White Matter Repair: Skin-Derived Precursors as a Source of Myelinating Cells

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ABSTRACT: Stem cell based therapies hold great promise for repair and functional restoration following neurological injury and disease. Skin-derived precursors (or "SKPs") are a novel, multipotent somatic stem cell that resides within the mammalian dermis. SKPs persist within the skin throughout adulthood and yet intriguingly, exhibit many similarities to embryonic neural crest stem cells (NCSCs). For example, SKPs give rise to both neural and mesodermal cell types, and the former appear biased to peripheral nervous system fates. As such, SKPs are capable of generating Schwann cells, the myelinating glial cell of the peripheral nervous system. Here we discuss our current understanding of the biological origin of SKPs and specifically the potential therapeutic utility of SKPs as a highly accessible and autologous source of Schwann cells for remyelination and repair of the injured or diseased nervous system.

RÉSUMÉ: Réparation de la substance blanche : précurseurs dérivés de la peau comme source de cellules myélinisantes. Les traitements à base de cellules souches pour la réparation et le rétablissement fonctionnel suite à une lésion neurologique ou à une maladie sont très prometteurs. Les précurseurs dérivés de la peau (PDP) sont de nouvelles cellules souches somatiques pluripotentes qu'on retrouve dans le derme des mammifères. Les PDP persistent dans la peau pendant toute la vie adulte et, ce qui est intriguant, présentent plusieurs caractéristiques similaires à celles des cellules souches de la crête neurale embryonnaire. À titre d'exemple, les PDP donnent naissance à des cellules de type nerveux et de type mésodermique et les premières semblent biaisées en faveur du système nerveux périphérique. Elles sont donc capables de générer des cellules de Schwann qui sont les cellules gliales myélinisantes du système nerveux périphérique. Nous discutons ici de notre compréhension actuelle de l'origine biologique des PDP et spécifiquement de leur utilité thérapeutique potentielle comme source autologue très accessibles de cellules de Schwann pour la remyélinisation et la réparation du système nerveux malade ou qui a subi une lésion.

Over the last two decades there has been growing interest in stem cell (SC) biology. Their ability to self renew and their coincident ability to generate multiple differentiated cell types are features which could potentially be exploited for cell replacement therapies following nervous system injury or disease.

SKPs are multipotent stem cells that reside in the dermis

Skin-derived precursors (SKPs) are a somatic stem cell derived from the dermis of the skin[1,2]. Dissociation of dermis to single cells and subsequent exposure to basic fibroblast growth factor and epidermal growth factor in vitro results in formation of spherical colonies of dividing cells, which we have termed "SKPs" (Figure 1A)[2]. Skin-derived precursors can be expanded indefinitely *in vitro*, forming new spherical colonies with each passage. Importantly, single SKP cells from embryonic or adult skin can be grown clonally[1-4], giving rise to millions of progeny, thus demonstrating their ability to self renew. Skin-derived precursors can be isolated from developing mouse skin as early as embryonic day 15 (E15) and persist within the skin throughout adulthood[1] although it is thought that their numbers may decline with increasing age[5]. It is not known how SKPs arrive within the dermis, but it has been speculated that they

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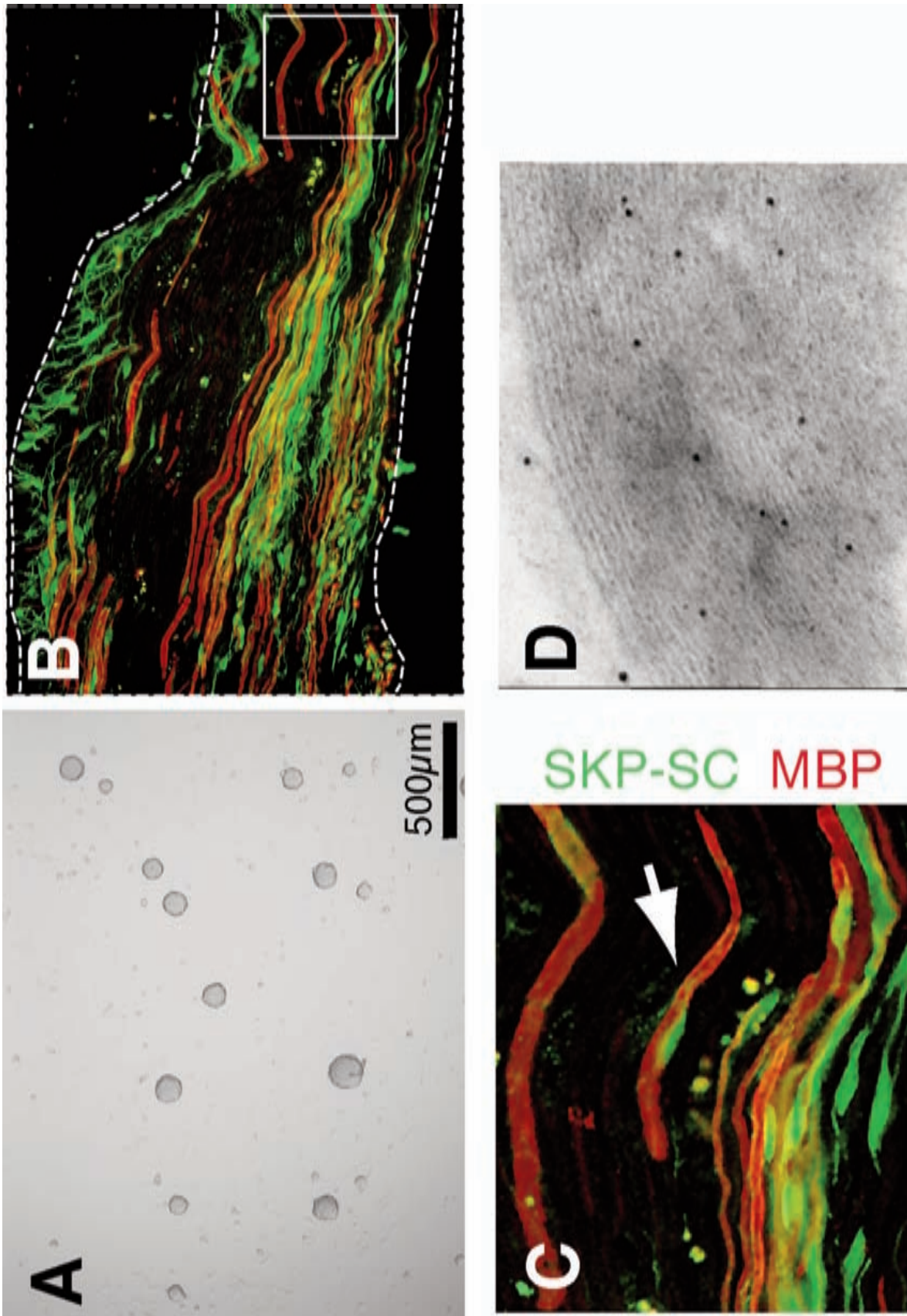
Figure 1: *SKP generate Schwann cells that myelinate the injured nervous system. A, Photomicrograph showing adult clonally-derived SKPs in vitro (10X magnification). B,C, Image showing longitudinal section of the injured sciatic nerve of a shiverer mutant mouse containing SKP-derived Schwann cells (green) six weeks after transplantation. Note that because shiverer mice are deficient for the MBP gene, only donor SKP-SC myelin are capable of myelin basic protein expression (MBP; red). Nerve is outlined by hatched lines. 10x magnification. C, High magnification of inset of boxed area in panel B, showing an MBP-positive (red) SKP-derived Schwann cell (green). D) Electron micrograph depicting a SKP-derived myelin sheath containing following immuno-EM for MBP. Gold-labeled particles (arrows) indicate the presence of MBP, which can only be from SKP-derived Schwann cells. Scale bar is 100nm. Reproduced with permission from The Journal of Neuroscience (2006) 14;26(24);6651-60.*

might migrate along cutaneous nerves or vasculature[6], or alternatively may be induced as a result of inductive interactions between epithelial and mesenchymal tissues within the developing skin. Indeed, one niche for SKPs is within the specialized mesenchymal compartment of hair follicles, called the dermal papilla and contiguous dermal sheath which surrounds the follicle[1].

One of their most intriguing properties is their ability to generate a varied array of cell types, both mesodermal and neural[2]. Upon exposure to cell culture medium containing high concentrations (5-10%) of serum, or in defined conditions designed to induce/support specific cell fates, these spherical SKP colonies attach to a laminin or fibronectin substrate, and the cells within them begin to extend processes and eventually give rise to smooth muscle cells, adipocytes, dermal fibroblasts, osteocytes, chondrocytes and even neural cell types[1-4, 6-9]. Within days of exposure to neuronal differentiation conditions, SKPs generate neuronal-like cells that express high levels of the neural precursor marker nestin, with subsequent expression of more definitive markers of neuronal fate such as βIII tubulin and neurofilament-M[1,3,7,10]. Interestingly, SKP-derived neurons appear to be biased toward peripheral neuron fates, expressing only catecholaminergic phenotypes (dopamine β-hydroxylase-positive, or tyrosine hydroxylase-positive) and not exhibiting glutamatergic, serotonergic, or cholinergic phenotypes[10].

Similarly, when differentiated under appropriate conditions, SKPs also generated glial cells that exhibited a bipolar, spindle-shaped morphology and in vitro immunostained positively for glial fibrillary acidic protein (GFAP), p75 neurotrophin receptor and s100β, all consistent with a peripheral glial cell called a Schwann cell. Schwann cells are exclusive to the peripheral nervous system and can be pro-myelinating or non-myelinating, a binary decision that is at least partially determined by the local availability of axon-secreted factors, such as neuregulin (NRG)[11]. Large diameter peripheral neurons rely on Schwann cells to wrap axons with a repeating sheath of myelin, thereby providing increased membrane resistance, decreased membrane capacitance and ultimately, more efficient transmission of electrochemical signaling between cells.

SKPs exhibit properties of neural crest stem cells

The finding that postnatal SKPs were capable of generating both mesodermal and peripheral neural cell types is intriguing, and highly reminiscent of an embryonic stem cell population originating from a transient structure called the neural crest. The neural crest arises at the dorsal surface of the neural tube as a result of inductive cellular interactions between neural tube and the overlying epithelium. Neural crest cells delaminate from the neural tube and migrate throughout the developing embryo. The fate of these migrating neural crest cells is sculpted by interactions with tissue-derived cues giving rise to sensory, autonomic and and enteric nervous systems as well as many mesodermal cell types such as melanocytes, smooth muscle cells within the ouflow tract of the heart, and craniofacial bones, adipose, cartilage and connective tissue[12]. Neural crest stem cells also exhibit a unique set of genes, many of which are also expressed by SKPs (ie. Sox10, Snail, Slug, Twist). Futhermore, SKPs derived from craniofacial skin originate from the neural crest, as demonstrated by lineage tracing studies using the Wnt-1cre: Rosa26 mice, which express β-galactosidase in all neural crest-derived tissues[1].

SKP-derived Schwann cells myelinate the injured peripheral nervous system

Based on their similarity to NCSCs, we hypothesized that factors involved in NCSC fate determination may similarly influence differentiation of SKPs progeny. Classic experiments on avian and rodent neural crest cells had highlighted a number of key factors involved in specification of the Schwann cell fate (reviewed in[12]). One such factor, neuregulin (NRG), is a key player in most aspects of Schwann cell development, survival, proliferation and myelination[11,13-16]. More specifically, NRG was found to be a potent regulator of glial fate determination for isolated rat NCSCs[17,18]. Remarkably, addition of NRG to SKP differentiation medium also resulted in a >5-fold increase in number of Schwann cells compared to control conditions[4,19]. These cells could be readily expanded in culture and could be purified to >95% purity under these defined culture conditions. Purified cells also actively migrated to and associated with axons when co-cultured with explanted dorsal root ganglia explants, and expressed myelin-specific proteins such as peripheral myelin protein 22, and myelin basic protein.

But are SKPs generating bona fide functional Schwann cells? That is, are SKP-derived Schwann cells capable of myelinating axons in vivo? If so, SKP-derived SCs might represent a highly accessible and renewable source of autologous myelinating cells which are not associated with ethical issues, and do not require invasive surgical procedures to obtain them. To test this possibility, we utilized a crush injury model of the sciatic nerve, transplanting SKPs distal to the crush site following the injury. In order to track the fate of donor cells, SKPs were generated from mice ubiquitously expressing green fluorescent protein. In addition, the recipient mice were a transgenic strain called shiverer which are deficient in the myelin basic protein gene (MBP). This way, only transplanted SKP-derived SCs that had successfully generated myelin, and not endogenous Schwann cells, could express MBP within the injured nerve. Examination six weeks later showed that many GFP-tagged SKP-derived SCs had generated MBP-positive myelin (Figure 1B,C). Quantification showed that ~70% of donor SKP- SCs within the nerve also stained positively for MBP, suggesting that surviving SKP-derived SCs efficiently generated myelin. Using immuno-electron microscopy for MBP we confirmed that SKP-derived Schwann cells generated true compact myelin containing characteristic repeating dense lines with the myelin sheath (Figure 1D).

Other groups have since extended these findings, demonstrating that SKP-derived SCs also support axon regeneration and improved functional outcome after peripheral nerve injury. Specifically, in experiments where the chronically denervated peripheral nerve was supplemented with SKP-derived SCs, there was a significant improvement in motor neuron regeneration that resulted in greater target muscle re-innervation[20]. Thus, SKPs differentiate into bona fide myelinating Schwann cells, and these SKP-derived SCs are capable of promoting anatomical and function recovery in the peripheral nerve, a finding with significant therapeutic implications.

Cell replacement for spinal cord injury: Why Schwann cells?

Tramautic spinal cord injury results in direct damage to axon tracts and cell bodies at the site of impact and is followed by a series of secondary events that lead to further neuronal death and demyelination of surrounding white matter tracts. Contusion-type injuries typically generate a cystic cavity that is encapsulated by meningeal fibroblasts, microglia and a wall of reactive astrocytic scar tissue[21]. Endogenous repair following spinal cord injury is limited and current treatments are insufficient.

More than three decades ago, Canadian neuroscientist Albert Aguayo and colleagues first demonstrated the regenerative properties of the peripheral nerve environment by grafting segments of peripheral nerve into the lesioned CNS[22,23]. Further experiments went on to show that it was the peripheral glial cells, Schwann cells, that were largely responsible for these growth-promoting effects[22]. This, along with their ability to re-myelinate demyelinated axons and their proliferative capacity *in vitro*, have made Schwann cells an exciting candidate for treating diseases such as multiple sclerosis and spinal cord injury.

Several experimental studies have demonstrated significant functional gains following transplantation of Schwann cells into acute or subacute spinal cord injured animals[24-26]. Although promising, these experiments also highlighted several limitations to using mature (peripheral nerve-derived) Schwann cells as a source of cell replacement and repair. Mature human Schwann cells are difficult to expand and once transplanted to the spinal cord show poor survival, failure to integrate within the astrocyte rich area of the CNS, and only short-range migration within the CNS. In addition, procurement of Schwann cells requires an invasive surgical procedure often resulting in lasting sensory deficits.

SKP-derived Schwann cells for spinal cord injury

Based on their capacity to remyelinate the injured or genetically dysmyelinated peripheral nerve, we asked whether SKP-derived Schwann cells could improve repair and functional recovery following a contusion spinal cord injury. We hypothesized that SKPs would provide an accessible and autologous, adult source of Schwann cells that could be used to treat spinal cord injury or other CNS demyelinating disorders. Moreover, we reasoned that because of their stem cell derivation, SKP-derived Schwann cells were likely to be similar to developing, immature Schwann cells and/or even Schwann cell precursors [reviewed in[27]], which may provide greater proliferative capacity and ultimately improved repair as compared to the mature nerve-derived SCs utilized in previous studies.

SKP-derived SCs remyelinate the injured adult spinal cord

To test this idea, we performed a series of spinal cord transplantations studies. Subacute transplantation (seven days post-injury) of neonatal SKP-derived SCs into the lower thoracic contusion cavity of adult rats resulted in significant survival and repair in comparison to either brain-derived neural precursors, naïve SKPs, or medium control treatment (Figure 2A)[28]. Both naïve SKPs and SKP-derived SCs filled in the lesion cavity and exhibited moderate survival. More impressive was the finding

that SKP-derived SCs formed "tissue bridges" across the cavity, most likely by fasciculating upon spared axon fibres. This was surprising since previous studies had demonstrated that the injured spinal cord is an unfavorable environment for stem cell survival, particularly when cells were injected directly into the lesion, with most cells dying in this environment[29]. In contrast, when neural precursors are transplanted into regions adjacent to, but not within the cavity, they have shown significantly greater survival and widespread migration, and have even myelinated demyelinated axons within intact areas of the cord[30].

In addition to the "bridge" formation that was observed with SKP-derived SCs in these experiments, neuroanatomical analysis 11 weeks post-transplantation revealed significant peripheral myelination within the lesion cavity and in the surrounding spared rim (Figure 2,B,C,D). SKP-derived SCs (which were genetically tagged with green fluorescent protein; GFP) had ensheathed central axons and exhibited a myelinating phenotype as indicated by their expression of the peripheral myelin protein P0 (Figure 2A). Coincidently, these SKP-derived SCs decreased their expression of both glial fibrillary acidic protein (GFAP) and the neurotrophin receptor p75, consistent with a myelinating phenotype. Evidence that this was *bona fide* myelin formation came from studies showing that myelinating SKP-derived SCs interacted with host oligodendrocytes and/or Schwann cells to allow re-assembly of essential paranodal and juxtaparanodal proteins at nodes of Ranvier, as indicated by the expression pattern for the myelin-associated adhesion molecule contactin associated protein (CASPR) and the KV1.2 potassium ion channel[28].

Recruitment of host SCs

While these experiments demonstrated that SKP-derived SCs themselves had the capacity to promote neuroanatomical repair of the spinal cord, one unexpected finding was that they appeared to enhance endogenous repair mechanisms. In this regard, injury to the spinal cord normally leads to infiltration of Schwann cells from the peripheral nerves in an apparent attempt at endogenous repair. In our experiments, one of the most striking effects of SKP-SC treatment was their impact on endogenous SC recruitment in the lesioned area and surrounding rim. Quantification of GFP-negative P0-positive (host) Schwann cells demonstrated that their numbers were dramatically increased following SKP-SC treatment. Remarkably, this augmented migration is also observed following transplantation of SKP-derived SCs into the chronically injured spinal cord (J. Sparling, J. Biernaskie, T. Morano, F. Miller, W. Tetzlaff, unpublished observations). It is not known whether the presence of SKP-derived SCs is stimulating proliferation or promoting survival of host SCs within the cord, or indirectly facilitating their migration. Future studies will need to explore the factors which may be regulating host SC recruitment and survival within the spinal cord, as a further adjunct therapy to maximize repair and functional outcome.

SKP-derived SCs are neuroprotective

In addition to their effects on axonal myelination, our analyses demonstrated that transplantation of SKP-derived SCs provided robust protection of the compromised white matter
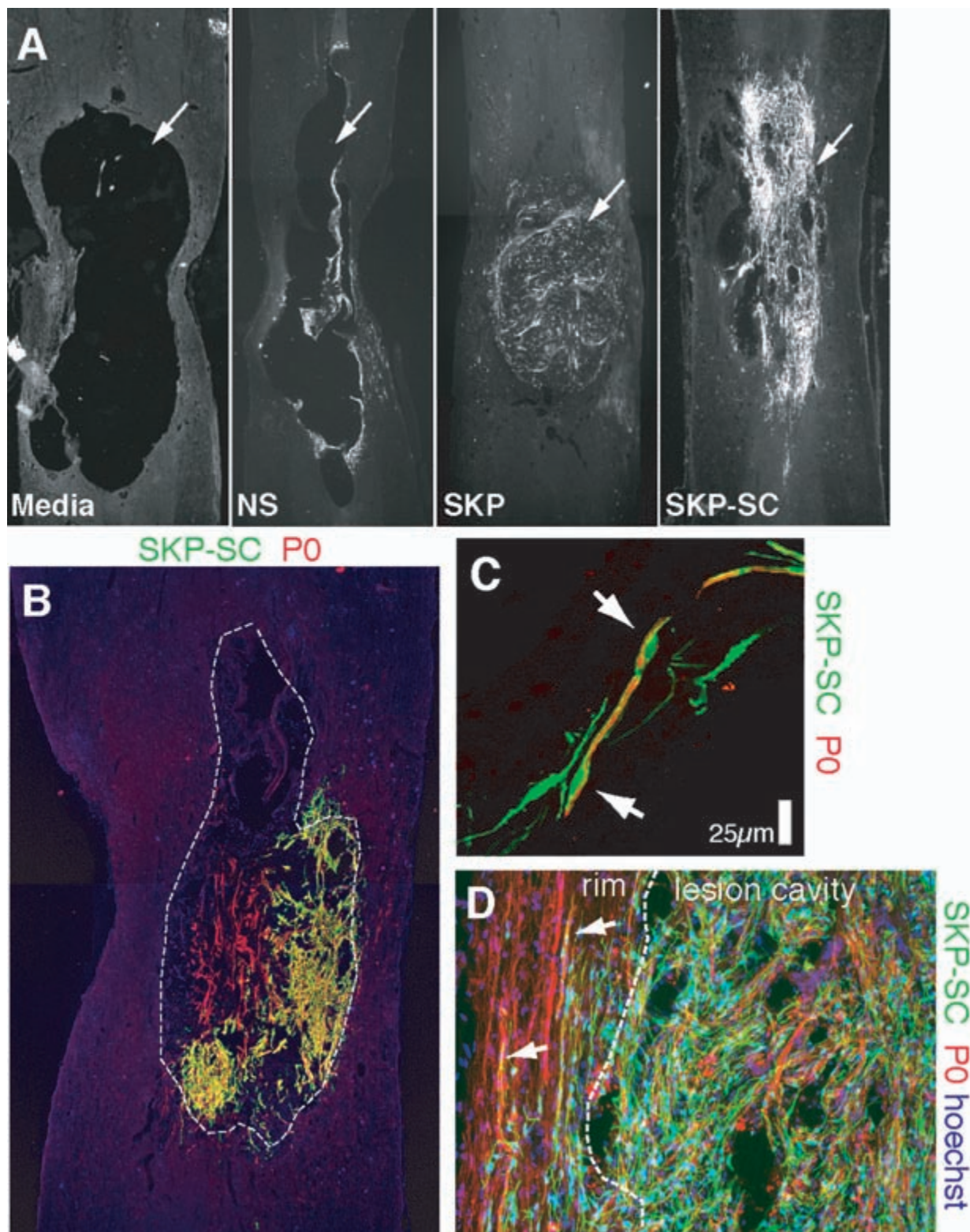
Figure 2: SKP-derived Schwann cells remyelinate the injured spinal cord. Low magnification (10x) images depicting typical transplant volume and cell survival at 11 weeks post transplant to the contused spinal cord. Donor cells are shown in white. Note that SKP-derived SCs survive and form cellular bridges within the cavity. B, Low magnification (5X) image depicting SKP-derived SCs (green) with the contused spinal cord, 11 weeks after transplant. P0 immunostaining (red) depicts peripheral myelin. C, High magnification (40x) image showing SKP-derived Schwann cells (green) co-express the peripheral myelin protein P0 (red). D, 20X image of contused spinal cord 11 weeks post-transplant, showing SKP-derived SCs (green) that have migrated from into the spared rim, many of which co-express peripheral myelin protein P0 (red; arrows). Reproduced with permission from The Journal of Neuroscience (2007) 5;27(36):9545-59.

tracts. Neuroanatomical analysis 11 weeks after the transplant revealed a 40% reduction in cavity volume following SKP-SC transplantation relative to the medium control treatment. These findings suggest that SKP-derived SCs may be either secreting trophic factors that promote the survival[31] and maintenance of glial cells and axons and/or they modify the relatively destructive inflammatory response that occurs following spinal cord injury. These findings are particularly intriguing given the recent interest in the use of bone marrow stromal cells to provide trophic support and reduce the immune response[32,33], since they suggest that SKPs may have the ability to perform similar functions.

SKP-derived SCs promote regeneration

For many decades one of the main goals of spinal cord injury studies was to promote long-distance axonal regeneration. More recently, the field has come to appreciate that even short-distance axonal sprouting and/or regenerative growth may promote neural plasticity that could ultimately result in some functional recovery. In this regard, Schwann cells have long been known to promote CNS axon growth, and our studies with SKP-derived SCs confirmed and extended this conclusion. Specifically, examination of spinal cord sections one and two weeks following transplantation go SKP-derived SCs showed that descending serotonergic, β-adrenergic fibers and retrogradely labeled sensory fibers were all significantly increased following SKP-SC transplant versus control treatments. Furthermore, we implemented a time-course experiment to assess the state of axonal growth within the lesion during the subacute to chronic stages of recovery. The relative absence of fibers within the lesion at one week, followed by a significant increase in fiber numbers at two weeks post-transplant, verified that SKP-SC treatment had stimulated regeneration of severed axons rather than simply protecting pre-existing axon tracts. Importantly, SKP-derived SCs also maintained a rostral-caudal orientation such that axon growth was directed through the cavity toward the distal end of the lesion[4]. Interestingly, the opposite effect was observed following naïve SKP transplant which resulted in excessive and highly disorganized axon growth. Thus, SKP-derived SCs provide not only neuroprotection of spared fibers, but also provide an effective scaffold that supports regenerative growth of severed axons.

How do SKP-derived SCs enhance axonal growth? Although the exact mechanism remains unclear, several pieces of evidence suggest that, in addition to providing a highly conducive growth environment within the cavity, SKP-derived SCs likely enhance growth by modifying the local environment surrounding the lesion. One of the hallmarks of the injured spinal cord is infiltration of reactive astrocytes and the subsequent formation of a glial scar[21]. In addition to forming a physical barrier to axonal growth, reactive astrocytes also produce growth-inhibitory chondroitin sulfate proteoglycans[34]. Several therapies have been directed toward preventing or removing these 'roadblocks to growth' with some success. Infusion of chondroitinase, an enzyme directed at degrading CSPGs)[35,36], or antibodies targeting inhibitory proteins such as Nogo, have provided modest benefit[37]. In our experiments, immuno-histochemical analysis of spinal cord sections confirmed the presence of reactive astrocytes surrounding the contusion cavity,

and their expression of chondroitin sulfate proteoglycans such as neurocan. Surprisingly, SKP-SC transplant caused a significant reduction of neurocan in the surrounding parenchyma, and an enrichment of growth-permissive molecules like laminin. This effect was most evident in areas where SKP-derived SCs were found interspersed with GFAP-positive astrocytes. This intermingling of SKP-derived SCs with astrocytes is in contrast with previous reports using nerve-derived Schwann cells[38] which typically form a clear dermarcation between the two populations. It is likely that these differing findings reflect a difference in developmental status of adult nerve-derived SCs versus SKP-derived SCs. Whether or not SKP-derived SCs can also modify the astrocytic scar formed in chronic spinal cord contusions is a key question for the future.

Future directions and obstacles to clinical use and future considerations

One major issue facing the use of Schwann cells and, indeed any exogenous cell, for white matter repair is cell survival. Implantation of Schwann cells into the acute, subacute, or chronic stage of spinal cord injury results in massive cell death[24,39,40]. One recent study using adult nerve-derived Schwann cells[39] found 78% cell loss, most of which occurred within 48 hours after transplant. Although much of this early death was reportedly due to necrosis[41] rather than apoptic cell death, pharmacologic intervention to rescue cells within vulnerable regions of the cord may be a necessary adjunct to promoting repair. In our experiments, survival of SKP-derived Schwann cells following transplant into the injured sciatic nerve or spinal cord range between 5% and 30%[4,28]. Interestingly, cell survival exceeding one month appears to be unchanged, suggesting that once SKP-derived Schwann cells have found a functional niche, (ie. successfully myelinated an axon) they are maintained, likely via axonal secretion of neuregulin or other trophic molecules. One contributing factor may be "anoikis", a term referring to cell death resulting from loss of contact or adhesive cell signals with the surrounding extracellular matrix. Subacute and chronic spinal cord injuries are typically cystic cavities. In our experiments with SKP-SC implants, surviving cells appeared to be those successfully navigating to spared or regenerating axon fibers within the cavity or demyelinated rim, or those SKP-derived SCs that had penetrated into the field of reactive astrocytic glia and assumed a non-myelinating phenotype[28]. Future studies will examine the effect of combining SKP-derived SCs with supportive extracellular matrices to restore adhesion-derived cues and thereby hopefully improving survival.

Many cell transplantation therapies have utilized undifferentiated adult stem cell populations for cell therapy. However, our studies characterizing the differentiation of SKPs within different injury environments argues that, while this may be a feasible strategy in environments that heal and/or regenerate normally (such as fractured bone), this is unlikely to be the strategy-of-choice for tissues that do not have sufficient endogenous repair mechanisms, such as the injured spinal cord. In particular, we found that, when naïve SKPs were transplanted into the injured spinal cord, an environment where there many growth factors and inflammatory cytokines are present, SKPs differentiated not only into Schwann cells, but also into

inappropriate mesenchymal cell types such as fibroblasts and adipocytes[28]. The presence of such cells may have considerably negative effects on repair and may actually worsen outcome. In this regard, other studies have reported that transplantation of undifferentiated neural stem cells into the injured spinal cord led to aberrant growth of endogenous sensory fibers and the onset of allodynia[42]. Therefore, considerable safeguards will need to be in place, as for any stem cell treatment, in order to prevent generation of inappropriate cell types, uncontrolled growth, or indirect effects on the integrity of the remaining nervous system circuitry.

One unanticipated positive result of our transplants was the finding that, relative to mature nerve-derived SCs, SKP-derived Schwann cells showed improved integration within the spared rim of the spinal cord and astrocytic glial scar. As discussed above, one explanation is that SKP-derived SCs, because they have originated from a neural crest-like stem cell, may be more similar to developing immature Schwann cells or Schwann cell precursors. Support for this idea comes from a recent study using purified embryonic glial populations which demonstrated that embryonic SCPs provided superior repair, remyelination, neuroprotection, and axonal growth relative to mature SCs)[43]. Our observations of sustained proliferation in culture and intermingling with reactive astrocytes are consistent with the behavior of an immature Schwann cell/Schwann cell precursor. Indeed, a recent study reported that transplanted embryonic Schwann cell precursors provided a 'smooth interface' with host astrocytes, and were observed navigating long distances through normal CNS environment. Interestingly, embryonic Schwann cell precursors also homed to demyelinated lesions, in which they formed new myelin[43]. These findings, in addition to the fact that Schwann cells do not appear to be targeted by the immune system in multiple sclerosis patients, suggest that SC precursors and potentially SKP-derived SCs may be a reasonable source of cells for treating spinal cord injury, multiple sclerosis, and perhaps other demyelinating disorders.

We originally identified and characterized SKPs with the hope that their highly accessible location in the skin and their persistence throughout adulthood would circumvent the ethical issues and invasive surgical procedures required to obtain alternative sources of neural stem cells or progenitors. Moreover, since SKPs could ideally be derived from the same patient, this would ultimately circumvent issues of immune rejection. In this regard, although further studies will be required to optimize their isolation and differentiation from adult human skin, and to promote their survival and integration following transplantation, it is our long-term hope that SKP-derived SCs may represent a valuable source of myelinating cells for nervous system therapies.

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