

Neural Stem Cells and Induced Neurons for Nerve Injury Repair

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Abstract

Cell transplantation can relieve the symptoms of or even reverse neurodegenerative diseases and repair nerve injuries. Fibroblast growth factor 1 promotes neuronal survival and stimulates axonal growth. A combination of fibroblast growth factor 1 and cell-based therapy is promising for nerve repair. Developers of future cell-based treatment should consider several key concerns: (1) the source of cells should be autologous, (2) consistent methods and protocols for cell isolation should be used, (3) the treatment should be tested in suitable animal models, and (4) the microenvironment of cells implanted should be optimally characterized. In addition, developing high temporal and spatial resolution images for cell tracking is crucial for evaluating the efficacy of cell transplantation. In this paper, we summarize recent progress in cellular reprogramming, such as induced neural stem cells and induced neurons, and the development of future cell-based therapy for peripheral nerve and spinal cord injury that includes conduits and growth factors.

Keywords: Cell therapy; Neural stem cells; FGF1; Induced neurons; Peripheral nerve injury

Fibroblast Growth Factor 1 for Nerve Injury Repair

In total, 22 mammalian fibroblast growth factors (FGFs) exist, grouped into seven subfamilies on the basis of differences in sequence homology and phylogeny. Notably, FGF1 and FGF2 share sequential and structural similarities and belong to the FGF1 subfamily [1]. The FGF ligands execute diverse functions by binding and activating the FGF receptor (FGFR) family of tyrosine kinase receptors with heparan sulfate proteoglycans. In total, four FGFR genes (FGFR1–FGFR4) encode receptors consisting of three extracellular immunoglobulin domains (D1–D3), a single-pass transmembrane domain, and a cytoplasmic tyrosine kinase domain [2]. Several FGFR isoforms exist because of exon skipping that removes the D1 domain and/or the acid box in FGFR1–FGFR4 [3]. Alternative splicing in the second half of the D3 domain of FGFR1–FGFR3 yields b (FGFR1b–FGFR3b) and c (FGFR1c–FGFR3c) isoforms that have distinct FGF-binding specificities [4] and are predominantly present in epithelial and mesenchymal cells, respectively. Each FGF binds to epithelial or mesenchymal FGFRs, with the exception of FGF1, which activates both spliced isoforms [3].

The involvement of FGF signaling in human diseases has been thoroughly documented. Deregulated FGF signaling can contribute to pathological conditions through gain- or loss-of-function mutations in the FGFRs. Because both *Fgf1*^{-/-} and *Fgf1*^{-/-}*Fgf2*^{-/-} mice are fertile and apparently normal [5], the physiological roles of FGF1 and FGF2 remain to be explored. However, FGF1 and FGF2 likely play a physiological role in the maintenance of the vascular tone because FGF1 and FGF2 administration lowers the blood pressure in rats [6] and can restore the nitric oxide synthase activity in spontaneously hypertensive rats [7]. In addition, blood vessels isolated from *Fgf2*^{-/-} mice exhibit a reduced response to

vasoconstrictors. Although *Fgf2*^{-/-} mice had hypotension caused by reduced smooth muscle contractility [8], their blood pressure could still be regulated [9]. Interestingly, FGF1 has been shown to be crucial in the remodeling process of adipose tissues [10]. Fibroblast growth factor 1 is induced in the gonadal white adipose tissue of a high-fat-diet-fed animal model [10]. Furthermore, *Fgf1*^{-/-} knockout mice developed a phenotype of profound diabetes when fed with a high-fat diet [10], suggesting the importance of FGF1, particularly when challenged with various nutritional conditions. A similar situation was also observed in collagen VI null mice that exhibited metabolic dysregulation and adipose tissue fibrosis only when fed with a high-fat diet [11,12].

The therapeutic potential of FGF1 has been demonstrated for cardiovascular disorders. Phase I trials have revealed that the intramyocardial injection of FGF1 during coronary artery bypass graft surgery improves collateral artery growth and capillary proliferation [13]. The beneficial effects of FGF1 on the peripheral circulation have also been shown. Injecting a plasmid encoding FGF1 into the leg improved the perfusion of end-stage lower-extremity ischemia in a Phase I trial [14] and reduced amputations in patients with critical limb ischemia by 2-fold in a recent Phase II study [15]. Interestingly, the distal blood and oxygen pressure were similar after the injection of FGF1 plasmid or a placebo [16], and the mechanism of FGF1 action might not have been primarily angiogenic.

FGF1, which can repair nerve injuries, enabled the functional regeneration of transected spinal cords in rats [17] and restored some motor functions in the paralyzed limbs of six months old boy with brachial plexus avulsion [18]. In addition, it benefited patients with chronic transverse myelitis [19]. Administering FGF1 and a combination of nerve grafts with FGF1 treatment partly restored ambulation in a paraplegic [20]. A combination of peripheral nerve grafts and FGF1 restored hind limb locomotor function in spinal cord-transected rats [17,21,22]. The expression of arginase I, the macrophage M2 marker, and the recruitment of M2 macrophages were observed in the repaired site [23]. Furthermore, FGF1 and nerve grafts induced IL-4 and NGF/BDNF expression in the repaired site, respectively. Therefore, developers of an ideal repair strategy should consider the beneficial effects of both FGF1 and nerve grafts [23]. We have previously demonstrated that a combination of FGF1, neural stem cells (NSCs), and micropatterned poly(D,L-lactide) conduits facilitated nerve repair and functional recovery in rats [24,25], and we are continually analyzing the efficacy of combinations of various growth factors and adult NSCs in peripheral nerve regeneration [26].

Neural Stem Cells for Peripheral Nerve Injury Repair

Sources of neural stem cells for clinical applications

Cell transplantation is expected to relieve symptoms of or even

reverse the progression of various neural diseases. The efficacy of mesenchymal, embryonic, or brain stem cells was evaluated in various animal models, such as those of Parkinson, Huntington, and Alzheimer disease, as well as in those with multiple sclerosis and cerebral ischemia [27]. In addition to involving direct cell transplantation, ideal therapeutic approaches should stimulate endogenous stem cells and induce the expression of active molecules in situ simultaneously. Several key concerns should be considered: (1) the source of cells should be autologous, (2) consistent methods and protocols for cell isolation should be used, (3) the approaches should be tested in suitable animal models, and (4) the microenvironment of cells implanted should be optimally characterized. In addition, developing high temporal and spatial resolution images for cell tracking is crucial for evaluating the efficacy of cell transplantation [26].

Patient-derived cells are ideal NSC sources for autologous cell transplantation because they can prevent immune rejection. Potential cell sources for treating neural diseases include brain tissue-derived NSCs [28,29], blood- or bone marrow-derived mesenchymal stem cells [30-32], skin- or blood-derived induced pluripotent stem cells (iPSCs) [33-36], skin- or urine-derived induced neural stem cells (iNSCs) [37-43], and skin-derived induced neurons (iNs) [44-53].

Isolation and characterization of neural stem cells

Flow cytometry and fluorescence-activated cell sorting have been applied extensively in stem cell biology, such as in stem and progenitor cell isolation from the hematopoietic and nervous systems. Neural stem cells have been isolated from brain tissues according to the NSC-specific cell surface marker CD133 and NSC-specific genes, such as Sox1, Sox2, nestin, and FGF1 [54,55]. Thus, NSCs can be isolated using such approaches and then cultured to form neurospheres, which are indicators of self-renewal. For determining the multipotency, EGF and FGF2 were withdrawn from the culture, or other inducing factors were added [56].

The human *FGF1* gene was first cloned in our laboratory [57]. Fibroblast growth factor 1 is expressed in neurons in various regions, including the ventral cochlear nucleus, olfactory bulbs, and hippocampus [58]. The *FGF-1B* promoter is brain-specific [59,60]. Interestingly, *FGF-1B* mRNA is elevated in the hippocampal neurogenic region for supporting NSCs during exercise-induced neurogenesis [61]. Furthermore, the *FGF-1B* promoter-driven GFP reporter (F1B-GFP) (USA patent No. 6984 518; 7045 678; and 7745 214) was used to isolate NSCs from human [55] and mouse brains [54,55]. The F1B-GFP-selected NSCs exhibited significant repair efficacy in the damaged sciatic nerves of paraplegic rats. A combination of nerve conduits, NSCs, and FGF1 repaired peripheral nerve injuries in animals [25,62]. Our laboratory has also demonstrated applying ultrananocrystalline diamond, a novel material, as a biomaterial for NSC transplantation in peripheral nerve injuries [63,64]. Recently, we demonstrated that F1B-GFP-selected NSCs with nerve conduits significantly improved the functional recovery of sciatic nerve injuries in mice (Figure 1) through the secretion of a cytokine (unpublished data). As a proof of concept, the direct combination of this cytokine with F1B-GFP NSCs in nerve conduits improved motor function recovery, promoted nerve regeneration, and increased the diameter of newly regenerated nerves by as much as 4.5-fold. Our data suggested a reduced likelihood of the administration of an immune factor in clinical settings for sciatic nerve injury repair.

Cellular Reprogramming for Induced Neurons and Induced Neural Stem Cells

Promising cellular sources for regenerative medicine should be

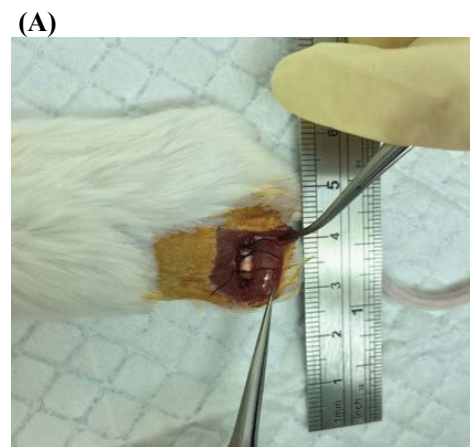


Figure 1: Combination of neural stem cells and nerve conduit in a mouse model of sciatic nerve injury.

(A) Before nerve transection, the sciatic nerve was fixed on the gluteus maximus by using 6-0 nylon microsutures, with a 5 mm distance between the proximal and distal fixed sites. A 3 mm nerve segment was transected in the medial site. A 5 mm conduit with or without neural stem cells (NSCs) and/or growth factors was interposed into the 3 mm nerve defect. The proximal and distal ends of the severed nerve were anchored to the conduit with 1 mm of residual nerve. The proximal and distal ends of the implanted conduit were tied using 6-0 nylon microsutures. After nerve conduit implantation, the muscle was sutured using 6-0 insoluble microsutures, and the skin was sutured using 6-0 nylon microsutures. (B) To investigate whether NSCs could facilitate the repair process after sciatic nerve injury, we injected 20 μ L of PBS with or without suspended NSCs into the adjacent muscle of the transected sciatic nerve. The sciatic nerve was one-cut severed in the medial site, without damaging the epineurium and connective tissue, which connected to the muscle. (C) Three weeks after the nerve injury, the NSC-injected mouse could extend the toes, which indicated functional recovery after the nerve injury. Compared with the NSC-injected mouse, the PBS-injected mouse failed to extend the toes, indicating a dysfunction of the sciatic nerve after transection.

personalized. Ideal sources are the somatic cells of patients, such as skin fibroblasts or peripheral blood cells. These personalized sources were reprogrammed into iPSCs [65]. Although the generation of iPSCs by patients with amyotrophic lateral sclerosis demonstrates the accessibility of patient-derived iPSCs [66], iPSCs have also been shown to have sizeable genetic and epigenetic abnormalities [67]. A recent direct reprogramming approach provides a straightforward, rapid, and reliable platform for producing various functional cells. Reports on the cellular reprogramming of myocytes [68], macrophages [69], cardiomyocytes [70], and hepatocytes [71] are summarized in Table 1. For example, a combination of *miRNA124*, *Brn2*, and *Myt11* has been used to reprogram human fibroblasts into functional neurons [49,72]. Notably, these functional cells expressed various mature neuronal markers and could fire action

potentials. Recent reports on the cellular reprogramming of neurons are summarized in Table 2. Recently, we showed that a signaling adaptor protein, SH2B1, enhanced the neurite outgrowth of iNs. These enhanced iNs expressed mature neuronal markers, such as NeuN, synapsin, GABA, vGluT2, and tyrosine hydroxylase. Notably, these SH2B1-enhanced iNs exhibited accelerated reprogramming (Figure 2) [48]. Our results will facilitate applying iNs in the disease modeling and treatment of neural diseases. Most recently, NSC-specific transcription factors, particularly SOX2, have been reported to reprogram mouse and human fibroblasts into multipotent iNSCs with a self-renewing ability [37-41]. Future studies are required for adapting this iNSC protocol and eventually substituting viral NSC factors using nonintegrating delivery modes such as Sendai viruses [73,74] or small molecules. We anticipate that iNSCs can provide

Authors	Davis et al., 1987	Feng et al., 2008	Ieda et al., 2010	Huang et al., 2011
Original cell type	Human and mouse fibroblast cells	Mouse fibroblast cells	Mouse fibroblast cells	Mouse fibroblast cells
Transduction Factors	MyoD	C/EBPa, PU.1	Gata4, Tbx5, Mef2C	Gata4, HNF1a, HNF4a, Foxa1, Foxa3
Cell type	Myocytes	Macrophages	Cardiomyocytes	Hepatocytes
References	67	68	69	70

Table 1: Cellular reprogramming of induced myocytes, macrophages, cardiomyocytes, and hepatocytes.

Authors	Ambasudhan et al., 2011	Pan et al., 2011	Yoo et al., 2011	Pfisterer et al., 2011	Caiazzo et al., 2011	Falk et al., 2012	Hsu et al., 2014
Original cell type	Human primary fibroblast cells, including BJ and foreskin dermal fibroblast cells	Human embryonic and postnatal fibroblasts	Human neonatal and adult dermal fibroblast cells	Human embryonic fibroblasts	Human fetal and adult and Parkinson's disease adult fibroblasts	Human adult fibroblasts	Human fetal fibroblasts
Transduction Factors	miR-124, Brn2, Myt11	Ascl1, Brn2, Myt11, NeuroD1	miR-9/9*, miR-124, NeuroD2, Ascl1, Brn2, Myt11	BAM, Lmx1a, FoxA2	Brn2, Myt11, Lhx3, Hb9, Isl1, Ngn2, NeuroD1	Ascl1, Ngn2	SH2B1, miR-124, Brn2, Myt11
Efficiency	Postnatal: 4-8% of plated cells Adult: 1.5-2.9% of plated cells	2-4% of the plated cells	10% of the plated cells	10% of plated cells	3% of infected cells	13.2% of infected cells	20-30% of the plated cells
References	46	48	49	50	51	52	47

Table 2: Cellular reprogramming of human induced neurons (iNs).

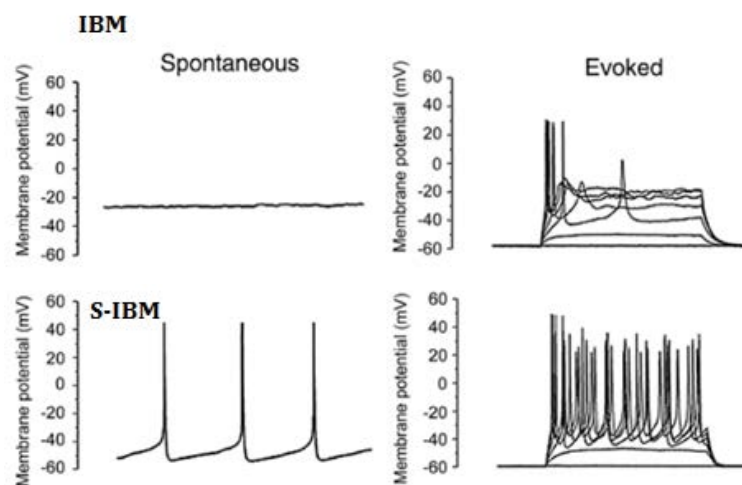


Figure 2: Human foreskin fibroblasts were infected with 3 factors (IBM; upper panels) or 3 factors plus SH2B1 (S-IBM; lower panels). Traces of spontaneous (left panels) and evoked (right panels) action potentials were recorded in the current-clamp configuration. To measure evoked action potential firing in the current clamp, membrane potentials were held at 260 mV. Membrane potential in response to five current steps (in 10-pA increments) from the holding membrane potential was recorded. To measure evoked firing, cells were recorded at 260 mV, and then, a +50-pA current was injected to elicit spikes. To measure spontaneous firing, the cell membrane potential was held at 250 or 260 mV without current stimulation. Data was collected from three independent experiments at day 14 after cellular reprogramming. *Abbreviations:* IBM, microRNA miR124 and transcription factors BRN2 and MYT11; SH2B1, SH2B adaptor protein 1 β ; S-IBM, SH2B1-enhanced IBM iNs

Author	Ring et al., 2012	Han et al., 2012	Corti et al., 2012	Wang et al., 2013	Zou et al., 2014	Castañó et al., 2014	Wang et al., 2013
Original cell type	Human foreskin fibroblast cells	Mouse fibroblast cells	Human cortical astrocytes	Human urine epithelial-like cells	Human fetal fibroblasts	Human CD133(+) Peripheral blood cells	Human CD34(+) peripheral blood cells
Transduction Factors	SOX2	Brn4, Sox2, Klf4, c-Myc, E47	OCT4, SOX2, ANOG	OCT4, SOX2, SV40LT, KLF4 and microRNA cluster MIR302-367	SOX2, c-MYC, BRN2 or BRN4	SOX2, c-MYC	OCT4, SOX2, c-MYC, KLF4
Induction Days	> 60	28-35	14-21	25	11	15	7
References	39	40	41	42	43	73	74

Table 3: Cellular reprogramming of human induced neural stem cells (iNSCs).

a safe and robust cellular platform for generating patient-specific neural cells for nerve injury repair. Recent reports on iNSCs are summarized in Table 3.

Future Prospects

Combination of biomaterials, growth factors, induced neural stem cells/induced neurons for peripheral nerve injury repair, natural polymers, including gelatin, collagen, chitosan, or chitin, and synthetic biodegradable polymers, such as poly(D,L-lactide) or poly(D,L-lactic-coglycolic acid), are commonly used for nerve regeneration because of their neurocompatible properties [75]. However, nondegradable materials should not be used for long-term neural repair to prevent nerve damage and chronic inflammation. Therefore, biodegradable materials are more acceptable for neural repair [76]. When used as the material for nerve conduits [77], poly(glycerol sebacate) exhibited surface-erodible and elastomeric properties. Notably, neurotrophic factors were linked to nerve conduits by using new techniques such as electrospinning or polymer blending [78], thus increasing the neural compatibility of the conduit surfaces [79].

Growth factors in situ play numerous crucial roles in regulating local neural and nonneural cells after an injury. Although endogenous growth factors secreted by neural cells in the distal nerve stump can support axonal regeneration, the supportive action may not be sustained indefinitely because of a decline in the cellular production of growth factors with time; hence, a continuous supply of growth factors is essential, mainly through the addition of exogenous growth factors. During tissue remodeling, growth factors can initiate signaling pathways involved in repair. In addition to stimulating endogenous neural stem/progenitor cells, some growth factors are also critical for their differentiation into various neural cells [80]. Nerve conduits used for nerve repair also provide a channel for growth factor diffusion [81]. A combination of liposomes and neurotrophic factor genes used in another approach effectively facilitated nerve injury repair [82,83]. Furthermore, the release of acidic products when poly(D,L-lactic-coglycolic acid) is used as microspheres for carrying growth factors may result in protein inactivation [84]. To control the delivery of growth factors, an electrospun nanofibrous scaffold is the ideal delivery vehicle because it can serve as a scaffold and provides optimal contact guidance [85].

Possible application in spinal cord and traumatic brain injury

To enhance the outcome of peripheral nerve regeneration by using scaffolds alone, efforts have been focused on optimally incorporating biochemical cues, including supporting cells, growth factors, and/or cytokines, within biomaterials. A combination of FGF1, biomaterials, and iNSCs/iNs has promising clinical applications. Notably, peripheral nerve grafts and FGF1 have not only repaired the hind limb of adult paraplegic rats [17,21] but also

exerted therapeutic effects on patients with spinal cord injury (SCI) and common peroneal nerve lesions [86,87]. Thus, direct FGF1 treatment may benefit patients. Notably, Chen et al. developed an FGF1-based SCI repair strategy and designed a clinical trial to test the efficacy and safety of using FGF1 in combination with surgical intervention in human SCI. Through the clinical trial, they demonstrated that using FGF1 is safe and feasible for treating SCI [87]. Significant improvements were observed in American Spinal Injury Association motor and sensory scale scores, ASIA impairment scales, neurological levels, and the functional independence measure 24 mo after treatment [87]. Accumulating evidence suggests that FGF1 can be used to treat SCI and peripheral nerve injury. Future efforts for generating FGF1-expressing iNSCs or iNs can further the potential treatment of peripheral nerve injury and central nervous system diseases, such as SCI and traumatic brain injury.

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