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Autocrine Production of IGF-I Increases Stem Cell-Mediated Neuroprotection

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder resulting in motor neuron (MN) loss. There are currently no effective therapies; however, cellular therapies using neural progenitor cells protect MNs and attenuate disease progression in G93A-SOD1 ALS rats. Recently, we completed a phase I clinical trial examining intraspinal human spinal stem cell (HSSC) transplantation in ALS patients which demonstrated our approach was safe and feasible, supporting the phase II trial currently in progress. In parallel, efforts focused on understanding the mechanisms underlying the preclinical benefit of HSSCs in vitro and in animal models of ALS led us to investigate how insulin-like growth factor-I (IGF-I) production contributes to cellular therapy neuroprotection. IGF-I is a potent growth factor with proven efficacy in preclinical ALS studies, and we contend that autocrine IGF-I production may enhance the salutary effects of HSSCs. By comparing the biological properties of HSSCs to HSSCs expressing sixfold higher levels of IGF-I, we demonstrate that IGF-I production augments the production of glial-derived neurotrophic factor and accelerates neurite outgrowth without adversely affecting HSSC proliferation or terminal differentiation. Furthermore, we demonstrate that increased IGF-I induces more potent MN protection from excitotoxicity via both indirect and direct mechanisms, as demonstrated using hanging inserts with primary MNs or by culturing with organotypic spinal cord slices, respectively. These findings support our theory that combining autocrine growth factor production with HSSC transplantation may offer a novel means to achieve additive neuroprotection in ALS. STEM CELLS *2015;33:1480–1489*

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease resulting in upper and lower motor neuron (MN) loss. There are no effective treatments and death typically occurs within 3–5 years of onset [1]. One obstacle facing therapeutic development is the complex, unclear etiology and several mechanisms have been proposed, including oxidative stress, mitochondrial dysfunction, a toxic microenvironment, astrocytic and glial dysfunction, loss of distal neuromuscular junctions, and a breakdown in local neurocircuitry within the spinal cord [2]. In addition, reductions in trophic factors within the spinal cord microenvironment in ALS further enhance MN susceptibility to the disease process [3–5]. Given this complexity, it is likely that a multifaceted treatment approaches may be warranted.

Cellular therapies are a new therapeutic avenue for ALS; they provide de novo neural tissue to support neurocircuitry and represent a source for in situ production of neuroprotective growth factors [6, 7]. Recently, we completed a phase I clinical trial examining intraspinal transplantation of human spinal stem cells (HSSCs) in 15 ALS patients, validating the feasibility and safety of this cellular therapy approach [8–13]. Phase II of the trial examining dosing and efficacy is ongoing, and in parallel, investigations in the laboratory are focused on understanding the mechanisms underlying how HSSCs support MNs in ALS. Previous work in G93A-SOD1 ALS rats confirmed that HSSCs integrate into the spinal cord, form synapses with host tissue, maintain MN numbers, and produce several neuroprotective growth factors, including glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and insulin-like growth factor-I (IGF-I) following intraspinal transplantation [14–18]. Of these growth factors, IGF-I is the most highly expressed, suggesting it may contribute to HSSC-mediated neuroprotection.

IGF-I has potent neurotrophic and neuroprotective effects and extensive preclinical evidence supports the attenuation of MN loss and maintenance of neuronal synapses and neuromuscular junctions by IGF-I [19]. We and others have shown that viral-mediated IGF-I delivery

increases MN numbers, improves grip strength, delays progression, and prolongs survival in ALS rodents [20–23]. A phase III trial investigating subcutaneous IGF-I in ALS patients, however, did not successfully demonstrate therapeutic efficacy, although this was likely due to failure of IGF-I to reach the spinal cord and confer continuous protection to diseased MNs [24]. The ability of cellular therapies to provide localized neurotrophic support directly to MNs within the spinal cord microenvironment may circumvent the issues encountered in earlier IGF-I trials.

In this study, we developed a novel stem cell line expressing increased IGF-I levels, HSSC:IGF-I, to examine the potential contributions of autocrine and paracrine IGF-I production to neuroprotection within the neuromuscular axis. Specifically, we assess the differences between HSSCs expressing lower levels of IGF-I and HSSC:IGF-I, focusing on growth factor production and the potential actions of IGF-I on cell proliferation, migration, and differentiation. We further explore the additive role of IGF-I in neuroprotection by examining the ability of HSSC:IGF-I to provide MN protection against excitotoxicity compared to HSSCs. We anticipate these studies will demonstrate how autocrine and paracrine growth factor production contributes to cellular therapy neuroprotection and lend further support to our contention that HSSCs confer multifaceted therapeutic benefits in ALS.

Materials and Methods

HSSC and HSSC:IGF-I Culture

HSSCs (NSI-566RSC) were prepared from spinal cord tissue obtained from a single 8-week human fetus following an elective abortion as described previously [17, 25]. To generate HSSC:IGF-I, HSSCs were exposed to replication-defective recombinant lentivirus engineered to overexpress a preproisoform of human IGF-I cDNA driven by the human ubiquitin C (Ubc) promoter. The resulting cells were propagated as a single cell line without further selection and named NSI-566RSC.Ubc_IGF-I. Using a control construct expressing green fluorescent protein (GFP) under the same promoter, approximately 90%–95% of the proliferating cells were GFP-positive.

Cell culture followed established protocols [18, 26, 27] using culture reagents obtained from Sigma (St. Louis, MO, www.sigmaaldrich.com), unless otherwise noted. Briefly, culture vessels were coated with 100 μg/ml poly(D-lysine) (PDL; Millipore, Billerica, MA, www.emdmillipore.com) in HEPES buffer and incubated for 24 hours at room temperature. Surfaces were then washed three times with sterile water and allowed to completely dry under the hood before coating with 25 μg/ml fibronectin in phosphate buffered saline (PBS) for 1 hour. Fibronectin solution was aspirated and vessels were used immediately without drying. HSSC and HSSC:IGF-I were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Life Technologies, Carlsbad, CA, www.lifetechnologies.com/us/en/home/brands/gibco.html) supplemented with 100 mg/l human plasma apo-transferrin, 25 mg/l recombinant human insulin, 1.56 g/l glucose, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite. For maintenance in a progenitor state, 10 ng/ml fibroblast growth factor (FGF) was added to the growth media. For differentiation, cells were cultured in differentiation media comprised of DMEM without FGF, supplemented with 4 mM L-glutamine, 20 μM L-alanine, 6 μM L-asparagine, 67 μM L-proline, 250 nM vitamin B12, 25 mg/l insulin, 100 mg/l transferrin, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite, for 7 days, with a 50% media change every other day. Cells were analyzed in their undifferentiated state (D0) or after 3 or 7 days (D3 or D7, respectively) of differentiation.

ELISA, Dot Blot Analysis, and Western Blotting

To confirm that lentiviral transduction led to increased stable expression of IGF-I, conditioned media were collected after 10 passages and analyzed by ELISA. To collect conditioned media, flasks were washed with PBS, 10 ml growth (D0) or differentiation (D3, D6, and D7) media without insulin was added, and cells were cultured for 24 hours prior to media collection. Conditioned media were concentrated 10-fold to 1 ml using Centricon filters (3 kDa cut off; Millipore) following the manufactures' guidelines and IGF-I levels at D0 and D6 were quantified using a human-specific IGF-I ELISA (R&D Systems, Minneapolis, MN, www.rndsystems.com). For dot blotting, concentrated media (200 μl) from D0 and D7 cultures were applied to nitrocellulose membranes using a Whatman Schleicher & Schuell Minifold I filtration manifold (Sigma) and membranes were exposed overnight at 4°C to primary antibodies, including GDNF (Abbiotec, San Diego, CA, www.abbiotec.com), VEGF (Santa Cruz Biotechnology, SantaCruz, CA, www.scbt.com), and BDNF (Santa Cruz Biotechnology), followed by 1 hour incubation at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Antibody binding was developed with LumiGLO Reagent and Peroxide (Cell Signaling, Danvers, MA, www.cellsignal.com) and exposed to Kodak BioMax XAR film (Sigma).

Western blotting on D0, D3, and D7 cell lysates was performed as previously described [27, 28]. Equal amounts of protein were loaded on either 10% or 12%, polyacrylamide gels, dependent on the size of the protein of interest. Polyvinylidene difluoride membranes were incubated with primary antibody overnight at 4°C and with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Primary antibodies were from Cell Signaling unless otherwise indicated and included IGF-I receptor (IGF-IR)-beta (IGF-IRβ, Tyr1135/1136), VEGF receptor (VEGFR), and GAPDH (Millipore). Antibody binding was visualized as described above.

Quantitative Real-Time RT-PCR

Total RNA was extracted from HSSC and HSSC:IGF-I at D0 and D7 using an RNeasy Kit (Qiagen, Valencia, CA, www.qiagen.com/us) following the manufacturers' instructions. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, www.bio-rad.com). Quantitative real-time RT-PCR (QPCR) analysis was performed using Power SYBR Green PCR Master Mix and a StepOnePlus thermal cycler (Applied Biosystems, Life Technologies, Grand Island, NY, www.lifetechnologies.com/us/en/website-overview/ab-welcome.html) using primers for IGF-IR, VEGF, GDNF, BDNF, vGlut1, vGlut3, GluR2, and GAD67 (Table 1; [27]). mRNA expression levels were calculated by the cycle threshold (Ct) value using StepOne v2.2.2 system software and normalized to an endogenous reference gene (GAPDH: ΔCt) and then relative to a control group ($\Delta\Delta$Ct), and were expressed as $2^{-\Delta\Delta Ct}$. Averages were calculated from two runs per sample and then averaged for three biological replicates.

Table 1. Quantitative real-time RT-PCR primer sequences

	Forward	Reverse
IGF-IR	CAATAAGTTCGTCCACAGAGACC	CCTCCTTTCCGGTAATAGTCTGT
VEGF	ATGGCAGAAGGAGGAGG	ATTGGATGGCACTAGCTGCG
GDNF	CTGACTTGGGTCTGGGCTATG	TTGTCACTCACCAGCCTTCTATTT
BDNF	CCAAGGCAGGTTCAAGAGG	TCCAGCAGAAAGAGAAGAGGA
vGlut1	AAGTTCCAGGTTTCTGTTCCTTT	ACCATGACTACCATTGTGAGGTT
vGlut3	GGGGTGTTGGTGCAGTACATT	CCCCTCTCCTATGCTTGTCTCTA
GAD67	GCCAGACAAGCAGTATGATGT	CCAGTTCCAGGCATTTGTTGAT

Abbreviations: BDNF, brain-derived neurotrophic factor; GAD67, glutamate decarboxylase; GDNF, glial cell-derived neurotrophic factor; IGF-IR, insulin-like growth factor-I receptor; VEGF, vascular endothelial growth factor; vGlut1, vesicular glutamate transporter 1; vGlut3, vesicular glutamate transporter 3.

Cell Proliferation and Migration

Cell proliferation was measured using a Click-iT EdU Kit (Invitrogen, Life Technologies, Carlsbad, CA, www.lifetechnologies.com/us/en/home/brands/invitrogen.html). Briefly, D0 undifferentiated and D3 differentiated HSSC and HSSC:IGF-I were incubated with 10 μM 5′-ethynyl-2′-deoxyuridine (EdU) for 2 hours and cells were fixed and processed according to the manufacturers' protocols. EdU incorporation was detected by the presence of fluorescence, and images were captured using an Olympus BX-51 microscope equipped with a digital camera. EdU-positive cell numbers represent quantification of a minimum of 2,000 cells from five images in three independent experiments.

Cell migration was assessed using a semipermeable transwell system by seeding cells onto 8 μm pore transwell inserts (BD Biosciences, Bedford, MA, www.bdbiosciences.com) at D0 or D7 at a concentration of 1×10^6 cells per milliliter. Differentiation media plus 10% fetal bovine serum with or without IGF-I (final concentration of 10 nM) was added to the lower chambers and transwell inserts containing HSSC or HSSC:IGF-I were placed over the chambers. After 24 hours, cells that had migrated through the insert were stained using the QCM 24-Well Colorimetric Cell Migration Assay (Millipore) and quantified by colorimetric measurement using LabSystems Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA, www.thermofisher.com/en/home.html) at optical densities of 530 and 590 nm.

Immunocytochemistry and Neural Index Assays

Immunocytochemistry was performed as previously described [27, 29]. HSSC/HSSC:IGF-I were grown on PDL and fibronectin-coated glass coverslips in 24-well plates for 0, 3, or 7 days, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton/PBS, and then blocked in 5% normal donkey serum/0.1% Triton/PBS. Primary antibodies for TUJ1 (Neuromics, Edina, MN, www.neuromics.com), GAD65/67 (Millipore), VGLUT2 (Millipore), or IGF-IRβ (Sigma) were incubated overnight at 4°C. Following incubation with Cy3, Cy5, or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, Westgrove, PA, www.jacksonimmuno.com), coverslips were mounted on glass slides using ProLong Gold with DAPI (Molecular Probes, Life Technologies, Carlsbad, CA, www.lifetechnologies.com/us/en/home/brands/molecular-probes.html). Images were collected using an Olympus BX-51 microscope. To measure the extent of neural differentiation, TUJ1 and DAPI-labeled images were analyzed following our published neural index protocol [27]. Briefly, TUJ1-labeled and corresponding DAPI images were opened in MetaMorph (Molecular Devices, Sunnyvale, CA, www.moleculardevices.com). Cell number was counted on DAPI images using the "count nuclei" plug-in, with manual adjustments to correct for any miscounted cells. Thresholds on TUJ1 images were then adjusted and the area of neurite coverage was measured using region statistics. The neural index was expressed as neurite area/cell (μm^2/cell).

Primary MN Coculture for Excitotoxicity Assays

Primary embryonic rat MNs were isolated according to our previously published protocol [27, 30] and cultured on PDL-coated glass coverslips in a 24-well plate. After 24 hours, the MNs were fed with 250 μl differentiation media for coculturing with HSSC or HSSC:IGF-I, which were seeded onto PDL-coated 3 μm pore transwell inserts (Corning, Tewksbury, MA, www.corning.com/lifesciences/worldwide.aspx) and differentiated for 4 days prior to coculturing. Cocultures were left for 3 days prior to beginning the excitotoxicity assay. All culture reagents were purchased from Gibco, Life Technologies unless otherwise indicated.

Excitotoxic stress was induced by adding 100 μM glutamate to the coculture media for 24 hours. The contribution of paracrine IGF-I production to protection was assessed by adding the IGF-IR inhibitor, NVPAEW541 (1 μM), 2 hours prior to glutamate. After glutamate treatment, MNs were fixed in 4% paraformaldehyde for 10 minutes and TUNEL was used to detect DNA fragmentation [27, 30]. Samples were labeled with digoxigenin-dUTP and stained with horseradish peroxidase-conjugated antidigoxigenin antibody using the ApopTagPlus In Situ Apoptosis Peroxidase Detection Kit (Chemicon). Alternatively, fluorescent TUNEL processing was carried out as described above with a Fluorescein-labeled conjugate using the ApopTagPlus In Situ Apoptosis Fluorescein Detection Kit (Chemicon). Fluorescent signal was detected and recorded using an Olympus BX-51 microscope. TUNEL-positive cells were counted in at least 10 representative fields per condition by a blinded investigator, per our published protocol, for an average total of approximately 1,000–2,000 MNs per condition [27].

Organotypic Spinal Cord Slice Cultures for Excitotoxicity Assays

Organotypic spinal cord slice cultures were prepared from P5–8 rat pups [31]. Briefly, lumbar spinal cords were isolated, membranes were removed, and spinal cord tissue was mounted in 7% agarose for Vibrotome sectioning. Sections (300 μm) were collected in ice cold PBS and three spinal cord slices were added to each Millicell Cell Culture Insert (0.4 μm pore, 300 mm diameter; Millipore) in a six-well plate. Wells were flooded with slice growth media containing 50% minimal essential medium, 25 mM HEPES, 25% heat-inactivated horse serum, and 25% Hanks' balanced saline solution, supplemented with 25.6 mg/ml D-glucose and 2 mM glutamine, at a final pH of 7.2. Cultures were incubated at 37°C in 5% CO_2 for 2 weeks, with media changes every 2 days. For coculture experiments, 2 μl of HSSC or HSSC:IGF-I cell suspension (6×10^3 cells per milliliter) was placed over the ventral horn of the slices 2 days after plating.

To induce excitotoxic stress, culture media were supplemented at day 7 with 100 μM threo-hydroxyaspartate (THA; Sigma) to inhibit glutamate transport until day 14. Slices were fixed in 4% PFA and processed for standard immunocytochemistry as described above, using SMI-32/ChAT (Millipore) to label large alpha MNs and HuNu (Millipore) to confirm HSSC and HSSC:IGF-I placement on the spinal slices. Fluorescent images were captured using an Olympus BX-51 microscope and SMI-32/ChAT-labeled MNs were quantified in the ventral horns of each slice. The total remaining MNs were quantified from nine slices per condition from three independent experiments.

Statistical Analyses

All results are representative of at least three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test or linear regression analysis (GraphPad Prism, La Jolla, CA, www.graphpad.com).

Results

Characterization of Growth Factor Production by HSSC:IGF-I

To examine the contribution of IGF-I production to HSSC neuroprotection, a lentivirus encoding full length human IGF-I was used to generate HSSC:IGF-I. ELISA on conditioned media from HSSC and HSSC:IGF-I cultures demonstrated that HSSCs produce low basal levels of 0.2 fg/cell per day IGF-I, a level that was consistent throughout the first week of differentiation (Fig. 1A). HSSC:IGF-I exhibited a sixfold increase in IGF-I production, averaging approximately 1.2 fg/cell per day (Fig. 1A), and this increased IGF-I production was maintained as the cells underwent differentiation. We next assessed the levels of several growth factors typically expressed by HSSCs using dot blot analysis on conditioned media from undifferentiated cells (D0) and cells after 7 days (D7) of differentiation. VEGF secretion was comparable in HSSC and HSSC:IGF-I and decreased similarly as both cell lines differentiated, while BDNF levels were consistent between cell lines and time points (Fig. 1B). GDNF levels were also comparable at D0; however, at D7, higher levels of GDNF were detected in conditioned media from HSSC:IGF-I compared to that from HSSCs (Fig. 1B). As previous studies have shown that HSSCs respond to IGF-I signaling, we also explored how growth factor receptor levels are regulated by paracrine IGF-I expression. IGF-IR protein levels in both cell lines throughout differentiation were reduced in HSSC:IGF-I relative to HSSCs at each time point examined (Fig. 1C). Given the association between IGF-I expression and VEGF signaling, we also investigated the effect of increased IGF-I expression on VEGFR levels. VEGFR is only expressed by HSSC and HSSC:IGF-I at D0; however, basal levels of VEGFR were considerably lower in HSSC:IGF-I compared to HSSC (Fig. 1C). Finally, to validate our protein expression results, we performed QPCR and similarly observed no measurable difference in the expression levels of IGF-IR (Fig. 1D), VEGF (Fig. 1E), or BDNF (Fig. 1G) between cell lines at the time points examined, although we did detect a significant increase in D7 HSSC:IGF-I GDNF transcript levels relative to levels in D7 HSSCs (Fig. 1F), a finding consistent with our protein expression analyses. Together, these results demonstrate significantly increased IGF-I production by HSSC:IGF-I that is maintained through 7 days of differentiation and is also accompanied by reduced early VEGFR expression and increased D7 GDNF expression.

Effects of IGF-I on Cellular Behavior and Differentiation in HSSC:IGF-I

IGF-I can modify cell behaviors such as proliferation and migration [32, 33]; therefore, we measured EdU incorporation to assess the effect of enhanced IGF-I production on HSSC:IGF-I proliferation rates. There were no significant differences in the proliferation rates of undifferentiated D0 HSSC:IGF-I compared to HSSC (Fig. 2A–2C) and no differences were observed between the cell lines at D3 of differentiation (Fig. 2A, 2D, 2E), suggesting that IGF-I does not maintain proliferation in HSSC:IGF-I. Alternatively, examination of the effect of IGF-I expression on the migratory potential of undifferentiated D0 HSSC:IGF-I revealed that HSSC:IGF-I had approximately double the migratory potential of HSSCs (Fig. 2F); however, no significant migration was detected in either cell line at D7 of differentiation (data not shown).

Previously, we demonstrated that exogenous IGF-I treatment enhanced HSSC neural differentiation [27]; therefore, we next examined how autocrine production of IGF-I affected HSSC:IGF-I differentiation. We first analyzed differentiation using our published neural index pipeline, which accounts for both neurite number and length relative to the number of cells present [27], and observed a twofold increase in the neural index of HSSC:IGF-I compared to HSSCs (52 μm^2/cell vs. 24 μm^2/cell, respectively; Fig. 3A), a difference that is clearly visible in the representative TUJ1 images (Fig. 3B, 3C). Next, we evaluated the expression of vGlut1, vGlut3, GluR2, and GAD67 using QPCR to characterize terminal differentiation, as HSSCs exhibit terminal phenotypes including both glutamatergic and GABAergic neurons by D7 of differentiation [18]. While no significant differences in the total percentage of differentiated cells were observed for either lineage, a significant increase in vGlut1 levels was present in D7 HSSC:IGF-I compared to HSSC (Fig. 3D). Additionally, decreased vGlut3 expression was seen in D7 HSSC:IGF-I compared to HSSCs (Fig. 3E), and GluR2 and GAD67 expression level differences between the two cell lines were not significant (Fig. 3F, 3G). These data demonstrate that increased IGF-I expression enhances the early migratory potential and neuronal differentiation of the cells, but does not significantly alter HSSC proliferation or behavioral properties following differentiation, thus maintaining the potential safety profile of HSSC:IGF-I for future translational applications.

Neuroprotective Effects of HSSC:IGF-I

To determine how increased IGF-I expression affects HSSC neuroprotection, we first modeled glutamate-induced excitotoxicity associated with ALS by exposing primary rat embryonic MNs to glutamate in vitro, a treatment that induces approximately 77% cell death (Fig. 4A). To assess the benefits of only secreted factors, we cocultured HSSCs or HSSC:IGF-I in a compartment adjacent to MNs. While HSSCs significantly reduced glutamate-associated MN death to 42%, HSSC:IGF-I further reduced cell death to under 20%, a level comparable

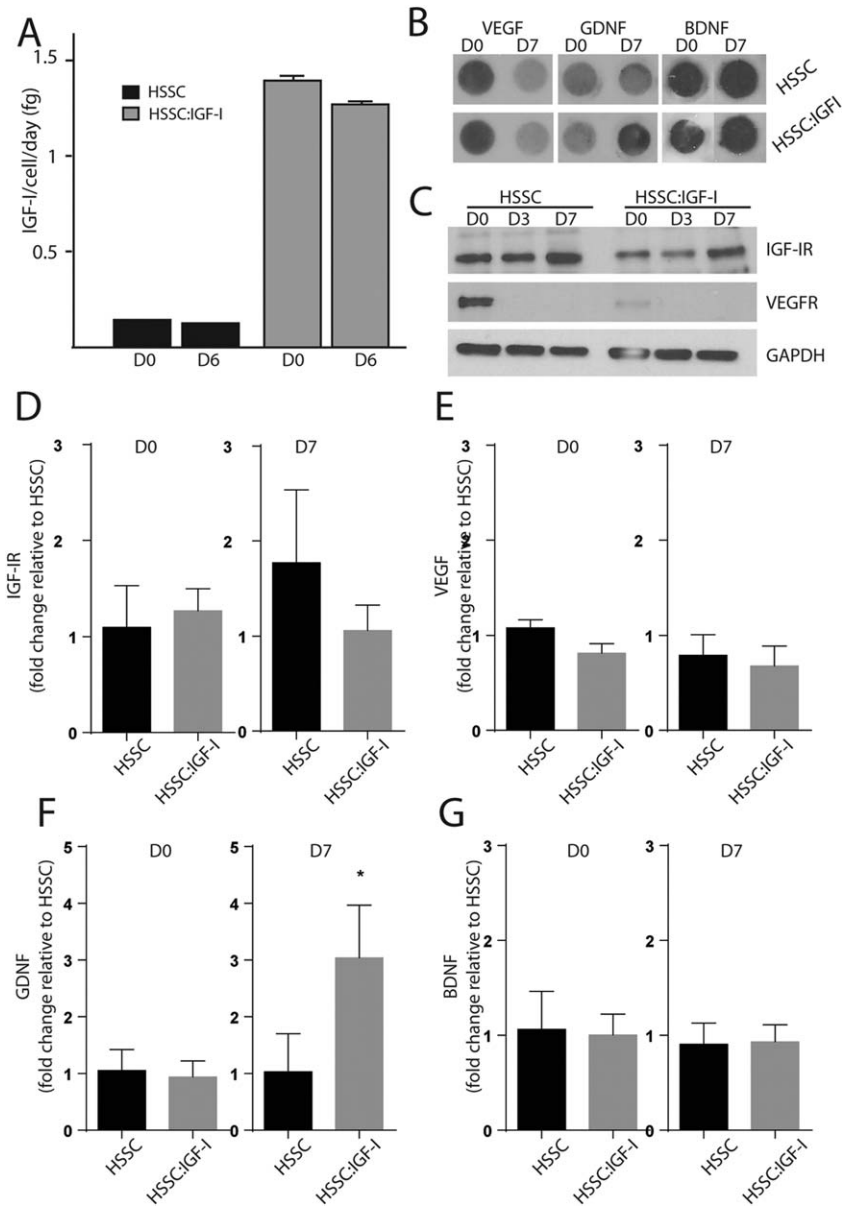

Figure 1. HSSC and HSSC:IGF-I growth factor and receptor profiles. **(A):** ELISA on HSSCs and HSSC:IGF-I conditioned media demonstrates the levels of IGF-I production in undifferentiated (D0) cells and in cells on D6 of differentiation. **(B):** Dot blot comparison of VEGF, GDNF, and BDNF levels in conditioned media from HSSCs and HSSC:IGF-I at D0 and D7. **(C):** Western blot analysis of growth factor receptor levels for IGF-IR and VEGFR in HSSC and HSSC:IGF-I lysates at D0, D3, and D7. **(D–F):** Quantitative real-time RT-PCR (QPCR) analysis of IGF-IR (D), VEGF (E), GDNF (F), and BDNF **(G)** expression at D0 and D7 in HSSCs and HSSC:IGF-I. QPCR data were normalized to GAPDH and presented as a fold-change from D0 HSSCs. Data are presented as mean + SD (*, $p < .05$) and are representative of at least three independent experiments. Abbreviations: BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; HSSC, human spinal stem cell; IGF-IR, insulin-like growth factor-I receptor; VEGF, vascular endothelial growth factor.

to that of the nontreated control group (Fig. 4A; data not shown). Furthermore, the addition of the IGF-IR inhibitor NVPAEW541 prevented the additional neuroprotection conferred by HSSC:IGF-I, reverting protection back to 39% death, a level comparable to that of HSSCs (Fig. 4A). These findings indicate that there is a measurable contribution of IGF-I to HSSC neuroprotection. Second, we analyzed the impact of secreted and cell contact-mediated HSSC and HSSC:IGF-I protection by plating the cells directly over spinal cord organotypic cultures subjected to ALS-associated excitotoxic stress. Cell placement over the spinal cord ventral horn was confirmed by stem cell-specific HuNu staining (Fig. 4C). Induction of glutamate toxicity using THA resulted in the survival of only 26% of the MNs in the spinal cord slices (Fig. 4B, 4E) relative to untreated controls; however, the addition of HSSCs increased MN survival to 50% in the presence of THA (Fig. 4B, 4F) and the addition of HSSC:IGF-I significantly increased MN survival beyond that observed with HSSCs, resulting in 78% of MNs per ventral horn surviving THA treatment (Fig. 4B, 4F, 4G). Overall, these studies demonstrate that IGF-I expression offers additive benefits to the therapeutic potential of HSSCs.

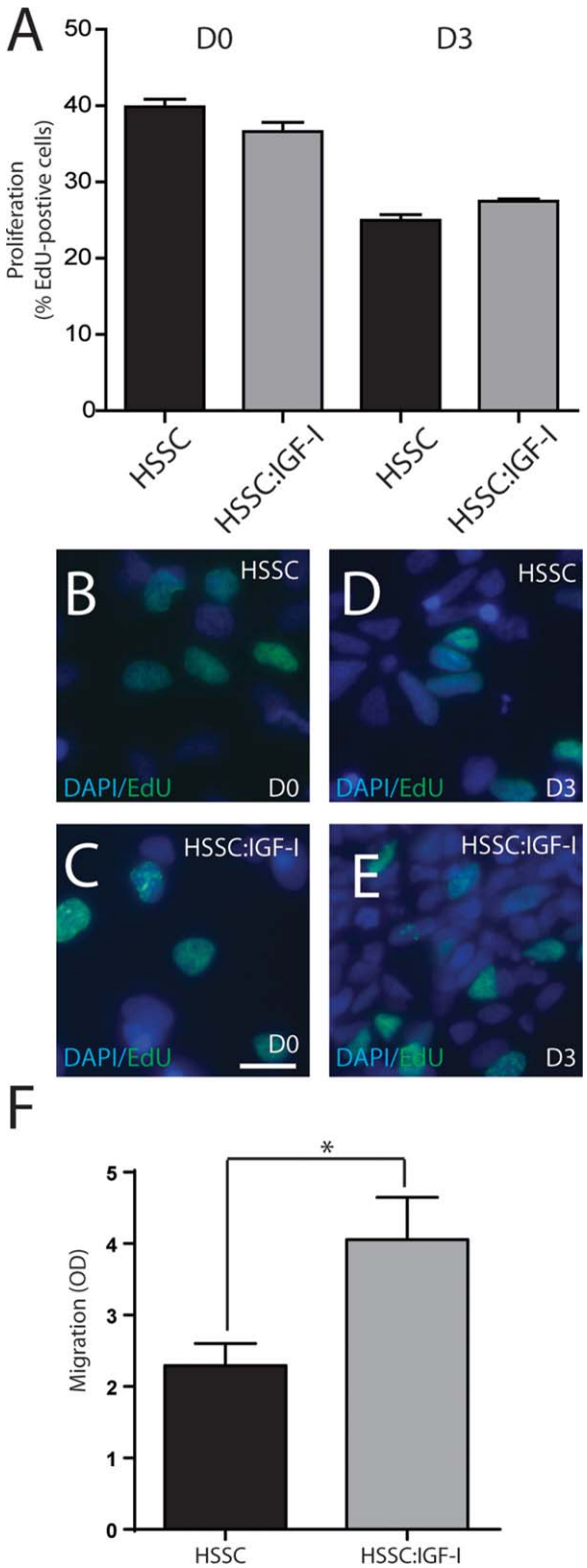

Figure 2. HSSC and HSSC:IGF-I proliferation and migration. **(A–E):** Representative images of the percentage of EdU-positive HSSCs and HSSC:IGF-I at D0 (B and C, respectively) and D3 (D and E, respectively) for measuring proliferation (A). **(F):** Relative migration levels of undifferentiated D0 HSSCs and HSSC:IGF-I, expressed as OD. Data are presented as mean + SD (*, $p < .05$) or are representative images (B–E, scale bar = 20 μm) of at least three independent experiments. Abbreviations: HSSC, human spinal stem cell; IGF-I, insulin-like growth factor-I; OD, optical density.

Discussion

ALS is a fatal, progressive neurodegenerative disease with no effective treatment. Characterized by a complex and elusive etiology that may not lend itself to conventional targeted drug development strategies, multifaceted treatments may be necessary to achieve meaningful outcomes. Stem cell-based therapies have gained momentum as a potential ALS therapy, as they offer a novel means to confer multifaceted protection [8, 12]. We recently completed a phase I clinical trial establishing the safety and feasibility of intraspinal HSSC transplantation in ALS patients, and the resulting phase II clinical trial assessing therapeutic dosing and efficacy is underway [9–13]. As we continue through the phases of this trial to establish stem cell-based therapies as a viable ALS treatment option, parallel efforts are focused on understanding the mechanisms underlying cellular therapy efficacy. This study describes the development and characterization of HSSC:IGF-I, a cell line that has the potential to offer the additional neurotrophic and neuroprotective benefits of the growth factor IGF-I. We demonstrate that HSSC:IGF-I produce robust levels of IGF-I, that this modification does not induce any detrimental alterations in cell behavior, and that IGF-I expression enhances HSSC neuroprotection against ALS insults relative to unmodified HSSCs. Together, these data support an additive role for autocrine and paracrine growth factor signaling in cellular therapy neuroprotection in ALS and suggest that the continued development of cellular therapies producing growth factors, including IGF-I, is warranted.

We contend that IGF-I production by HSSCs will confer additive benefits to the salutary effects of HSSC transplantation for ALS, as both IGF-I and HSSCs have proven efficacy in preclinical studies. HSSCs naturally produce an array of growth factors, including BDNF, GDNF, VEGF, and IGF-I, and upon intraspinal transplantation into G93A-SOD1 ALS rats, they differentiate into glutamatergic and GABAergic neurons, synapse with host MNs, and attenuate disease progression in ALS rodent models, as evidenced by delayed onset and progression rates and improved survival, especially when transplanted into multiple spinal cord regions [14–18]. Positive effects include a 27% increase in MN protection in the vicinity of HSSC grafts and transient functional improvements; however, no long-term effects on the disease course and little efficacy in regions away from the grafts, reflected in the approximate 50% decrease in total MN numbers compared to control rats and loss of descending motor tract conduction, were noted in a subsequent study [14]. Thus, despite the localized benefits of HSSC transplantation, there is a critical need to maintain neurocircuitry along the entire neuromuscular and corticospinal axis in order to achieve functional outcomes in ALS; a feat that could be achieved by autocrine and paracrine neurotrophic and neuroprotective growth factor production by cellular therapies targeting upper MNs.

Growth factors have long been considered a potential therapy for ALS, and extensive preclinical and clinical data support the utility of IGF-I in ALS [19]. In vitro studies demonstrate that IGF-I treatment activates the neuroprotective p44/42 MAPK and PI3K/Akt signaling pathways to attenuate glutamate-induced cell death in primary embryonic MN cultures [30], and viral-mediated IGF-I transfection of SHSY-5Y cells protects not only transfected cells against glutamate

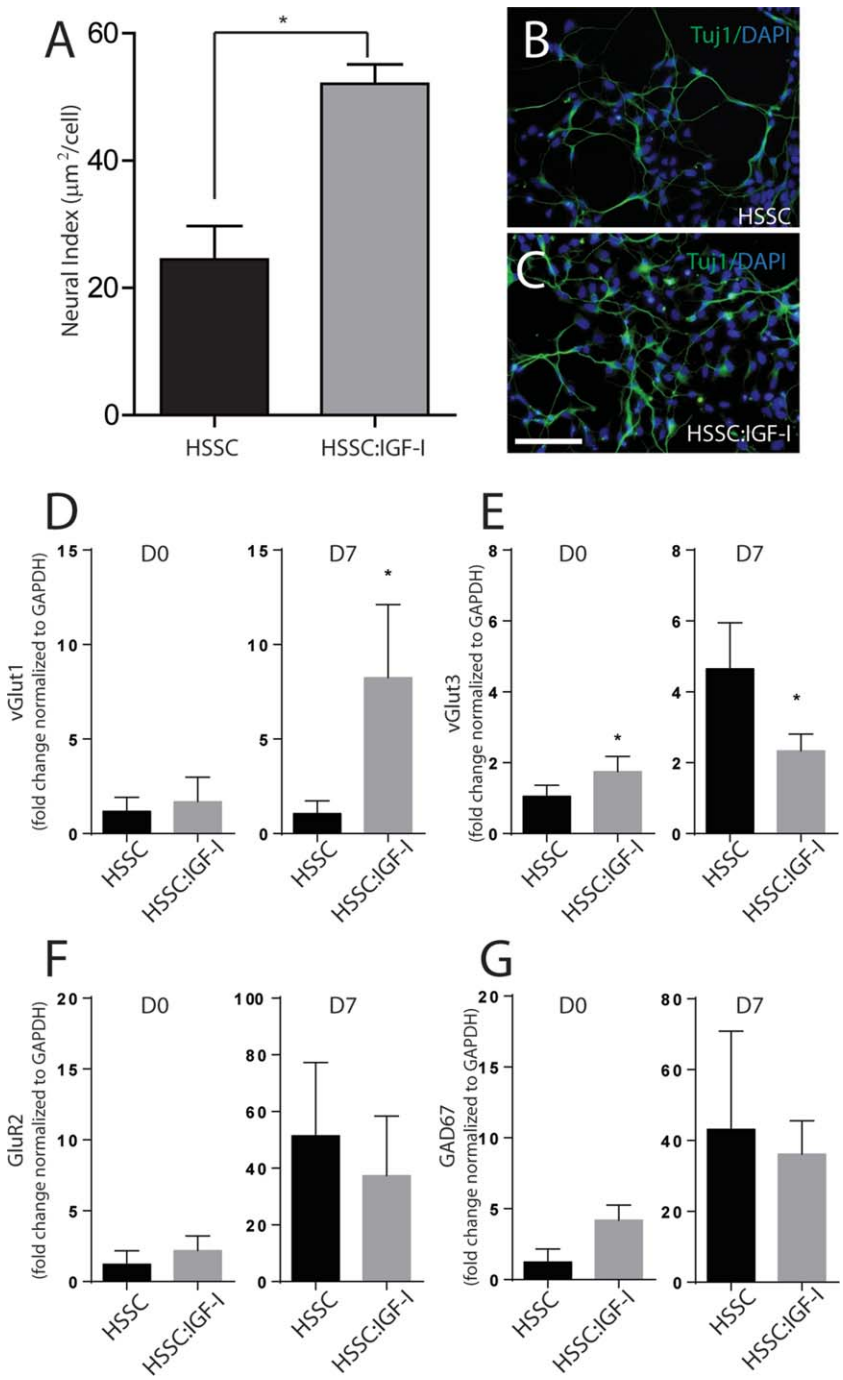

Figure 3. HSSC and HSSC:IGF-I differentiation. **(A–C):** Representative images of TUJ1 and DAPI-labeled HSSC (B) and HSSC:IGF-I (C) for calculation of the neural index (A) at D7. **(D–G):** Quantitative real-time RT-PCR (QPCR) analysis of phenotypic markers of differentiated neurons including vGlut1 (D), vGlut3 (E), GluR2 (F), and GAD (G). QPCR data were normalized to GAPDH and presented as a fold-change from D0 HSSC. Data are shown as mean + SD (*, $p < .05$) or are representative images (B, C, scale bar = 100 μm) of at least three independent experiments. Abbreviations: HSSC, human spinal stem cell; IGF-I, insulin-like growth factor-I.

toxicity but also neighboring cells, suggesting that growth factor production is capable of both autocrine and paracrine effects [28]. Numerous in vivo studies also provide further justification for IGF-I treatment in ALS, with IGF-I exerting beneficial effects on MNs and at the level of the neuromuscular junction and spinal cord microenvironment [19–22]. Furthermore, the potential clinical utility of IGF-I is further supported by the significantly reduced levels of IGF-I observed in the cerebrospinal fluid of ALS patients [4]. The lack of significant efficacy in the phase III clinical trial assessing subcutaneous IGF-I in ALS patients, however, was likely attributable to failure of IGF-I to reach vulnerable MNs following the used subcutaneous delivery approach [24, 34–36]. Notably, this limitation can be overcome using cellular therapy strategies to deliver growth factor-producing cells directly into the brain or spinal cord milieu.

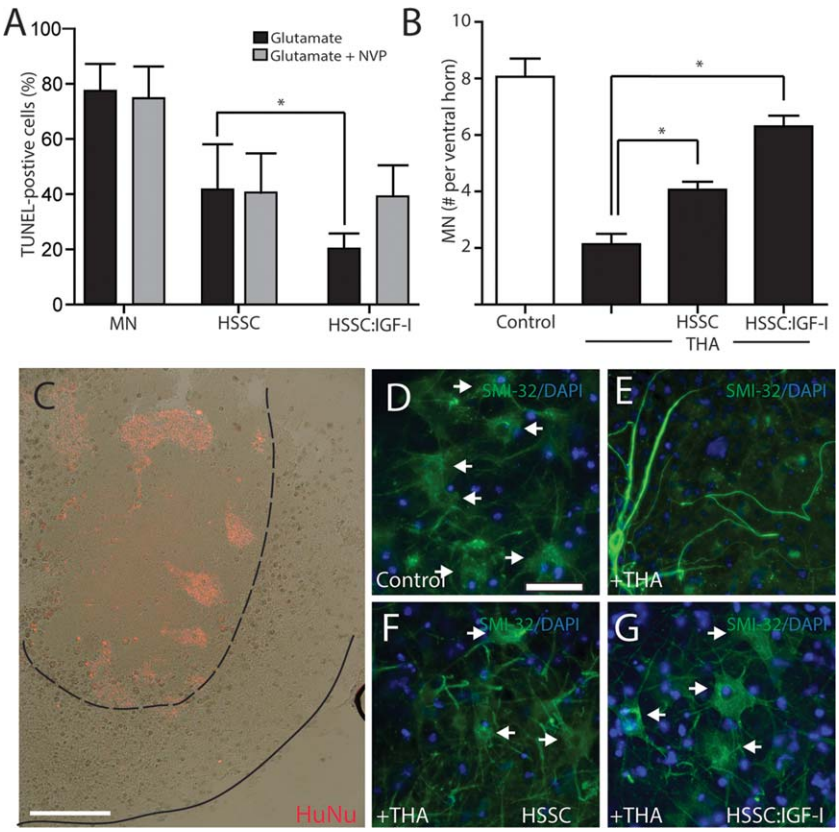

Figure 4. Neuroprotective potential of HSSCs and HSSC:IGF-I. **(A):** Quantification of MN death using the TUNEL assay following exposure to glutamate (100 μM) in the absence or presence of HSSCs or HSSC:IGF-I cocultured using transwell inserts. IGF-IR inhibition by NVPAEW541 (1 μM) was used to determine the contribution of paracrine IGF-I production to neuroprotection. Data are presented as mean + SD (*, $p < .05$ for HSSC vs. HSSC:IGF-I). **(B–G):** Quantification of MN survival (B) in rat spinal cord organotypic slices directly cocultured with HSSCs or HSSC:IGF-I following treatment with THA (100 μM) to induce toxicity. Representative images confirming of the presence of HuNu-positive HSSCs cultured on top of spinal slices (C; dashed line delineates the outline of the ventral horn, solid line delineates the extent of the white matter) and of ChAT/SMI-32 MN staining in the ventral horns of a nontreated control slice (D), a THA-treated slice (E), a THA-treated slice cocultured with HSSCs (F), and a THA-treated slice cocultured with HSSC:IGF-I (G). Arrows depict representative MNs within the spinal cord slices. Data are presented as mean + SD (*, $p < .05$ vs. slice + THA) or are representative images (C, scale bar = 100 μm; D, scale bar = 50 μm) of at least three independent experiments. Abbreviations: HSSC, human spinal stem cell; IGF-I, insulin-like growth factor-I; MN, motor neuron; THA, threo-hydroxyaspartate.

In this study, we engineered HSSCs to produce increased levels of IGF-I to establish HSSC:IGF-I as a novel tool to evaluate the potential effects of IGF-I expression in neuroprotection. These cells exhibit a sixfold increase in IGF-I in both their progenitor cell state and following differentiation, a finding consistent with previous reports demonstrating 6.4-fold increases in IGF-I production following lentiviral transfection of postnatal subventricular zone neural progenitor cells [37]. In addition to increasing IGF-I expression, we also identified increased levels of GDNF following differentiation in HSSC:IGF-I, an observation supporting the premise that neurotrophic factor production may offer additive benefits to HSSC efficacy. While we have previously demonstrated that HSSCs are an incredibly safe cell line [9, 11, 13, 14], IGF-I has the potential to increase cell proliferation and impact migration [37–41]; therefore, we verified that enhancing IGF-I expression in HSSCs does not adversely alter cell behavior. In line with our previous studies assessing the impact of exogenous IGF-I on HSSC behavior, this data indicate that while HSSC:IGF-I express the IGF-IR, IGF-I does not act as a mitogen for HSSC, thus confirming the inherent safety profile of HSSC:IGF-I for future translational applications. Specifically, we see no significant increases in HSSC:IGF-I proliferation at any time point, a finding that may likely be attributed to the reduced expression of IGF-IR observed in HSSC:IGF-I relative to HSSCs. In addition, we see no notable migration of HSSC:IGF-I following differentiation. This is consistent with our in vivo experiences demonstrating that HSSCs only migrate to a minimal extent following intraspinal transplantation, with the majority of cells remaining in a large bolus at the injection site [14, 42, 43]. For HSSC:IGF-I cells, however, the observed increase in migratory potential prior to differentiation may be advantageous, whereby lower numbers of intraspinal injections may be sufficient achieve adequate coverage across the desired spinal cord segments [16, 43, 44]. Together, these behavioral HSSC:IGF-I characteristics support their utilization to assess the potential additive contributions of IGF-I production to HSSC-mediated neuroprotection.

Growth factor expression alters the differentiation profile of umbilical cord stem cells [45]; therefore, we examined how IGF-I affects terminal differentiation in HSSCs. In line with previous reports and our initial studies using exogenous IGF-I [17, 18, 27], HSSC:IGF-I have a higher neural index than HSSCs, but the majority of terminally differentiated cell types

remain similar between the lines. These findings correlate with other studies demonstrating increased TUJ1 expression in response to IGF-I overexpression in differentiating neural progenitor cells [37]. Furthermore, no differences in astrocyte or oligodendrocyte ratios were seen between cell lines, and both glutamatergic and GABAergic neurons were predominately represented. Given the reported loss of inhibitory interneurons and the critical need to maintain neurocircuitry along the entire neuromuscular axis [14, 46], the generation of glutamatergic and GABAergic neurons further validates the therapeutic potential of HSSCs in ALS. Thus, the observed differentiation profile of HSSC:IGF-I maintains that IGF-I production by HSSCs can support MNs and also provide critical infrastructure to spinal cord neurocircuitry.

As further shown by these studies, HSSC:IGF-I confer neuroprotection via both direct and indirect mechanisms, suggesting that IGF-I may enhance HSSC cell contact-mediated neuroprotection as well as elicit paracrine effects on MNs and surrounding cells within the spinal cord microenvironment through secreted factors. Notably, the increased levels of IGF-I likely underlie the enhanced protection conferred by HSSC:IGF-I, as evidenced by both the increased protective capacity of HSSC:IGF-I relative to HSSCs, as well as the attenuated protection in the presence of IGF-IR inhibition. Similar to this approach, gene therapy has been used to develop a number of stem cell-based combinatorial cellular therapy modalities expressing neuroprotective factors for ALS [12]. Intracerebroventricular injection of mesenchymal stem cells expressing the antioxidant protein GLP-1 into G93A-SOD1 mice improved disease onset, motor performance, and survival [47], and modifying mesenchymal stem cells to express GDNF and VEGF provided neuroprotection in G93A-SOD1 rats [48, 49]. GDNF-expressing glial progenitors also protected MNs following intraspinal transplantation in G93A-SOD1 rats; however, protection was not seen with unmodified cells [50]. These studies support a similar role for autocrine and paracrine IGF-I production as a means to augment the therapeutic potential of HSSCs in ALS [14, 16, 27, 48].

Conclusions

Extensive preclinical data justify the therapeutic use of HSSCs and IGF-I individually [8, 12, 19] and the safety of both individual treatment strategies has been established in clinical trials [11, 24]. Most recently, the successful completion of our phase I trial examining intraspinal HSSC transplantation in ALS patients has paved the way for the ongoing phase II trial assessing the therapeutic dosing and initial efficacy of this groundbreaking treatment. In parallel, our quest to elucidate how IGF-I expression influences cellular therapy-mediated neuroprotection in ALS prompted the derivation of HSSC:IGF-I. We demonstrate that IGF-I expression does not enhance cell proliferation, but does increase the early migratory capacity, neural differentiation, and neuroprotective potential of HSSC:IGF-I. These data support IGF-I-mediated neurotrophism as a means to achieve additive neuroprotection, and suggest that transplantation of HSSC:IGF-I in addition to this HSSC therapeutic approach may offer further MN protection in ALS. Notably, transplantation of HSSC:IGF-I into the brain has the potential to confer protection to upper MNs by harnessing the stable, permanent expression of diffusible IGF-I within the central nervous system, thus achieving critical neuroprotection at multiple sites along the neuromuscular axis. With continued progress through our ongoing clinical trial using HSSCs, along with the completion of future studies establishing the feasibility and efficacy of growth factor-producing cellular therapies in ALS models, we have incredible potential to establish an effective, multifaceted MN protection strategy for ALS, and potentially for other neurological disease applications as well.

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Author Contributions

J.S.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; S.A.S.: data analysis and interpretation, manuscript writing, and final approval of manuscript; L.M.M.: collection and/or assembly of data, data analysis and interpretation, and final approval of manuscript; C.P.: collection and/or assembly of data and final approval of manuscript; T.G.H. and K.J.: provision of study material or patients and final approval of manuscript; E.L.F.: conception and design, financial support, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

T.G.H. and K.J. are employees of Neuralstem, Inc. E.L.F. is an unpaid consultant to Neuralstem, Inc. All other authors have nothing to declare.

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