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

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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 p75NTR. 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-secreting cells, 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.

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[Key words: Mesenchymal stem cells; Schwann cells; Brain-derived neurotrophic factor; Peripheral nerve regeneration; Neuroprotection; Neuregeneration; 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. Epineurial 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, neurona 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 (trans-differentiated) 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 neurorgenereation (25).

For this study, we chose to focus on BDNF delivery because it has been shown to provide neuroprotection and facilitate the rescue
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). Previous 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 changes in proliferation and expression patterns in surface markers (50).

**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; 12400-053; 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), and were centrifuged at 500 rpm for 5 min. MSCs were successfully transdifferentiated MSCs into an SC-like phenotype and quantified their morphological, molecular, and functional changes.

**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 transcription (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 (IMDM; 12400-053; Invitrogen, Carlsbad, CA, USA), 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% conversion rate of undifferentiated MSCs (uMSCs) to iMSCs 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 plated in Transdifferentiation Media 1 (TDM1) that consisted of IMDM, 5% FBS, 5% equine serum, and 35 ng mL−1 recombinant nerve growth factor (BDNF; R2625; Sigma–Aldrich). Finally, cells were plated in TDM3 for 8–20 days. TDM3 consisted of IMDM, 5% FBS, 5% equine serum, 14 μM forskolin (FSK, 344270; EMD Millipore, Billerica, MA, USA), 5 ng mL−1 basic fibroblast growth factor (rFGF, basic; G5071; Promega, Madison, WI, USA), and 200 ng mL−1 recombinant heparulin (1 HEP20; Gibco, 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 changes in proliferation and expression patterns in surface markers (50).

**Immunocytochemistry to assess SC marker proteins in iMSCs vs. uMSCs** A panel of antibodies was used for immunocytochemistry (ICC) analysis to compare uMSCs vs. iMSCs (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 (36–39). Cells were allowed to attach for a total of 48 h, re-attached to the plate for 90 min. 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 (8511; 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 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 (4′,6-diamidino-2-phenylindole-1H-indole-6-carboxamidine) (D3571; Invitrogen, 1:2000) and mounted 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 protein expression in iMSCs vs. uMSCs** Once confocal microscopy 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 scanned into the ImageXpress Micro and allowed to image for 37°C. Plates were imaged using the 20× objective with 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 (495 nm), and DAPI (385 nm). Images were analyzed via a multichannel analysis (MCA) software tool to detect the Cy3 fluorescence levels higher than the threshold was set based upon the presumption that iMSCs 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 used 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 49–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 Software (La Jolla, CA). A p value of 0.05 was considered significant. Error bars in graphs represent the standard error.

**ELISA to determine secretion of BDNF from iMSCs 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 iMSCs 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 Emax 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
collected and immediately frozen at −20°C. The ELISA was performed as per the kit manufacturer’s instructions.

### PC12-TrkB cell neurite outgrowth assay to assess bioactivity of secreted BDNF

PC12-TrkB cells were used to assess the bioactivity of BDNF released from MSCs. The original PC12 cells are a clonal cell line derived from a rat pheochromocytoma, which projects long neurites when exposed to NGF. The PC12-TrkB cell line was genetically programmed to over-express the BDNF neurotrophin receptor, TrkB (54). We utilized this assay to ensure that the BDNF being secreted by cells was still biologically active and could induce neurite outgrowth. We assumed that actual neurite length would correlate with the amount of BDNF production and thus used PC12-TrkB cells to assess the bioactivity of MSC-secreted BDNF.

PC12-TrkB cells were provided by Dr. Moses Chao (Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute for Biomolecular Medicine, New York Univ.). Cells were maintained in RPMI-1650 (ATCC Cell Biology; Manassas, VA) containing 10% heat-inactivated equine serum (SH30074; Hyclone, South Logan, UT) and 5% fetal bovine serum. Cells were incubated at 37°C with 5% CO2. To observe neurite outgrowth, PC12-TrkB cells were plated at 3000 cells per well of a 96 well plate. Using the condition media collected from MSCs (see above), PC12-TrkB cells were grown in a 50:50 mixture of condition media and PC12 maintenance media (MM). Twenty ng of human recombinant BDNF in PC12 MM was used as a control (rhBDNF; 248-BD005; R&D systems, Minneapolis, MN). Cells were allowed to adhere for 48 h and were subsequently fixed with 4% paraformaldehyde in 0.1 M PO4 buffer. Neurite outgrowth was visualized with an Anti-Beta III tubulin Cy3 conjugated antibody (AB15708C3; EMD Millipore, Billerica, MA; 1:100). For image analysis, 25 microscopic fields in each well were taken randomly using a 20x objective on the ImageXpress Micro high content screening system. The MetaXpress 4.0 software’s neurite outgrowth module was used to calculate the average neurite length per cell (microns) for each condition.

### Morphometric analysis to compare morphological changes between uMSCs vs. tMSCs

Schwann cells are known to have an elongated bipolar, spindle-shaped morphology and similar morphology has been observed in tMSCs by previous transdifferentiation studies. Once submitted to TDM media, our MSC line will elongate and appear more spindle-shaped (see Results, Fig. S1). To quantify this cellular elongation, we looked at a parameter known as the aspect ratio and total cell area. Several studies have demonstrated that the aspect ratio of MSCs may affect their lineage commitment, and a larger aspect ratio is commonly seen with tMSCs (36,55). The aspect ratio is the ratio of a cell’s length (longest dimension) to its breadth (width perpendicular to the length) and is expected to be a value of one or greater. A value of one is seen for objects whose length and width are the same, such as circles/regular polygons. A fibroblastic cell, such as an MSC, which has a rounded morphology with no defined directionality is expected to have an

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<th>Primary antibody</th>
<th>Dilution</th>
<th>Marker</th>
<th>Source</th>
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<tr>
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RESULTS

Characterization of uMSCs  Genetically modified MSCs used in this study were characterized previously using a high throughput imaging system and automated image analysis (32). The viability and proliferation of BDNF-GFP and GFP MSCs were compared to the original population of non-genetically modified MSCs using a number of in vitro assays such as propidium iodide staining, Ki67 immunolabeling, and cellular migration via time-lapse digital imaging. The results demonstrated no significant differences between genetically modified MSCs and unmodified MSCs, confirming the normal health of BDNF-GFP and GFP-expressing MSC lines.

Effect of chemical transdifferentiation on cell morphology  Many previous studies have qualitatively described morphological changes to MSCs when they were trans-differentiated to SC-like cells. Transdifferentiated cells appeared bipolar and assumed a spindle cell appearance similar to SC morphology, whereas undifferentiated cells exhibited a typical fibroblast-like morphology (Fig. S1). This observation was analytically supported via automated morphometric analysis (Fig. 2). The aspect ratio, an indicator of cell elongation, was calculated to compare the phenotype of transdifferentiated cells to control cells. A ratio close to one indicates cells with a regular polygonal shape with no directionality (fibroblast-like morphology), whereas a ratio >1 is produced by cells with a bipolar, elongated morphology. All four of our cell types had aspect ratios greater than 1, indicating cell length is greater than breadth. The aspect ratio of GFP tMSCs (4.1 ± 0.32) was significantly higher than uMSCs (1.8 ± 0.33), indicating that transdifferentiated cells are more elongated and have a bipolar morphology (Fig. 2A). BDNF tMSCs also had a higher aspect ratio of 3.4 as compared to uMSCs (3.07); however, the difference was not significant (Fig. 2A). Genetic modification of uMSCs using lentiviral vectors seemed to have an influence on morphology of the cells, and BDNF uMSCs are elongated as compared to GFP uMSCs. Because of the already higher aspect ratio of BDNF uMSCs, only a slight increase in aspect ratio of BDNF-tMSCs was observed which was not significant. Average total cell area was also compared between the cells grown in MM and TDM. Average cellular area was: BDNF uMSCs ~ 939.5 μm², BDNF tMSCs ~ 13471 μm², GFP uMSCs ~ 1106.6 μm², and GFP tMSCs ~ 1192.5 μm². While both types of tMSCs had a higher average area, there was no significant difference between any of the cell types (Fig. 2B), which means that while cells undergo a morphological change during transdifferentiation, they do not shrink or expand in their size.

Evaluation of Schwann cell-like phenotype  To determine aspect ratios (length of cell/width of cell) and cellular areas of undifferentiated and differentiated MSCs using the MetaXpress software Morphometric Analysis program (36). A student’s t-test was used to compare means within the same cell type on GraphPad prism v6 software (GraphPad Software, Inc., La Jolla, CA, USA). A p-value ≤ 0.05 was considered significant. Error bars in graphs represent the standard error.

TuJ1 for any neuronal expression (56–59). To assess cell proliferation, anti-Ki67 was also used. Plates were visualized in the ImageXpress Micro high content screening system and subsequently analyzed with the MetaXpress 4.0 software using the multi-wavelength cell scoring module.

After 8 days’ growth in TDM3, a significantly larger number of BDNF tMSCs were positively labeled by anti-S100, anti-S100β, and anti-p75NTR. Specific staining was not seen for GFAP or TuJ1 for any cell type confirming that cells were less astrocytic or neuronal-like. S100 immunolabeling was identified in the cytoplasm with only minor staining seen in the nucleus, consistent with expected findings (Fig. S2A and B). Transdifferentiated BDNF cells showed approximately a six-fold higher percentage of S100 immunolabeling compared to uMSCs (Fig. 5A): 42% ± 9.9 versus 6.9% ± 4.4, respectively. Anti-S100β binds to the Schwann cell specific S100β protein and is a more exclusive marker than anti-S100, which is expressed by many different cell types (60). S100β staining was only seen in the cytoplasm (Fig. 3A and B). Again, BDNF tMSCs demonstrated higher levels of staining (29.1% ± 1.9) than uMSCs (4.7% ± 1.3), with a six-fold difference observed (Fig. 5A). Up to

FIG. 2. Morphometric analysis. Transdifferentiated cells appeared bipolar and elongated as compared to undifferentiated cells. Morphometric analysis was conducted to determine aspect ratios (length of cell/width of cell) and cellular areas of undifferentiated versus transdifferentiated MSCs. (A) Aspect ratio of GFP and BDNF cells. A ratio greater than one implies cellular elongation. GFP tMSCs had an aspect ratio significantly greater than GFP uMSCs, implying a greater average cell length and morphology similar to Schwann cells. (B) Average total cellular area of GFP and BDNF MSCs. There were no significant differences between cell types indicating that elongation of cells did not affect the total cellular area of the MSCs. Error bars represent standard error of the mean. N = 30 cells per condition, four independent transdifferentiation experiments carried out. A total of 120 cells per condition. *Significantly different at p ≤ 0.05.
52 ± 10% of BDNF tMSCs expressed p75NTR Neurotrophin receptor (Fig. 4A and B, and 5). Ki67 was similar for both BDNF cell types, with approximately 25% of cells showing active proliferation, demonstrating results similar to those found by Sharma et al. 2015 (32) (Fig. S3A, B).

Transdifferentiated GFP cells showed markedly different results from the BDNF cells. GFP MSCs showed only a significant difference in antibody immunolabeling for S100β (Fig. 3A). Approximately 23.9% ± 5.2 of GFP tMSCs were immunolabeled by the S100β antibody, while only 6.1% ± 0.5 of uMSCs showed labeling. GFP tMSCs had a higher average percentage of cells immunolabeled for S100 and p75NTR, but the variability between replicates was too great to be significant. Again, Ki67 was similar for both cell types, with 30% of cells showing immunolabeling (Fig. S3C, D). In general, both GFP cell types appeared to proliferate more than their BDNF counterparts.

At 20 days’ growth in TDM3, cells were additionally tested to assess cell proliferation and cell marker profiles. After 20 days’ growth in TDM3, BDNF cells appeared largely unchanged when compared to day 8 cells. BDNF tMSCs continued to express significantly higher levels of S100, S100β, and p75NTR (Fig. 5B). Again, immunolabeling for GFAP and TuJ1 was not noted for any cell type. When compared to Day 8 there was a decrease (42 vs. 27%) in S100 immunolabeled BDNF-tMSCs on Day 20; however, levels were still significantly higher than BDNF uMSCs. A similar drop was noted for BDNF tMSC levels of p75NTR (52% on Day 8; 34% on Day 20). S100β remained consistent from Day 8 to Day 20 for BDNF tMSCs (29% vs. 32%). The BDNF uMSCs continued to express minimum levels of Schwann cell markers (5–8%). Ki67 immunolabeling demonstrated no significant difference between BDNF cell types and remained close to ~25% as on Day 8.

Post 20 days TDM3, GFP cells appeared further transdifferentiated to resemble a Schwann cell-like phenotype. A significant difference was noted between GFP tMSCs and uMSCs for S100 and p75, however, no difference was seen for S100β (Fig. 5B). GFP tMSC expression of S100 increased by approximately 10% (20%
on Day 8; 30% on Day 20). The opposite effect was noted for p75 expression, with a drop-in expression of 10%. Again, Ki67 levels remained at roughly 25%

Combined, the ICC results demonstrate that both GFP and BDNF MSCs assumed a Schwann cell-like phenotype, based off the increased immunolabeling for specific markers. In general, BDNF cells showed a significant change in their immunolabeling profile faster than GFP cells, and at higher levels. Both cell types retained their SC-like phenotype for up to 20 days in TDM3.

Quantification of BDNF production from MSCs ELISAs were performed using conditioned media samples, to quantitatively determine BDNF levels. After 8 days’ growth in TDM3, BDNF cells were secreting significantly higher levels of the neurotrophic factor than the GFP control cells. Secretion of BDNF from control GFP uMSCs was 2.74 ± 2.7 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹). Levels of BDNF were undetectable from the conditioned media collected from the GFP tMSCs (Fig. 7A). BDNF uMSCs and tMSCs secreted significantly higher amounts of BDNF than both GFP cell types (45.16 ± 14.0 and 39.8 ± 6.3 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹), respectively). A Tukey–Kramer test revealed significant differences between both BDNF cell types and GFP cells. There was no significant difference between BDNF uMSCs and tMSCs. ELISAs conducted after 20 days’ growth in TDM3 revealed continued BDNF production and secretion by the BDNF uMSCs (71.32 ± 17.78 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹) and BDNF tMSCs (102.26 ± 30.37 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹) (Fig. 7A). While a small increase in the amount of BDNF secretion was observed from BDNF uMSCs from 45.16 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹ on day 8 to 71.32 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹ at day 20, a considerably higher difference was observed in the secretion from BDNF tMSCs from 39.8 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹ on day 8 to 102.26 ng mL⁻¹ (10⁶ cells⁻¹ day⁻¹ on day 20. Again, there was no significant difference between BDNF cell types. There was no significant difference in BDNF levels between 8 vs. 20 days in TDM3. GFP uMSCs and tMSCs appeared to be producing similar levels of BDNF,
approximately 16.9 ng mL\(^{-1}\) (10\(^6\) cells)\(^{-1}\) day\(^{-1}\). A Tukey–Kramer test revealed significant differences between BDNF tMSCs and both GFP cell types. A student’s t-test calculated a significant p-value of 0.04 for the difference between BDNF uMSCs and GFP tMSCs. The difference between BDNF uMSCs and GFP uMSCs was very close to statistical significance, with a p-value of 0.054. These results demonstrated that the transdifferentiation process did not alter or decrease the ability of the BDNF MSCs to produce and secrete significant quantities of BDNF.

**BDNF bioactivity: PC12-TrkB neurite outgrowth assay** The PC12-TrkB cell assay was utilized to assess the bioactivity of secreted BDNF. The original PC12 cells are a clonal cell line derived from a rat pheochromocytoma, which projects long neurites when exposed to NGF (54). The PC12-TrkB cells were genetically programmed to over-express the BDNF Neurotrophin receptor, TrkB (61). By comparing neurite length between conditions, PC12-TrkB cells were used to assess the bioactivity of MSC-secreted BDNF. Immunolabeling for TuJ1 showed the extent of neurite outgrowth for PC12-TrkB cells cultured in the following conditioned medias: GFP uMSC (Fig. 6A), GFP tMSC (Fig. 6B), PC12 growth media (Fig. 6C) BDNF uMSC (Fig. 6D), BDNF tMSC (Fig. 6E), and 20 \(\mu\)g mL\(^{-1}\) rhBDNF control (Fig. 6F).

Eight-day conditioned media from both BDNF uMSCs and tMSCs visibly enhanced PC12-TrkB neurite outgrowth when compared to GFP conditioned media (Fig. 7B), with an average neurite...
Peripheral nerve injury limits mobility and sensation in up to 2.8% of trauma patients and often results in an unsatisfactory return of function (9,62). The gold standard for severe transected peripheral nerve damage involves microsurgery replacement with an autologous nerve graft. However, due to donor site morbidity, many studies have shifted focus to alternative strategies such as nerve guidance conduits and glial cell transplantation. Schwann cells are the primary glial cells of the peripheral nervous system and are necessary for nerve damage repair and regeneration. Specifically, SCs remove myelin debris and guide the directed growth of regenerating axons by undergoing dedifferentiation, proliferation, and migration (63,64). Additionally, SCs produce neurotrophic factors such as BDNF, NGF, NT-3, and NT-4/5, which are necessary for neuronal growth and survival (65). Unfortunately, SCs can only be obtained by sacrificing a healthy nerve, and the process of cell culture is often arduous (66). In search of an alternative to Schwann cells, many studies have looked at mesenchymal stem cells, especially the process of transdifferentiation into a Schwann cell-like phenotype (56–58). For our purposes, we chose to study MSCs not only for their plasticity but also because of our past success in genetically modifying these cells as delivery vehicles to hypersecrete neurotrophic factors (8,32,35). Since neurotrophic factors such as BDNF promote nerve regeneration, they hold great therapeutic potential. Current clinical use of BDNF is limited, however, due to the absence of safe and reliable delivery systems that can provide sustainable effective concentrations over time (67). As a novel alternative to traditional nerve regeneration therapies, we combined dual strategies of using MSCs as BDNF delivery vehicle as well as the production of SC-like cells from MSCs via transdifferentiation. In order to accomplish this, we first successfully created a BDNF hypersecreting MSCs cell line which stably produces BDNF over multiple passages and thus can be used for creating a large pool of autologous MSCs for transplantation. It has been observed before that, after differentiation of stem cells, the proliferation of cells decrease which causes difficulties in generating a large population of transplantable cells. Hence, differentiating from a large pool of autologous BDNF hypersecreting SC-like cells could be a cost-effective strategy as compared to genetically modifying a population of a differentiated cell to hypersecrete neurotrophic factors.

In the current study, we subjected both BDNF and GFP expressing mouse MSCs to a transdifferentiation protocol and subsequently characterized the morphological and molecular changes. Previous studies have shown that, after transdifferentiation, MSCs become bipolar and spindle in shape as compared to the normal fibroblastic structure of MSCs and also start expressing SCs specific protein markers such S100B (59,68). Consistent with the literature, in this study too, MSCs became elongated and spindle shaped, with tMSCs demonstrating a larger on average aspect ratio. A larger aspect ratio implies cells were longer in one direction in tMSCs as compared to no specific directionality in undifferentiated cells. Several other studies have found similar morphological changes (34,36,37). Furthermore, transdifferentiated cells had higher average cellular markers such S100β directionality in undifferentiated cells. Several other studies have found similar morphological changes (34,36,37). Furthermore, transdifferentiated cells had higher average cellular markers such S100β and 5.5±1.8 μm for GFP MSCs. No statistical difference was found between day 8 and day 20 of BDNF uMSCs, a slight increase was observed in the neurite length for day 20 BDNF tMSCs as compared to day 8 BDNF tMSCs. However, no significant differences were observed at p≤0.05. The neurite outgrowth for BDNF uMSCs vs. tMSCs was 43.7±4.2 μm and 54.3±7.3 respectively, vs. 5.0±1.1 and 8.5±1.9 μm for GFP MSCs. Together these results demonstrate that BDNF MSCs (both uMSCs and tMSCs) can produce and secrete bioactive BDNF with potent neurite outgrowth promoting activity. Furthermore, these results demonstrate that the transdifferentiation process itself did not alter or reduce the production and secretion of bioactive BDNF from the tMSCs.

DISCUSSION

Peripheral nerve injury limits mobility and sensation in up to 2.8% of trauma patients and often results in an unsatisfactory return of function (9,62). The gold standard for severe transected peripheral nerve damage involves microsurgery replacement with an autologous nerve graft. However, due to donor site morbidity, many studies have shifted focus to alternative strategies such as nerve guidance conduits and glial cell transplantation. Schwann cells are the primary glial cells of the peripheral nervous system and are necessary for nerve damage repair and regeneration. Specifically, SCs remove myelin debris and guide the directed growth of regenerating axons by undergoing dedifferentiation, proliferation, and migration (63,64). Additionally, SCs produce neurotrophic factors such as BDNF, NGF, NT-3, and NT-4/5, which are necessary for neuronal growth and survival (65). Unfortunately, SCs can only be obtained by sacrificing a healthy nerve, and the process of cell culture is often arduous (66). In search of an alternative to Schwann cells, many studies have looked at mesenchymal stem cells, especially the process of transdifferentiation into a Schwann cell-like phenotype (56–58). For our purposes, we chose to study MSCs not only for their plasticity but also because of our past success in genetically modifying these cells as delivery vehicles to hypersecrete neurotrophic factors (8,32,35). Since neurotrophic factors such as BDNF promote nerve regeneration, they hold great therapeutic potential. Current clinical use of BDNF is limited, however, due to the absence of safe and reliable delivery systems that can provide sustainable effective concentrations over time (67). As a novel alternative to traditional nerve regeneration therapies, we combined dual strategies of using MSCs as BDNF delivery vehicle as well as the production of SC-like cells from MSCs via transdifferentiation. In order to accomplish this, we first successfully created a BDNF hypersecreting MSCs cell line which stably produces BDNF over multiple passages and thus can be used for creating a large pool of autologous MSCs for transplantation. It has been observed before that, after differentiation of stem cells, the proliferation of cells decrease which causes difficulties in generating a large population of transplantable cells. Hence, differentiating from a large pool of autologous BDNF hypersecreting SC-like cells could be a cost-effective strategy as compared to genetically modifying a population of a differentiated cell to hypersecrete neurotrophic factors.

In the current study, we subjected both BDNF and GFP expressing mouse MSCs to a transdifferentiation protocol and subsequently characterized the morphological and molecular changes. Previous studies have shown that, after transdifferentiation, MSCs become bipolar and spindle in shape as compared to the normal fibroblastic structure of MSCs and also start expressing SCs specific protein markers such S100B (59,68). Consistent with the literature, in this study too, MSCs became elongated and spindle shaped, with tMSCs demonstrating a larger on average aspect ratio. A larger aspect ratio implies cells were longer in one direction in tMSCs as compared to no specific directionality in undifferentiated cells. Several other studies have found similar morphological changes (34,36,37). Furthermore, transdifferentiated cells had higher average cellular areas than uMSCs, though no significant differences were observed.
Cells were further characterized by immunostaining using Schwann cell markers such as S100, S100β, p75NTR, GFAP, and TuJ1. 30–50% of BDNF tMSCs were preferentially immunolabeled for Schwann cell markers such as S100, S100β, and p75NTR after 8 days in TDM3, and even out to 20 days in TDM3, with minimal decreases in expression seen. Zaminy et al. (45) and Ladak et al. (44) reported significantly higher neurite outgrowth as compared to both GFP uMSC and tMSC conditioned media. Astrocyte marker GFAP and microglia marker p75NTR, GFAP, and TuJ1. After exposure to TDM conditions, BDNF production was quantified by ELISA, and its biological activity tested via the PC12-TrkB bioassay. The ELISA results showed that BDNF production was well above the GFP baseline both after 8 and 20 days in TDM3. In a previous study on a different BDNF MSC cell line, Harper et al. (8) found that unaltered BDNF MSCs secrete approximately 41 ng mL⁻¹ (10⁶ cells)⁻¹ day⁻¹ of BDNF. This is very similar to our findings of BDNF secretion of 45.2 ng mL⁻¹ (10⁶ cells)⁻¹ day⁻¹ at day 8 by BDNF uMSCs. Furthermore, by day 20, BDNF secretion for BDNF uMSCs increased to 71.32 ng mL⁻¹ (10⁶ cells)⁻¹ day⁻¹. While uMSCs showed a smaller increase in BDNF secretion from day 8 to day 20, a considerably higher difference was observed in the secretion from BDNF tMSCs from 39.8 on day 8 to 71.33 ng mL⁻¹ (10⁶ cells)⁻¹ day⁻¹ at day 20, a considerably higher difference was observed in the secretion from BDNF tMSCs from 39.8 on day 8 to 71.33 ng mL⁻¹ (10⁶ cells)⁻¹ day⁻¹ at day 20. These results are very promising, given that Harper’s results were obtained from unaltered cells, signifying that the process of transdifferentiation does not repress BDNF secretion and increases the amount of BDNF secreted as the culture time increases. The bioactivity of secreted BDNF was verified by the increased neurite outgrowth of PC12-TrkB cells by both BDNF uMSCs and BDNF tMSCs. There were no significant differences observed in the amount of neurite outgrowth which suggests that the amount of BDNF secreted from BDNF uMSCs is enough to cross the BDNF threshold required to initiate sufficient neurite outgrowth from PC12-TrkB cells. Reducing the number of BDNF cells cocultured with PC12-TrkB, can reduce the amount of BDNF secreted and thus can be used to find out if BDNF secreted from

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uMSCs and tMSCs enhances the neurite outgrowth differently or not. These findings suggest that not only is BDNF actively secreted by our cells, but it was bioactive and had the capacity to promote neurite sprouting and regeneration. Were these cells to be used for in vivo studies, we believe they could increase axonal outgrowth and survival. Given the fact that BDNF production remained stable for over 20 days, we believe these cells have the capacity to produce sustainable, effective BDNF concentrations over prolonged time periods.

Our research group has previously shown that lentiviral-induced BDNF MSCs have the capacity to survive and protect neuronal function within the retina (8). For future studies, we hope to transplant BDNF TMCs within a biodegradable conduit into a rat sciatic nerve gap model to assess the in vivo effects on peripheral nerve regeneration. In addition to peripheral nerve transections, BDNF hyper-secreting MSCs could be used in the treatment of spinal cord trauma (71), ischemic stroke (72), Parkinson’s (73), and many other neurodegenerative disorders. We hope our results from this study encourage the future use of transdifferentiated genetically modified MSCs as a reliable and effective system for delivery of neurotrophic or other therapeutic factors.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2017.05.014.

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