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Transdifferentiation of brain-derived neurotrophic factor (BDNF)-secreting mesenchymal stem cells significantly enhance BDNF secretion and Schwann cell marker proteins

Metzere Bierlein De la Rosa,[1] Anup D. Sharma,[2,4] Surya K. Mallapragada,[2,4] and Donald S. Sakaguchi[1,3,4,*]

Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA,[1] Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA,[2] Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA,[3] and Neuroscience Program, Iowa State University, Ames, IA 50011, USA[4]

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The use of genetically modified mesenchymal stem cells (MSCs) is a rapidly growing area of research targeting delivery of therapeutic factors for neuro-repair. Cells can be programmed to hypersecrete various growth/trophic factors such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) to promote regenerative neurite outgrowth. In addition to genetic modifications, MSCs can be subjected to transdifferentiation protocols to generate neural cell types to physically and biologically support nerve regeneration. In this study, we have taken a novel approach by combining these two unique strategies and evaluated the impact of transdifferentiating genetically modified MSCs into a Schwann cell-like phenotype. After 8 days in transdifferentiation media, approximately 30–50% of transdifferentiated BDNF-secreting cells immunolabeled for Schwann cell markers such as S100β, S100, and $p75^{NTR}$. An enhancement was observed 20 days after inducing transdifferentiation with minimal decreases in expression levels. BDNF production was quantified by ELISA, and its biological activity tested via the PC12-TrkB cell assay. Importantly, the bioactivity of secreted BDNF was verified by the increased neurite outgrowth of PC12-TrkB cells. These findings demonstrate that not only is BDNF actively secreted by the transdifferentiated BDNF-MSCs, but also that it has the capacity to promote neurite sprouting and regeneration. Given the fact that BDNF production remained stable for over 20 days, we believe that these cells have the capacity to produce sustainable, effective, BDNF concentrations over prolonged time periods and should be tested within an *in vivo* system for future experiments.

[**Key words:** Mesenchymal stem cells; Schwann cells; Brain-derived neurotrophic factor; Peripheral nerve regeneration; Neuroprotection; Neuroregeneration; High content screening; Morphometric analysis; Neurite outgrowth; Cellular area]

Peripheral nerve injuries occur as the result of sudden trauma and can lead to loss of sensory and motor function to peripheral limbs (1). Many surgical procedures are available to halt the propagation of nerve damage, and the adoption of a procedure depends on the extent of the injury. Epineural sutures are considered the standard of care in the case of transection injuries (2). Another surgical procedure, autologous nerve grafting, is widely used in cases of gap formation (2–4). Although these surgical procedures provide many benefits, there are still considerable limitations associated with them such as donor site morbidity, neuroma formation, fascicle mismatch, and scarring (5). To overcome such restrictions, researchers have explored various avenues to improve post-surgical outcomes (6–11). The most commonly studied methods include: cell transplantation, delivery of growth factors which stimulate regenerating axons and implanting nerve regeneration conduits containing replacement cells at the site of injury (7,12–14). Replacement cells which offer maximum benefits for the treatment of peripheral nerve injuries are Schwann cells (SCs), which are peripheral glial cells responsible for clearing out debris from the site of injury. Additionally, they release growth factors to stimulate myelination and axonal regeneration (15,16). Both primary SCs (17) and genetically modified SCs (18) enhance nerve regeneration in animal models; however, there is no good source for extracting SCs and the only method to obtain SCs is by sacrificing a healthy nerve. To overcome such challenges, various cell types including embryonic stem cells (19), umbilical cord-derived stem cells (20), bone marrow-derived mesenchymal stem cells (MSCs) (21), adipose-derived stem cells (22), olfactory ensheathing cells (23), and dental pulp-derived stem cells (24) have been used as an alternative to lost native SCs, and each has reported enhanced nerve regeneration. Mesenchymal stem cells, in particular, are preferred due to benefits such as autologous transplantation, routine isolation procedures, and paracrine and immunomodulatory properties (17–19). Mesenchymal stem cells have been transplanted at the site of injury either directly in their native form (undifferentiated) or in an SC-like form (transdifferentiated) and have been shown to significantly enhance nerve regeneration. In addition to transdifferentiated MSCs, some studies have also transplanted *ex-vivo* genetically modified MSCs that hypersecrete growth factors to improve neuroregeneration (25).

For this study, we chose to focus on BDNF delivery because it has been shown to provide neuroprotection and facilitate the rescue

* Corresponding author at: 505 Science Hall II, Iowa State University, Ames, IA 50011, USA. Tel.: +1 515 294 3112; fax: +1 515 294 8457.
E-mail address: dssakagu@iastate.edu (D.S. Sakaguchi).

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and repair of damaged neurons (26–29). BDNF is responsible for neurogenesis and helps with survival and growth of various types of neurons such as dorsal root ganglion neurons and cortical neurons. It is also widely explored as a therapeutic agent to target various neurodegenerative conditions (30,31). Previously, we were able to generate a stable brain-derived neurotrophic factor (BDNF) hypersecreting MSC line (32), which continued to secrete BDNF across multiple passages. Thus, our BDNF MSC line can be used as a large-scale source of autologous cell transplantation. Additionally, we successfully transdifferentiated MSCs into an SC-like phenotype on micropatterned substrates (34). For this study, we planned to combine the two separate strategies of transdifferentiation and growth-factor hypersecretion into one cell line. Using these two techniques together provides us with a large pool of SC like cells, which not only provide growth factor therapy but may also provide distinct advantages shown by transdifferentiated MSCs, such as enhanced neurotrophic factor secretion, enhanced neurite outgrowth (33), and myelination (34) as shown by various studies.

To the best of our knowledge, our group is the first to study transdifferentiation of genetically engineered BDNF hypersecreting MSCs, in order to create a large pool of SC-like cells hypersecreting a neuroprotective growth factor. These transdifferentiated MSCs showed various SC-like characteristics such as bipolar spindle-shaped morphology, expression of SC marker proteins (S100, S100β, and $p75^{NTR}$) and increased release of BDNF. In this study, we successfully transdifferentiated BDNF-hypersecreting MSCs into an SC-like phenotype and quantified their morphological, molecular, and functional changes.

MATERIALS AND METHODS

Mesenchymal stem cell isolation and culture Mesenchymal stem cells (MSCs) isolated from adult mice were obtained from the Texas A&M Health Science Center College of Medicine, Institute for Regenerative Medicine. MSCs were maintained as an adherent cell line in Iscove's Modified Dulbecco's Medium (IMDM; 12440-053; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; SH30071.03; Hyclone, South Logan, UT), 10% donor equine serum (SH30074; Hyclone), 2 mM L-glutamine (25030-081; Invitrogen), and antibiotic-antimycotic solution (1%, 15240-096; Invitrogen; 10,000 U mL^{-1} penicillin, 10,000 μg mL^{-1} streptomycin, 25 ng mL^{-1} amphotericin B). Cells were incubated at 37°C with 5% CO_2 humidified atmosphere. When cultures reached 75% confluence, MSCs were harvested from the flask using 0.25% trypsin and 1 mM EDTA solution (25200-056; Invitrogen) and were centrifuged at 500 rpm for 5 min. MSCs were subsequently plated into T-25 culture flasks (25 cm^2) at 75–150 cells cm^{-2}. Fresh media was added every other day to feed the cells.

Lentiviral vectors were used to engineer MSCs to produce and secrete brain-derived neurotrophic factor (BDNF; human cDNA) as well as green fluorescent protein (BDNF-GFP-MSCs), as previously reported (8,35). A similar method was utilized to generate the GFP-expressing mouse MSC line (GFP-MSCs). These cells were obtained from a previous study (32). Fig. 1 shows the schematic of the experimental design and the differences observed after study.

***In vitro* transdifferentiation of MSCs into SC-like cells** Sub-confluent MSCs were subjected to a three-step chemical transdifferentiation following a previously established induction protocol (36) (modified from Dezawa et al. (37)). There are many well-established protocols for the transdifferentiation of mesenchymal stem cells including transdifferentiation via direct transplantation (38,39), co-culture with dorsal root ganglion cells (40) or olfactory ensheathing cells (41), conversion via overexpression of master control genes (42), and even electrical transdifferentiation (43). However, these methods may require prolonged periods of time, produce a low yield of transdifferentiated MSCs (tMSCs), and may require viral expression of exogenous transcription factors, making these methods impractical within a clinical setting. Using the method described below by Dezawa et al., over a 50% conversation rate of undifferentiated MSCs (uMSCs) to tMSCs can be achieved in as little as 12 days (36,44,45). This brief timeline gives our particular transdifferentiation protocol an advantage over some of the newer, more labor-intensive protocols, which may take double the amount of time (46). Studies indicate that ideal time for neural stem cell transplant is approximately one week after nerve injury (47), which would make our protocol a more clinically feasible option.

First, for 24 h, cells were placed in Transdifferentiation Media 1 (TDM1) that consisted of IMDM and 1 mM β-mercaptoethanol (BME; M6250; Sigma–Aldrich, St. Louis, MO, USA). Subsequently, for 72 h, cells were placed in TDM2 that consisted of IMDM, 5% FBS, 5% equine serum, and 35 ng mL^{-1} all-trans retinoic acid (ATRA; R2625; Sigma–Aldrich). Finally, cells were placed in TDM3 for 8–20 days. TDM-3 consisted of IMDM, 5% FBS, 5% equine serum, 14 μM forskolin (FSK, 344270; EMD Millipore, Billerica, MA, USA), 5 ng mL^{-1} platelet-derived growth factor (PDGF; H8291-10UG; Sigma–Aldrich), 10 ng mL^{-1} basic fibroblast growth factor (rhFGF, basic; G5071; Promega, Madison, WI, USA), and 200 ng mL^{-1} recombinant heregulin β1 (HRG, PF048-50UG; Calbiochem, EMD Millipore). In addition to being grown in TDM media for a total of 12 days, cells were also kept in transdifferentiation media for a total of 32 days (20 days in TDM3). Several studies have demonstrated that long-term culture can alter the genetic composition of MSCs (48,49), and cause changes in proliferation and expression patterns in surface markers (50). We kept cells in media for 32 days in order to observe the long-term effects of transdifferentiation media on MSC replication rates, and ability to express Schwann cell markers. After 8 and 20 days, *in vitro* (DIV), cells were counted and plated at 2000 cells/well in a 96 well plate (655090; Greiner Bio-One, Monroe, NC, USA). Immunocytochemistry was used to characterize the control, undifferentiated MSCs (uMSCs), and transdifferentiated MSCs (tMSCs).

Immunocytochemistry to assess SC marker proteins in tMSCs vs. uMSCs A panel of antibodies was used for immunocytochemistry (ICC) analysis to compare uMSCs vs. tMSCs (Table 1). The antibodies chosen are common Schwann cell and glial cell markers whose expression levels were used to support conversion from an MSC to a Schwann cell-like state in various previous transdifferentiation studies (36,51–53). Cells were allowed a total of 48 h to re-attach and proliferate within the 96-well plate. After this time, all wells were rinsed twice with 0.1 M PO_4 buffer and fixed for 20 min with cold 4% paraformaldehyde in 0.1 M PO_4 buffer. Then, cells were rinsed 3 times with filtered phosphate buffered saline (PBS; BP2944100; Fisher-Scientific, Waltham, MA, USA) every seven minutes. Cells were incubated in blocking solution consisting of PBS with 5% normal donkey serum (NDS; 017-000-001; Jackson ImmunoResearch, West Grove, PA, USA), 0.4% bovine serum albumin (A9647; BSA; Sigma), and 0.2% Triton X-100 (85111; Fisher Scientific).

The primary antibodies were diluted in blocking solution and cells were incubated overnight at 4°C. Following incubation, cells were rinsed with PBS 4 times every 8 min, and incubated in secondary antibodies diluted in blocking solution. The following secondary antibodies were used: Donkey-α-Mouse Cy3 (715-165-51; Jackson ImmunoResearch Labs, 1:500) and Donkey-α-Rabbit Cy3 (711-165-152; Jackson ImmunoResearch Labs, 1:500). Cell nuclei were stained with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine) (D3571; Invitrogen, 1: 2000) and incubated at room temperature in the dark for 60–90 min. Cells were then rinsed with PBS 3 times every 7 min. Controls included cells incubated without any primary or secondary antibodies, as well as cells with only secondary antibody applied.

High throughput image acquisition and image analysis to quantify SC marker proteins in tMSCs vs. uMSCs Once immunocytochemistry (ICC) was performed, cells were imaged on the ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA, USA). The use of this system is advantageous as it provides thousands of images (6144 images per wavelength) in a short time period, allowing for extensive data analysis. After the ICC, each 96-well plate was loaded into the ImageXpress Micro and allowed to equilibrate for 20 min at 37°C. Plates were imaged using the 20× objective and a total of 64 microscopic fields per well were taken, for a total of 6144 images per wavelength, per plate. Three wavelengths were selected for our experiments: Cy3 (550 nm), GFP (395 nm), and DAPI (358 nm). Images were analyzed via a multiwavelength cell scoring module on the MetaXpress 4.0 software (Molecular Devices). A threshold of intensity level above local background was set based upon the presumption that uMSCs would express minimum fluorescent levels of the antibody analyzed. Cells with fluorescence levels higher than the threshold were marked as positive. Other parameters such as minimum and maximum cell width, minimum stained area, and cytoplasmic vs. nuclear staining were considered during the analysis. For a more detailed procedural description, please refer to Sharma et al. (36). The percentage of positively stained cells was calculated by dividing the number of cells immunoreactive to each antibody by the total number of DAPI-stained nuclei per image. Every Cy3 and DAPI image was analyzed to calculate the average percentage of Cy3 expressing cells per well. Subsequently, the average percentage of Cy3 expression was calculated according to cell type and averaged across a total of four 96-well plate replicates. The Tukey–Kramer Corrections or a Student's *t*-test was used to compare means for all data analysis using R open software and GraphPad open software. A p-value ≤ 0.05 was considered significant. Error bars in graphs represent the standard error.

ELISA to determine secretion of BDNF from tMSCs vs. uMSCs An enzyme-linked immunosorbent assay (ELISA) was used to quantify BDNF release from the genetically modified MSCs (GFP-MSCs and BDNF-MSCs). The ELISA data was used to not only ensure that tMSCs were still secreting BDNF but to measure absolute BDNF levels. We originally hypothesized that the process of transdifferentiation would increase BDNF production, so the ELISA was used to compare ng/cell production. Additionally, we used this data to compare BDNF levels between cells grown in TDM3 for 8 days vs. 20 days, to determine if longer TDM3 exposure would have detrimental effects on production. The E_{max} Immunoassay was used (G7610; Promega, Madison, WI) to measure levels of BDNF in conditioned media from BDNF-MSCs and GFP-MSCs for 48 h. Cells were plated at 10,000 cells per well in a six-well plate and allowed to grow for 48 h. Conditioned media was

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