of passage 6, and all the experiments were performed concurrently from the same source population of cells.

Cell seeding

A total of 25 human nerve allografts were seeded with clinical-grade human MSCs using a dynamic bioreactor as previously described.[27] Briefly, nerve allografts were combined with MSCs in a 15 mL TubeSpin® Bioreactor tube containing 10 ml cell culture medium and 1 million cells per nerve (maximum of 4 nerves per tube). Tubes were secured on a bioreactor rotator system (Revolver™ Labnet, Edison, USA) with a rotation axis of 30° at a fixed speed of 18 rpm. The rotating device was placed in an incubator for 12 h at 37 °C and 5% CO_2. After incubation, seeded nerves were placed in a culture dish containing advanced minimum essential medium (MEM) (Life Technologies, Grand Island, NY) containing a solution of 5% human platelet lysate, 2 mmol/L Glutamax, 2 U/ml heparin, and 1% penicillin/streptomycin

Table 1 mRNA primer sequences.

Gene ID	Biology	Forward primer	Reverse primer	Literature reference
GAPDH	Reference gene	CCCGGTACACCACGTTCTTC	TGTGGTCATGAGTCCTTCCA	Dudakovic et al. (2017)[30]
AKT1	Reference gene	ATGGCGCTGAGATTGTGTCA	CCCGGTACACCACGTTCTTC	Staal (1987)[36]
NGF	Neurotrophic marker	ATACAGGCGGAACCACACTCAG	ATACAGGCGGAACCACACTCAG	Kingham et al. (2014)[14]
BDNF	Neurotrophic marker	AGAGGCTTGACATCATTGGCTG	CAAAGGCACTTGACTACTGAGCATC	Kingham et al. (2014)[14]
GDNF	Neurotrophic marker	CACCAGATAAACAAATGGCAGTGC	CACCAGATAAACAAATGGCAGTGC	Kingham et al. (2014)[14]
PTN	Neurotrophic marker	ACTGGAAGTCTGAAGCGAGC	CTTCTTCTTAGATTCTGCTTGAGGT	Tezuka et al. (1990)[37]
GAP43	Neurotrophic marker	GTCCACTTTCCTCTCTATTTC	TGTTCATTCCATCACATTGA	Kingham et al. (2014)[14]
VEGFA	Angiogenic marker	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCACGAAG	Kingham et al. (2014)[14]
PECAM/CD31	Angiogenic marker	AACAGTGTTGACATGAAGAGCC	AACAGTGTTGACATGAAGAGCC	Albeda et al. (1991)[28]
MPZ	Myelination marker	GAGGAGGCTCAGTGCTATGG	GCCCGCTAACCGCTATTTCT	Shy (2006)[35]
PMP22	Myelination marker	GTTAAAGGGAACGCCAGGA	AGTTTCTGCAGCCCAAAGGA	Li et al. (2017)[32]
MBP	Myelination marker	GGCTGTGCAACATGTACAAGGA	GGACAGTCCTCTCCCCCTTTCC	Zhou et al. (2017)[39]
COL1A1	ECM protein	GTAACAGCGGTGAACCTGG	CCTCGCTTTCCTTCCTCTCC	Malfait et al. (1993)[33]
COL3A1	ECM protein	TTGAAGGAGGATGTTCCCATCT	ACAGACACATATTTGGCATGGTT	Chiarelli et al. (2018)[29]
LAMB2	ECM protein	ACACGCAAGCGAGTGTATGA	AATCACAGGGCAGGCATTCA	Naba et al. (2017)[34]
FBLN1	ECM protein	AGAGCTGCGAGTACAGCCT	CGACATCCAAATCTCCGGTCT	Naba et al. (2017)[34]
CASP3	Apoptotic protein	GGACCTGTGGACCTGAAAAA	AGTTCGGCTTTCCAGTCAG	Kingham et al. (2014)[14]
EGR1	Transcription factor	ACCCCTCTGTCTACTATTAAGGC	TGGGACTGGTAGCTGGTATTG	Wang et al. (2018)[38]
CD96	Immunoglobulin	AGATTGTGTGATGAAGGACATGG	AGATTGTGTGATGAAGGACATGG	Islam et al. (2018)[31]

solution. Cell culture media were changed every 3–4 days. The impact of seeding on MSC survival has been previously determined to not affect the cell viability over a 7-day study period.[27]

For the control group, 250,000 cells/well were suspended in 6-well plates (Thermo Fisher Scientific, Waltham, MA) containing 2.5 ml cell culture medium. The plates were placed in an incubator at 37 °C and 5% CO_2 for up to 21 days. Media were changed every 3–4 days.

Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

To quantify changes in relative gene expression profiles of MSCs in culture and MSCs seeded onto the decellularized nerve allografts, qRT-PCR was performed on days 3, 7, 14, and 21. Total RNA was extracted from 5 samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA), and RNA yield was evaluated using a NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA), followed by reverse transcription into cDNA by RT-PCR using SuperScript III (Invitrogen, Carlsbad, CA) under the following conditions: 3 min at 65 °C, 1.5 h at 37 °C, and 5 min at 95 °C. The resulting cDNA libraries were analyzed by qRT-PCR (C1000 Touch Thermal cycler, Bio-Rad, Hercules, CA) using SYBR Green detection, and specific primers for the panel of genes are given in Table 1. All genes were chosen from the literature because of their importance in nerve regeneration.[14,28–39] All MSC-only samples were analyzed in triplicate, and all MSC-seeded nerves were analyzed in groups of five. Results were normalized to the reference gene *AKT1* within each sample and to the value of MSCs only on day 0. *AKT1* was selected as a reference gene because it remains constant among a larger number of tissues, cells, and conditions better than other commonly used normalizing genes (e.g., *GAPDH* and *HPRT1*; pers. obs.). In this study, the suitability of both *GAPDH* and *AKT1* as normalization markers was determined and validated using a comprehensive online tool using four different algorithms (geNorm, NormFinder, BestKeeper, and Delta-Ct).[40–42] *AKT1* was identified as the most stable reference gene (Figure 2). Decellularized nerve allografts without MSCs ($n = 5$) were used as a negative control. The differences in gene expression levels were quantified using the comparative delta crossover threshold ($2^{-\Delta\Delta Ct}$) method.[24,43,44]

Protein isolation and enzyme-linked immunosorbent assay (ELISA)

Production of growth factors by MSCs was evaluated by analysis of cell supernatants by enzyme-linked immunosorbent assay (ELISA). Cell supernatants were collected on days 3, 7, 14, and 21 and centrifuged at 1500 rpm for 10 min at 4 °C. Aliquots were stored at −80 °C. As ELISA is a costly technique, four sandwich ELISA kits were selected according to relevance and availability: NGF, GDNF, VEGF (catalog numbers: EHNGF, EHGDNF, KHG0111, respectively, Thermo Fisher Scientific, Waltham, MA), and BDNF (catalog number: MBS355324, MyBioSource, San Diego, CA). All reagents, samples, and standards were prepared as per the manufacturer's instructions. Briefly, samples and standards were loaded in triplicates onto wells and incubated at room temperature for 2.5 h. Plates were rinsed four times and incubated with 100 μl biotinylated antibody for 1 h. Plates were rinsed again four times and incubated with 100 μl streptavidin HRP reagent and subsequently rinsed four more times. The HRP reaction was initiated by addition of 100 μl TMB substrate to each well and incubated in the dark for 30 min. Reactions were terminated by addition of 0.2 M sulfuric acid, and absorbance was measured at 450 nm on a SpectraMax190 microplate reader (Molecular Devices, Inc., Sunnyvale, CA). Samples were analyzed in triplicate, and

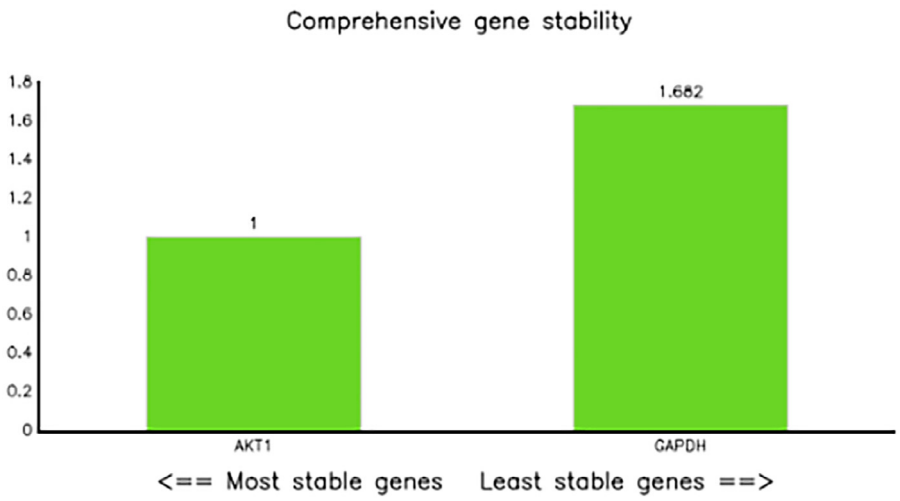

Figure 2 Ranking of candidate reference genes based on their expression stability calculated by comprehensive ranking. Comprehensive gene stability of the reference genes AKT1 and GAPDH in human tissue. The *y*-axis represents the geometric mean of results obtained from geNorm, NormFinder, BestKeeper, and Delta Ct. The *x*-axis represents ranking from least to most stable gene expression, and lower values indicate more stable gene expression.

absorbance values of advanced MEM were used as negative controls. A standard curve was plotted to determine concentrations (pg/ml).

Statistical analysis

The Kolmogorov–Smirnov test was applied to test for a normal distribution. To detect differences in gene expression between sampled groups, data were analyzed using the Mann–Whitney *U* test. A value of $p < 0.05$ was considered statistically significant. All results are reported as mean ± standard deviation.

Results

Quantitative real-time polymerase chain reaction (qRT-PCR)

As a negative control, we first evaluated the gene expression of the decellularized nerve graft without cells. No RNA levels were detectable at time point 0, and therefore, no comparisons were made for baseline expression (with the MSC group).

Figure 3(a)–(d) demonstrates the relative MSC gene expression levels clustered by genes that share a common function. For individual time points, there were significant differences for the following genes: *NGF, BDNF, GDNF, PTN, GAP43, VEGF, CD31, PMP22, MBP, COL1A1, FBLN1, EGR1,* and *CD96*. The majority of these differences occurred in the first week (D0-D7). No significant differences were found in *MPZ, COL3A1, LAMB2,* and *CASP3* expression (Figure 3(d)).

The genes that showed significant changes in gene expression over 21 days were *BDNF* ($p = 0.021$), *GDNF* ($p = 0.043$), *VEGF* ($p = 0.021$), *CD31* ($p = 0.021$), and *MBP* ($p = 0.021$). Neurotrophic-, angiogenic-, and myelination-related (*BDNF, VEGF, CD31,* and *MBP*) gene expression values were significantly upregulated in the seeded MSCs, while the neurotrophic marker *GDNF* was significantly upregulated in the MSC group only (Figure 3(d)).

Protein isolation and enzyme-linked immunosorbent assay (ELISA)

Figure 4 depicts the production of three factors analyzed by ELISA over 21 days. Only the NGF protein production in the MSCs was significantly increased at individual time points: 3 days with 207.99 pg/ml ± 28.47 versus 45.02 pg/ml ± 12.73 ($p = 0.017$) and 7 days with 210.78 pg/ml ± 6.69 versus 11.42 pg/ml ± 12.73 ($p = 0.003$).

Production of VEGFA protein in the seeded MSCs was low but increased per time point from 108.95 pg/ml ± 8.43 on day 3 to 190.10 pg/ml ± 19.61 on day 21, while only the MSCs produced stable significantly increasing ($p = 0.028$) levels of the VEGFA protein over the 21 days from 590.84 pg/ml ± 7.57 on day 3 to 692.78 pg/ml ± 72.23 on day 21.

ELISA revealed a significant ($p = 0.011$) upregulated BDNF protein production over the time course of 21 days by the seeded MSCs (370.16 pg/ml ± 13.37 at 72 h compared to 18.73 pg/ml ± 7.21 in the MSC-only group), correlating with the significant upregulation of BDNF gene expression. ELISA growth factor analysis showed no detectable GDNF-protein production in either group.

Discussion

This study investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can increase mRNA and protein expression of growth factors that may support nerve regeneration.

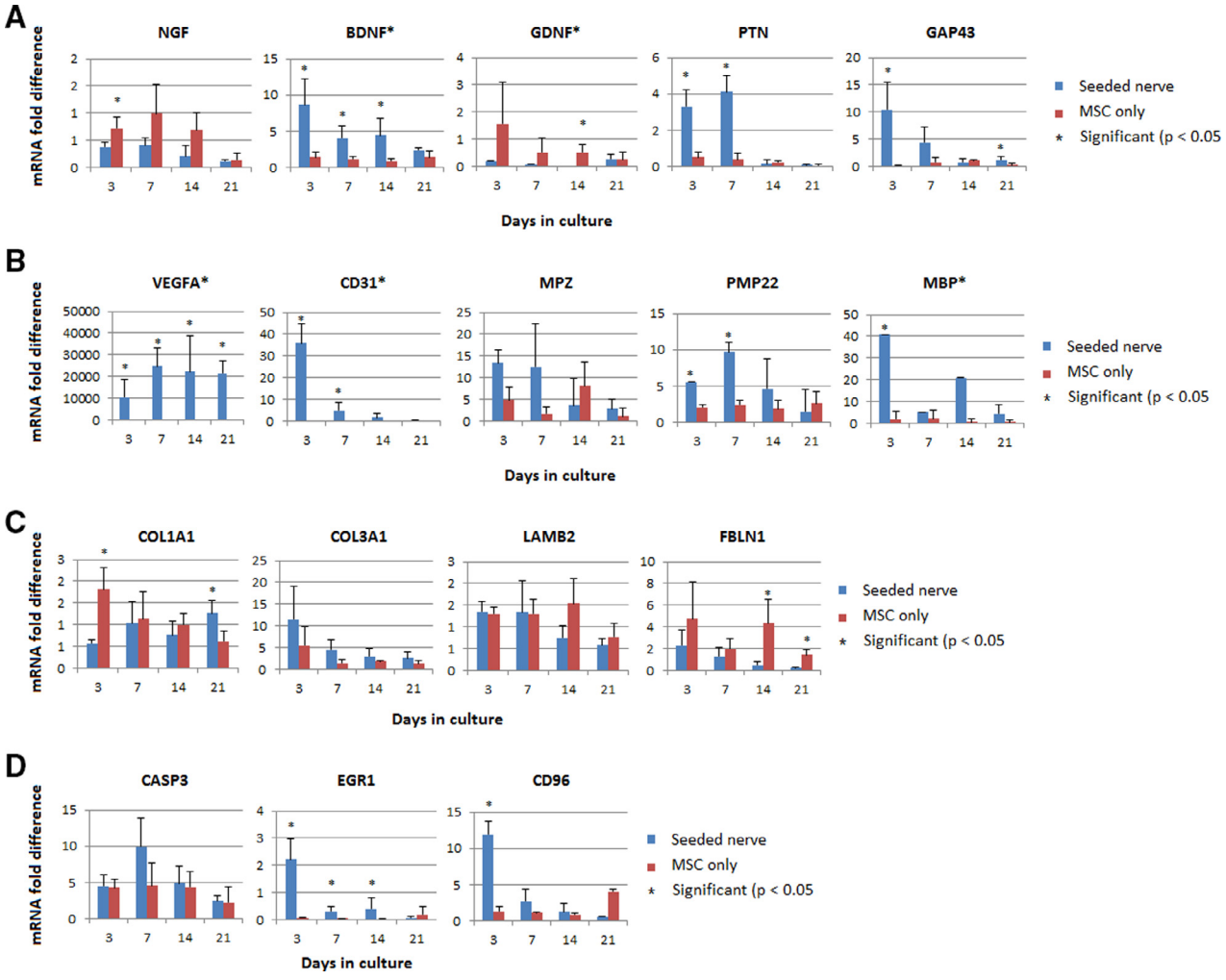

Figure 3 Gene expression clustered by genes that share a common function. The gene expression of each time point was normalized to AKT1, and the fold difference versus MSCs day 0 was calculated. The error bars represent the standard deviation ($n = 5$). * denotes $p < 0.05$ when compared to the gene expression in seeded nerves to MSCs only at that time point. (A) Neurotrophic factors (B) Angiogenic factors (C) Extracellular matrix (ECM) factors (D) Other factors: apoptotic protein (CASP3), transcription factor (EGR1), and immunoglobulin (CD96).

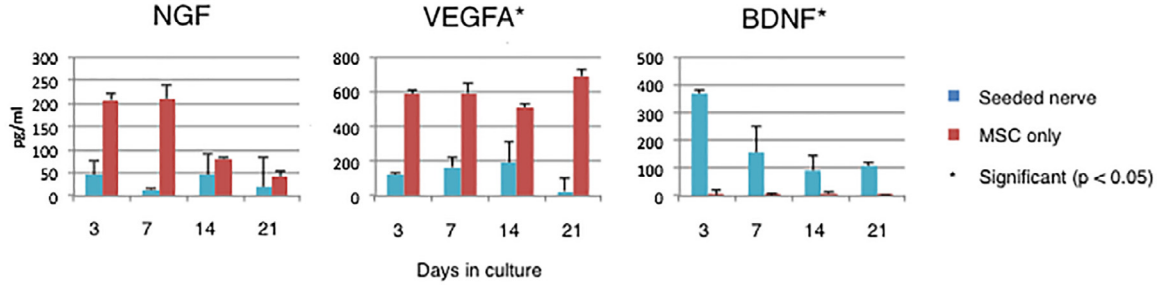

Figure 4 Growth factor production. ELISA results of the cell supernatants for 21 days are depicted.

qRT-PCR and ELISA results

In the decellularized nerve grafts, no RNA levels were detectable, which was expected. The purpose of the decellularization process is to render a scaffold devoid of cells that does not elicit an immune response. Further, the decellularization process most likely caused degradation of RNA in cells that produced the ECM. Although we did not confirm the presence of the residual ECM, previous studies have repeatedly demonstrated preservation of the ECM after nerve decellularization.[8,22]

Many soluble growth factors are secreted by MSCs, and the interaction of MSCs with the allograft may alter the expression of nerve-related growth factors or angiogenic

Table 2 Summary of seeding-induced changes of growth factor production.

Gene ID	Biology	mRNA	Protein
NGF	Neurotrophic marker	↓	↓
BDNF	Neurotrophic marker	↑↑*	↑↑*
GDNF	Neurotrophic marker	↓*	
PTN	Neurotrophic marker	↑	
GAP43	Neurotrophic marker	↑	
VEGFA	Angiogenic marker	↑↑*	↓↓*
PECAM/CD31	Angiogenic marker	↑↑*	
MPZ	Myelination marker	↑	
PMP22	Myelination marker	↑	
MBP	Myelination marker	↑*	
COL1A1	ECM protein	↓	
COL3A1	ECM protein	↑	
LAMB2	ECM protein	↓	
FBLN1	ECM protein	↓	
CASP3	Apoptotic protein	↑	
EGR1	Transcription factor	↑	
CD96	Immunoglobulin	↑	

Results are summarized and arrows indicate an increased or decreased expression in comparison to the unseeded MSCs.

* While there were significant differences for individual time points, overall, only the factors BDNF, GDNF, VEGFA, CD31, and MBP showed significant difference between groups.

proteins that are collectively conducive for allograft rejuvenation. Therefore, we examined whether seeding MSCs altered the expression of select growth factors and cell adhesion molecules linked to neurogenesis or angiogenesis. The interaction between the regeneration-associated genes is likely to be a very dynamic process, and therefore, multiple time points were studied (Figure 3(a)-(d)). Three of the five neurotrophic markers and all myelination and angiogenic markers were increased in MSCs seeded onto a decellularized nerve graft when compared to MSCs alone (Table 2).

NGF is important for the development and maintenance of the sympathetic and sensory nervous systems.[12] NGF mRNA levels were downregulated when MSCs were seeded on a decellularized (motor) nerve graft, suggesting that upon adhesion to the substrate, MSCs become less involved in sensory nerve growth.

We measured GDNF to assess the potential of MSCs in preventing motor neuron apoptosis (induced by axotomy), and qPCR analysis revealed that GDNF mRNA levels significantly decreased when MSCs were seeded, suggesting that the beneficial effect of seeded MSCs is not caused by the prevention of motor neuron apoptosis.[45] The reduced expression of NGF and GDNF in the seeded MSCs can be caused by multiple factors. For some growth factors, upregulation might be triggered by not only the nerve allograft structure but also factors from the wound environment that were not included in our in vitro model.[46] Furthermore, we used motor nerve allografts, and NGF has been previously found to be upregulated in sensory nerves but not in motor nerves.[47]

The BDNF gene promotes the survival and differentiation of selected neuronal populations of the peripheral nervous system and participates in axonal growth.[48] qPCR analysis showed a significant upregulation of the BDNF maker upon seeding, suggesting that the interaction between MSCs and the nerve allograft could stimulate axonal growth.

The PTN-gene induces neurite outgrowth and has significant roles in cell growth and survival.[49] qPCR analysis showed that PTN mRNA levels were increased in allografts seeded with MSCs.

GAP43 is expressed at high levels in neuronal growth cones during development and axonal regeneration and is considered a crucial component of an effective regenerative response.[49] qPCR analysis showed that GAP43 levels were upregulated upon seeding. These results suggest that axonal regeneration and neurite outgrowth are promoted upon adhesion to the graft. However, the effect of genes upregulated in MSCs on axonal growth should be further evaluated, for example, in neuronal co-culture models including the seeded nerve allograft.

The VEGFA growth factor plays a role in angiogenesis.[50] CD31 is involved in leukocyte migration, angiogenesis, and integrin activation.[51] MPZ is required for the proper formation and maintenance of myelin. PMP22 is produced by Schwann cells and helps develop and maintain myelin. MBP is an integral component of myelin formation and stabilization.[52] All of the above-mentioned markers were highly expressed in the MSC-seeded nerves but were scarcely detected in the MSCs alone, suggesting that upon adhesion, MSCs increase their capacity for angiogenesis and myelination.

In vitro expansion of the MSCs resulted in high levels of ECM-related gene expression in both groups. COL1A1 and COL3A1 encode collagen types 1 and 3 that are found in most connective tissues. LAMB2, also an ECM marker, is typically upregulated in cells during adhesion, differentiation, migration, and signaling, as well as neurite outgrowth and regulation of Schwann cell development. Furthermore, FBLN is known to promote cell adhesion and migration within the ECM.[53] Observed upregulation of these markers suggests that seeded MSCs retain their adhesion, migration, and proliferation potential. High levels of ECM-related gene expression in both groups demonstrate that seeded MSCs retain cell adhesion, collagen anabolic activity, and cell-to-cell communication abilities. Influencing stem cell fate by interaction with a specific matrix, to create a so-called stem cell niche, is not a new concept. Indeed, cell adhesion molecules are capable of activating signaling pathways associated with promotion of MSC self-renewal and retention of "stemness."[54]

Of the other genes that were evaluated, CASP3 was measured to assess the potential of seeded MSCs to mediate cell apoptosis.[55] EGR1 functions as a transcriptional regulator and plays a role in the regulation of cell survival, proliferation, and cell death.[56] CD96 is involved in the late phase immune responses. qPCR analysis revealed a moderate upregulated expression of these markers over 21 days in the seeded MSCs (Figure 3(d)), suggesting that upon adhesion, the remaining nerve ECM can trigger cell apoptosis and cell survival while eliciting a late immune response.

To assess whether changes in mRNA levels for secreted factors in MSCs are reflected by corresponding changes in proteins secreted into the medium, we performed ELISA (Figure 4).

Similar to qPCR results, we found a decreasing trend for the NGF protein, suggesting that regulation at the protein

level (e.g., translation, protein stability, or secretion) is altered in MSCs attached to nerve allografts. The significant upregulation of GDNF gene expression in the MSC-only group did not correlate with an increased GDNF protein production.

Discrepancies in protein and mRNA levels, such as we found for VEGF, could be due to translational control, as well as selective differences in protein stability or secretion. For GDNF, ELISA results showed no detectable protein production in either group. Therefore, we were unable to verify whether GNDF gene expression upregulation increased GDNF protein production. In both groups, GDNF expression measured by qPCR was very low, which might have caused the undetectable protein levels. Another (technical) explanation could be that there were large amounts of cell supernatants (1–10 ml) stored frozen, while for the ELISA analysis, only small amounts (microliters) were used; this could have resulted in low concentrations despite adequate centrifugation. Additionally, there are many post-transcriptional mechanisms involved in converting mRNA into protein, and proteins may differ substantially in their half-lives.[57]

Collectively, the results (summarized in Table 2) show that interactions of MSCs with decellularized nerve allografts modulate gene expression in MSCs including the stimulation of mRNAs for key proteins that support nerve growth and/or homeostasis.

Strengths and limitations

The combination of decellularized allografts with MSCs was attempted by Hu et al.,[58] who used decellularized allografts injected with bone marrow-derived MSCs to repair 40 mm ulnar nerve gaps in rhesus monkeys, obtaining good electrophysiological and immunohistochemistry results. However, other measures of functional outcomes (muscle force and mass) were not evaluated. It remains unclear whether cell seeding strategies will enhance the in vivo performance of nerve allografts, and the source of seeded cells is the topic of considerable debate (both topics that need resolution before clinical translation of these strategies can be accomplished). For example, Wang et al.[59] and Fan et al.[60] tried seeding MSCs that were differentiated into Schwann cell-like cells. Wang et al. found that in comparison to Schwann cells, results of differentiated MSCs seem promising. Fan et al.[60] compared differentiated MSCs to undifferentiated MSCs and found that differentiated cells yielded better outcomes after nerve reconstruction. Kingham et al.[14] showed that predifferentiation of MSCs resulted in increased secretion of neurotrophic and angiogenic factors. Most levels of these factors measured in the undifferentiated seeded cells in this study were also enhanced, suggesting that cells might not have to be predifferentiated before surgery. Other researchers hypothesized that once stimulation factors are removed, the cells tend to revert back to their original phenotype.[61] A strength of this study is the use of a previously validated nontraumatic seeding technique.[27] Another major strength of this study is that only human tissues were used; results are therefore translatable to clinical studies. Moreover, the MSCs and the nerve allograft were obtained from different donors, which is comparable to the potential future situation where banked nerve allografts could be seeded with a patient's own MSCs.

A limitation of this study is the lack of biological replicates, which is due to the scarcity of eligible nerve allograft donations available for research purposes. We, however, do not expect to find any difference within allograft donors, as all grafts are decellularized by the same protocol. Second, ELISA was not performed for all known growth factors. As ELISA is a costly technique, only four ELISA kits were selected based on priority from the literature. Moreover, ELISA data did not incorporate the potential for differences in cell biology (e.g., growth and proliferation) among the seeded and control cell groups. Another limitation of this study is that no evidence of cell survival at later time points is demonstrated (in vitro); this would theoretically help to reassure the robustness of the useful induction of growth factors seen by seeding undifferentiated MSCs on decellularized nerves. In contrast, cells cultured for more than 21 days become extremely unpredictable because of the number of times they have divided to cover the substrate. Furthermore, we focused on the clinical translatability. Cells that have been cultured for weeks would not be used in the clinical setting. In addition, we recently published an in vivo rat study demonstrating that implanted MSCs, seeded onto a decellularized nerve allograft, survived up to 29 days. Gradually diminishing signals were observed in the first week following implantation, concluding that MSCs have a finite survival after implantation.[62]

In this vitro model, we investigated the direct interaction between nerve allografts and MSCs. Other factors such as wound healing responses will likely influence MSC behavior.[46] Therefore, future studies should focus on the need for predifferentiation and on the specific role of MSCs seeded onto nerve allografts in an in vivo model of nerve regeneration. Long-term in vivo studies will allow direct assessment of whether the seeded MSCs and their secreted factors also provide better functional nerve repair outcomes.

Conclusion

This study demonstrates that seeding of undifferentiated adipose-derived MSCs onto processed nerve allografts permits the sustained secretion of neurotrophic and angiogenic factors that can stimulate nerve regeneration. Our results suggest that for clinical translation, cells may not need differentiation before surgery and that the trophic functions in tissue repair occur in the first week after surgery. The preoperative combination of autologous MSCs and readily available processed nerve allografts is an exciting avenue for additional research aimed at customization of peripheral nerve repair in individualized medicine.

Acknowledgments

We thank the American Donor Services for providing fresh human cadaveric motor nerves. We also thank the members of the orthopedic research laboratories and particularly Patricia F. Friedrich for assistance with procedures and Anthony Windebank, M.D., Allan Dietz, Ph.D., Amel Dudakovic,

Ph.D., and Roman Thaler, Ph.D. for their expertise and guidance.

Funding for this study was received from the Mayo Clinic Center for Regenerative Medicine Bridge funding and the Michael van Vloten Foundation (personal funding N. Rbia and L.F. Bulstra).

Conflict(s) of interest

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

References

1. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. *Microsurgery* 2012;**32**:1–14.
2. Cho MS, Rinker BD, Weber RV, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. *J Hand Surg Am* 2012;**37**:2340–9.
3. Giusti G, Willems WF, Kremer T, et al. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). *J Bone Joint Surg Am* 2012;**94**:410–17.
4. Moore AM, MacEwan M, Santosa KB, et al. Acellular nerve allografts in peripheral nerve regeneration: a comparative study. *Muscle Nerve* 2011;**44**:221–34.
5. Rbia N, Shin AY. The role of nerve graft substitutes in motor and mixed motor/sensory peripheral nerve injuries. *J Hand Surg Am* 2017;**42**:367–77.
6. Szynkaruk M, Kemp SW, Wood MD, Gordon T, Borschel GH. Experimental and clinical evidence for use of decellularized nerve allografts in peripheral nerve gap reconstruction. *Tissue Eng Part B Rev* 2013;**19**:83–96.
7. Chernousov MA, Carey DJ. Schwann cell extracellular matrix molecules and their receptors. *Histol Histopathol* 2000;**15**:593–601.
8. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Eng* 2004;**10**:1346–58.
9. Hoyng SA, De Winter F, Gnavi S, et al. A comparative morphological, electrophysiological and functional analysis of axon regeneration through peripheral nerve autografts genetically modified to overexpress BDNF, CNTF, GDNF, NGF, NT3 or VEGF. *Exp Neurol* 2014;**261**:578–93.
10. Lee JY, Giusti G, Friedrich PF, Bishop AT, Shin AY. Effect of vascular endothelial growth factor administration on nerve regeneration after autologous nerve grafting. *J Reconstr Microsurg* 2016;**32**:183–8.
11. Piquilloud G, Christen T, Pfister LA, Gander B, Papaloizos MY. Variations in glial cell line-derived neurotrophic factor release from biodegradable nerve conduits modify the rate of functional motor recovery after rat primary nerve repairs. *Eur J Neurosci* 2007;**26**:1109–17.
12. Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. *Mol Neurobiol* 2003;**27**:277–324.
13. Hundepool CA, Nijhuis TH, Mohseny B, Selles RW, Hovius SE. The effect of stem cells in bridging peripheral nerve defects: a meta-analysis. *J Neurosurg* 2014;**121**:195–209.
14. Kingham PJ, Kolar MK, Novikova LN, Novikov LN, Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair. *Stem Cells Dev* 2014;**23**:741–54.
15. Salgado AJ, Reis RL, Sousa NJ, Gimble JM. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr Stem Cell Res Ther* 2010;**5**:103–10.
16. Ladak A, Olson J, Tredget EE, Gordon T. Differentiation of mesenchymal stem cells to support peripheral nerve regeneration in a rat model. *Exp Neurol* 2011;**228**:242–52.
17. Safford KM, Hicok KC, Safford SD, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* 2002;**294**:371–9.
18. Moore AM, Wood MD, Chenard K, et al. Controlled delivery of glial cell line-derived neurotrophic factor enhances motor nerve regeneration. *J Hand Surg Am* 2010;**35**:2008–17.
19. Pfister LA, Papaloizos M, Merkle HP, Gander B. Nerve conduits and growth factor delivery in peripheral nerve repair. *J Peripher Nerv Syst* 2007;**12**:65–82.
20. Armstrong SJ, Wiberg M, Terenghi G, Kingham PJ. ECM molecules mediate both Schwann cell proliferation and activation to enhance neurite outgrowth. *Tissue Eng* 2007;**13**:2863–70.
21. di Summa PG, Kalbermatten DF, Raffoul W, Terenghi G, Kingham PJ. Extracellular matrix molecules enhance the neurotrophic effect of Schwann cell-like differentiated adipose-derived stem cells and increase cell survival under stress conditions. *Tissue Eng Part A* 2013;**19**:368–79.
22. Hundepool CA, Nijhuis TH, Kotsougiani D, et al. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. *Neurosurg Focus* 2017;**42**:E4.
23. Crespo-Diaz R, Behfar A, Butler GW, et al. Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. *Cell Transpl* 2011;**20**:797–811.
24. Dudakovic A, Camilleri E, Riester SM, et al. High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. *J Cell Biochem* 2014;**115**:1816–28.
25. Mader EK, Butler G, Dowdy SC, et al. Optimizing patient derived mesenchymal stem cells as virus carriers for a phase I clinical trial in ovarian cancer. *J Transl Med* 2013;**11**:20.
26. Mahmoudifar N, Doran PM. Osteogenic differentiation and osteochondral tissue engineering using human adipose-derived stem cells. *Biotechnol Prog* 2013;**29**:176–85.
27. Rbia N, Bulstra LF, Bishop AT, van Wijnen AJ, Shin AY. A simple dynamic strategy to deliver stem cells to decellularized nerve allografts. *Plast Reconstr Surg* 2018;**142**:402–13.
28. Albelda SM, Muller WA, Buck CA, Newman PJ. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J Cell Biol* 1991;**114**:1059–68.
29. Chiarelli N, Carini G, Zoppi N, Ritelli M, Colombi M. Transcriptome analysis of skin fibroblasts with dominant negative COL3A1 mutations provides molecular insights into the etiopathology of vascular Ehlers-Danlos syndrome. *PLoS One* 2018;**13**:e0191220.
30. Dudakovic A, Gluscevic M, Paradise CR, et al. Profiling of human epigenetic regulators using a semi-automated real-time qPCR platform validated by next generation sequencing. *Gene* 2017;**609**:28–37.
31. Islam Z, Jahan I, Ahammad RU, et al. FAS promoter polymorphisms and serum sFas level are associated with increased risk of nerve damage in Bangladeshi patients with Guillain-Barre syndrome. *PLoS One* 2018;**13**:e0192703.
32. Li LX, Dong HL, Xiao BG, Wu ZY. A novel missense mutation in peripheral myelin protein-22 causes Charcot-Marie-Tooth disease. *Chin Med J (Engl)* 2017;**130**:1779–84.

33. Malfait F., Wenstrup R., De Paepe A. Classic Ehlers-Danlos Syndrome 1993. In: Adam M.P., Ardinger H.H., Pagon R.A., Wallace S.E., Bean L.J.H., Stephens K., Amemiya A., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2019. 2007 May 29 [updated 2018 Jul 26].
34. Naba A, Pearce OMT, Del Rosario A, et al. Characterization of the extracellular matrix of normal and diseased tissues using proteomics. *J Proteome Res* 2017;**16**:3083-91.
35. Shy ME. Peripheral neuropathies caused by mutations in the myelin protein zero. *J Neurol Sci* 2006;**242**:55-66.
36. Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc Natl Acad Sci USA* 1987;**84**:5034-7.
37. Tezuka K, Takeshita S, Hakeda Y, et al. Isolation of mouse and human cDNA clones encoding a protein expressed specifically in osteoblasts and brain tissues. *Biochem Biophys Res Commun* 1990;**173**:246-51.
38. Wang LF, Liu YS, Yang B, et al. The extracellular matrix protein mindin attenuates colon cancer progression by blocking angiogenesis via EGR-1-mediated regulation. *Oncogene* 2018;**37**:601-15.
39. Zhou Y, Simpson S Jr, Charlesworth JC, et al. Variation within MBP gene predicts disease course in multiple sclerosis. *Brain Behav* 2017;**7**:e00670.
40. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004;**26**:509-15.
41. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**:0034.1-0034.12.
42. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;**64**:5245-50.
43. Jesuraj NJ, Santosa KB, Macewan MR, et al. Schwann cells seeded in acellular nerve grafts improve functional recovery. *Muscle Nerve* 2014;**49**:267-76.
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402-8.
45. Skaper SD. Neurotrophic factors: an overview. *Methods Mol Biol* 2018;**1727**:1-17.
46. Clements MP, Byrne E, Camarillo Guerrero LF, et al. The wound microenvironment reprograms Schwann cells to invasive Mesenchymal-like cells to drive peripheral nerve regeneration. *Neuron* 2017;**96**:98-114 e7.
47. Hoke A, Redett R, Hameed H, et al. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. *J Neurosci* 2006;**26**:9646-55.
48. Karpova NN. Role of BDNF epigenetics in activity-dependent neuronal plasticity. *Neuropharmacology* 2014;**76 Pt C**:709-18.
49. Blondet B, Carpentier G, Lafdil F, Courty J. Pleiotrophin cellular localization in nerve regeneration after peripheral nerve injury. *J Histochem Cytochem* 2005;**53**:971-7.
50. Shibuya M. Vascular Endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies. *Genes Cancer* 2011;**2**:1097-105.
51. DeLisser HM, Christofidou-Solomidou M, Strieter RM, et al. Involvement of endothelial PECAM-1/CD31 in angiogenesis. *Am J Pathol* 1997;**151**:671-7.
52. Li J, Parker B, Martyn C, Natarajan C, Guo J. The PMP22 gene and its related diseases. *Mol Neurobiol* 2013;**47**:673-98.
53. Barros CS, Franco SJ, Muller U. Extracellular matrix: functions in the nervous system. *Cold Spring Harb Perspect Biol* 2011;**3**:a005108.
54. Abdal Dayem A, Lee S, H YC, Cho SG. The impact of adhesion molecules on the in vitro culture and differentiation of stem cells. *Biotechnol J* 2018;**13**(2) Epub 2017 Dec 27. doi:10.1002/biot.201700575.
55. Reid AJ, Sun M, Wiberg M, et al. Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis. *Neuroscience* 2011;**199**:515-22.
56. Calogero A, Arcella A, De Gregorio G, et al. The early growth response gene EGR-1 behaves as a suppressor gene that is down-regulated independent of ARF/Mdm2 but not p53 alterations in fresh human gliomas. *Clin Cancer Res* 2001;**7**:2788-96.
57. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* 2003;**4**:117.
58. Hu J, Zhu QT, Liu XL, Xu YB, Zhu JK. Repair of extended peripheral nerve lesions in rhesus monkeys using acellular allogenic nerve grafts implanted with autologous mesenchymal stem cells. *Exp Neurol* 2007;**204**:658-66.
59. Wang Y, Zhao Z, Ren Z, et al. Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. *Neurosci Lett* 2012;**514**:96-101.
60. Fan L, Yu Z, Li J, Dang X, Wang K. Schwann-like cells seeded in acellular nerve grafts improve nerve regeneration. *BMC Musculoskelet Disord* 2014;**15**:165.
61. Keilhoff G, Goihl A, Langnase K, Fransa H, Wolf G. Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. *Eur J Cell Biol* 2006;**85**:11-24.
62. Rbia N, Bulstra LF, Thaler R, et al. In vivo survival of mesenchymal stromal cell-enhanced decellularized nerve grafts for segmental peripheral nerve reconstruction. *J Hand Surg Am* 2018 Oct 6. pii: S0363-5023(17)32175-5[Epub ahead of print]. doi:10.1016/j.jhsa.2018.07.010.