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Fate of stem cell transplants in peripheral nerves

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Abstract While damaged peripheral nerves demonstrate some potential to regenerate, complete functional recovery remains infrequent, owing to a functional loss of supportive Schwann cells distal to the injury. An emerging solution to improve upon this intrinsic regenerative capacity is to supplement injured nerves with stem cells derived from various tissues. While many of these strategies have proven successful in animal models, few studies have examined the behavior of transplanted stem cells in vivo, including whether they survive and differentiate. In previous work, we demonstrated that cells derived from neonatal rodent dermis (skin-derived precursor cells, or SKPs) could improve regenerative parameters when transplanted distal to both acute and chronic nerve injuries in Lewis rats. The aim of this work was to track the fate of these cells in various nerve injury paradigms and determine the response of these cells to a known glial growth factor. Here, we report that SKPs survive, respond to local cues, differentiate into myelinating Schwann cells, and avoid complete clearance by the host's immune defenses for a minimum of 10 weeks. Moreover, the ultimate fate of SKPs in vivo depends on the nerve environment into which they are injected and can be modified by inclusion of heregulin-1β.

Introduction

Rationale for stem cells in peripheral nerve lesions

Stem cells have attracted considerable interest of late for their potential applicability to nervous system injury and disease. Lesions of the peripheral nervous system are frequent and debilitating, and only the mildest of injuries are associated with acceptable functional recovery (Humphreys et al., 2007; Noble et al., 1998). The capacity of the denervated distal nerve to support axonal regeneration depends on proliferating SCs within the basal lamina tube (Bungner, 1891) which are essential for guiding regenerating axons to their denervated targets (y Cajal, 1928). Over time of denervation, however, Schwann cells become refractory to neurotrophins and neuregulins, downregulate the required receptors, and ultimately fail in their ability to interact with axons and other non-neuronal cells (You et al., 1997; Li et al., 1997). Though transplantation of Schwann cells (SCs) into nerve and spinal cord injuries is an obvious and biologically effective strategy (Guenard et al., 1992; Guest et al., 1997), it is made somewhat impractical due to their limited expansion potential, lengthy culture times, and morbidity associated with nerve harvest to obtain sufficient numbers of transplantable cells. Several alternative sources of cellular therapy have thus been examined, including bone

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marrow, adipose tissue, amniotic fluid, hair follicle, and dermis. Many of these precursor cell strategies have demonstrated utility in treating peripheral nerve lesions in experimental animals (for review, see Walsh and Midha, 2009). However, how precursor cells might respond to and modify the nerve environment and thereby confer regenerative advantage is largely unknown.

Skin-derived precursor cells

The skin dermis contains neural crest-related precursor cells (skin-derived precursors, or "SKPs") that can differentiate into neural crest cell types in vitro when supplied with the appropriate cues, including those with characteristics of peripheral neurons and SCs (Fernandes et al., 2004; Toma et al., 2001, 2005). Specifically, when SKPs are cultured with heregulin-1β (a known differentiation factor of neural crest cells destined to become SCs), they take on an apparent SC phenotype ("SKP-SCs") and can be transplanted distal to injuries of rodent peripheral nerve injuries (McKenzie et al., 2006; Walsh et al., 2009, 2010). In previous work, we utilized SKP-SCs as a source of exogenous Schwann cells to seed isogeneic acellular grafts bridging defects in rodent peripheral nerve. Regeneration of nerves treated with SKP-SCs was significantly improved over diluent control, and comparable to autograft (the current best available method for nerve repair) (Walsh et al., 2009). We next delivered SKP-SCs into the distal segment of a chronically denervated tibial nerve and found that we could enhance the typically barren distal nerve environment toward one that was more growth-supportive (Walsh et al., 2010). While these in vivo data strongly supported the use of SKP-SCs as a potential source of autologous Schwann cells for peripheral nerve transplantation, the fate of these cells in vivo and their long-term contribution to the nerve environment has yet to be fully elucidated. This knowledge will be of crucial interest as we consider the clinical translation of cell therapy for human nerve repair. Given the ability of SKPs to survive and differentiate into SCs distal to nerve crush (McKenzie et al., 2006) we hypothesized that they would retain this ability in all nerve environments. However, as the environments vary with respect to nerve components such as basal lamina and host Schwann cells, survival might be expected to differ between cell differentiation states as well as nerve injury paradigms.

Present study

In this study, we examined the survival and differentiation of SKPs at different timepoints following transplantation into intact nerve, acutely denervated nerve, and chronically denervated nerve. We found that the ultimate fate of SKPs in vivo depends on the nerve environment into which they are injected. Though their survival tends to be poorer on average than differentiated SKP-SCs, naïve SKPs are instructed by the microenvironment of the nerve to take on a SC phenotype. Moreover, while SKPs did migrate several millimeters from the injection site, they did not demonstrate significant levels of proliferation after 1 week in the nerve. It also appears that the differentiation state of SKP-SCs is such that they are sensitive to exogenous growth factors: supplying heregulin-1β at the time cell injection decreases the level of apoptosis occurring immediately after cell transplant. Given this knowledge regarding the behavior of SKPs in peripheral nerve tissue, we hope to be able to modify various aspects of the cells' biology to improve their utility for nerve transplantation.

Results

SKP phenotype in vitro

Within 1–2 weeks of culture in bFGF and EGF, SKP cells isolated from dissociated skin samples formed floating spheres (Fig. 1A), while cells with fibroblastic morphology attached to the dish and could be discarded by selectively passaging only the floating cell population. Culturing aliquots of dissociated SKP spheres in heregulin-1β and forskolin-containing media resulted in adherent monolayers of cells with a bipolar morphology identical to that observed in nerve-derived SC cultures (Morrissey et al., 1991) (Fig. 1B). The SC phenotype was further confirmed via positive immunostaining for GFAP, S100β, ErbB3, and p75NGFR (Fig. 1C). Of note, some cells in the impure culture without this morphology did not express SC markers. Therefore we identified SKP-SCs as those that a) demonstrated a bipolar, spindle shape *and* b) expressed GFAP. The entire process of differentiation and purification of SKP-SCs takes on average of 2 weeks. We used only purified SKP-SCs meeting those characteristics in further experiments requiring differentiated cells.

Survival and differentiation of SKPs within the injured nerve environment

Naïve SKPs were transplanted distal to nerve transection and followed by immunohistochemistry of frozen sections obtained at the follow-up timepoints (2 and 8 weeks). Labeled (YFP+) cells could be found existing as a tract within the endoneurium at each timepoint and condition assessed. They migrated 2–3 mm from the injection site and were distributed over roughly 20% of the cross sectional area of the nerve. At the 2 week follow-up (Table 1), survival was higher (~10.5%) when SKPs were injected into an acutely injured nerve versus after chronic denervation (~5.8%). A two-way ANOVA run on the data in Table 1 demonstrated that nerve environment had a significant effect on survival data ($F(2,18)=5.60$, $p<0.05$). Interestingly, the fewest number of cells could be counted from the intact nerve, indicating clearance or lack of survival within this environment. At the 8 week follow-up, the number of cells retained decreased in all groups, such that there was no more than ~4% long-term survival when transplanting naïve SKPs. Naive SKPs differentiated into GFAP positive SCs in the peripheral nerve environment. Though SKPs implanted into chronically denervated nerves had low survival, they remarkably demonstrated high levels of differentiation into SCs (73.3% and 76.5% at 2 and 8 weeks, respectively).

Since the chronically denervated nerve appeared to be poorly supportive for SKP survival, we used this environment to test whether survival could be improved by differentiating

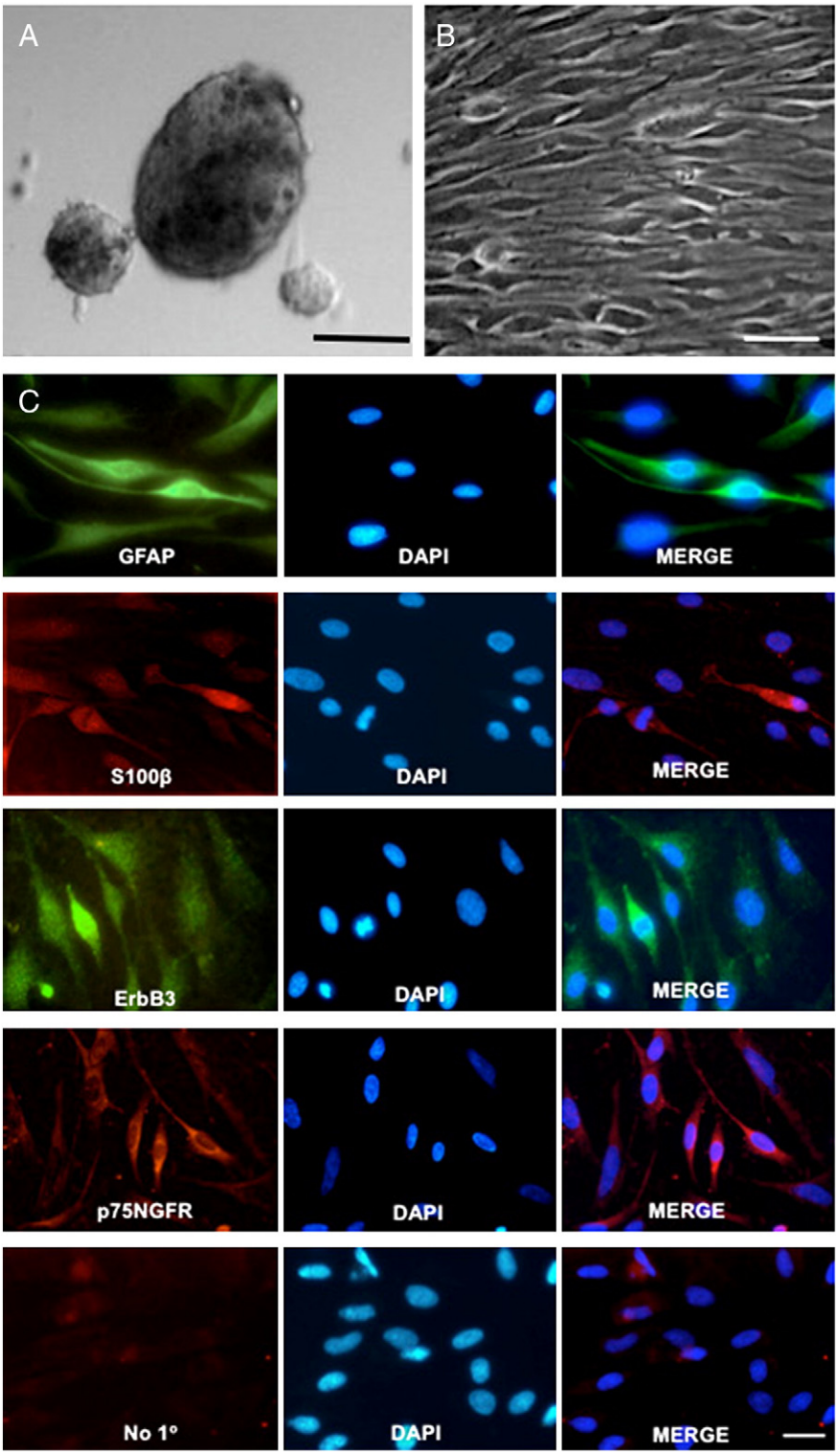

Figure 1 In vitro phenotype of SKP-SCs. A: SKPs cultured in DMEM/F12 media supplemented with EGF and bFGF form spheroid colonies within 1 week of culture initiation. Phase contrast microscopy, 200× original magnification; scale bar = 50 μm. B: when differentiated in forskolin and heregulin-1β, SKPs attach to the culture substrate and differentiate into a Schwann cell phenotype, forming confluent cultures of aligned bipolar cells (SKP-SCs). Phase contrast microscopy, 100× original magnification; scale bar = 50 μm. C: after 2 weeks of differentiation, impure cultures of SKP-SCs were re-plated and stained with various Schwann cell markers. Note that only the bipolar, spindle shaped cells demonstrate robust and positive immunofluorescence in most cases, while other cell morphologies tend not to demonstrate staining. Omission of primary antibody (no 1°) served as a staining control. Fluorescent micrograph, original magnification 400×. Scale bar = 20 μm.

Table 1 Survival and differentiation of SKPs in various nerve environments.

Time of SKP injection, harvest	Mean number YFP-positive cell bodies	% Survival	Proportion GFAP-labeled
Immediate, 2 weeks	31,440	10.48 ± 2.9% [c]	33.42 ± 3.22% [b,c]
Immediate, 8 weeks	12,190	4.06 ± 0.59%	22.92 ± 1.79% [b,c]
Chronic denervation, 2 weeks	17,300	5.83 ± 0.71%	73.31 ± 3.41% [a]
Chronic denervation, 8 weeks	11,640	3.92 ± 0.72%	76.54 ± 8.48% [a,c]
Uninjured, 2 weeks	9360	3.13 ± 0.66% [a]	67.32 ± 2.38% [a]
Uninjured, 8 weeks	8000	2.68 ± 0.14%	44.2 ± 1.89% [a,b]

Naïve GFP-labeled SKP spheres were dissociated and 300,000 cells were injected distal to a sciatic nerve transection injury in immunosuppressed Sv129 mice either immediately after injury or following 8 weeks of chronic denervation. Histological assessment of nerve sections harvested 2 weeks post transplant demonstrated a survival percentage of 10.48% in the acutely injured nerve, whereas when delivered into a chronically denervated nerve, the number of detected SKPs decreased to 5.8%. Of note, however, was that the proportion of GFAP-differentiated SKP-SCs increased in the chronically denervated nerve which had lower numbers of cells overall. Survival was poorest in uninjured nerves. Data represent mean ± SEM of n = 4/group.

[a] Significantly different from immediate injection group at the equivalent timepoint.

[b] Significantly different from chronic denervation group at the equivalent timepoint.

[c] Significantly different from uninjured group at the equivalent timepoint (one-way ANOVA with post-hoc Tukey's multiple comparison test).

SKPs into SCs prior to injection (Fig. 2). When SKP-SCs were implanted into chronically denervated nerve, survival was greater than that of naïve SKPs (6.0% versus 3.8%; $p<0.05$, Student's t test).

Proliferation and apoptosis of SKPs in vivo

SKPs maintained a basal low level of proliferation in vivo over various time-points assessed. BrdU positive profiles formed a low percentage of total labeled SKPs (4.2% at 1 week, 4.5% at 2 weeks and 3.6% at 3 weeks), and there was no evidence of tumorigenesis throughout all experiments with either SKPs or SKP-SCs, as demonstrated by both gross and microscopic examination of transplanted tissues.

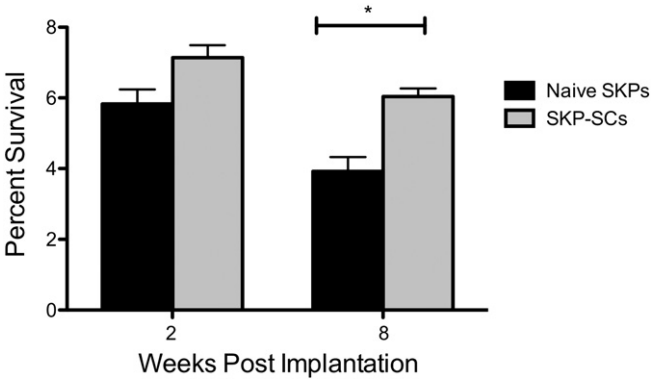

Figure 2 Survival of naive versus SC-differentiated SKPs in the chronically denervated nerve. GFP-labeled naive or forskolin/Hrg-1β-differentiated SKPs were injected at a concentration of 100,000 cells/ul distal to a sciatic nerve transection injury in Sv129 mice following 8 weeks of chronic denervation, followed by histological assessment of surviving YFP+ cells within the distal nerve. After 2 weeks, 7.1% of differentiated cells versus 5.8% of undifferentiated cells were found to survive. This difference was significantly different at 8 weeks with 6% survival in differentiated cells and 3.8% survival in undifferentiated cells. *$p<0.05$ (two-tailed Student's t test).

Because survival was particularly poor in cells transplanted into intact nerves versus transected nerves, we asked whether they were being cleared by apoptosis early after transplantation. We repeated the injection techniques described above, and transplanted DiI-labeled naïve SKPs into either an intact rat nerve or one that had been transected immediately prior to cell delivery, and quantified caspase 3 positive SKPs in representative sample sections. At 24 h, 36.9% of the SKPs counted from the intact nerve were undergoing apoptosis. Distal to transection injury, 23.05% of the cells transplanted were apoptotic (Fig. 3). This difference was not statistically significant.

Maintenance of SC fate in peripheral nerve

We next wanted to assess whether differentiated SKP-SCs remained in the injured nerve and retained their phenotype over the longer term, or whether they were subject to engulfment by macrophages secondary to a prolonged inflammatory response. When Lewis rat SKP-SCs, pre-labeled in vitro with lipophilic DiI, were implanted within a isogeneic acellular graft, they retained a SC phenotype for at least 10 weeks (Fig. 4A). In this environment, 38% of all counted cells were S100β/DiI positive, i.e. SKP-SCs. However, 20% of the counted cells were DiI positive only (accounting for 34% of total DiI positive cells), indicating a population of cells that had either de-differentiated or expanded from contaminating cells in the original injection. While there were ED-1 positive macrophages in the grafts at this timepoint, few appeared to double-label with SKPs (11%), demonstrating minimal ongoing immune response (Fig. 4B). Moreover, there appeared to be a spatial separation between the column of transplanted SKPs and the infiltrating macrophages, with macrophages tending to favor the extremities of the graft and the SKPs forming a continuous cord in the center of the graft.

Finally, SKP-SCs show the capacity to produce myelin both in vitro (Fig. 5A) and in vivo. The SKP-SCs retained within the nerve took on a myelinating phenotype once axonal contact was re-established, as they enveloped regenerating axons and expressed myelin proteins.

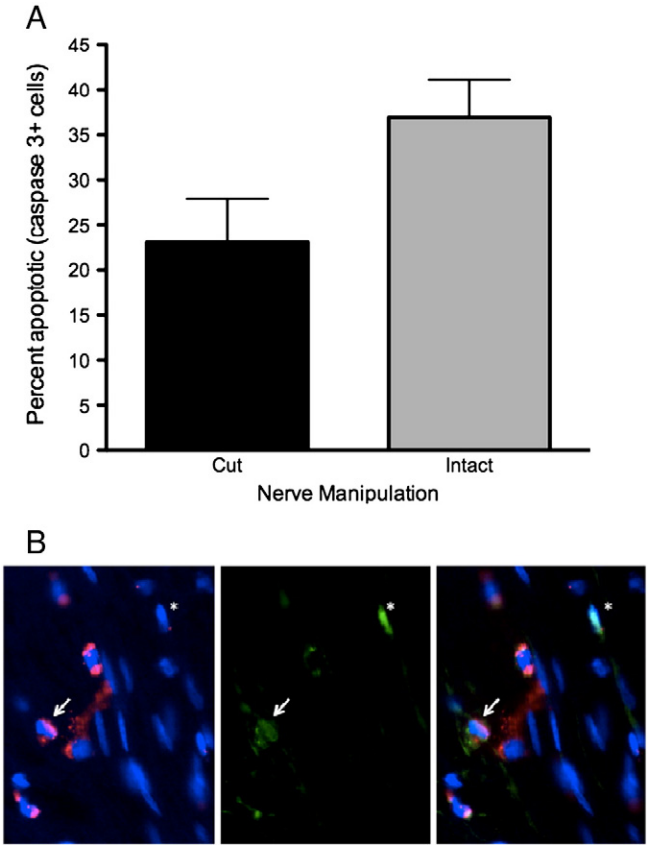

Figure 3 Apoptosis of SKPs following transplantation into the nerve. SKPs were either transplanted into the intact sciatic nerve or distal to transection injury (n=4/group). Nerves were harvested at 24 h, at which point the number of caspase 3 positive (apoptotic) SKP cells in the sampled area was quantified. A: a mean of 36.90±4.2% of SKP cells were caspase positive in the intact nerve, while 23.05 4.8% of SKPs were caspase positive distal to transection. Data represent mean±SEM; $p>0.05$ by two-tailed Student's t test. B: fluorescent micrograph demonstrating nuclear caspase 3 staining (green) of SKP-SCs (red). Nuclei are labeled with Hoescht. Arrow: caspase 3 positive SKP-SC. Asterisk: caspase 3 positive, non-SKP cell. 400× original magnification.

Effect of neuregulins on limiting SKP apoptosis

Finally, we asked whether neuregulins may be an important director of SKP fate. It appeared that in vitro, SKP-SCs were sensitive to heregulin-1β, as removal of this component from the culture media resulted in an approximate doubling of apoptosis in these cells (Fig. 6A; $p<0.001$, Student's t test). We then found that early apoptosis of SKPs in nerve tissue could be minimized by application of exogenous heregulin-1β concomitantly with cell injection (Fig. 6B). In each animal, cells in the presence of heregulin-1β were injected into the sciatic nerve of the right leg, and those in DMEM alone were injected into the left leg. The treatment with 500 ng/ml heregulin-1β resulted in significantly fewer apoptotic SKPs after 24 h ($p<0.01$, Student's t test).

Discussion

Major findings

The major findings of this study were: (1) skin-derived precursor cells (SKPs) demonstrate variable survival and capacity for differentiation within peripheral nerves, depending on the nerve injury environment, (2) Schwann cell predifferentiation of SKPs improves their survival in nerve lesions and (3) SKP-SCs retain their SC fate over time and myelinate regenerating axons, forming a permanent component of the regenerated nerve. Finally (4), SKPs are sensitive to neuregulins, and injecting them concomitantly with heregulin-1β significantly decreases the level of apoptosis occurring in these cells upon transplantation into nerve tissue

Tracking survival of stem cell transplants

Recently, investigators have placed greater emphasis on the retention and differentiation of stem cells transplanted into the nervous system. However, is long term survival of most of the transplanted cells an absolute requirement for their therapeutic activity? Some investigators have noted recovery from various lesions with few remaining stem cells and in the absence of their overt differentiation, suggesting alternative mechanisms of support, such as enhancing host repair mechanisms (Prockop, 2007; Marcus et al., 2008). In this study, we saw roughly 10% survival at 2 weeks, meaning that at minimum, 90% of the cells are lost before full functional

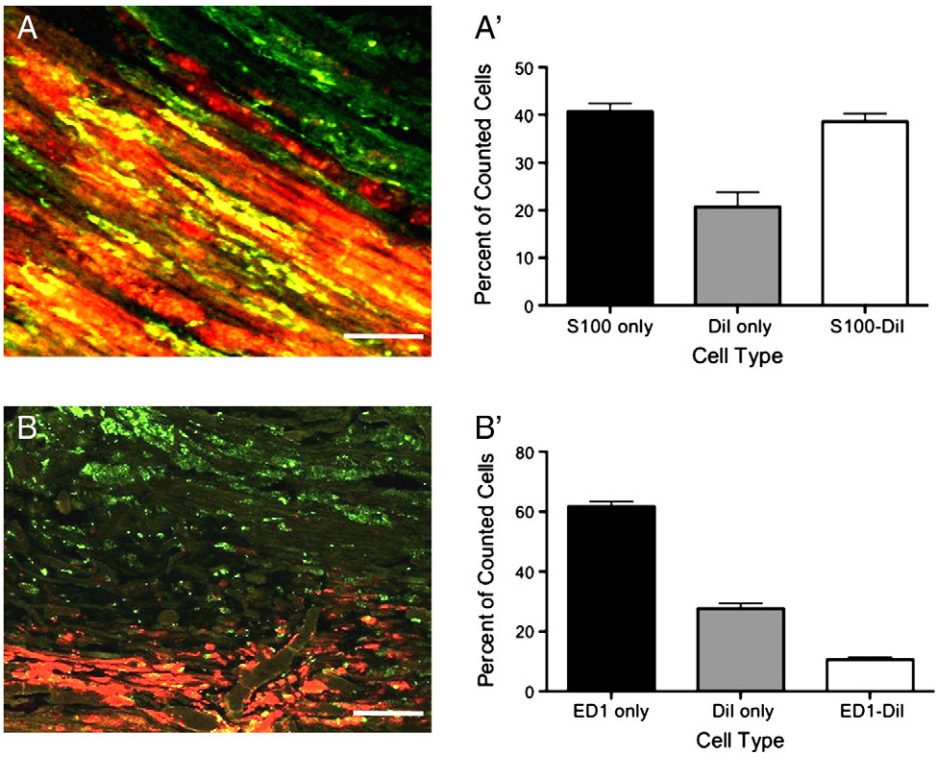

Figure 4 Long term fate of SKP-SCs within an acellular graft bridging a rodent sciatic nerve lesion. A: SKP-SCs retain their S100β + phenotype for at least 10 weeks following transplantation into acellular nerve grafts. Red = DiI labeled cells, green = S100β staining, yellow = double labeled cells. A′: immunofluorescent quantification of randomly selected fields of sectioned grafted areas determined that, of the cells counted, 20% were DiI positive only (SKPs), 40% were DiI and S100β positive (SKP-SCs), and 40% were S100β + only (host SCs). B: SKP-SCs avoid long-term immune engulfment by resident macrophages within the acellular graft. Confocal image, red = DiI labeled cells, green = ED-1 staining, yellow = double labeled cells. B′: immunofluorescent quantification of graft sections demonstrated that 27% of counted cells were DiI + only, 11% were DiI and ED-1 + (SKPs engulfed by macrophages), and 61% were ED-1 + only. A, B: 100× original magnification; scale bar = 100 μm. "C": center graft; "P": periphery of graft.

recovery is expected to take place. While we have shown that the cells undergo apoptosis fairly early, there may be other reasons to explain absence of cells within the nerve. For this reason, it is particularly important for investigators to standardize injection techniques, ensure that the passage of cells through the needle does not cause them damage, and dissociate cells adequately into single cell suspension prior to injection. These errors were minimized in these experiments by checking viability before and after passage through the needle, and by incorporating Fast Green dye in

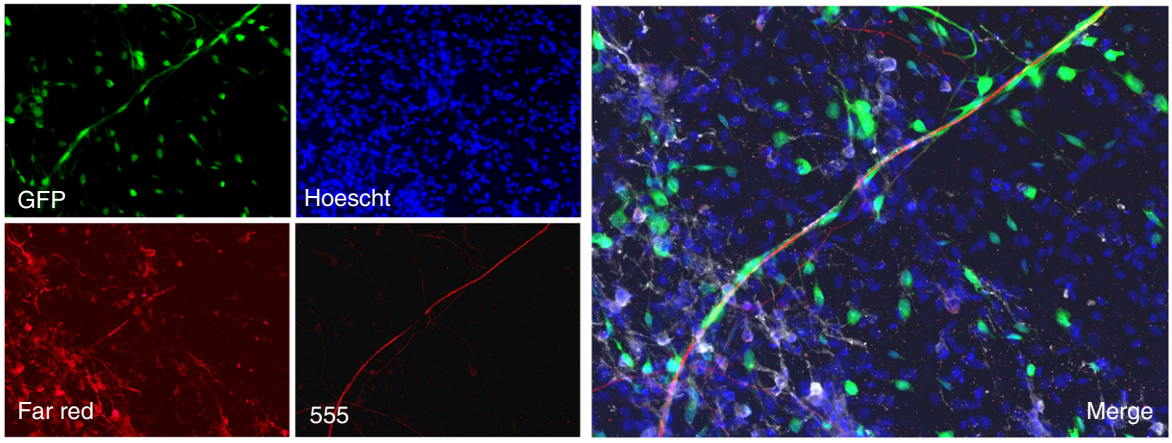

Figure 5 SKP-SCs demonstrate a myelinating phenotype in vitro. GFP lentivirus transduced neonatal SKP-SCs (GFP) envelop DRG neurites (far red) and express myelin basic protein (555). All nuclei visualized with Hoescht (blue). Merge demonstrating intimate alignment of SKP-SCs along neurites (pseudocolored as gray for presentation purposes).

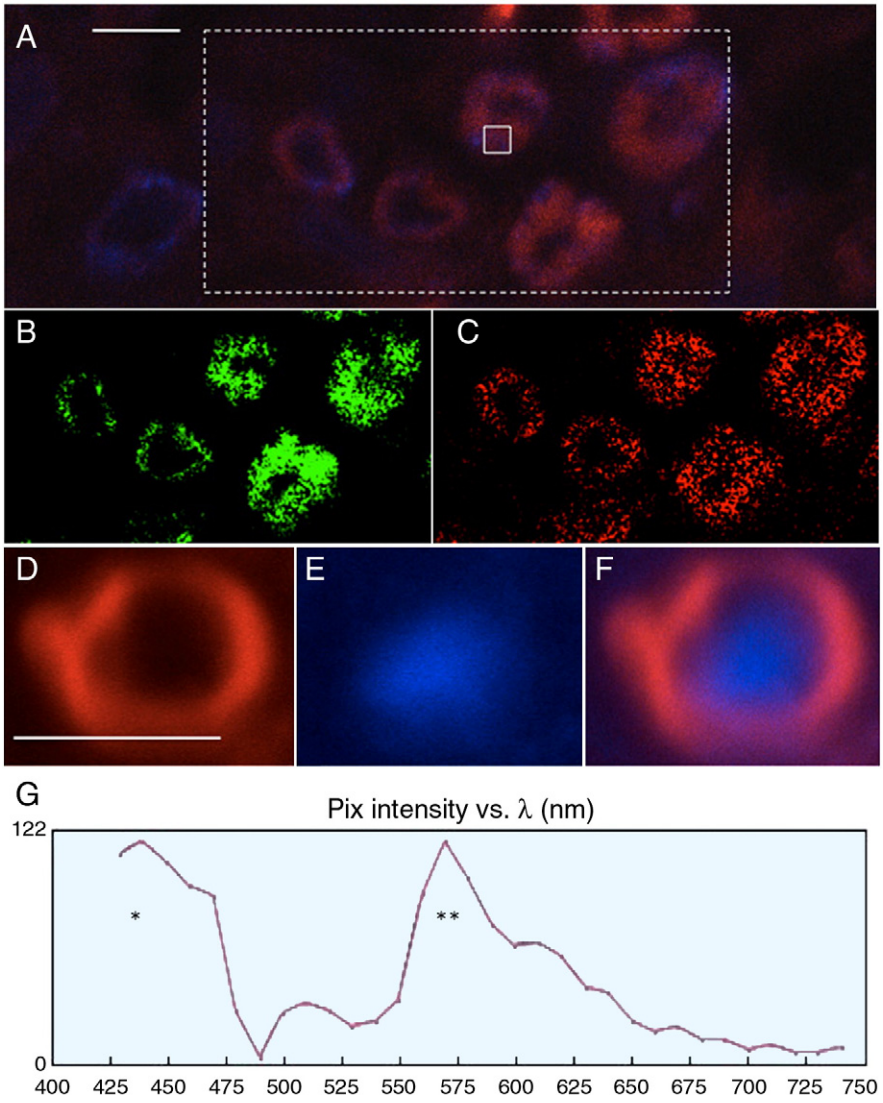

Figure 6 SKP-SCs myelinate regenerating axons in vivo. A–C: spectral confocal image of several myelin figures, labeled for MBP (blue, Alexa 405). A: the MBP co-localizes with DiI fluorescence (red) of SKP-SC produced myelin. B: spectral unmix of DiI fluorescence from the dashed-line box of the previous figure (pseudocolor green). C: spetral unmix of Alexa 405 fluorescence (MBP, psueodocolor red) of the same area above. D–F: high magnification cross-section view of a single axon from a different staining preparation, demonstrating myelination by a SKP cell. SKP-SCs were pre-labeled with DiI (D), and axons were stained with alexa 405 for neurofilament (E). F: merge of blue and red signals. G: intensity versus wavelength graph demonstrating two distinct and specific emission peaks for myelin and SKP cells. *: Alexa 405 (MBP), **: DiI (SKPs), as detected from the solid box area of A).

pilot experiments to ensure the cell infusion reached the endoneurial target. Of note, the ideal number of cells for transplant in peripheral nerve injuries has not been thoroughly investigated, with numbers as few as 4×10^3 cells and as many as 2×10^7 cells reported in the literature (Hu et al., 2007; Aquino et al., 2006). We chose $3–5\times10^5$ cells for our study as this was an intermediate value comparable to that used in previous studies employing SKPs (McKenzie et al., 2006). These survival data imply that further studies must be undertaken to determine a) if this is in fact enough cells to maximize any therapeutic benefit they may have, and b) whether cell survival and long term retention can be improved somehow. Moreover, the results highlight the need for careful consideration and refinement of cell delivery techniques for therapeutic use.

One of the main requirements of any study involving stem cells is a reliable method to track the transplanted cells over the long term. Here, we have employed two techniques: deriving cultures from fluorescent transgenic animals and exposing cultured cells to the lipophilic carbocyanine derivative CM-DiI. Though the use of fluorescent cells derived from transgenic animals is an ideal method to ensure homogenous staining, label retention, and cell viability, it necessitated immunosuppression of the recipient animals. Immunosuppression with cyclosporine A, aside from being nephrotoxic, also has variable effects on nerve regeneration itself (Meirer et al., 2002). In order to work with a somewhat better-characterized model of nerve regeneration and avoid immunosuppression, we switched to inbred Lewis rats for both the cell source and recipient animals. These isogenous

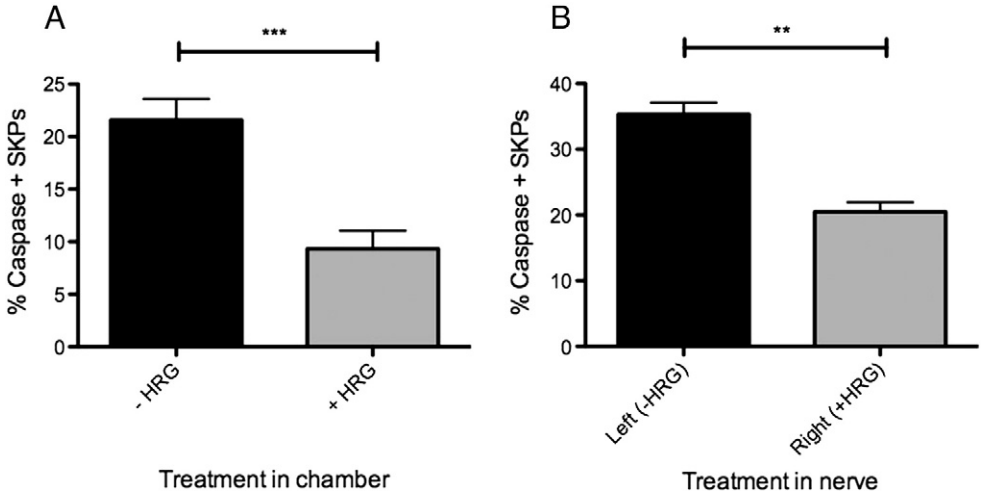

Figure 7 Heregulin-1β modifies the fate of SKPs. A: growth of newly-differentiated SKP-SCs for 48 h in media containing 10 ng/ml heregulin-1β resulted in significantly less apoptosis than growth in complete culture media lacking this factor, as assessed by immunocytochemistry for apoptotic marker cleaved caspase 3. B: inclusion of heregulin into SKPs' diluent prior to injecting into peripheral nerves significantly decreases number of apoptotic SKPs seen at 24 h. Data represent mean ± SEM; **$p<0.01$, ***$p<0.001$, Student's t test.

rat-derived cells were labeled with CM-DiI with no evidence of dilution or loss of signal for at least 10 weeks following transplantation. Following a standard 20 min staining procedure, roughly 95% of cells are stained, with no evidence of cell damage which can occur with other labeling methods. These dyes have the advantage of being technically simple to use, rapid, and resistant to leakage to nearby cells (Nishiura et al., 2004). Our laboratory has also had recent success using lentiviral techniques to introduce fluorescent proteins for robust labeling of stem cells prior to transplant or in vitro use.

Importance of differentiation state

Very few studies compare survival and efficacy of naive versus pre-differentiated cells within a lesion site, despite the fact that each should be considered a separate cell type with different survival requirements. In this study, SKP-SCs showed significantly better survival in the chronically denervated nerve than naïve SKPs. Mature Schwann cells are known to survive in the absence of axons by establishment of an autocrine signaling loop, whereas those taken from embryonic nerves rely on axonal cues for survival (Meier et al., 1999). It may be that immature precursor cells such as the naive SKPs do not possess the appropriate signaling mechanisms to survive in the chronically denervated environment, which is essentially devoid of viable axons. This is corroborated by the finding that, of those originally naive SKPs that did survive, many had differentiated into GFAP positive SCs, perhaps conferring them with this autocrine survival strategy (Table 1). Thus, to be considered a practical strategy, care should be taken with respect to the differentiation state of the cells: either transplant more naive cells in expectation of their loss, or transplant cells in a pre-differentiated state. Another argument for transplanting cells that are differentiated towards the cell type needed in the environment is the assumption that they would exhibit more of the desirable traits of the native SCs and fewer less desirable traits commonly associated with undifferentiated stem cells, such as tumor formation or allodynia (Hofstetter et al., 2005; Amariglio et al., 2009). For example, in a model of spinal cord injury, SKP-SCs outperformed naïve SKPs with respect to remyelination, neural protection, and reducing reactive gliosis (Biernaskie et al., 2007). For this reason, and because of the survival data presented here, we chose to use differentiated SKP-SCs for our studies of nerve regeneration (Walsh et al., 2009, 2010).While we did not assess the phenotype of SKPs *not* differentiating into SCs, the aforementioned study found that they might differentiate into smooth muscle or adipocytes (Biernaskie et al., 2007).We must therefore carefully consider the impact that such inappropriate differentiation would have on the regenerating nerve and the success of SKP-based therapies.

Of upmost importance is the function of these cells in the nerve environment, specifically their ability to myelinate regenerating axons, which is paramount to the appropriate functioning of grafted SCs from any source. In this study, the SKP-SCs retained within the nerve indeed took on a myelinating phenotype (Fig. 6). In previous work, Mckenzie et al. used immunoelectron microscopy to demonstrate that SKPs produced compact myelin in a *Shiverer* mouse model (McKenzie et al., 2006). We are currently undertaking experiments using confocal and electron microscopy to determine what percentage of retained SKPs may be generating bona-fide myelin, and what the functional and electrophysiological implications of this myelin may be in the regenerated nerve.

Influence of nerve environment

Given the ability of naive SKPs to differentiate in vivo, it appears that the microenvironment of the injured nerve is

instructive of transplanted cell fate. Following nerve injuries, neurons convert from a "transmitting" mode to a "growth" mode (Fenrich and Gordon, 2004), during which they express genes associated with growth cone elongation as well as those that mediate their interaction with surrounding Schwann cells. These include growth factor receptors, cell adhesion molecules, and neuregulins (Fu and Gordon, 1997). Other candidates include the cytokines and growth factors produced by cells in the nerve microenvironment, such as FGF, TGFβ, and PDGF (Chandross et al., 1995; Reynolds and Woolf, 1993).

That SKPs demonstrated poorest survival in the intact nerve was very interesting, and corroborates findings of other groups studying stem cellular supplementation of nervous system injuries (Han et al., 2004; Liu et al., 2000; Franklin et al., 1996). Though the mechanism for such exclusion is not entirely known, Franklin and colleagues have suggested that stem cells transplanted into the intact spinal cord are perhaps considered supernumerary and are cleared by apoptosis in a recapitulation of developmental events. Indeed, in our studies cells were shown to be undergoing a very high degree of apoptosis within 24 h after transplantation, which supports this hypothesis.

Effect of exogenous neuregulins

One of the most interesting findings of this work was that the fate of transplanted stem cells is exquisitely sensitive to the environment in which they are placed with respect to their retention, differentiation, and death. Therefore, in addition to the common practice of enhancing stem cells' supportive phenotype, considerations regarding requirements for survival in a particular environment must also be made. Pan and colleagues found that administration of G-CSF to animals receiving transplants of amniotic fluid mesenchymal stem cells not only improved survival of transplanted cells but also augmented nerve regeneration over that of a primarily cell-based approach (Pan et al., 2008). Here, we attempted to utilize a similar strategy to improve retention of SKPs within the peripheral nerve. Recent evidence from Biernaskie et al. (2007) and our laboratory has suggested that SKP-SCs are similar to Schwann cell precursors (SCPs) with respect to their secretion of trophic factors and activity within the environments of injured peripheral nerve and spinal cord. SCPs are the main glial cell within the rodent peripheral nervous system at E14. The period between E14/15 and E17/18 is critical in nerve development, since during this period SCPs mature into Schwann cells, an event that is identified by a loss of reliance on axonal support and neuregulins for survival (Dong et al., 1995; Grinspan et al., 1996). Indeed, removal of heregulin-1β from the cell culture media of SKP-SCs resulted in a significant increase in apoptosis (Fig. 7A). We wondered if this loss of trophic support might explain the considerable amount of apoptosis that occurs in SKPs 24 h after their transplantation (particularly in the intact nerve, where neuregulins are minimal). We found that addition of heregulin-1β significantly decreased the number of apoptotic SKPs at 24 h. However, what is not known from these data is whether preventing early apoptosis will translate to larger numbers of cells retained over the long term and also whether this will positively impact subsequent nerve regeneration. We are currently exploring these questions in our laboratory.

Impact of transplanted cells on nerve environment

Finally, just as the SKP cell's phenotype is affected by the microenvironment, these cells are also likely modifying their milieu. We showed previously that SKP-SCs secrete various neurotrophic factors capable of inducing neurite outgrowth in vitro (Walsh et al., 2009). While a certain proportion of SKP-SCs injected into peripheral nerve lesions can be expected to interact directly with regenerating axons, preliminary evidence has suggested that they are acting in an indirect manner as well, especially since the survival indices of SKP-SCs in chronically injured nerves are only in the order of around 6% (Fig. 2). Studies transplanting SKP-SCs into the injured spinal cord resulted in a significant increase in the recruitment of host Schwann cells into the cord relative to neural stem cells types or diluent alone (Biernaskie et al., 2007). Although the mechanism of such recruitment is unknown, this suggests that at least part of their contribution to repair may involve enhancing endogenous repair mechanisms via host Schwann cell recruitment to the site of injury. Current studies are underway to determine if this is the case in the peripheral nerve as well. Additionally, SKP-SCs have been shown in the model of spinal cord injury to modify their local environment within the glial scar by reducing expression of inhibitory extracellular matrix components such as neurocan (Biernaskie et al., 2007). It is possible that SKP-SCs exhibit similar effects in the chronically denervated nerve, also thought to be inhibitory to axon elongation (Heine et al., 2004). Our laboratory is currently exploring the incorporation of extracellular matrix degradation capability within SKP-SC therapies to further improve regeneration through chronically denervated nerves.

Conclusion

The findings of this study outline the need for greater consideration of cell fate following transplantation into the nervous system. By further understanding cell attributes in vivo such as survival, proliferation, death and differentiation, investigators can manipulate the relevant variables with the expectation of enhancing their utility in nerve regeneration.

Methods

Cell culture and characterization

For initial survival experiments in the mouse model, skin-derived precursor cells (SKPs) were generated from dermis of postnatal day 2 transgenic YFP-expressing mice [(Jax lab; Stock: Tg(GFPU)5Nagy/J)] and cultured according to published protocols (Toma et al., 2001; Biernaskie et al., 2006). For subsequent experiments where a rat model was used, cells were obtained from postnatal day 2 Lewis rats (Charles River, QC). Regardless of cell source, cultures were handled identically. Briefly, pups were quickly decapitated and skin on the dorsal torso was sterilized with a 70% EtOH swab prior to removal with sterile scissors. Collected

tissue was minced in Hank's Balanced Salt Solution (HBSS [GIBCO, Burlington ON]) on ice and then incubated for approximately 45 min in 0.1% collagenase at 37 °C. Skin pieces were mechanically dissociated, washed in cold DMEM and passed through a 40 μm cell strainer. Filtrate was centrifuged at 1200 rpm and the pellet was triturated and resuspended to a concentration of 50,000 cells/ml in culture media (DMEM-F12 [GIBCO] 3:1, 1% penicillin/streptomycin [Sigma, Oakville ON]) containing B-27, 20 ng/ml EGF and 40 ng/ml bFGF (all from Gibco-BRL). Cells were cultured and passaged three times as undifferentiated spheres in 25 cm^2 tissue culture flasks (Corning, Corning NY) in a 37 °C, 5% CO2 tissue-culture incubator. To induce differentiation towards Schwann cells, spheres were triturated and replated on poly-D lysine/laminin coated culture dishes (Corning) in DMEM/F12 media with 4 μM forksolin, 10 ng/ml heregulin-1β, and 1% N2 supplement (Gibco). After incubation for 1 week, cells appearing under phase contrast to have bipolar SC morphology (confirmed with glial fibrillary acidic protein (GFAP) staining of sister cultures) were isolated with cloning cylinders and expanded in the same medium until >95% purity was achieved. Schwann cells were obtained from sciatic nerve of the same P2 neonates used for SKP generation as previously described (Walsh et al., 2009).

Immunocytochemistry

Cultured SKP-SCs were fixed in ice-cold absolute methanol for 20 min and blocked with goat serum for 1 h. Cells were then probed with rabbit anti-GFAP (1:200; Sigma), rabbit anti-S100β (1:100, Santa Cruz), rabbit anti-ErbB3 (1:200, Santa Cruz) and rabbit anti-p75 NGFR (1:200, Santa Cruz), and visualized with anti-rabbit 488 or anti-rabbit 555 (Molecular Probes, Inc, Burlington, ON). Nuclei were labeled with Hoescht 33342 (1:5000, Invitrogen, Burlington, ON). Visualization was carried out under a fluorescence microscope (Olympus BX51, Center Valley, PA)

In vitro myelination

Dorsal root ganglia (DRGs) from P10 rat pups were grown in Lab Tek chamber slides (Nunc, Rochester NY) with nerve growth factor (NGF; 50 ng/ml) and 2.5% FBS in neurobasal media with antibiotics. After 2 days, 7 μg/ml cytosine arabinoside was given for 96 h to eliminate endogenous Schwann cells. After day 6 GFP lentivirus-transduced SKP-SCs (150,000 cells/well) were seeded on DRGs. SKP-SCs were allowed to associate with axons for 5 days followed by induction of myelination with 50 μg/ml ascorbic acid. After 14 days, DRG/SKP-SC cocultures were fixed and stained for myelin (MBP, 1:200; visualized with 555) axons (TUJ1, 1:1000, Santa Cruz; visualized with far red) and nuclei (Hoechst 1:500).

Animals

Behavior of SKPs in peripheral nerve injury was first investigated in a mouse model in order to correlate with the original data published by McKenzie et al. (2006). For these experiments, pharmacologically immunosuppressed (as described below) adult male Sv129 mice (Jackson Laboratories, Bar Harbor ME) were used. We used a Lewis rat model for all subsequent experiments because finer surgical repair techniques required larger nerves, and the ability to use an inbred strain and isologous transplants eliminated the need for immunosuppression. Animals were maintained in a temperature and humidity controlled environment with a 12 h light/dark cycle. Food (Purina, Mississauga, ON) and water were available ad libitum. Surgical interventions were carried out under inhalation anesthetic (Isofluroane, 99.9% Halocarbon Laboratories, River Edge, NJ) and pain control was provided by means of intraperitoneal or oral administration of buprinorphene. Surgical procedures were carried out aseptically, and standard microsurgical techniques were used with an operating microscope (Wild M651; Wild Leitz, Willowdale, ON). Animals were sacrificed at endpoint under deep anesthesia using an overdose of intracardiac Euthanol (Bimeda-MTC, Cambridge, ON). The protocol was approved and monitored by the University of Calgary animal care committee and adhered strictly to guidelines set by the Canadian Council on Animal Care.

Survival and differentiation of SKPs in nerve injury

Sv129 mice immunosuppressed with cyclosporin A (20 mg/kg/day) served as recipients of SKPs or SKPs-SCs from YFP+ mice for these experiments (Midha et al., 1994). Immunosuppression was required in this case as the donor animals were from an outbred stock. The sciatic nerve was exposed in the proximal thigh and transected or left intact. In the first experiment, 3 μl of naïve SKP cells (100,000 cells/μl) were injected deep to the epineurium of the nerve using a 30 gauge needle fitted to a Hamilton syringe either immediately or following 8 weeks of chronic denervation. Chronic denervation was established by cutting the sciatic nerve in the proximal thigh and capping both the distal and proximal stumps with silicone-occluded silastic tubing to prevent reinnervation. The second experiment also employed the chronic denervation paradigm, however animals were given either naïve SKPs or differentiated SKP-SCs in order to compare their survival. In all experiments, the transected nerve was left unrepaired in order to assess viability of SKPs independently from ingrowing axons and Schwann cells. Cell viability of SKPs prior to and after injections was verified to be >90% by Trypan blue dye exclusion in each experiment from an aliquot from the same suspension. To determine SKP survival in the absence of ingrowing axons and proximal Schwann cells, nerves were left unrepaired for the duration of the study. Animals (n=4/group/time-point) were sacrificed at 24 h, 2 weeks or 8 weeks post-transplant and the distal nerve was collected for immunohistochemistry and morphometry. A subset of animals received intraperitoneal injections of BrdU (5-bromo-2′-deoxyuridine, Sigma) at 100 mg/kg 4 h before harvesting at 1 week in order to assess proliferation of transplanted cells (Chen et al., 2005). Isolated segments were fixed whole in 4% paraformaldehyde overnight, cryoprotected in 20% sucrose, embedded in O.C.T compound (VWR, Mississauga, ON) and then longitudinally sectioned at 16 μm with a cryostat (Leica Microsystems Inc, Richmond Hill ON) at −22 °C and mounted on Superfrost slides (Fisher Scientific). Sections were blocked with 1% BSA and incubated overnight in primary antibody (rabbit anti-GFAP, 1:500; Sigma, mouse anti-BrdU, 1:200; Abd Serotec, Oxford, UK,

or rabbit anti-cleaved caspase 3 [a marker of apoptosis], 1:100; Cell Signaling Technology, Boston MA). Myelination by SKP-SCs was confirmed by staining axons with neurofilament (1:400) and Myelin Basic Potein (MBP 1:200) both from Santa Cruz. Following a wash with PBS, slides were incubated with secondary antibody (anti rabbit or anti mouse 405, 555 or 488; 1:400; Molecular Probes) for 2 h. Slides were then washed and coverslipped using Fluorosave reagent (Calbiochem, San Diego, CA) and viewed under a fluorescence microscope (Olympus BX51, Center Valley, PA). Omission of primary or secondary antibody was used as negative control for the staining process. SKPs (YFP+ or YFP/GFAP+) were counted in every eighth section and counts were multiplied by 8 to adjust for sampling frequency (McKenzie et al., 2006) in order to assess survival and quantify non-differentiated cells and SC-differentiated cells within each environment presented. Myelin was visualized using the fluorescent hydrophobic probe Nile red (Arnaud et al., 2009).

Maintenance of SC phenotype in acellular grafts

In order to assess long-term maintenance of the SC phenotype of SKP-SCs used in acellular graft repair and assess their overall contribution to SC count in the nerve, we carried out an experimental paradigm analogous to what we have previously reported (Walsh et al., 2009). We created a 12 mm gap in the sciatic nerve of Lewis rats, and subsequently repaired it with an isogenic graft rendered acellular by repeated freezing and thawing (Ide et al., 1983; Hall, 1986). At this time, 3 μl of DiI (Invitrogen) labeled rat SKP-SCs were injected into both the distal and proximal 3 mm of the acellular grafts, giving a total of 5×10^5 cells delivered in 6 μl of media. Controls consisted of isografts receiving media alone or autograft repair (the current gold standard of treatment). At 10 weeks post-transplant, nerves were harvested for immunohistochemistry as described above, and every section throughout the thickness of nerve was collected. Primary antibodies used were rabbit anti-S100β (1:100; Santa Cruz), and mouse anti-macrophage/monocyte clone ED-1 (1:200; Chemicon, Billerica, MA). Secondary antibodies were anti-rabbit or anti-mouse 488 (1:400; Molecular Probes, Inc). Sections were viewed with a confocal microscope (Zeiss Scanning system, LSM 510) in order to assess co-localization of fluorescence signals. Three random high-powered fields/section were photographed, taking 3 sections throughout the thickness of the nerve (9 images/nerve) in order to assess phenotype of injected SKP-SCs. Identical images were obtained in each channel and then merged to identify DiI signal only (SKPs), DiI+488 signals (SKPs+macrophage or SKP-SC), or 488 signal only (host SC or host macrophage). An observer blinded to treatment groups used this method to compare the number of SCs in SKP-SC treated grafts, media treated grafts and autografts, and the counts expressed are based on a sampling area of 12,000 μm^2.

Regulation of SKPs' early apoptosis by heregulin-1β

From the experiments above, we noted that SKPs showed poorest survival in the intact nerve and the chronically denervated nerve—two environments that are potentially devoid of accessible neuregulins. Moreover, a considerable amount of apoptosis in transplanted cells likely occurs within 24 h, likely due to the fact that cultured cells lose both their in vitro attachment and trophic factors when dissociated and placed in vivo. We first tested the reliance of SKP-SCs on neuregulins in vitro by growing newly-differentiated SKP-SCs in the presence or absence of heregulin-1β for 48 h. Cells were plated at equal density in each chamber of two 4-well Chamber Slides (Nunc, Rochester NY). Half of the wells received heregulin-1β (10 ng/ml) and the other half received its diluent (PBS). After 48 h, cells were fixed in methanol and processed for immunocytochemistry as described above. Apoptotic cells were labeled with anti-cleaved caspase 3 (1:250) (detected with anti-rabbit 488). Percentage of SKPs undergoing apoptosis was counted from three random fields of view per well (at 1000× magnification) by an observer blinded to the treatment.

We next sought to determine whether inclusion of heregulin-1β into the injection cocktail could decrease apoptosis observed at 24 h. For this experiment, surgeries to expose the nerve of adult Lewis rats were performed bilaterally, as described above. 300,000 DiI labeled SKP-SCs (suspended in DMEM with or without 500 ng/μl heregulin-1β) were injected distal to the injury site. Twenty four hours later, nerves were harvested and processed for immunohistochemistry, labeling with cleaved caspase 3 (1:100) and detected with anti-rabbit 488. The analysis method described above for percentage differentiated cells was used to quantify caspase positive cells in this experiment. Fluorescence microscopy was employed, using the appropriate filters to detect DiI labeled cells (SKP-SCs) and 488 signal (caspase 3 labeled, apoptotic cells). Images were merged using ImageJ (NIH) in order to quantify the percentage of apoptotic SKP-SCs.

Statistical analysis

Differences between differentiated versus naïve cell survival and apoptosis with or without heregulin were compared using a two-tailed Student's t-test. A two-way ANOVA was performed to detect whether there was an effect of nerve environment on survival. Statistical significance was accepted at $p<0.05$, with all results presented as the mean ± SEM.

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