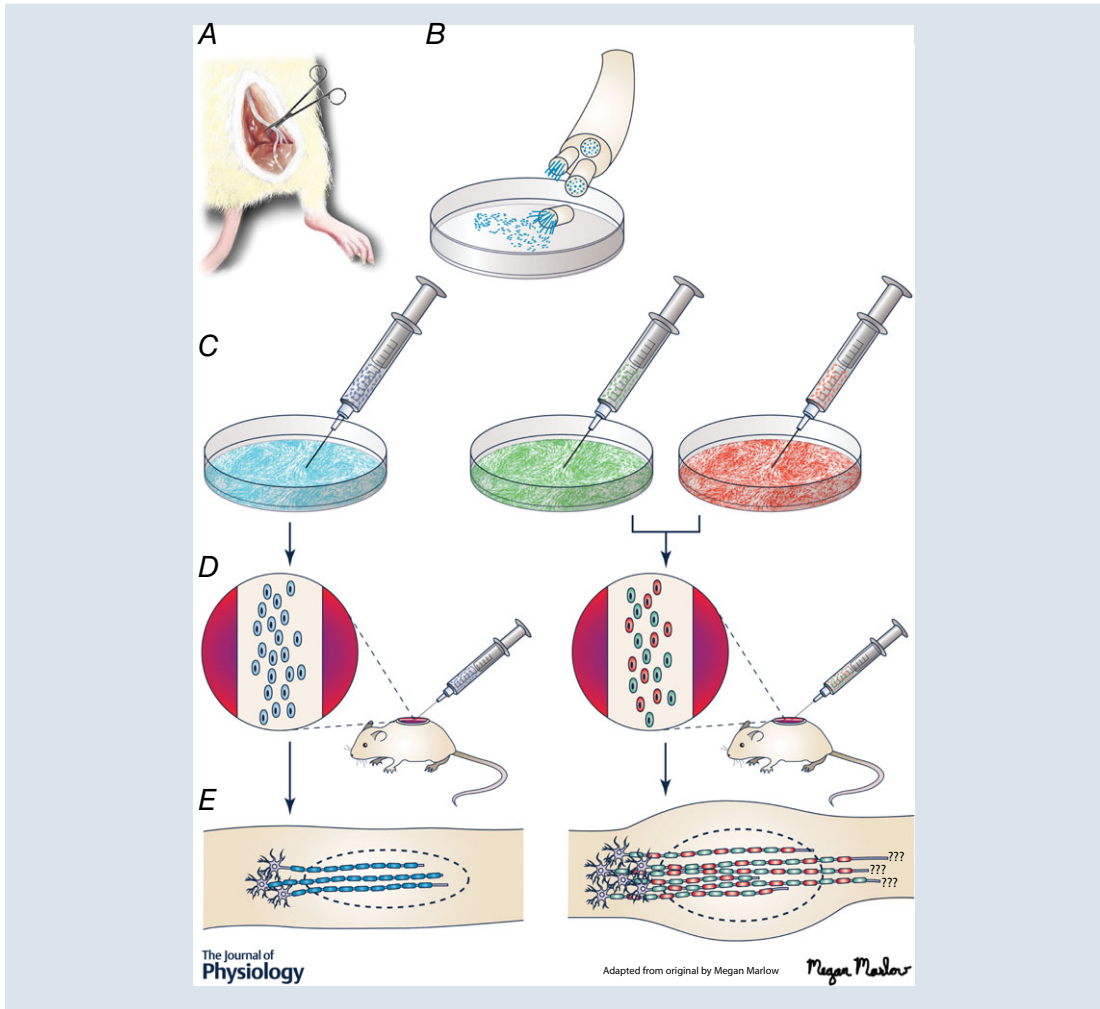


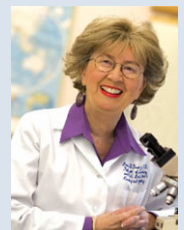
Efficacy of Schwann cell transplantation for spinal cord repair is improved with combinatorial strategies

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Abstract When cells (including Schwann cells; SCs) of the peripheral nervous system (PNS) could be purified and expanded in number in tissue culture, Richard Bunge in 1975 envisioned that the SCs could be introduced to repair the central nervous system (CNS), as SCs enable axons to regenerate after PNS injury. Importantly, autologous human SCs could be transplanted into injured human spinal cord. Availability of the new culture systems to study interactions between sensory neurons, SCs and fibroblasts increased our knowledge of SC biology in the 1970s and '80s. Joining the Miami Project to Cure Paralysis in 1989 brought the opportunity to use this knowledge to initiate spinal cord repair studies. Development of a rat complete spinal cord transection/SC bridge model allowed the demonstration that axons regenerate into the SC bridge. Together with study of contused rat spinal cord, it was concluded that implanted SCs reduce cavitation, protect tissue around the lesion, support axon regeneration and form myelin. SC transplantation efficacy was improved when combined with neurotrophins, elevation of cyclic AMP levels, olfactory ensheathing cells, a steroid or chondroitinase. Increased efficacy meant higher numbers of axons, particularly from the brainstem, and more SC-myelinated axons in the implants and improvement in hindlimb movements. Human SCs support axon regeneration as do rat SCs. Astrocytes at the SC bridge–host spinal cord interfaces play a key role in determining whether axons enter the SC milieu. The SC work described here contributed to gaining approval from the FDA for an initial autologous human SC clinical trial (at the Miami Project) that has been completed and found to be safe.

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Abstract figure legend Cartoons illustrating both the general procedures to prepare Schwann cells (SCs) for transplantation and the more specific plan to examine the effect of transplanting both neurotrophin- and chondroitinase-secreting SCs (green and red, respectively). The blue SCs represent non-transduced control cells. The nerve is removed (A) and the fascicles dissected to liberate SCs (B). Enzyme and mitogen treatment further results in a large number of purified populations of SCs (C). In some cases they are transduced with viral vectors carrying DNA for green fluorescent protein/neurotrophin and mCherry/chondroitinase before transplantation; here both are combined in some animals (D). In contusion/implant sites, blue SC-myelinated axons are present in the implant but are greatly increased in number when green and red SCs are transplanted (E). See text for an expanded explanation of the specific experiment.

In 1975, Richard Bunge suggested that glial cells from the nervous system could be isolated and purified in tissue culture and then used to repair the central nervous system (CNS) (Bunge, 1975). This was a time when the techniques to purify and isolate glia had become available. When he joined the faculty at Washington University School of Medicine in 1970, he along with Patrick M. Wood, set out to develop techniques to culture purified populations of cells from dorsal root ganglia (sensory neurons, Schwann cells (SCs) and fibroblasts) singly or in combination. These techniques enabled us to gain more information about SC biology. For example, we discovered that there is a molecular signal on the surface of axons that causes SCs to proliferate (Wood & Bunge, 1975). We found that SCs require contact with axons to form basal lamina (Clark & Bunge, 1989) which is required for SCs to form myelin (Eldridge *et al.* 1989). We also learned that SCs need ascorbic acid to form myelin (Eldridge *et al.* 1987). We observed that SCs generate extracellular matrix components in addition to those in the basal lamina such as various types of collagens (Bunge *et al.* 1980). All this earlier work has been reviewed by Bunge *et al.* (1983,

1990) and Bunge & Bunge (1983). We also proved that perineurium is formed from fibroblasts not SCs (Bunge *et al.* 1989).

In 1980, a landmark paper from the Aguayo laboratory in Montreal was published revealing that a piece of peripheral nerve inserted into a complete gap in the adult rat spinal cord engendered regeneration of axons from host neuronal somata (Richardson *et al.* 1980). This was a very important discovery because it demonstrated that if the environment is appropriate, CNS neurons will regenerate axons in damaged spinal cord. Moreover, it became known that when axons in peripheral nerve regenerate, they do so into tunnels of basal lamina harbouring surviving SCs. The message was clear that SCs are key to axon regeneration in the peripheral nervous system (PNS). Why not, then, introduce them into the CNS? Because they were available using the new culture techniques, we decided to transplant purified populations of SCs rather than pieces of peripheral nerve into transected spinal cord.

In addition to the findings mentioned above, SCs were likely transplant candidates because they are easily accessible in peripheral nerve and they produce growth

factors and extracellular matrix molecules that promote axon growth (Bunge, 1994). Also, they could provide a requisite scaffold for the regrowth of axons across the area of injury. SCs myelinate axons in the CNS (Gilmore & Sims, 1993) and, by doing so, restore conduction activity (Blight & Young, 1989). As would be found later, large numbers of rat and human SCs can be generated in culture. Furthermore, they can be genetically engineered to generate additional growth factors. The strongest impetus for us to pursue SC transplantation was that they could be autologously transplanted into a spinal cord injury site following removal of a piece of peripheral nerve from the injured person's leg and the SCs then purified and expanded in number in culture (Bunge & Wood, 2012). More recently, the exhaustive evaluation of additional SC-transplanted rats for the Food and Drug Administration (FDA) demonstrated clearly that tumours are not formed.

Upon transplantation of Richard, Pat and myself to the Miami Project to Cure Paralysis in 1989, SC implant studies began in earnest. Fortunately Dr Xiao-Ming Xu joined our group as a postdoctoral fellow for it was Dr Xu who developed a new model of a SC bridge positioned in a complete gap in the rat spinal cord. After achieving a complete transection gap, the stumps were inserted into a polymer channel that contained a cord of SCs. The channel held the SCs and stumps in place. One month later, the SCs had bridged the gap between the transected stumps of the spinal cord (Xu *et al.* 1997). By examining tissue sections from either side of the SC bridge after immunostaining for axons, numerous axons were observed to have entered the SC bridge. Looking at cross sections of the bridge, the appearance resembled peripheral nerve in that SCs had myelinated or ensheathed the regenerated axons. A summary of these initial SC bridge/complete spinal cord transection experiments is that axons grew onto the SC bridge from both stumps, there were 1990 ± 594 myelinated axons on the bridge and there were eight times more non-myelinated but ensheathed axons on the bridge (Xu *et al.* 1997).

Due to clinical relevance, contusion studies were also initiated. A result of contusion injury in both rats and humans often is the formation of large cysts due, at least in part, to the removal of lesioned tissue by macrophages. Transplantation of SCs led to reduction in cyst size, protection of spinal cord tissue from secondary damage, and support of axon growth into the SC graft (5212 ± 1783 SC-myelinated axons) (Takami *et al.* 2002). There was a modest improvement in locomotion (Takami *et al.* 2002). These results were promising in that in both injury models transplanted SCs led to higher numbers of SC-myelinated axons in the implant. When SCs are not transplanted, endogenous SCs are consistently observed to enter lesions and myelinate axons (2125 ± 697 in the Takami *et al.* 2002 study) (see also Pearse *et al.* 2004b; Kanno *et al.* 2014).

The necessity for combinatorial strategies came to mind because of the variety of changes that occur in spinal cord injured tissue. Secondary tissue damage must be halted, as many nerve cells as possible around the injury need to be saved, inflammation needs to be curbed, scar formation needs to be reduced, inhibitory factors blocking axon growth need to be neutralized, nerve cells need to be awakened to regrow axons, sustenance for nerve cells needs to be supplied, axon growth across the injury needs to be promoted and axon exit from the SC implant needs to be fostered. Combination strategies, therefore, were initiated in our laboratory.

SC implantation was combined with the administration of a steroid, methylprednisolone (Chen *et al.* 1996); a variety of growth factors (primarily neurotrophins; Xu *et al.* 1995; Menei *et al.* 1998; Blits *et al.* 2003; Golden *et al.* 2007; Enomoto *et al.* 2013; Kanno *et al.* 2014); another cell type, olfactory ensheathing cells (Takami *et al.* 2002; Plant *et al.* 2003; Pearse *et al.* 2004a, 2007; Fouad *et al.* 2005); an enzyme, chondroitinase (Fouad *et al.* 2005; Kanno *et al.* 2014); and elevation of cyclic AMP (Pearse *et al.* 2004b). In some cases genes were introduced into SCs before transplantation to provide more neurotrophin (Menei *et al.* 1998; Golden *et al.* 2007; Flora *et al.* 2013; Enomoto *et al.* 2013; Kanno *et al.* 2014). All these combinations were more effective than transplantation of SCs alone: more SC-myelinated axons appeared in the graft, more axons from neurons above the spinal cord grew into the graft, more axons exited the graft into the spinal cord and locomotion of the paralysed rats improved (reviewed in Fortun *et al.* 2009; Tetzlaff *et al.* 2011).

An example of one of the combination strategies was the insertion of cDNA into the SCs before implantation to generate a bifunctional neurotrophin, D15A (Golden *et al.* 2007). D15A excites both trk B and C receptors, mimicking the activity of both brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). In the presence of D15A there was a fivefold increase in graft volume and SC number and up to a fivefold increase in myelinated axons. Total axons (i.e. myelinated and ensheathed) were estimated to reach 75,000. Noradrenergic fibre length was significantly increased in the SC graft. There was no improvement in Basso, Beattie and Bresnahan Locomotor Scale (BBB) scores, however, possibly because regenerated descending axons did not re-enter the spinal cord caudally.

The D15A cDNA was further modified to generate, after introduction into SCs, a neurotrophin with less affinity for the p75 receptor (Enomoto *et al.* 2013). The neurotrophins activate not only the trk receptors but also the p75 receptor, which when activated signals apoptosis. The question was whether the modified D15A with less affinity for the p75 receptor would be more efficacious than D15A, because the goal was to enhance survival and growth of nerve cells and axons without apoptosis. As usual, 2 million SCs were transplanted 1 week after

contusion injury (NYU impactor) of adult female Fischer rats (Enomoto *et al.* 2013). With reduced affinity for the p75 receptor, SC survival was improved 10-fold and the number of myelinated axons in the implant was increased sixfold. Proprioceptive afferents were increased in and around the transplant as were serotonergic fibres. Again there was no apparent improvement in locomotion on the basis of BBB scoring. These findings precipitated the next experiment, to provide both D15A and chondroitinase in the SC implant to not only increase axon growth into the implant but also to modify the cord–implant interface to improve axon exit from the implant (Kanno *et al.* 2014).

One million SCs expressed either green fluorescent protein (GFP) or GFP and D15A; another million SCs expressed either mCherry or mCherry and chondroitinase (to modify chondroitin sulphate proteoglycans and thus reduce their inhibitory effect on axon growth). One million of each were transplanted into each animal (Kanno *et al.* 2014). GFP⁺ SCs and mCherry⁺ SCs served as control implants. To ensure that the chondroitinase was biologically active, not only *in vitro* but also *in vivo*, sections from every animal group were immunostained for the proteoglycans; immunostaining was not seen in the animals receiving chondroitinase, showing that the enzyme had been biologically active after transplantation. The implants were most dense following the full combination strategy. Myelinated axons were significantly higher in number in the full combination compared to chondroitinase alone. These counts were also higher in the D15A implants, but not as high as in the D15A + chondroitinase implants. More neurons above the spinal cord responded to the full treatment. Walking scores (BBB) were improved and pain in the hindlimbs was reduced. The borders of SC implants containing chondroitinase were more interdigitated than without the enzyme, raising the possibility that this had led to more 5HT-labelled axons in the implant and 500 μm caudal to the implant in the full combination. Because the injury was a contusion rather than a complete transection, we do not know if this increase in fibres was due to regeneration, sprouting or improved sparing in the tissue in and around the implant.

Throughout our complete transection/SC bridge studies, sometimes the border between the glial fibrillary acidic protein (GFAP)-positive spinal cord and the GFP⁺ SC implant was very sharply delineated; in this case there was little growth of axons into the SC bridge (Williams *et al.* 2015). At other times, however, this border was very irregular due to interdigitation of astrocyte processes and the SC implant; in this case there was growth of regenerated axons into the SC implant. When we introduced SCs in fluid rather than gelled Matrigel, more cases of interdigitation and regenerated fibres were observed in the SC implant. Furthermore, the more numerous the astrocyte

processes in the SC implant, the more axons entered the implant. Often there was a close positioning of the astrocyte processes, SCs and regenerated axons in the implants. Electron microscopy revealed a number of images of a basal lamina surrounding this triumvirate, suggesting that the formation of these tunnels aided the growth of axons across the SC implant. It will be important to better understand under what conditions astrocyte processes enter the SC implant, thereby enhancing axon regeneration, rather than inhibiting axon growth.

Human SC implants also are effective in supporting axon regrowth as demonstrated in a nude athymic rat, complete transection model (with administration of methylprednisolone) (Guest *et al.* 1997). SC-myelinated axons were found in the SC bridge along with a variety of axon types (propriospinal, sensory, motor neuronal, noradrenergic and serotonergic). Some axons re-entered the spinal cord. Two locomotion tests, BBB scoring and the inclined plane, revealed significant improvements. It is interesting to note that interdigitated interfaces between the spinal cord and the SC bridge were observed.

This work along with work by others led to the approval by the FDA of an open labelled, unblinded, non-randomized and non-placebo dose escalation clinical trial looking at safety of transplantation of autologous human SCs with long term follow-up (Guest *et al.* 2013). *Pari passu* with the non-human transplantation work was an effort in the Miami Project to prepare human SCs by FDA guidelines for transplantation into human injured spinal cord. The cells were prepared in a facility with extensive experience producing clinical grade cells. The plan was to obtain a piece of sural nerve from a spinal cord injured person within 1 month of the injury and then place the nerve in culture to isolate, purify and expand the number of SCs. Then the SCs would be injected into the epicentre of the lesion. The trial was approved in July 2012, the first subject was transplanted in December 2012, and the trial was completed and determined to be safe in 2015. A second trial to test autologous human SC transplantation in chronically spinal cord injured subjects is at present underway in the Miami Project.

Substantial progress has been made in experimental animal models to discover interventions that improve outcomes after SCI. Promising strategies are now reaching human clinical trial status. At present, combination therapeutic strategies seem necessary. One component will be cell transplantation to fill the spinal cord cysts that often form after injury and to provide a permissive scaffold for regenerating axons to cross the lesion site. Important contributions are anticipated from biomedical engineers who are developing valuable matrices to enhance transplanted cell survival and to provide slow release of supportive molecules such as growth factors. Electrical stimulation and rehabilitation approaches will

undoubtedly be a part of a combinatorial therapy. The next years promise to be very productive and exciting as we build on what is now a sound foundation of knowledge acquired to promote repair.

In ending this article, the author would like to quote an African proverb. 'If you want to go quickly, go alone. If you want to go far, go together'. The challenge we have to restore full function after SCI is daunting and complex and will require the input of many investigators with varied expertise working together.

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Additional information

Competing interests

The author claims no conflict of interest.

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