Research report

Co-transplantation of mesenchymal and neural stem cells and overexpressing stromal-derived factor-1 for treating spinal cord injury

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Abstract

Genetic engineering of mesenchymal stem cells (MSCs) and neuronal stem cells (NSCs) has been used to treat spinal cord injuries (SCI). As a mechanism of therapy, MSCs secrete high amounts of trophic factors, while NSCs can differentiate into neuronal lineages and aid in tissue replacement. Additionally, the forced overexpression of secreted proteins can enhance the secretome of transplanted cells, which can increase therapeutic efficacy. This study utilized a combinational treatment consisting of MSCs, NSCs, and the forced overexpression of the chemokine stromal-derived factor-1 (SDF-1) from MSCs (SDF-1-MSCs) as treatment in a rat model of SCI. Transplants occurred at 9-days post-injury, and motor functions were evaluated for 7-weeks post-injury. White matter sparing and axon densities surrounding the lesions were quantified. Findings from this study demonstrate that co-transplanting SDF-1-MSCs with NSCs improved motor functions and enhanced axon densities surrounding the lesion. However, no improvements in white matter sparing were found and tumors were found in some of the animals that received co-transplantations with either SDF-1-MSCs and NSCs or unmodified-MSCs and NSCs, but not in any animal treated with a single cell type. This study offers evidence that providing SDF-1 to NSCs, through the forced expression from MSCs, can enhance the therapeutic potential of the graft, but developing a safe means of doing this requires further work.

1. Introduction

Deficits arising from spinal cord injuries (SCI) result in permanent disability, and no treatment options are available to restore motor functions. Cellular replacement therapies have potential as treatment, as positive pre-clinical outcomes have been found in several models of central nervous system (CNS) dysfunction (Abrams et al., 2009; Crane et al., 2014; Kumagai et al., 2013; Kumamaru et al., 2013; Lu et al., 2005). The use of different cell sources, such as mesenchymal stem cells (MSCs) or neural stem cells (NSCs), contain unique properties that offer different therapeutic mechanisms for promoting repair when transplanted into an injured spinal cord (Li and Lepski, 2013; Yasuda et al., 2011). MSCs have also demonstrated abilities to secrete high quantities of neurotrophic factors, which promote tissue survival in compromised environments (Abrams et al., 2009; Crane et al., 2014; Ropper et al., 2017; Rossignol et al., 2009; Song et al., 2014). Transplantations of NSCs, unlike transplanting MSCs, more readily differentiate into CNS lineages that can functionally integrate into the neural architecture (Nishimura et al., 2013; Yasuda et al., 2011).

Abbreviations: BBB, Basso, Beattie, and Bresnahan scale for locomotor recovery; CXCR4, chemokine receptor-4; GFP, green fluorescent protein; MSCs, mesenchymal stem cells; NF-70, neurofilament-70kD; NSCs, neuronal stem cells; SCI, spinal cord injury; SDF-1, stromal derived factor-1; tdTomato, fluorescent tandem-dimer tomato protein.

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Given that both trophic support and CNS lineage differentiation can improve behavioral outcomes post-SCI, co-transplantation with MSCs and NSCs may offer a potential synergistic effect. This was supported in vitro, as co-culturing MSCs and NSCs will enhance stemness of NSCs (Haragopal et al., 2015). This suggests that co-transplantation of MSCs and NSCs may function synergistically to further improve functional outcomes when used to treat SCI.

When MSCs are used as a treatment for SCI, the trophic secretions have been attributed as the primary mechanism underlying the therapeutic effects. Efforts to further enhance these secretions are made by forcing the overexpression of key trophic factors from the transplanted cells (Crane et al., 2014; Dey et al., 2010; Kumagai et al., 2013; Lu et al., 2005; Sasaki et al., 2009; Shang et al., 2011; Taylor et al., 2006). In this study, stromal derived factor-1 (SDF-1) was overexpressed in MSCs and its therapeutic effects were compared with unaltered MSCs, when co-transplanted with NSCs. SDF-1 is a small-secreted chemokine that acts on both the chemokine receptor-4 and 7 (CXCR4 and CXCR7; Li and Ransohoff, 2008; Lieberam et al., 2005; Luker et al., 2012) and has been linked to increases in mitogen signaling, cellular survivability, cytoskeletal re-arrangement and cellular motility/migration on cells that possess the CXCR4/CXCR7 receptors, such as NSCs (Gong et al., 2006; Holgado et al., 2013; Krathwohl and Kaiser, 2004; Lopez-Ilasaca et al., 1997; Merino et al., 2015; Vila-Coro et al., 1999; Wu et al., 2009). The SDF-1/CXCR4 axis plays a critical role in the ongoing neurogenesis of the olfactory system and repair processes in response to injury, including axon growth and regeneration (Bodea et al., 2014; Chalasani et al., 2003; Dziembowska et al., 2005; Filippo et al., 2013; Li and Ransohoff, 2008; Lieberam et al., 2005; Orimo et al., 2005; Toba et al., 2008; Xue et al., 2014; Zendedel et al., 2012). These interactions suggest that a co-therapy using SDF-1 and NSCs could further improve outcomes after SCI specifically through the influence of SDF-1 on the transplanted NSCs. For this study, SDF-1 was overexpressed from MSCs, which we hypothesize will modulate the local environment, and provide a source of its constitutive release to support the NSC graft.

We co-transplanted embryonic NSCs with either bone marrow-derived MSCs (MSC/NSCs) or MSCs that were engineered to over-express SDF-1 (SDF-1/MSC/NSCs). These groups were compared with rats given transplants of NSCs-only, MSCs-only, or SDF-1-MSCs-only, as potential treatments for SCI. Behavioral measures utilized the Basso, Beattie and Bresnahan (BBB) scale for locomotor recovery (Basso et al., 1995) to assess functional abilities weekly for 7-weeks post-injury. The viability of transplanted NSCs was assessed using in vivo bioluminescence imaging. Immunohistochemistry was performed on tissue sections to identify NSC survival, white-matter pathology, and spared axonal fibers surrounding the lesion.

2. Results

2.1. Immunocytochemistry and characterization of MSCs and NSCs by flow cytometry

Prior to transplantation, NSCs were analyzed for the expression of CXCR4 (Fig. 1). This NSC cell line demonstrated positive labeling for both the neuronal stem cell marker, Nestin, as well as CXCR4, confirming that this cell line possesses the receptor to SDF-1. Both Nestin and CXCR4 labeling were found to be localized in the soma and in the developing neurites. Next, MSCs were transduced with a gene coding for the green fluorescent protein (GFP), while NSCs were transduced with a gene coding for the tandem-dimer Tomato fluorescent protein (tdTomato) as well as the firefly luciferase protein, to allow for evaluating the survivability of the cells post-transplantation.

Characterization of MSCs with flow cytometry revealed positive expression for GFP (52.2%), CD90 (95.3%), CD44 (90.0%), CD105 (76.3%), and CXCR4 (92.8%), while negative expression for CD34 (21.0%), CD11b (8.1%), and MHC-II (26.2%). Similarly, SDF-1-MSCs labeled positive for GFP (85.9%), CD90 (98.5%), CD44 (91.2%), CD105 (87.9%), and CXCR4 (88.6%), and negative expression for CD34 (15.6%), CD11b (5.5%), and MHC-II (28.2%). NSCs labeled positive for tdTomato (92.1%), Nestin (96.8%), Sox-2 (88.0%), CD133 (87.99%), and CXCR4 (74.68%). Together, this data suggests that the MSCs and SDF-1-MSCs express traditional MSC markers, while the NSCs express neural lineage stem cell markers (Fig. 2).

To evaluate the ability of MSCs or SDF-1-MSCs to enhance the stemness of NSCs in vitro, co-cultures were performed in media conditions that restricted growth factors, to encourage spontaneous differentiation of NSCs. Following 1-week of culturing NSCs without growth factors, a reduction in the expression of stem cell markers was found for Nestin (2%), Sox-2 (15.3%) and CD133 (5.2%), whereas the expression of CXCR4 remained positive, with only mild reductions (40.7%) compared to NSCs maintained in growth factors. Co-culturing NSCs with either SDF-1-MSCs or GFP-MSCs preserved the expression of Sox-2 (57.7% and 48.4% respectively) within the NSC population. Only mild increases in the expression of Nestin (10.0% and 11.6%), CD133 (12.7% and 11.7%), and CXCR4 (50.8% and 47.2%) were found to be correlated with tdTomato in the co-cultured samples. These results suggest that removing growth factors from the media allows NSCs to begin to differentiate, and that the paracrine effects from the co-cultured MSCs or SDF-1-MSCs can partially rescue the stem cell potential of the NSCs (Fig. 3).

2.2. In vitro reporter gene expression and in vivo confirmation of neurosphere transplantation

In order to evaluate for NSC viability, post-transplantation, NSCs were transfected with the fluorescent reporter gene coding for tdTomato, as well as the firefly luciferase gene for in vivo imaging. Following transfection, a clonal selection was performed to establish a homogenously expressing cell line (Fig. 4A–C). MSCs were similarly transfected with a retrovirus expressing a gene that produces the green fluorescent protein (GFP) alone, or a gene that produces the poly-cystic expression of both SDF-1 and GFP. Successfully transfected MSCs were purified using an antibiotic selection with puromycin.

The successful transplantation of these NSC neurospheres was confirmed using in vivo bioluminescent imaging. Bioluminescent signals observed at 24-h, 1-, and 3-weeks post-transplantation suggest that the transplantation was successful, and that the graft is capable of surviving for at least 3 weeks in vivo (Fig. 4D–F). Further imaging was not performed due to the low bioluminescent signal from the graft at 3 weeks post-transplantation, so the final analysis of stem cell viability was performed using immunohistochemistry.

2.3. Behavioral analysis

A repeated-measures analysis of variance (ANOVA) was used as an overall outcome measure for the 7 weeks of BBB testing, and revealed significant between-group differences (F(6,75) = 16.12, p < 0.001). Fisher’s protected least significant differences (PLSD) post-hoc analysis was used for the repeated measures ANOVA and revealed differences between the SDF-1-MSC/NSC- and vehicle-treated rats (p < 0.05), as well as between the non-injured controls and all other groups (p < 0.001). To determine
when significant differences were found between groups, individ-
ual Kruskal-Wallis ANOVAs were ran at each time-point post-
injury, and revealed between-group differences at every week
\( p < 0.05 \). Pair-wise comparisons between experimental groups
were done using individual Mann-Whitney \( U \) tests, which revealed
significant differences between SDF-1-MSC/NSC- and vehicle-
treated rats at 3- \( (U = 46, p < 0.05) \), 4- \( (U = 41, p < 0.05) \), 6-
\( (U = 40.5, p < 0.05) \), and 7-weeks post-injury \( (U = 37, p < 0.05) \). Dif-
fences were also found between SDF-1-MSC/NSC- and SDF-1-
MSC-treated rats at 7-weeks post-injury \( (U = 35.50, p < 0.05) \).
Fig. 3. Flow cytometry analysis of MSC and NSC Co-culture. NSCs were cultured without growth factors for 1-week to promote differentiation, and were analyzed with flow cytometry for neuronal lineage markers when grown either alone, in co-culture with SDF-1-MSCs or in co-culture with GFP-MSCs. The characterized populations were compared to NSCs grown in NSC growth media containing growth factors. The NSC population was analyzed for positive co-labeling against tdTomato and Nestin, Sox-2, CD133, and CXCR4. Following 1-week of co-culture a decrease in Nestin, Sox-2, and CD133 was observed, however co-culturing NSCs with either SDF-1-MSCs or GFP-MSCs partially preserved the expression of Sox-2 in the NSC population.
Fig. 4. NSC expression of tdTomato and luciferase. NSCs were transfected to express tdTomato (A–C) and luciferase for in vivo detection following injections with D-luciferin (D–F). Bright-field imaging of neurospheres (A), with fluorescent imaging using filtered light at 550 nm (B), revealed a homogenous tdTomato expression in the transplanted cells. These tdTomato-expressing NSCs could be detected in vivo for up to 3 weeks (D–F), with little change in bioluminescent expression between 1- (D), and 7-days (E), but a noticeable drop between 7- and 21-days (F), post-transplantation. Scale bar represents 100 μm.

(Fig. 5). No other treatment group showed significant improvements over vehicle controls. Significant differences were observed between non-injured controls and all other treatment groups at every week post-injury ($p < 0.001$).
As will be discussed later, 2 rats in the SDF-1-MSC/NSC treatment group, and 3 rats in the MSC/NSC treatment group developed tumors. Surprisingly, only 1 rat in the MSC/NSC treatment group showed behavioral abnormalities that could be attributed to the aberrant growth in the spinal cord. This rat dropped 5 BBB points between week 5 and week 6 post-injury, from a score of 14 to a score of 9, and maintained this level of function until euthanasia. All other rats that developed tumors maintained normal patterns of functional recovery until 7-weeks. The behavioral statistics were ran again with the removal of this one rat, however still no significant improvements could be found between the MSC/NSC- and vehicle-treated rats.

2.4. White matter sparing

Cross sections at 13 locations surrounding the lesion epicenter were stained with eirochrome cyanine, and the area of spared white matter surrounding the lesion was measured. The lesion epicenter was defined as the section containing the least spared white matter in each spinal cord, and the sections were aligned with respect to this objectively defined position. A repeated-measures ANOVA was used to evaluate the area of white matter sparing and found no significant between-group differences ($p = 0.714$; Figs. 6 and 7).

2.5. Neurofilament around lesion

Cross-sections were labeled using immunohistochemistry against the neurofilament-70 kD (NF-70) axonal protein for quantifying axon densities surrounding the lesion. A one-way ANOVA was used to compare positive pixel staining for NF-70 labeled sections, and revealed significant between-group differences on measures taken from the white matter near the anterior horn ($F(5,35) = 6.07, p < 0.001$), as well as near the rubrospinal tract ($F(5,35) = 3.626, p < 0.05$). Fisher’s PLSD revealed that labeling in SDF-1-MSC/NSC-treated rats and NSC-only-treated rats were significantly different from that of every other SCI group, for measures taken at the anterior horn and for measures taken near the rubrospinal tract (Figs. 8 and 9).

2.6. Tumor formation and cell survivability

Unexpectedly, tumors were observed in the spinal cords of two of the rats that received SDF-1-MSC/NSC-co-transplants and three of the rats that received MSC/NSC-co-transplants. However, no tumors were found in any other treatment group, including the NSC-only, MSC-only, or SDF-1-MSC-only groups. Positive staining for both GFP and tdTomato was found in the tumor mass. However, the bulk of the mass labeled positive for NF-70 and glial fibrillary acidic protein (GFAP), leading to the interpretation that the tumors originated from the NSCs, and only when co-transplanted with MSCs (Figs. 10 and 11). Interestingly, no immunohistochemical labeling against GFP was detected, nor was any tdTomato expression, in any animal except those animals that developed tumors. This suggests a poor viability of the transplant at 7-weeks post-injury.

2.7. Correlations of outcome measures

A two-tailed Pearson’s correlation was used to evaluate the relationships between the 7-week end point BBB scores and the labeling of NF-70 images from the anterior horn and the rubrospinal tract regions, as well as for the area of white matter sparing at the lesion epicenter. Significant positive correlations were found.

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Fig. 6. White matte sparing. No treatment resulted in a sparing effect of white matter. Cross sections were stained with eirochrome cyanine, and the area of white matter was measured for each slice. The slice with the least area was determined as the lesion center and sections were aligned accordingly in the rostral-caudal direction.
between the end point BBB scores and the white matter sparing ($R = 0.372, p < 0.05$), as well as the images taken near the rubrospinal tract ($R = 0.320, p < 0.05$), but not for images taken from the anterior horn ($R = 0.209, p = 0.22$).

3. Discussion

The major findings from this study are: (1) only the co-transplantation with SDF-1-MSC/NSCs significantly enhanced behavioral function when compared to vehicle controls; (2) only the SDF-1-MSC/NSCs and NSCs-alone treatment groups showed sparing of NF-70 axonal fibers surrounding the lesion; and (3) transplantation of SDF-1-MSC/NSCs or MSC/NSCs increased the risk of forming tumors.

Only transplants of SDF-1-MSC/NSCs conferred significant improvements in motor functions relative to vehicle-treated controls. However, the combinational treatment of SDF-1-MSC/NSCs did not improve BBB scores beyond those of other treated SCI groups, except for the SDF-1-MSC-only-treated rats. This finding suggests that the effects derived from this combinational therapy are a result of providing SDF-1 to the NSC transplant, rather than to MSC transplants, or by combining NSCs with unaltered MSCs.

Testing motor functions in severe SCI rat models is complicated by a lack of suitable and objective behavioral assays. In order to effectively use other behavioral outcomes, such as the horizontal ladder foot slip analysis, balance beams, cat-walk, foot print analysis, and/or rotarod, it is necessary for all the rats in the study to be capable of performing consistent weight-supported stepping. Rats that are not capable of consistent weight supported stepping will often utilize compensatory behaviors that can result in false outcomes. For example, rats unable to perform weight-supported stepping may straddle a balance beam and traverse further distances compared to the rats that attempt to weight support but still possess a lack of trunk stability during gait. Similarly, rats that
Finding a significant correlation between BBB scores at end-point and NF-70 labeling at the ventral-corticospinal tract and the rubrospinal tract, because NF-70 in these regions suggests a possible mechanism, whereby the transplanted cells within the lesion site. In our previous study with MSC/NSC co-transplants, both the MSCs and the NSCs were limited to just four collections of lateral-corticospinal-, rubrospinal-, raphespinal-, as well as reticulospinal-tract fibers in rats. Previous research has demonstrated the importance of descending fibers, specifically derived from the raphespinal tract, for promoting motor functional recovery after SCI (Ropper et al., 2017). Finding a significant correlation between BBB scores and NF-70 labeling at the rubrospinal tract regions holds particular significance due to the cluster of descending motor axons in this region of the spinal cord. This generalized region of the white matter in the spinal cord holds collections of lateral-corticospinal-, rubrospinal-, raphespinal-, as well as reticulospinal-tract fibers in rats. Previous research has demonstrated the importance of descending fibers, specifically derived from the raphespinal tract, for promoting motor functional recovery after SCI (Ropper et al., 2017). Finding a significant correlation between BBB scores and NF-70 labeling at the rubrospinal tract regions, but not near anterior horn, which is more involved in trunk stability, is consistent with the importance of sparing motor fibers for improving locomotor abilities. However, it is important to note that this study was unable to distinguish between axons that have been spared from those which are sprouting in response to the transplanted cells within the lesion site.

Although it is difficult to discern whether the presence of SDF-1 on the NSCs was most critical in improving function, the combination of the SDF-1-MSCs and NSCs expanded the therapeutic reach beyond what either of these provided individually. While it is possible that the delayed transplant surgery may have also delayed the therapeutic effect, a clear effect of the SDF-1 on the co-transplanted animals was observed at 12-days post-transplant. The ability for SDF-1 to promote survival and function of NSCs, in vivo, has been well characterized in previous studies (Addington et al., 2014; Filippo et al., 2013; Kokovay et al., 2010; Merino et al., 2015; Peng et al., 2012), yet the inability to detect surviving cells at 6-weeks post-transplantation leaves the therapeutic mechanisms in question. When provided acutely following injury, a greater amount of trophic secretions, such as the SDF-1 released from our engineered MSCs, can aid in suppressing the propagation of necrosis that occurs following an SCI (Kritsakiarana et al., 2016). The finding that SDF-1-MSC/NSC-treated rats contained more NF-70 labeling surrounding the lesion is consistent with this hypothesis that the therapeutic effects may have been derived from trophic secretions that either enhanced sprouting of spared fibers around the lesion, or preserved fibers from being lost due to secondary injury cascades. If this proves to be the case, this combinational therapy may be further enhanced by using immunosuppression to reduce rejection of transplanted cells, which would further take advantage of cellular replacement as a mechanism of intervention. In order to promote better viability of the NSC transplant, NSCs were transplanted as neurospheres, instead of in single cell suspension. Although this transplantation strategy may improve viability, a question remains regarding whether the transplantation of neurospheres, rather than single cell suspensions, could have contributed to the formation of tumors found in this study.

The development of tumors in some animals that received co-transplantations of either MSC/NSCs (in 3 of 11 rats) or SDF-1-MSC/NSCs (in 2 of 11 rats) is of concern. These tumors were analyzed using immunohistochemistry against both neuronal markers NF-70 and GFAP, as well as for the fluorescent markers distinguishing the MSCs (GFP) from NSCs (tdTomato) in the graft. Although GFP expression was detected within the tumor, the bulk of the tumors stained positive for NF-70, as well as GFAP. Additionally, in rats that developed tumors following co-transplants with the fluorescently labeled NSCs, tdTomato expression was found within, and at the margins of the growth. As tumors were only found in co-transplanted animals, and were labeled predominantly with neuronal lineage markers, it is hypothesized that they formed as a result of a complex interaction between the MSCs and NSCs, in vivo, and also that the source of the uncontrolled growth was likely coming from the transplanted NSCs. This was a surprise finding, since no tumors were observed in previous studies from our lab using co-transplantations of MSCs and NSCs into the striatum of rats (Rossignol et al., 2014).

The use of NSCs from embryonic tissue, and the relatively high passage number from both the NSCs and MSCs that were used in this study may have contributed to the formation of tumors. Prolonged time spent in culture has been associated with chromosomal instabilities of stem cells, which may increase the likelihood of developing tumors (Foudah et al., 2009; Moon et al., 2011; Redaelli et al., 2012). In our previous study with MSC/NSC co-transplants, both the MSCs and the NSCs were limited to just four passages prior to transplantation (Rossignol et al., 2014). Alternatively, elevated levels of reactive-oxygen species (ROS), such as those produced following SCI (Jia et al., 2012; Zhang et al., 2016) have been linked to an increase in chromosomal damage and possible transition of healthy cells into oncogenic cells (Mahalingaiah et al., 2015; Song et al., 2016). Again, our previous study involved the use of rats carrying the transgene for Huntington’s disease.
which may not have the same levels of ROS as observed in rat models of SCI. The risks of developing tumors from co-transplantation strategies may not be comparable between different neurotraumatic and neurodegenerative conditions. Clearly, these issues need further study before co-transplantations of MSCs with NSCs can translate into safe and effective therapies.

Of similar concern is the oncogenic potential following the use of genetic manipulations to the transplanted stem cells. This study utilized a genetic engineering approach to introduce SDF-1 to the transplanted MSCs and NSCs for two reasons. First, overexpression from the transplanted cells can provide a constitutive expression of the SDF-1. As well, the transplantation of the MSCs that overexpress SDF-1 allows for the local administration, which can provide intra-tissue gradients, and more selectively modulate the immediate transplantation environment. It should be emphasized that the use of high-passage cells, or the use of genetic manipulations to overexpress SDF-1, GFP, or tdTomato, was not enough to promote tumors following transplantation, as transplants of SDF-1-MSCs-only, MSCs-only, or NSCs-only were tumor-free. Similarly, it should be emphasized that providing SDF-1 to NSCs did not increase the frequency of tumor formation and that the functional and histological outcomes from this study should be taken as separate and independent findings. It is unlikely that the tumor formation in this study is linked to the combination of SDF-1 with NSCs, but is probably more related to the co-transplantation of MSCs with NSCs following SCI.

Fig. 9. NF-70 labeling. NF-70 labeled sections were imaged using a 20× objective positioned over a 500 × 500 μm square section of tissue. The measures were taken bilaterally from the white matter near the anterior horn and near the rubrospinal tract. Positively labeled pixels, which exceeded an empirically established threshold, were counted. Images are representative for each group, and indicate increased staining in the NSC-only and SDF-1-MSC/NSC groups. Scale bar represents 100 μm.
Insights into the potential mechanism behind the tumors found in this work can be derived from the flow cytometry experiment performed in this study. The results from the flow cytometry experiment found that co-culturing NSCs with either MSCs or SDF-1-MSCs had a preservative effect on Sox-2 expression, which was otherwise decreased when NSCs were cultured in conditions that promote differentiation. Sox-2 is a transcription factor associated with the maintenance of potency in stem cell populations (Ellis et al., 2004; Liu et al., 2013; Pevny and Nicolis, 2010; Vinci et al., 2016; Zhou et al., 2016). As such, it may be possible that co-transplanting MSCs with NSCs functioned to maintain the potency of the grafted NSCs, resulting in a continual proliferation of stem cells, rather than a malignant transformation of the transplant. One of the primary functions of endogenous MSCs in the bone-marrow is to support the haematopoetic stem cell niche by keeping haematopoetic stem cells alive, quiescent, and capable of mitotic activity (Greenbaum et al., 2013; Méndez-Ferrer et al., 2010; Walenda et al., 2010). This endogenous function of MSCs is provided through the high quantities of secreted trophic factors, growth factors, cytokines and chemokines by these cells (Greenbaum et al., 2013; Méndez-Ferrer et al., 2010; Walenda et al., 2010). Not coincidentally, these properties are what attract the use of MSCs as a therapy for treating SCI, as this secretome can have restorative effects on the nervous system following trauma (Abrams et al., 2009; Shang et al., 2011; Song et al., 2014). The transplantation of MSCs into the spinal cord likely induced a microenvironment similar to that produced in endogenous conditions. However, the receptiveness of the co-

![Fig. 10. Tumors found in co-transplantation groups. Cross sections of injured spinal cords were stained with 100% hematoxylin and 0.1% stock of eosin, which revealed substantial atrophy at regions closer to the lesion in NSC-only- and MSC-only-treated rats. However, in the animals that developed tumors following co-transplantation with either MSC/NSCs or SDF-1-MSC/NSCs, the cross sectional diameter increased, demonstrating a neoplastic gross morphology (A). The boundaries of the tumor show a dense growth, which contrasts with the less-clearly defined lesion boundaries in NSC-only or MSC-only-treated rats. Within the lesion, the tumor tissue contained a larger nucleus-cytoplasm ratio, a characteristic of mutated and tumorigenic tissue, while this ratio is normalized within NSC-only and MSC-only transplant lesions (B). Scale bar represents 2 mm (A).](image-url)
transplanted NSCs to this permissive environment may have interacted to produce uncontrolled proliferation in some of the co-transplanted animals. Although further study is needed to fully understand the etiology of the observed tumors, our findings do point to an important rule: when using living biological tissue as a therapy, aggressive combinational approaches to treatment may increase both the therapeutic efficacy, but also an increased risk of forming tumors.

3.1 Conclusions

In conclusion, the results of this study suggest that providing SDF-1 to transplanted NSCs, through the forced overexpression from co-transplanted MSCs, can enhance the therapeutic efficacy of the graft. However, future work needs to improve upon methods to enhance the viability of the transplanted cells, while also limiting the risk of unwanted cell proliferation.

4. Experimental procedure

4.1 Study design

Five experimental groups, a vehicle-control group, and a non-injured control group, were analyzed for behavioral outcomes every week for 7-weeks post-injury. Groups contained either SDF-1-MSCs with NSCs (SDF-1-MSC/NSCs, n = 11), MSCs with NSCs (MSC/NSCs, n = 11), NSCs alone (NSCs-only, n = 11), SDF-1-MSCs
alone (SDF-1-MSCs, n = 14), MSCs alone (MSCs-only, n = 14), HBSS for vehicle-controls (n = 16), or non-injured controls (n = 5).

Animals received transplantsations into the lesion epicenter 9 days after receiving a contusion spinal cord injury, and were examined using the BBB weekly for 7-weeks post-injury. At 1-week post-injury, prior to transplantation, the motor function was monitored using the BBB and rats were assigned to groups with equal baseline scores, and homogeneity of variance. The rats were given lethal doses of pentobarbital at 7-weeks post-injury, with equal baseline scores, and homogeneity of variance. The rats were examined using the BBB weekly for 7-weeks post-injury. At 1-9 days after receiving a contusion spinal cord injury, and were

Prior to transplantation, SDF-1-MSCs and MSCs-only were transduced with SDF-1-2A-GFP sequence or GFP-alone. GFP expression could be detected in the MSCs after 24-h post-transduction, and a clonal selection was performed to analyze for the expression of CXCR4 and Nestin to confirm the existence of NSCs and MSCs in vitro, a co-culture was performed in parallel with a GFPA-only control retrovirus, which was used to produce a second line of MSCs for the expression of stem cell lineage proteins using flow cytometry. For SDF-1-MSCs and GFP-MSCs, immunocytochemistry was performed to analyze the expression of CD90 (1:50, 554893, BD Biosciences, San Jose, CA), CD44 (1:250, ab24504, AbCam Co., Cambridge, UK), CD105 (1:100, ab23990, AbCam Co., Cambridge, UK), CD34 (1:50, ab8158, AbCam Co., Cambridge, UK), CD11b (1:100, ab8879, AbCam Co., Cambridge, UK), CCR4 (1:100, pab3-305, Invitrogen Co., Carlisle, CA), as well as for the expression of GFP. For NScs, immunocytochemistry was performed to analyze for the expression of Nestin (1:100, ab11306, AbCam Co., Cambridge, UK), Sox-2 (1:100, ab97959, AbCam Co., Cambridge, UK), CD133 (1:300, ab19898, AbCam Co., Cambridge, UK), CXCR4 (1:1000, pab3-305, Invitrogen Co., Carlisle, CA) as well as for tdTomato. Immunocytochemistry was revealed using Gt × IgG Alexafluor-594 (1:333; Invitrogen Co., Carlisle, CA) for MSC markers, and Gt × IgG Alexafluor-488 (1:333; Invitrogen Co., Carlisle, CA) for NSC markers.

A small sample of neurospheres, that did not express tdTomato, were plated on poly-D-lysine- (0.1 mg/ml; Sigma Aldrich Co., St. Louis, MO) coated coverslips 24-h before processing for immunocytochemistry. NSCs were labeled using immunocytochemistry for the expression of CXCR4 and Nestin to confirm the existence of the CXCR4 receptor on this population of NSCs. Primary antibodies used Rb × CXCR4 (1:1000, PA3-305; Invitrogen Co., Carlisle, CA), and Ms × Nestin (1:300, AB11306, AbCam Co., Cambridge, UK) while secondary antibodies included, Gt × IgG Alexafluor-488 (1:333; Invitrogen Co., Carlisle, CA), and Gt × Ms Alexafluor-594 (1:333; Invitrogen Co., Carlisle, CA).

To evaluate the effects of MSCs on NSCs in vitro, a co-culture was performed by plating either 1.0 × 10^5 MSCs or SDF-1-MSCs and 1.0 × 10^5 NSCs, or just 2.0 × 10^5 NSCs, into a 25-cm flask in differentiation conditions. Differentiation conditions consisted of NSC culture medium removed of N2, as well as the growth factors bFGF, and EGF. Cells were cultured for 1 week prior to preparation for flow cytometry. To analyze the co-culture with flow cytometry, the cells were prepared as described above and labeled for markers Nestin, Sox-2, CD133, as well as CXCR4 and revealed using the secondary antibody Gt × IgG Alexafluor-350 (1:333; Invitrogen Co., Carlisle, CA). To ensure that only NSCs were included in the analyses, cell counts were limited to positive co-labeling with both tdTomato and Alexafluor-350.

To evaluate the viability of NSC post-transplantation, a reporter gene coding for the firefly luciferase protein, fused through a P2A sequence to tandem-dimer tomato (tdTomato), was virally transduced into a sub-population of NSCs pCDH-EF1-Luc2-P2A-tdTomato was a gift from Kuzuhiko Oka (Addgene plasmid # 72486). These luciferase/td-tomato-expressing NSCs were transplanted in place of unmodified NSCs into 3 animals from each of the SDF-1-MSC/NSCs, MSC/NSCs, and NSCs-alone groups. The lenti-virus was packaged in immortalized human embryonic kidney cell-like 293-FT (HEK293FT) cells using three separate plasmids: the pCDH-EF1-Luc2-P2A-tdTomato (18.75 μg), the psPAX2 cassette (psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260); 13.125-μg), and the pCMV-VSV-G envelope (pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454); 5.625-μg) (Stewart et al., 2003). After 24-h post-transfection, the HEK293FT cells were washed twice, and further incubated in 8 ml NSC media for another 48 h prior to harvesting the viral containing supernatant. This supernatant was used to transduce the NSCs for 48-h. Reporter-gene expression could be detected by 24-h post-transduction, and a clonal selection was used to expand the brightest expressing neurospheres after 9-days in culture.

4.4. Characterization of NSCs and MSCs in vitro

Prior to transplantation, MSCs and NSCs were characterized for the expression of stem cell lineage proteins using flow cytometry. For SDF-1-MSCs and GFP-MSCs, immunocytochemistry was performed to analyze the expression of CD90 (1:50, 554893, BD Biosciences, San Jose, CA), CD44 (1:250, ab24504, AbCam Co., Cambridge, UK), CD105 (1:100, ab156756, AbCam Co., Cambridge, UK), MHC-II (1:100, ab23990, AbCam Co., Cambridge, UK), CD34 (1:50, ab8158, AbCam Co., Cambridge, UK), CD11b (1:100, ab8879, AbCam Co., Cambridge, UK), CCR4 (1:1000, pab3-305, Invitrogen Co., Carlisle, CA), as well as for the expression of GFP. For NSCs, immunocytochemistry was performed to analyze for the expression of the NYU MASCIS Model II Impactor was used to induce contusion injuries at the T8 vertebrae, on 3–4 month-old female Sprague Dawley rats, bred in house, weighing 250–350 g. All surgical proce-
dure was performed under sterile surgical conditions in a designated surgical suit. All procedures using animals followed NIH guidelines and were approved by the Central Michigan Universities Institutional Animal Care and Use Committee (protocol #1415, approved 6/1/2014). Rats were anesthetized and maintained with isoflurane (Med-Vet International, Mettawa, IL), and a laminectomy was performed at the T9 vertebrae. A conduction injury was induced by dropping the 10-g piston from a height of 25 mm above the surface of the dura. Following injury, the muscles, fascia, and fat were sutured. The incision site was closed using 12-mm auto clips, and the rat was given 0.02 mg/kg of Buprenorphine (Reckitt Benkiser Pharmaceuticals, Richmond, VA), as well as 1.0 mg/kg of Meloxicam (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO), and allowed to recover in a heated empty cage. Buprenorphine and Meloxicam were administered for 3-days post-injury, and 10 mg/kg of enrofloxacin (Baytril; Med-Vet International, Mettawa, IL) was administered when urinary tract infections occurred. Bladders of the rats receiving SCI were manually expressed 4-times per-day every 6 h, for the first week post-injury, and 2 times per day, and every 12 h, thereafter, until reflexive micturition returned.

At 9 days post-injury, the rats were again anesthetized using isoflurane, and the spinal cord was re-exposed. For transplantation into the spinal cord, 5 μL of either 3.0 × 10^5 total cells suspended in HBSS, or HBSS alone, were injected into the lesion epicenter at a rate of 0.5 μL/min. The cells transplanted into the spinal cords were either 3.0 × 10^5 MSCs, SDF-1-MSCs, or NSCs-alone, or 1.5 × 10^5 of the MSCs/SDF-1-MSCs with another 1.5 × 10^5 of the NSCs for co-transplantations. Following transplantation, the muscles, fat, and fascia were sutured, the incision was stapled using 12-mm auto clips, and the animal was allowed to recover. At post-transplantation, rats were given 1.0 mg/kg of Meloxicam 2 times per day for 3 days.

4.6. Behavioral examination

Functional ability was evaluated weekly for 7 weeks post-injury, using the BBB scale for locomotor recovery. Assessments were made by placing the rats in a circular 90-cm diameter plastic enclosure of, which allowed for exploration. Briefly, the BBB is a 21-point rating scale capable of capturing locomotor abilities in severe conditions of SCI, and functions by allowing rats with hind-limb deficits to explore an open field (Basso et al., 1995). Scores are assigned based on well-defined functional abilities, with scores ranging from 0, which represents no observable movement in the hind legs, to a score of 21, which resembles normal movements in rats. Rats were assessed in the circular enclosure for up to 4 min by two independent raters.

4.7. In vivo imaging

To confirm successful transplantation of neurospheres, one animal from each group receiving NSCs that express the firefly luciferase were imaged using the IVIS Lumina LT Series III (Perkin Elmer Co., Waltham, MA) optical imaging system for bioluminescence/fluorescence, at 24-h, 1-week, and 3-weeks post-transplantation. For this procedure, the rats were anesthetized using isoflurane, and were given intraperitoneal injections with 150 mg/kg of o-luciferin/Na+ dissolved in saline (Gold Biotechnology Inc., Olivette, MO). Rats remained anesthetized under isoflurane within the imaging for 45 min, and images were obtained every 5 min post-injection of the luciferin substrate. Exposure times for this imaging were 5-min, and were performed at a maximal binning of 16, with the camera iris completely open. Rats were imaged for only the first 3-weeks post-transplantation because the bioluminescence decreased beyond detectable levels in some of the rats by the third week of analysis. The final analysis for cell viability was determined by evaluating the tissue sections for GFP expression and/or tdTomato expression.

4.8. Histology

At 7 weeks post-injury, the rats were euthanized via overdose of Fatal Plus (Med-Vet International, Mettawa, IL) and transcardially perfused using a 4% paraformaldehyde (PFA; Sigma Aldrich Co., St. Louis, MO). The spinal cords were then extracted and post-fixed overnight in 4% PFA before being transferred through 24-h incubations in 10%, 20%, and finally to 30% sucrose at 4 °C (Sigma Aldrich Co., St. Louis, MO). Once tissue equilibrated to 30% sucrose the spinal cords were cut into 1-cm long sections and frozen in M-1 embedding matrix (Invitrogen Co., Carlsbad, CA) on dry ice and placed into short-term storage at −80 °C. Tissue was later cut into cross-sections at 30-μm thickness, and mounted with 360-μm spacing between sections.

Eriochrome cyanine staining (Sigma Aldrich Co., St. Louis, MO) was performed to evaluate white matter loss/pathology by evaluating the area of spared white matter on 6 tissue sections rostral, and 6 tissue sections caudal to the lesion epicenter. The lesion epicenter was identified based on the section containing the least amount of spared white matter, and data was aligned with respect to this defined epicenter and compared across groups at respective distances in the rostral-caudal direction. The area of spared white matter surrounding the lesion was traced and analyzed using Image J, with group identities being blinded to the experimenters. The following eriochrome-cyanine-stained sections were used for this analysis: vehicle (n = 7); MSCs-only (n = 5); SDF-1-MSCs (n = 4); NSCs-only (n = 9); MSC/NSCs (n = 9); and SDF-1-MSC/NSCs (n = 11).

To evaluate axonal sparing, fiber densities surrounding the lesion site were analyzed using immunohistochemical labeling against neurofilament-70kD (1:500, Ms × NF-70, MAB1615; Millipore Co., Billerica, MA), and visualized by labeling with Gt × Ms. Alexafluor-594 (1:333; Invitrogen Co., Carlsbad, CA). Images were acquired from 4 locations: left/right white matter near the anterior horn/ventral corticospinal tract region, as well as in the white matter of the rubrospinal tract region (Supplementary Fig. 2). Four tissue sections surrounding the lesion epicenter were assessed, using a 20× objective, while maintaining consistent exposure times and gain settings for each picture. Images were quantified using Image J, by analyzing a square 500-μm section of tissue, sampled bilaterally in white matter near the anterior horn, or near the rubrospinal tract region in the upper quartile of the tissue section. A threshold was set to remove background pixels that did not represent positive staining by having reviewers, who were blinded to experimental groups, sample the pixel intensities of three regions of high-background in each image, while avoiding any artifacts when tracing the labeled area. The average of the three sampled background intensities was used to set an appropriate threshold unique to each image. Pixel intensities were obtained from each pixel in the image, and all pixels above the set threshold were considered positive staining, and were counted for analysis. Images were taken bilaterally from both the white matter near the anterior horn, and rubrospinal tract regions, and both images from the respective locations were averaged, followed by averaging the values obtained from all 4-tissue sections from each animal. The data obtained was presented as a percentage of pixels labeled brighter than the set threshold from the area measured. Only rats that did not receive the tdTomato expressing NSCs were analyzed for NF-70, to prevent any false-positive staining that may arise from detecting the tdTomato expression in the transplanted cells. Analysis between groups for NF-70 stained sections was performed for...
the following groups: vehicle (n = 7); MSCs-only (n = 5); SDF-1-MSCs (n = 4); NSCs-only (n = 6); MSC/NSCs (n = 6); and SDF-1-MSC/NSCs (n = 8).

Cell viability was assessed by the presence of tdTomato to detect NSCs, and immunohistochemical labeling against GFP (1:3000, Ck) to detect NSCs, and immunohistochemical labeling against GFP (1:3000, Ck)

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4.9. Statistics

A repeated-measures ANOVA was used to evaluate differences between groups on the BBB over the 7-week testing period. Next, the Kruskal-Wallis non-parametric ANOVA was used to analyze the BBB scores at each week post-injury, with individual Mann-Whitney U tests used for planned pairwise comparison. Planned comparisons were made between vehicle-treated and all other groups. Additional planned comparisons were made between the SDF-1-MSC/NSC-treated and SDF-1-MSC-, NSC-only-, and MSC/NSC-treated rats. A repeated-measures ANOVA was used to evaluate differences in white matter sparing for the 13 sections analyzed that surround the lesion epicenter. A one-way ANOVA was used for comparing all other measures. Fisher's PLSD post-hoc analysis was conducted when appropriate. A two-tailed Pearson’s correlation was run between BBB scores obtained at 7-weeks post-injury and NF-70 labeling for images obtained near the rubrospinal tract and at the anterior horn, as well as for white matter areas obtained at the lesion epicenter. Statistical analyses were performed using the Statistical Package for the Social Sciences version 23, with the alpha value set at 0.05 for all analyses.

Author contributions

A.S., J.R. and G.D conceived and designed the experiments; A.S., G.K., Z.D., D.B., M.F., and K.C., provided technical support for surgical procedures, post-operative care, cell culture, and histological preparations including transcardial perfusions, tissue extractions, and tissue sectioning.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainres.2017.07.005.

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