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# METHODS

# Combined Action of GDNF and HGF Up-Regulates Axonal Growth by Increasing ERK1/2 Phosphorylation

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A stimulating effect of a combination of hepatocyte growth factor (HGF) and glial neurotrophic factor (GDNF) on the growth of neurites in the spinal ganglion model was demonstrated. The mechanism of neurite growth in the spinal ganglion model is associated with transactivation of HGF c-met receptor in the presence of both HGF and GDNF. The combination of HGF and GDNF significantly activated mitogenic signaling cascade mediated by protein kinases ERK1/2, which can be a mechanism for increasing the number of neurites. Our findings can be used for developing effective methods for restoring impaired peripheral nerve function after traumatic and ischemic injury using a combination of GDNF and HGF.

**Key Words:** *hepatocyte growth factor; glial neurotrophic factor; regeneration; peripheral nerve injury*

Regeneration and functional recovery of peripheral nerves after trauma is realized via a chain of sequential processes regulated by a combination of various bioactive molecules with neurotrophic, angiogenic, immunomodulating properties [9]. However, complete regeneration of the nerve due to only natural mechanisms is impossible in case of significant damage [11]. The most serious problem is the development of glial scar after induced inflammatory reaction [4]. The gold standard for surgical treatment of damaged nerves is autograft, but this approach also has limitations, and recovery of the sensory and motor function is achieved in only half of the patients [5]. Therefore, the development of strategies for restoring damaged peripheral nerves affecting the entire complex of processes associated with nerve regeneration or their obstruction remains an urgent problem.

A promising approach can be the use of combinations of growth factors that have pleiotropic effects on regenerative processes due to neurotrophic, neuroprotective, angiogenic, anti-inflammatory, and antifibrotic activities. Hepatocyte growth factor (HGF) along with angiogenic activity exhibits anti-inflammatory, immunomodulating, antiapoptotic, antifibrotic, and neuroprotective properties [8]. We have previously demonstrated that HGF overexpression in ischemic skeletal muscle or myocardium stimulates neovascularization and suppresses accumulation of inflammation cells and fibrosis [7]; in the model of traumatic injury of the peroneal nerve, HGF accelerates its functional and anatomical recovery [2].

In the present study, using the neurite outgrowth model from cultured mouse spinal ganglion cells, we explored the possibility of stimulating neurite outgrowth with a combination of HGF and glial neu-

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rotrophic factor (GDNF) that has a powerful neuroprotective effect promoting neuronal survival, axon growth and myelination, migration and proliferation of Schwann cells [1]. In addition to the influence of HGF and GDNF on almost the entire complex of processes providing nerve regeneration, the choice of this combination of factors is determined by the ability of GDNF to enhance the phosphorylation of the HGF-c-met tyrosine kinase receptor via activation of Src kinase [10]. We hypothesized that combined effect of HGF and GDNF can enhance stimulation of neurite growth and will promote effective regeneration of peripheral nerves after injury. This assumption is supported by the fact that the combined effect of HGF and GDNF enhances differentiation of mesenchymal stromal cells in the bone marrow of the neuronal cells [6].

## MATERIALS AND METHODS

**Animals.** The experiments were carried out on neonatal 3-day-old C57Bl/6 mice ($n$=8). Dorsal ganglia were isolated under aseptic conditions using microsurgical sterile instruments. The animals were decapitated, the spine was released from the surrounding tissues on the dorsal side and opened, the spinal cord was removed, and spinal ganglia were isolated cutting off the nerve branches as much as possible. Surgical manipulations and euthanasia were developed in compliance with National and European Union Directives and were approved by the Ethics Committee of the National Medical Research Center for Cardiology (permit No. 385.06.2009).

**Culturing of dorsal root ganglion explants.** The explants were plated on 35-mm dishes precoated with poly-D-lysine (0.1 mg/ml; Sigma-Aldrich) and cultured in DMEM (Gibco) containing 4,5 g/liter glucose, 10% fetal calf serum (HyClone), and 1% solution of penicillin-streptomycin (Gibco) at 37°C and 5% $CO_2$ for 4 h until explant attachment to the substrate. Then, the medium was replaced with DMEM containing 4.5 g/liter glucose, 1% penicillin–streptomycin, and recombinant mouse GDNF (100 ng/ml; Abcam), HGF (100 ng/ml, R&D System) or both 100 ng/ml GDNF and 100 ng/ml HGF. Serum-free DMEM containing 1% penicillin-streptomycin served as the control. The explants were cultured at 37°C and 5% $CO_2$ for 48 h; then, morphology of dorsal root ganglion explants was evaluated under an inverted microscope AxioObserver A1 (Carl Zeiss).

**Quantitative analysis of neurite outgrowth from mouse dorsal root ganglion explants.** For assay of neurite outgrowth, the explants were fixed in 4% formaldehyde in PBS (10 min at room temperature) and washed with PBS. Explants were analyzed by phase-contrast microscopy using an inverted microscope AxioObserver A1.

Neurite outgrowth was evaluated by 2 parameters: neurite number and length. Neurite length was calculated as follows: 10-15 neurites for each dorsal root ganglion was analyzed and the average length for 5 longest neurites was calculated in ImageJ. For neurites with common origin, only one neurite was measured. Number of neurites were evaluated by [15].

**Culture of Neuro2A mouse neuroblastoma cells.** Neuro2A cells were cultured in DMEM with 4.5 g/liter glucose, 10% fetal calf serum, 1% non-essential amino acids (HyClone), and 1% penicillin-streptomycin at 37ºC and 5% $CO_2$. For subculturing, the cells were treated with Versene solution (PanEco) and 0.05% trypsin (HyClone). The effect of HGF and GDNF were assessed after cell incubation with the growth factors for different times. Active concentrations of HGF and GDNF were 25 and 50 ng/ml, respectively. After incubation with growth factors, the cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl; pH 8.0) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Thermo Scientific).

**Immunoblotting.** Cell lysate were homogenized by passing through insulin syringe with 29G needles and centrifuged at 20,000$g$ and 4°C for 30 min. Supernatants containing protein fractions were collected and Laemmli SDS-PAGE electrophoresis was carried out under denaturing conditions. Then, the proteins were transferred to polyvinylidene fluoride membrane; non-specific binding was blocked by incubation in 5% non-fat dry milk in Tris buffer with 0.1% Tween-20 (TBST) for 1 h at room temperature. Then, the membranes were stained with primary antibodies anti-pHGFR (Tyr1234) AP20840PU-N (Acris Antibodies); anti-pERK1/2 (Thr185/Thr202) #sc9101 (Cell Signaling); anti-vinculin ab18058 (Abcam) at 4°C for 12 h, washed 3 times in TBST, and incubated with second antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Protein bands were detected using chemiluminescent substrate Clarity ECL (Bio-Rad). The results were analyzed using a FusionX gel-documenting system (Vilber Lourmat) and GelAnalyzer2010a software.

The results were processed statistically using Microsoft Excel 2010 software. The data obtained from spinal ganglia were presented as the $M\pm SEM$. Significance of differences was analyzed using Mann—Whitney $U$ test; the differences were significant at $p<0.05$. The data obtained on cell lines are presented as $M\pm SD$. Significance of differences was analyzed using the Student's $t$ test; the differences were significant at $p<0.05$.

## RESULTS

Quantitative analysis of neurites growing from dorsal root ganglion explants showed statistically significant increase in the number of neurites in response to 48-h treatment with 100 ng/ml GDNF in comparison with the control (Fig. 1).

Recombinant HGF produced more pronounced effect on the length of neurites than GDNF (Fig. 1, *b, e*). Combined action of HGF and GDNF produced maximum effect on the number of neurites (Fig. 1, *d*, *e*), which indicated mutual potentiation of the stimulating effects of HGF and GDNF. At the same time, analysis of neurite length revealed no stimulating effect of GDNF, while HGF significantly increased this parameter; the effect of their combination was comparable with effect of HGF alone (Fig. 1, *f*). Thus, the combination of HGF and GDNF produced a stimu-

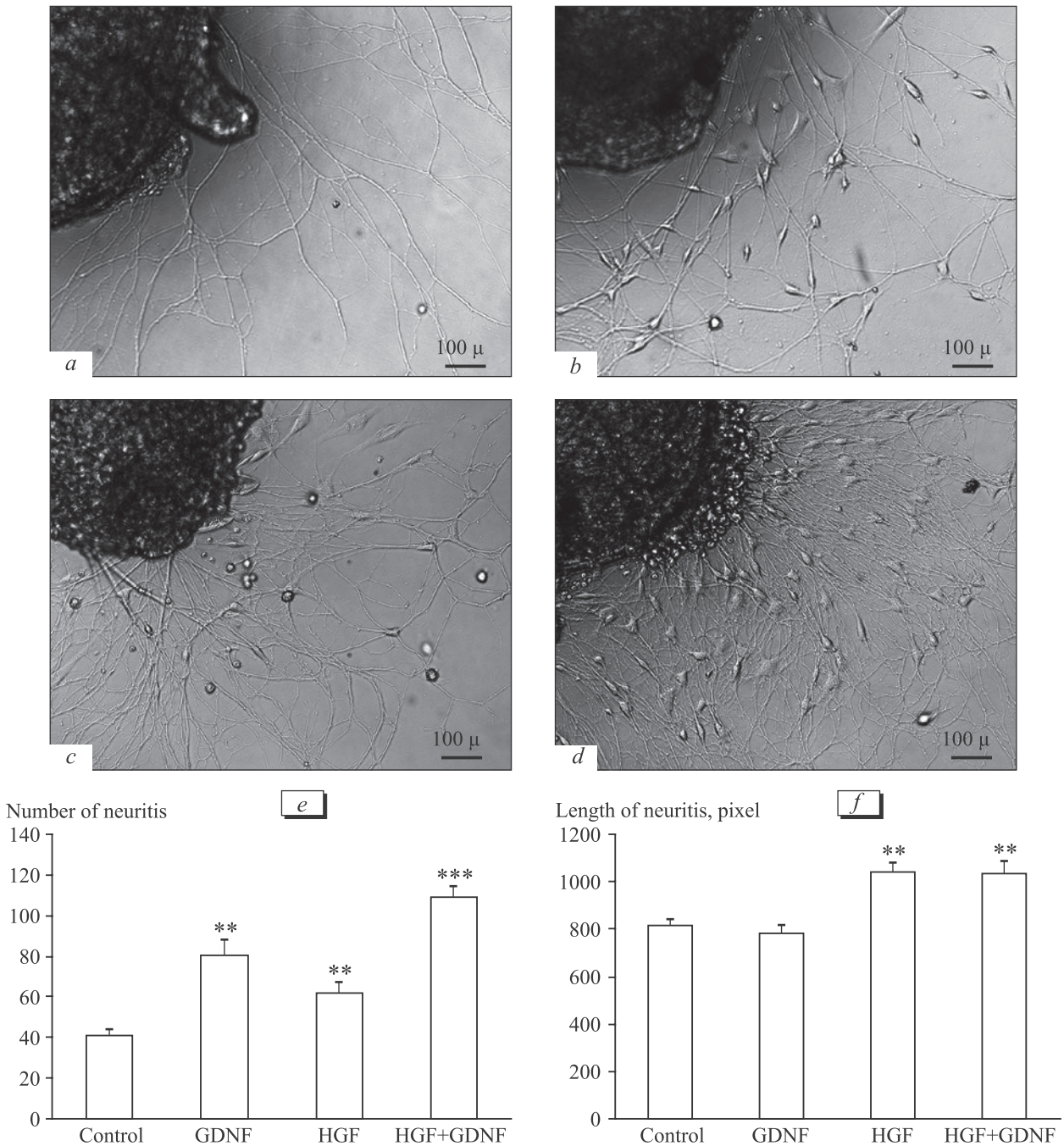


**Fig. 1.** HGF and GDNF combination increases the number and length of neurites. Representative images of dorsal root ganglia after 48-h culturing in a medium without growth factors (*a*) and in the presence of 100 ng/ml HGF (*b*), 100 ng/ml GDNF (*c*), and 100 ng/ml GDNF+ 100 ng/ml HGF (*d*). *e*) Number of neurites; *f*) length of neurites. *$p$<0.01, **$p$<0.001 in comparison with the control.

lating effect on both the number and length of neurites growing from spinal ganglia, which manifested in greater length of neurites attained at a certain time interval. Both factors complemented each other well and increased number and length of neurites and the rate of neurite growth.

Potentiation of the effect in combined application of HGF and GDNF was also shown for other processes. Combined application of HGF and GDNF produced more potent stimulating effect on neuronal differentiation in bone marrow MSC culture than individual application of these factors [6]. A possible molecular mechanism of HGF and GDNF cooperation is enhancement of intracellular mitogen-activated signaling. Receptor complex for GDNF includes specific receptor type 1α (GFRα1) and tyrosine kinase receptor Ret [12]. However, GDNF can activate intracellular signaling via GFRα1 independently of Ret [14]. On tubulogenesis model in Madin-Darby Canine Kidney cells that express GFRα1 and lack Ret, GDNF stimulated tubulogenesis and branching via phosphorylation of HGF receptor c-met [10]. Moreover, c-met activation was not caused by direct interaction of GDNF with c-met, but was mediated by Src kinase activation [12]. According to these data we hypothesized that potentiation of axonal growth from explants of dorsal root ganglia of neonatal mice in the presence of HGF and GDNF combination can also be a result of activation

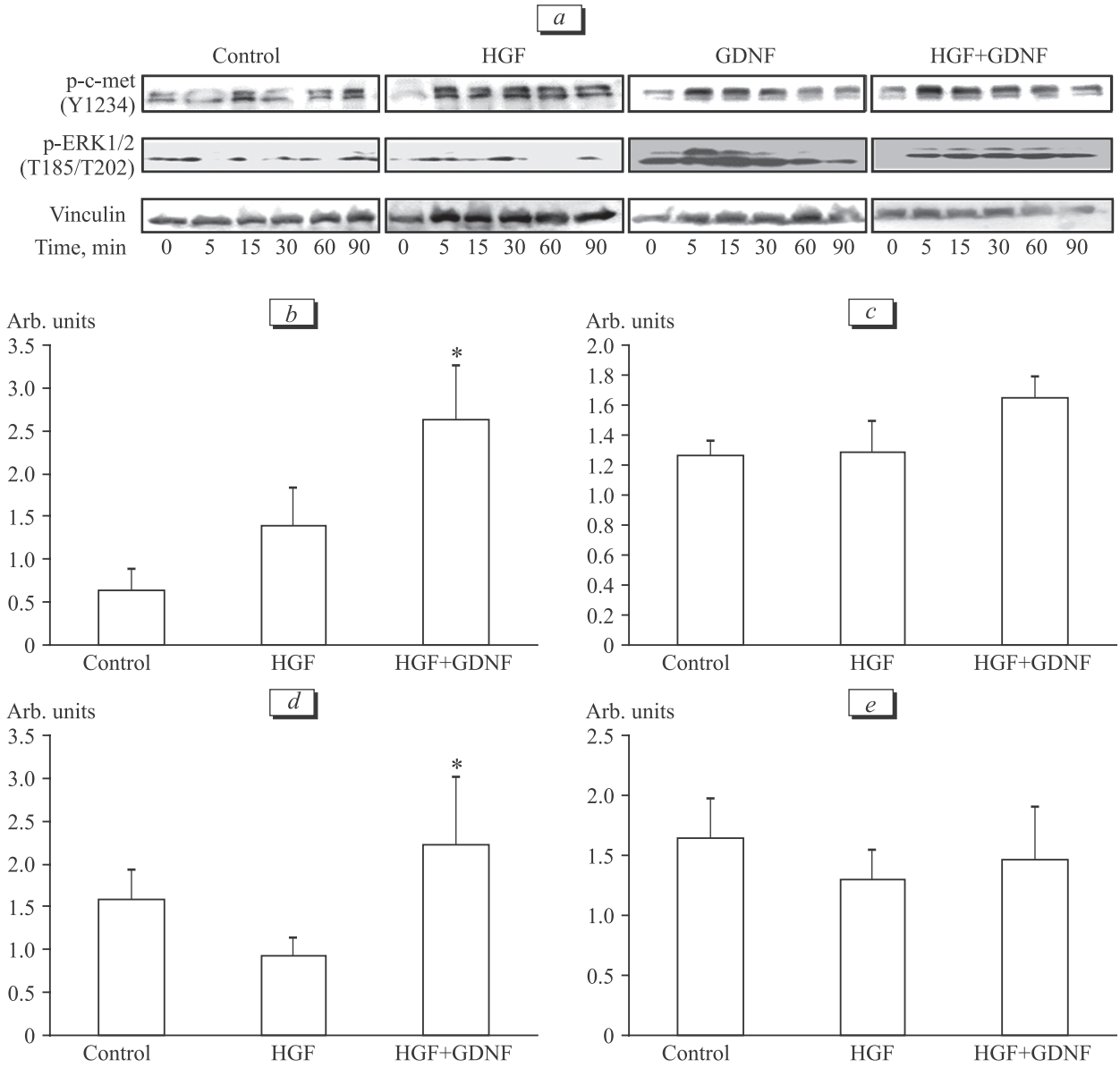


**Fig. 2.** HGF и GDNF combination activates receptor c-met and phosphorylation of ERK1/2. *a*) Representative images of immunoblots. Phosphorylation of p-c-met-Tyr1234 after incubation with growth factors for 5 (*b*) and 15 min (*c*). Phosphorylation of p-ERK1/2-Thr185/Thr202 after incubation with growth factors for 5 (*d*) and 15 min (*e*). *$p$=0.05 in comparison with HGF.

of c-met signaling. For verification of this assumption we analyzed the effect of HGF and GDNF on c-met phosphorylation and ERK1/2 activation in Neuro2A cells. Previously study has demonstrated that GDNF via interaction with GFRα1 stimulates neurites outgrowth through activation of ERK1/2, Rac1, and Cdc42 [14].

Our experiments showed that HGF activates c-met phosphorylation in Neuro2A cells; the effect peaked by the 5th min and lasted for 90 min (Fig. 2, *a*, *b*). At the same time, HGF did not activate ERK1/2 phosphorylation statistically significant, though densitometry of 3 representative immunoblots revealed a trend to increasing ERK1/2 phosphorylation after 5-min incubation followed by a return to baseline after 1 h. GDNF also stimulated phosphorylation of c-met in Neuro2A cells and stably activated ERK1/2 signaling (Fig. 2, *a*). Incubation of neuroblastoma cells with HGF and GDNF significantly enhanced c-met phosphorylation at Tyr1234 and ERK1/2 phosphorylation at Tyr185/Tyr202 (Fig. 2, *b*, *d*) in comparison with their individual effects with a maximum on the 5th min of incubation followed by return to baseline in 90 min, which demonstrated cooperative action of HGF and GDNF on ERK1/2-dependent signaling.

The results confirmed our assumption that more potent effect of HGF and GDNF combination in comparison with individual factors can be related to c-met transactivation with active GDNF-GFRα1 complex. Addition of isolated growth factors induces interaction of GDNF-GFRα1 complex with RET for GDNF signaling and interaction of HGF with c-met for HGF signaling. However, the mechanism of c-met transactivation with GDNF-GFRα1 complex via Src kinase was previously demonstrated in epithelial cells of the kidney [10], which indicates the possibility of potentiation of HGF and GDNF effects, including stimulation of neurite growth, through activation of HGF signaling, GDNF signaling, and Src signaling. All these signaling pathways promote activation of ERK-kinase that have a positive effect on axonal and neurite growth [13]. Moreover, previous studies showed the interaction between c-met and tyrosine kinase receptor of neurotrophine BDNF that promoted phosphorylation of β-catenin, growth and branching of axons [3]. We can hypothesize that activation of HGF signaling enhanced due to c-met transactivation through GDNF-GFRα1 complex contributes to neurite outgrowth via β-catenin signaling pathway. This hypothesis and the possibility of improving the efficacy of recovery of peripheral innervation through combined hyperexpression of HGF and GDNF will be tested in further experiments on the model of traumatic and ischemic injury to the peripheral nerve.

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## REFERENCES

1. Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK. GDNF, NGF and BDNF as therapeutic options for neurodegeneration. Pharmacol. Ther. 2013;138(2):155-175.
2. Boldyreva MA, Bondar IV, Stafeev IS, Makarevich PI, Beloglazova IB, Zubkova ES, Shevchenko EK, Molokotina YD, Karagyaur MN, Ratner EI, Parfyonova YV. Plasmid-based gene therapy with hepatocyte growth factor stimulates peripheral nerve regeneration after traumatic injury. Biomed. Pharmacother. 2018;101:682-690.
3. David MD, Yeramian A, Duñach M, Llovera M, Cantí C, de Herreros AG, Comella JX, Herreros J. Signalling by neurotrophins and hepatocyte growth factor regulates axon morphogenesis by differential beta-catenin phosphorylation. J. Cell Sci. 2008;121(Pt 16):2718-2730.
4. Deumens R, Bozkurt A, Meek MF, Marcus MA, Joosten EA, Weis J, Brook GA. Repairing injured peripheral nerves: bridging the gap. Prog. Neurobiol. 2010;92(3):245-276.
5. Lee SK, Wolfe SW. Peripheral nerve injury and repair. J. Am. Acad. Orthop. Surg. 1999;8(4):243-252. doi: 10.1097/00006534-198804000-00086.
6. Ma Q, Cai M, Shang JW, Yang J, Gu XY, Liu WB, Yang Q. In vitro neural differentiation of bone marrow stromal cells induced by hepatocyte growth factor and glial cell derived neurotrophic factor. Eur. Rev. Med. Pharmacol. Sci. 2016;20(22):4654-4663.
7. Makarevich PI, Dergilev KV, Tsokolaeva ZI, Boldyreva MA, Shevchenko EK, Gluhanyuk EV, Gallinger JO, Menshikov MY, Parfyonova YV. Angiogenic and pleiotropic effects of VEGF165 and HGF combined gene therapy in a rat model of myocardial infarction. PLoS One. 2018;13(5). ID e0197566. doi: 10.1371/journal.pone.0197566.
8. Nakamura T, Sakai K, Nakamura T, Matsumoto K. Hepatocyte growth factor twenty years on: Much more than a growth factor. J. Gastroenterol. Hepatol. 2011;26(Suppl. 1):188-202.
9. Navarro X, Vivó M, Valero-Cabré A. Neural plasticity after peripheral nerve injury and regeneration. Prog. Neurobiol. 2007;82(4):163-201.
10. Popsueva A, Poteryaev D, Arighi E, Meng X, Angers-Loustau A, Kaplan D, Saarma M, Sariola H. GDNF promotes tubulogenesis of GFRα1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. J. Cell Biol. 2003;161(1):119-129.
11. Reyes O, Sosa I, Kuffler DP. Promoting neurological recovery following a traumatic peripheral nerve injury. P. R. Health Sci. J. 2005;24(3):215-223.
12. Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. J Cell Sci. 2003;116(Pt 19):3855-3862.
13. Wang X, Wang Z, Yao Y, Li J, Zhang X, Li C, Cheng Y, Ding G, Liu L, Ding Z. Essential role of ERK activation in neurite outgrowth induced by α-lipoic acid. Biochim. Biophys. Acta. 2011;1813(5):827-838.
14. Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed. MOJ Orthop. Rheumatol. 2014;1(3). ID 00019. doi: 10.15406/mojor.2014.01.00019.
15. Zhang W, Li Z. The effects of target skeletal muscle cells on dorsal root ganglion neuronal outgrowth and migration in vitro. PLoS One. 2013;8(1). ID e52849. doi: 10.1371/journal.pone.0052849.