Advances in peripheral nerve regeneration as it relates to VCA

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ABSTRACT

Vascularized composite allotransplant (VCA) offers functional, social, and quality of life improvements for patients who have exhausted traditional reconstructive options. Unlike solid organ transplant, VCA success is not only based upon the quality of perfusion and level of immunosuppression, but also on the success of nerve regeneration within the transplanted part. This paper will summarize the present state of peripheral nerve regeneration in the context of VCA and will explore the latest research advances that will affect the future of the field. These advances offer promising future strategies to improve patient outcomes in VCA.

KEYWORDS

face transplant; hand transplant; nerve recovery; nerve repair; VCA

Introduction

Vascularized composite tissue allotransplantation (VCA) is a clinical option for patients who have exhausted the reconstructive options of local flaps and free tissue transfer. VCA procedures are life-changing, offering patients with no traditional reconstructive options the opportunity for return of function, social integration, and increase in quality of life.

Unlike solid organ transplant, VCA outcomes are not solely dependent on reperfusion of tissue and successful immunosuppression. Nerve regeneration is an essential component of functional success of VCA, enabling movement and sensation of the transplanted part. The risk-balance ratio of VCA depends upon return of function in exchange for the risks associated with life-long immunosuppression.1 There has been variable return of motor and sensory function following VCA, which is ultimately dependent on the success of nerve regeneration.2-4 Strategies to improve peripheral nerve regeneration are paramount to this field. This review discusses the current knowledge and practices in the field of peripheral nerve regeneration. We also explore research directed to advance the field, examining how these innovative approaches can affect the success of VCA, potentially improving patient outcomes.

Nerve injury and regeneration in VCA

In 1943, Seddon described 3 types of nerve injuries: neurapraxia, axonotmesis, and neurotmesis.5 Sunderland later further expanded these categories to 5 different classifications.6 In grade 1, there is a conduction disruption with axons intact. Grade 2 entails a disrupted axon with intact endoneurium. In Grade 3, there is disruption of the endoneurium but preservation of the perineurium. In Grade 4, the perineurium is disrupted but epineurium is preserved. In grade 5, the entire nerve trunk is transected. This review will focus on Sunderland Grade 5 peripheral nerve injuries, as VCA transplantation process entails complete transection of both donor and recipient nerves.

Normal axon physiology is tightly controlled by electrolyte homeostasis. When an axon is transected and the membrane compromised, the alteration in ion concentrations leads to a program of degeneration and regeneration of the axon. In the VCA recipient, the proximal segment of the axon degenerates back to the nearest adjacent node of Ranvier, a process called chromatolysis.7

In the donor graft, the peripheral nerve undergoes Wallerian degeneration from the transection point to its distal motor or sensory receptor.8 Schwann cells in both the donor and recipient segments then
phagocytose the axonal and myelin debris, leaving empty endoneural tubes as a scaffold for regeneration. Macrophages surround the area, which stimulates the Schwann cells to proliferate. The Schwann cells create long columns within the endoneural tubes called Bungner bands, which are required for successful regeneration. The cells then release neurotrophic factors that guide the regenerating axons to their distal target.

Axonal regeneration occurs from the most distal intact node of Ranvier of the recipient. 50–100 nodal sprouts mature into a growth cone, and follow neurotrophic signals to their distal targets. The axons then extend from the growth cone and connect with the motor endplates on the muscles. Axonal extensions that do not reach a distal muscle receptor undergo pruning. If either the receptor or endoneural tube is not reached by the growth cone and pruning does not occur, the cone grows in a disorganized way, which can lead to a clinical neuroma.

Current nerve regeneration outcomes in VCA

Comparing outcomes in both face and hand transplant is complicated by the varying pre-transplant defects among patients as well as the differing coaptation methods between surgeons. Each VCA surgery is tailored toward the specific patient, with unique challenges. Furthermore, particularly in hand transplant, different functional measurements and scoring systems between centers lead to difficulty in comparing outcomes. Despite this, there have been published post-operative motor, sensory, and functional results that can guide clinical expectations and practice.

Functional outcomes reported in face transplant include 10 reports of facial expression before and after face transplant. The timeline to muscle reinnervation and motor recovery varies, which is to be expected due to differing preoperative injury patterns and donor grafts. Reports include a patient who regained facial movement at 3 months post-op, a patient who demonstrated a symmetrical smile after 6 months, another patient with a symmetrical smile at 18 months, and 4 patients who showed spontaneous mimicry and emotional readability 5–12 months post-transplant. In one patient, a failed nerve anastomosis led to no reinnervation after 2 y. This particular patient’s original injury was caused by a bear attack, which led to an avulsed and scarred stylomastoid foramen and only the neural stem of the facial nerve remaining, leading to an inability to successfully perform the neurorrhaphy. Sensation in face transplant has been previously been published on 9 recipients, which generally begins from the edge of the allograft toward the center of the face. Light touch is generally detectable by the patient 3–12 months following transplant, and temperature after 6–8 months. Interestingly, Siemionow et al. reported that only one of 4 patients that she summarized underwent successful anastomosis of both the mental and infraorbital sensory nerves. However, all 4 patients regained full sensation comparable to the patient with successful coaptation. This led to the hypothesis of alternative paths to sensory reinnervation, including through trigeminofacial communications, nervi nervorum of the facial nerve, somatic afferents of the facial nerve, or adrenergic plexus of the vascular pedicle. An animal model of sensory reinnervation would be helpful in determining the common pathways to nerve regeneration in face transplant, but these results are reassuring should the surgeon be unable to connect the sensory nerves due to pre-existing trauma in the recipient.

Outcome metrics in hand transplant include the Disabilities of the Arm, Shoulder, and Hand (DASH) score, the Hand Transplant Registry Score System, the Chen Score for Limb Replantation, the Comprehensive Functional Score System, and the Carrol Score. Although the tests among centers vary, there is general improvement in patients in long-term follow-up as reported by the latest International Registry on Hand and Composite Tissue Transplantation report. The single greatest discrepancy in function between patients results from differences in level of transplantation. The more distal the level, the better functional recovery. The majority of patients recover protective sensation of the transplanted graft, with those generally with more distal transplants also obtaining 2-point discrimination. It has been published that the level of transplant greatly contributes to the amount of functional improvement that can be expected. Patients with distal transplants (wrist level) can expect to regain limited intrinsic function, the ability to 2-point discriminate, and better motor recovery and movement. Mid-forearm and proximal transplanted patients generally have more limited intrinsic function and protective sensation and weaker motor recovery. However, despite these limitations, these more proximally transplanted patients still have immense
improvement in functional activities that they are able to perform, including the ability to drive, address acts of daily living, and grasp small objects. Furthermore, the degree of functional recovery is also closely correlated with the length of time since surgery; patients further out from transplant generally have much better functional outcomes, with the best outcomes from patients several years following surgery. This stark difference between proximal and distal hand transplant recipients as well as the correlation of length of time to functional recovery demonstrates the need to address mechanisms to improve nerve regeneration in VCA.

**Current options for nerve repair**

**Direct nerve repair**
The ideal choice for treating a transected nerve remains direct repair of the severed ends. Various factors contribute to success of this technique, including timing of the repair, tension across the repair, atraumatic suture technique, status of the sutured ends, vascularity of the tissue bed, and internal matching of the fascicles.

Minimal tension across the repair site is a critical component of a successful repair. Undue tension has been shown to lead to nerve ischemia and mechanical failure of the repair in an animal model. There is no consensus on the best method to assess intraoperative nerve tension; one method, proposed by Medinaceli et al, determines that tension is inappropriate when a single 9–0 suture fails to approximate the repair. Careful microsurgical technique should be used with the use of magnification, preferably a surgical microscope.

Suturing healthy nerve ends is one of the most important factors for successful nerve recovery. Methods to assess the condition of the nerve vary according to the surgeon’s preference and include gross visual inspection as well as assessment under the microscope, feel and consistency, intraoperative histology, and nerve studies. In cases of upper limb VCA, this may be challenging when attempting to cut back terminal neuroma bulbs until a uniformly viable proximal nerve stump is identified. A healthy vascular tissue bed for the repair is another factor affecting nerve recovery. In VCA, it is crucial to cut back the recipient nerve proximally until healthy, scar-free nerve is reached. The donor nerve can be transected as distal as possible to prevent the need for a nerve graft; the coaptation site can then be more proximal to the recipient than the transplanted graft.

Alignment of the nerve must be performed with great care to match the fascicular components of the nerve. This is particularly challenging in the context of VCA, when the donor and recipient nerves may have differing fascicle number and size. Methods used to achieve precise alignment include visual coaptation under the microscope, aligning surface vessels, histology and selective staining, and electric stimulation. Although the more advanced techniques may seem more accurate, they have not provided superior results to the simpler visual methods. The usefulness of these techniques and the superiority of any of them over the others are yet to be studied in VCA models.

Nerve repair with epineural sutures with proper alignment is still the gold standard technique for nerve suturing. Fascicular repair using perineural sutures may allow for more precise alignment of the fascicles, but carries the disadvantage of more intraneural dissection and postoperative scarring. Grouped fascicular repair is another method that can be used in nerves with well-defined topography such as the distal median and ulnar nerves, where groups of fascicles surrounded by thickened epineural tissue can be sutured together to gain better alignment. Superiority of the results of the more sophisticated fascicular techniques has not yet been demonstrated.

**Nerve grafts**

While it is unlikely that nerve grafting would be required in cases of VCA, as the donor tissue can be procured with extra nerve length, consideration will be given to this topic as this could be encountered in complicated cases. If tension-free primary repair is not possible, nerve autografts are still considered the gold standard. In traditional nerve repair, an expendable nerve from the patient can be used to bridge the nerve gap. The sural nerve is the most widely used donor nerve and can provide a length of up to 40 cm to be grafted, but may be unavailable in cases of bilateral lower limb amputees. Other sources for nerve grafts include the medial and lateral cutaneous nerves of the forearm, dorsal cutaneous branch of the ulnar nerve, superficial peroneal nerve and the posterior and lateral cutaneous nerves of the thigh. The disadvantages of this technique are the donor site morbidity including sensory loss, possible neuroma
formation, and limited supply of donor nerve. In VCA, this graft can be instead be taken from the donor since the recipient is already on immunosuppression. This prevents nerve harvest site morbidity in the recipient and provides an excellent option should additional nerve length be required. The sural nerve is additionally far from the solid organ harvest sites. Thus, with coordination, harvesting of this nerve may be performed simultaneously alongside the solid organ teams.

**Strategies to improve nerve regeneration in the context of VCA**

Since VCA outcomes are largely based upon the success of nerve regeneration and functional recovery, research in this realm is of particular importance to the field. Current translational research strategies to improve nerve regeneration in VCA include finding alternatives to suture repair through welding at the coaptation site, improving regenerative capacity through introduction of growth factors, examining the relationship between immunosuppression and nerve regeneration, and using electrical stimulation, novel gene editing techniques, and advances in stem cell biology. These basic science research innovations will lead to improved clinical peripheral nerve regeneration and, hopefully, advances in VCA patient outcomes.

**Alternatives to sutures in nerve repair: Nerve welding**

Current microsurgical nerve repair options invariably use sutures in their approach, leading to several limitations. Suture repair does not reseal the nerve, thus failing to limit leakage of interneural fluids. Sutures have also been shown to lead to epineural scarring, which inhibits regeneration; indeed it has been proven that a greater number of sutures is correlated with a higher scar index. Suture repair also can lead to foreign body reactions at the repair site as well as additional trauma to the nerve, both of which increase the potential of neuroma formation. Thus, one important area of progress in peripheral nerve repair is the development of a suture-free repair.

**Nerve glue**

One option for suture-less nerve repair is by using glue rather than sutures. The ideal glue for nerve coaptation would provide adequate tensile strength at the coaptation site, not hinder regeneration, induce fibrosis, nor be cytotoxic. Fibrin glue has the longest history of utilization as a nerve glue and has been particularly vital in pediatric brachial plexus reconstruction. It has been shown to reduce operative time as well as enabling repair in the bony foramen of the proximal nerve root, where suture repair is impossible. A systematic review of the use of fibrin glue found mixed results but, in the majority of cases, glue repair was equal to or superior to suture repair. There are still clinical concerns about the tensile strength of the repair when fibrin glue is used as the sole coaptation technique. Biomechanical studies have demonstrated a lower load to failure when glue alone is compared with suture repair in a rabbit model. Further well-controlled clinical studies are necessary to determine the effectiveness of fibrin repair in comparison to standard microsurgical repair techniques.

An alternative option to fibrin glue is cyanoacrylates. These esters of cyanoacrylic acid harden on contact with weak basic substances. They have extremely fast cure rates and high tensile strength; however, the byproducts of this process are formaldehyde and cyanoacetates, which are toxic to tissues. Furthermore, if the cyanoacrylate seeps into the repair site between the coapted nerves, it can lead to tissue inflammation and hinder regeneration. Different formulations of cyanoacrylates have vastly different tensile properties as well as toxicity profiles. Future research may improve their profile as nerve repair glue if the mechanical strength, toxicity, and biocompatibility is balanced.

**Laser repair**

Another option for nerve coaptation is through laser welding. Previously, this was limited to thermal laser welding, in which tissue is bonded by heating it to denature proteins, which then anneal together upon cooling. This method was used to successfully coapt nerves in 1985, but concerns about a high dehiscence rate have prevented its wide-scale adoption. Furthermore, thermal welding also raises concerns about damage to axons, nerve tissue, and surrounding extraneural tissues and is a rather cumbersome coaptation method.

One exciting innovation that addresses these concerns is nonthermal laser bonding through photochemical tissue bonding (PTB). In this repair method, the severed nerve ends are approximated together and
the site is wrapped in an amnion wrap. A photoreactive dye is then added around the wrap and exposed to an Nd/YAG laser. The reaction of the dye with the laser leads to cross-linking of collagen in the amnion wrap with collagen in the epineurium of the nerve. This repair technique has been argued to be better than the current standard of microsuturing because it provides a means of sealing the intraneural fluids within the repair site while preventing the nerve from exposure to inflammatory modulators. Animal studies have demonstrated that this method leads to better functional outcomes and axon counts in both rat and rabbit models of nerve repair when compared with standard suture repair. This repair method has not yet been used clinically.

**Polyethylene glycol fusion**

Unlike mammals, peripheral nerves in invertebrates, particularly crayfish and earthworms, reconnect almost immediately after injury. This inhibits the Wallerian degeneration that hinders axonal regeneration in mammals, and instead leads to restoration of function. This natural phenomenon inspired research to fuse axons in mammals. Polyethylene glycol is a fusogen used in cell research to create hybridomas, or cells with more than one nucleus, through fusing membranes. The compound leads to membrane fusion by dehydrating the membrane, and has been shown to restore axonal continuity after the coaptation site is exposed. Sutures must be used in addition to polyethylene glycol fusion because the fusion itself does not have adequate tensile strength. A specific sequence of antioxidants and polyethylene glycol has now been developed to lead to optimal axonal fusion. This study demonstrated almost immediate restoration of electrophysiology (compound action potentials) across the repair site following fusion as well as dramatic improvement in functional recovery (gait function) in a rat model of sciatic nerve repair when polyethylene glycol fusion was used. This repair method was recently used in humans for the first time, in digit re-implantation, with positive results, but requires further clinical study to determine its long-term efficacy and widespread application.

**Minimizing time to recovery**

One of the most critical factors in nerve recovery is the time required for the regenerating nerve to reach the motor endplate. Following denervation, muscle fibrosis and atrophy begins immediately. This process plateaus at 4 months, when 60–80% of muscle volume has been lost. Motor endplate degeneration also occurs rapidly following nerve injury, with a critical loss of endplates occurring within 12 to 18 months following injury. In cases of upper arm transplantation, this time limit may create a scenario where there are few remaining motor endplates within the distal muscles of the hand by the time the regenerating nerve reaches the hand, thus resulting in minimal recovery of hand intrinsic muscle function. Multiple studies have sought to utilize novel techniques to accelerate nerve healing to maximize muscle recovery. This includes the application of growth factors and hormones, which have been shown to improve both the speed and quality of nerve regeneration. Particularly relevant to VCA is the effect of immunosuppression protocols on nerve regeneration. The usage of FK506 (Tacrolimus) has been demonstrated to accelerate peripheral nerve regeneration, improve Schwann cell migration, and decrease muscle denervation time. In animal models, this has led to quantifiable functional muscle improvement and lower rates of motor endplate deterioration. This section will further expand on the potential of these agents to facilitate nerve regeneration, thereby limiting the extent of motor endplate loss and preventing permanent muscle atrophy.

**Enhancing nerve regeneration through application of growth factors**

Neurotrophins are a complex of growth factors and cytokines that are released from nerve endings, particularly following nerve injury. They play a key regulatory role in the process of nerve regeneration, growth and differentiation. Neurotrophins support axonal elongation as well as stem cell migration and differentiation. Research on using these factors to improve nerve regeneration, in addition to their delivery method to the site of injury, is a topic of great interest to the neurobiology field. Neurotrophic factors can be divided into 3 main families; neurotrophins, glial cell line derived neurotrophic ligands (GFLs) and the neuroepoietic cytokines. Each member of the aforementioned neurotrophic families is thought to have a distinct function and characteristic with some existing overlaps between the different factors in terms of cellular responses and overall molecular effects.
Nerve Growth Factor (NGF)

Nerve Growth Factor (NGF) is one of the first isolated neurotrophins. It is present in low concentrations in healthy, intact nerves. Following nerve injury, NGF transcription is upregulated, especially in the distal nerve stump, and it is thought to play an important role in the survival of neurons and elongation of axons. NGF is mainly present in sensory neurons. Braun et al have found no effect of NGF on motor neuron growth and regeneration in an *in vitro* study. NGF was found to affect motor neurons only in the presence of astrocytes, which suggests that it might play an additive effect when present with other growth factors and cytokines.

Glial cell-derived neurotrophic factor (GDNF)

GDNF is a member of the GFL family. It was found to have regenerative and protective effects on axons following nerve injury. Oppenheim and colleagues determined that overexpression of GDNF increases the number of motor axons innervating the neuromuscular junction *in vivo*. Furthermore, the use of GDNF following facial nerve injury in rats was shown to increase the number of myelinated neurons, improve survival of injured axons and improve functional outcomes. Other nerve growth factors and cytokines include GGF, NT-3, CNTF, BDNF and NRG-1. Each of these factors was found to improve both axonal survival and regeneration to a certain extent.

Growth hormone

It has been hypothesized that administration of growth hormone can improve peripheral nerve regeneration. This hormone, normally secreted by the pituitary gland, acts directly as well as through insulin-like growth factor 1 (IGF-1) signaling to affect growth and prevent cellular apoptosis. In a rat model of sciatic nerve transection, it was shown that subcutaneous administration of growth hormone accelerated axonal regeneration and improved rates of axonal remyelination as well as reducing muscle atrophy, and enhancing muscle reinnervation. Furthermore, systemic growth hormone therapy also improved both the conduction velocity and amplitude of motor potentials in a rat ulnar nerve transection model. It is not known whether growth hormone affects the muscle fibers directly, thereby preserving motor endplates and fiber diameter, or if its effect is purely through acceleration of nerve regeneration rates. More research is therefore needed to fully understand the mechanism of this agent’s improvement of peripheral nerve regeneration. However, one exciting aspect of this research is that growth hormone is already approved by the Food and Drug Administration (FDA) and used for other clinical applications.

Chondroitin sulfate proteoglycans are in the extracellular matrix around peripheral nerves and, at high levels relative to growth-promoting signals, have demonstrated to inhibit axonal growth. Therefore, another avenue of research to enhance nerve regeneration has been to treat with chondroitinase to remove chondroitin sulfate proteoglycans at the injury site. This treatment option has been shown to lead to marked enhancement of peripheral nerve regeneration through acellular nerve grafts. Additionally, chondroitinase treatment has shown to improve nerve regeneration in both a rat model of nerve transection and crush injury. Chondroitinase has been shown to improve nerve regeneration in a rat orthotopic hind limb transplant model, with animals receiving the enzyme demonstrating statistically greater total number of fibers and nerve density compared with controls. These early animal studies demonstrate the promise of potentially using this enzyme in VCA to improve nerve regeneration by augmenting the extracellular matrix surrounding the nerve transection site. One large limitation of research on the effect of growth factor and hormone delivery on nerve regeneration is that delivery methods of these factors vary across studies, which may affect results greatly. Given the fact that we still do not have an ideal delivery system, it is likely that more studies will be needed to elucidate the exact effect that such factors have on nerve regeneration. Furthermore, the process of neurogenesis involves a precise synergy and a delicate balance between more than just one or 2 different cytokines, nerve growth factors, signaling proteins and cellular elements.

Immunosuppression and nerve regeneration

FK506 (Tacrolimus®) is a FDA approved immunosuppressant originally used to prevent solid organ allograft rejection in transplant patients. FK506 is also used as a systemic immunosuppressant to prevent nerve allograft rejection. The immunosuppressant
was found to have nerve regenerative properties when administered peripherally following injuries to peripheral nerves; subsequently several studies have demonstrated the neuroprotective and neurotrophic effects of Tacrolimus. Despite the promise of this research, well-controlled clinical studies are still necessary to demonstrate the effect of FK506 in nerve regeneration.

Tulaci et al have developed a rabbit model in which they assessed the histopathological effects of FK506 on facial nerve injuries. Interestingly, their study showed that FK506, given systemically for 2 months following end to end anastomosis of a transected facial nerve, resulted in a significant increase in axonal diameter, myelin sheath thickness, and a total increase in the number of myelinated axons compared with the control group. Additionally, FK506 has been shown in a mouse orthotopic limb allotransplantation model to improve Schwann cell migration and proliferation as well as leading to more robust axonal regeneration.

FK506 has been shown to enhance the effects of nerve growth factors by increasing cellular sensitivity to growth factors as well as reducing local inflammatory response. Combining FK506 with the delivery of growth factors could lead to even greater improvements in nerve regeneration than FK506 alone. Indeed, Labroo et al. showed that nerves treated with FK506 alone had improved neurite elongation compared with the non-FK506 control. Furthermore, nerve samples treated with a combination of FK506 and glial cell line-derived neurotrophic factors (GDNF) showed an even greater significant increase in neurite elongation and branching, which was dose dependent and occurred in the first 48 hours in vitro. The same group has worked to develop drug-release devices that aim to release optimal levels of FK506 at controllable rates for improved neurite growth and outbranching, but such devices require more study to assess their efficacy and effectiveness on nerve regeneration and functional recovery.

While FK506 has been shown to reduce inflammation and prevent rejection, it has been shown that a component of rejection may actually be beneficial to nerve recovery. In a rat nerve allograft model, it was shown that inducing the onset of acute rejection early (5 d post-transplant) versus late (8 d post-transplant) led to differing functional outcomes. The early group demonstrated improved functional outcomes and muscle re-innervation when compared with the late group. This indicates that inflammation and immunosuppression does influence nerve regeneration, although its mechanism is still not clear.

**Electrical stimulation**

Electrical stimulation has been explored as one potential means to speed up axonal regeneration as well as improve functional outcomes, including the extent of reinnervation. This includes research on electrically stimulating the distal muscle as well as the regenerating nerve stump. Direct muscle stimulation appears to enhance overall reinnervation and axonal regeneration. Electrical stimulation is thought to decrease the rate of muscle atrophy and improve skeletal muscle receptiveness to reinnervation. Fewer studies have looked at the effect of electrical stimulation of the proximal nerve stump, but the preliminary data on this topic provides exciting results. Al Majed et al. have reported that, in a rat model, one hour of immediate direct electrical stimulation of the proximal nerve stump resulted in a significant increase in the kinetics of targeted muscle reinnervation.

Furthermore, electrical stimulation of the proximal nerve was also associated with the upregulation of brain-derived neurotrophic factor and its receptors in motor neurons. The authors concluded that electrical stimulation accelerated axonal growth across the repair site. Most of the available data has looked at the effect of electrical stimulation on nerve regeneration in animal models; few clinical trials are available to demonstrate the clinical applicability in human subjects. Promising results have been reported by Gordon et al, who demonstrated improved functional outcomes in both sensory and motor nerve regeneration following median nerve compression in the carpal tunnel. Although these studies have provided valuable results and demonstrate improvement in functional outcome and axonal regeneration, more clinical trials are needed, especially in the context of VCA.

**Gene editing**

Gene or genome editing refers to a form of genetic engineering in which a DNA sequence is added, deleted or modified in the genome of an organism, resulting in controlled mutations. The aim is ultimately to alter protein transcription and translation in a way that leads to a desired outcome. This area of research is even more salient in the current area of
CRISPR/Cas9 editing technology, an innovation that makes it far easier and more straightforward to edit genes.

In short, CRISPR gene editing is based off of the defense systems of bacteria and other microorganisms. CRISPR uses a protein (called Cas9) that creates double stranded cuts in DNA, thus destroying the sequence at that site. Guide RNA, designed by the scientist, guides the Cas9 protein to a unique sequence in the DNA; in this way, the method can lead to extremely targeted deletion of a genetic sequence. CRISPR technology has also advanced to include alternative sequences to be used as a guide in the subsequent DNA repair process, thus enabling the repair of a mutated gene or insertion of a novel gene into the DNA. In this way, CRISPR/Cas9 has revolutionized the potential of genetic engineering and is often cited as the scientific advancement of the century. CRISPR/Cas9 editing has been effectively been performed on both animal and human cells in vitro. A clinical trial, currently being performed in China for patients with late-stage lung cancer, is the first to inject patients with cells modified in vitro with CRISPR/Cas9 in an attempt to provide targeted immunotherapy. Other animal studies have also concentrated on methods to deliver the CRISPR/Cas9 technology to cells in vivo in animal models. We predict that the next decade will lead to large advancements in this system and its introduction into clinical practice, including potentially in improving nerve regeneration. One application of gene editing to promote nerve regeneration is through the deletion of suppressor of cytokine signaling-3 (SOCS3). This molecule is associated with reduced nerve growth capacity following nerve injury. SOCS3 functions in a negative feedback loop to suppress the neuronal cells ability to activate the JAK-STAT signaling pathway, an important pathway that is needed for nerve repair, regeneration and dendritic branching and outgrowth. Expression of SOCS3 therefore plays a negative role in regulating neuronal cell survival and growth. Deletion of SOCS3 in adult retina ganglionic cells was found to promote significant optic nerve regeneration. Moreover, recent studies have shown that SOCS3 plays a negative role in regulating neuronal growth by inhibiting IL-6 induced activation of JAK/SOCS3 pathway. Park et al have recently shown that reduction of SOCS3 expression enhances neurite growth in Neuroscreen-1 cells (NS-1). The same team also showed that suppression of the SOCS3 pathway has a neuroprotective effect. Reducing SOCS3 expression after spinal cord injury led to reduced demyelination in areas distal to the injury.

Another important signaling protein that could be advantageous to gene edit is phosphatase and tensin homolog protein (PTEN). PTEN inhibits protein translation by repressing PI3 kinase activation and downstream signaling. Deletion of PTEN was found to enhance axonal regeneration and allow reactivation of PI3 kinase pathway, as demonstrated by Dun et al. Interestingly, co-deletion of PTEN and SOCS3 led to sustained axonal regeneration ability by retinal ganglionic cells. The same results were found to be true in corticospinal neurons as well. Additionally, co-deletion also led to a significantly improved visual and skilled locomotive abilities following injury. As evident from these findings, PTEN and SOCS3 play a role in inhibiting neurite outgrowth and axonal regeneration. Limitations of these studies include the cell types examined, which are primarily central nervous system cell types. Further studies are needed to evaluate the effect of PTEN/SOCS3 deletion on peripheral nerve regeneration potential. Furthermore, gene editing in vitro provides numerous exciting results but the challenge remains to find a way to obtain the same results in vivo through precise, targeted mechanisms to improve peripheral nerve regeneration following nerve injury. Undoubtedly, CRISPR/Cas9 technology offers new opportunities for gene editing, but these techniques still need to be validated in terms of clinical safety and effectiveness.

**Stem cell biology in peripheral nerve regeneration**

Unless axonal regeneration and re-innervation occur quickly, the growth-supportive environment established by resident Schwann cells is progressively “switched off,” leading to poor muscle reinnervation. It has been demonstrated that chronically denervated Schwann cells, which lack axonal interaction for extended periods of time, become quiescent and cease to proliferate and secrete neurotrophic factors. Another issue more specific to VCA is the possibility that allogeneic Schwann cells in the transplanted nerve may undergo rejection. Therefore, strategies to create an environment of supportive Schwann cells in the distal aspect of the regenerating nerve are of paramount research interest.
One strategy to increase peripheral nerve regenerative capacity is to augment the repair and regeneration location with Schwann cells to prolong the growth-promoting and regenerative environment. This was historically attempted by harvesting a biopsy of a healthy nerve from the patient, extracting the Schwann cells, expanding them in vitro, and then transplanting them into the nerve injury site. However, the harvest procedure is invasive, and Schwann cells require a lengthy expansion time in vitro to gain enough cells to transplant. Schwann cell-based therapy has previously been limited by these complicating factors.

Recent advances in stem cell biology have provided alternative cell sources for Schwann cell transplantation. The focus of stem cell biology in the context of peripheral nerve regeneration has been to find an accessible, noninvasive source of cells that can be rapidly expanded in culture. Stem cells of varying sources can then be transplanted in their undifferentiated state or can alternatively be differentiated into a Schwann cell-like phenotype in vitro before transplant. The differentiation protocols used vary among research groups, but generally involve exposing the cells to a series of factors that include β-mercaptoethanol, all-trans retinoic acid, fetal bovine serum, forskolin, recombinant human bFGF, recombinant human platelet derived growth factor-AA, and recombinant human heregulin β-1. Embryonic stem cells are one pluripotent cell option, and have been successfully differentiated into the neural lineage, including to a Schwann cell phenotype. These cells would prevent the need for an invasive biopsy, but do have distinct disadvantages, including tumorigenicity, immunogenicity, and ethical controversy. In 2006, Takahashi et al. presented a mechanism to induce adult fibroblasts to a stem cell-like state (iPS cells). These cells have been successfully differentiated into the neural lineage, including to a Schwann cell phenotype. However, concerns about the iPS cells still exist, including the difficulty of the reprogramming protocol. Thus, stem cell research in peripheral nerve regeneration has focused on using adult, multipotent progenitor cells.

**Bone marrow stromal cells**

There are several types of adult progenitor cells that have been used in peripheral nerve regeneration research. Bone marrow stromal cells harvested from long bones are one viable option. These cells can rapidly expand in culture and can be differentiated into a Schwann-like phenotype expressing appropriate markers, including S100 protein, GFAP, and p75. These cells have been shown to functionally myelinate cells in culture. However, when bone marrow stromal cells are introduced “undifferentiated” into the injury site in a rat model, only 5% were shown to spontaneously differentiate into a Schwann cell phenotype. The majority of reports have demonstrated that using BMSCs leads to at least equivalent results as cultured Schwann cells. However, BMSCs, in comparison to other sources, have lower in vitro proliferation and differentiation capacity and involve a painful and invasive harvest.

**Skin and hair bulge stem cells**

The skin and hair bulge present another source of adult stem cells. The hair bulge contains a large population of neural crest cells, which have the potential to differentiate into neurons, smooth muscle cells, and most importantly for this application, Schwann cells. Neural crest cells have been shown to differentiate in vivo into Schwann cells in the microenvironment of a rat peripheral nerve injury, and have been demonstrated to improve recovery in this model. However, the robustness of the differentiation is one area of concern, as only a small percentage of cells differentiate in the in vivo environment. Thus, it may be more clinically useful to differentiate the cells ex vivo before introducing them into the injury location. Skin dermis is another source of potential cells, as it contains neural crest-related precursor cells (SKPCs) that also have the potential to differentiate into Schwann cells. These cells have also been shown to differentiate in vitro as well as successfully myelinating axons in an in vivo model.

**Adipose-derive stem cells**

Perhaps of the most accessible source of multipotent stem cells are those found in fat. These cells can differentiate into Schwann cell phenotypes in vitro. Unfortunately, it has been demonstrated that these cells are not effective in differentiating in vivo, so in vitro differentiation is necessary.

Although stem cell biology presents innovative means to improve peripheral nerve regeneration, there are still issues that need to be addressed in this...
growing area of research. One question is the ideal number of cells to be transplanted into the injury site; numbers of cells transplanted varied widely between animal models. Thus, it is important to determine the ideal protocol in a clinical setting, as too few cells may be ineffective, but too many cells may be detrimental to recovery due to a need to compete for space and resources with the regenerating nerve in the cellular milieu. Furthermore, there is also a question of the state of differentiation of the transplanted cells. It is appealing to use cells in a less differentiated state – for example, the neural crest cells of the hair bulge – as these cells then have the potential to expand in the injury site before differentiating into the Schwann cell phenotype. However, it is not clearly known whether these cells will indeed differentiate in vivo into the correct cell type. Differentiating the cells in vitro before implantation enables the ability to control cell number and cell type before injection. Furthermore, multipotent cells could have the potential to be tumorigenic due to their undifferentiated status. Therefore, we believe that early clinical work in this realm should focus on using in vitro differentiated cells.

**Conclusion**

Success of VCA is largely dependent on return of muscle function and sensation, which requires peripheral nerve regeneration. Although current suture repair techniques have resulted in positive outcomes in both hand and face transplant, there is still immense opportunity for improvement in both quality and timing of nerve regeneration. Research to improve nerve regeneration in the unique context of immune suppression is of utmost importance. Advances in bioengineering, molecular biology, immune suppression therapy, genetic engineering, and stem cell biology present exciting new avenues of research that will impact the future of VCA patient outcomes; however, there are still challenges in understanding the underlying interplay between immunosuppression and nerve regeneration, determining novel drug and cell delivery techniques to improve regeneration, and meeting engineering challenges in alternatives to current suture neurorrhaphy.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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