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Eur Surg. Author manuscript; available in PMC 2014 June 01.

Published in final edited form as:
Eur Surg. 2013 June ; 45(3): . doi:10.1007/s10353-013-0205-0.

Tissue engineered constructs for peripheral nerve surgery

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Summary

Background—Tissue engineering has been defined as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". Traumatic peripheral nerve injury resulting in significant tissue loss at the zone of injury necessitates the need for a bridge or scaffold for regenerating axons from the proximal stump to reach the distal stump.

Methods—A review of the literature was used to provide information on the components necessary for the development of a tissue engineered peripheral nerve substitute. Then, a comprehensive review of the literature is presented composed of the studies devoted to this goal.

Results—Extensive research has been directed toward the development of a tissue engineered peripheral nerve substitute to act as a bridge for regenerating axons from the proximal nerve stump seeking the distal nerve. Ideally this nerve substitute would consist of a scaffold component that mimics the extracellular matrix of the peripheral nerve and a cellular component that serves to stimulate and support regenerating peripheral nerve axons.

Conclusions—The field of tissue engineering should consider its challenge to not only meet the autograft "gold standard" but also to understand what drives and inhibits nerve regeneration in order to surpass the results of an autograft.

Keywords

Peripheral nerve surgery; Tissue engineering; Nerve conduits; Acellular nerve allograft; Stem cells; Neural crest cells

Nerve injury and clinical need

It is important to understand the anatomy of peripheral nerves when determining the options for nerve repair or reconstruction after injury. In a nerve, myelinated axons of motoneurons, large sensory neurons, smaller nonmyelinated sensory nerves, and autonomic neurons are bundled into fascicles within the connective tissue layer of the epineurium. Each fascicle is surrounded by the perineurium (the level of the blood-nerve barrier) and each nerve fiber is contained within the endoneurium. Peripheral nerve injuries result from open or closed trauma and are often debilitating [1]. The nerve injury can involve damage to any of the tissue layers. Seddon has broadly classified nerve injuries into the three groups: local conduction block with or without demyelination (neurapraxia), axon transection and damage

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Conflict of interest
PJJ and SEM in the past have received research funding from Axogen Inc., the makers of the clinically available acellular nerve allograft. However, that funding was not used to produce the current manuscript and Axogen Inc., did not participate in the composition of this manuscript.

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with both perineurium and epineurium remaining intact (axonotmesis), and nerve transection (neurotmesis) where the continuity of the nerve is disrupted either by transection or scar [2]. In practical terms, nerve transection injuries require surgical repair, ideally performed by a coaptation of the proximal and distal nerve stumps without undue tension.

Unless nerve injuries are sustained close to the cell body, there is no motoneuron loss [3] but sensory neuron loss may be as high as 40 % regardless of the location [4–7]. Therefore, nerve regeneration is primarily driven by the ability of the surviving neurons to regenerate their axons from the proximal stump to the distal stump to reinnervate their end-organ target. To facilitate this process, axonal regeneration from the proximal stump into the distal stump is preceded by Wallerian degeneration in the distal stump, whereby the distal nerve stump axon fragments are broken down and phagocytosed by the resident Schwann cells (SCs) [8]. SCs are responsible for phagocytosis of axon debris in the first 3 days, the debris being mitogenic for the cells and thereby playing an important role in their proliferation post injury [9, 10]. Macrophages are also involved leading to overall removal of inhibitory factors to axonal extension and outgrowth [11]. After Wallerian degeneration is complete, SCs progressively assume long processes and align on the basal lamina of fibronectin and laminin (Bands of Bungner), providing a permissive growth environment for the regenerating axons that emerge from the proximal nerve stump [12].

Damage to a peripheral nerve often results in a defect, scar, or neuroma in continuity that leads to a gap between the proximal and distal nerve stumps that cannot be directly repaired without excessive tension and impeding or limiting regeneration [13–17]. Therefore, a guide or graft is used to bridge the damaged ends to reconnect the proximal and distal stumps. Nerve autografts remain the clinical standard of care for critical, and long, large diameter peripheral nerve defects. The autograft provides a scaffold and trophic support in the form of basal lamina, endoneural tubes, and SCs for the regenerating axons guiding nerves to the distal stump [18]. Donor nerves that are commonly used as autografts are expendable sensory nerves such as the sural nerve or the medial antebrachial cutaneous nerve [17–19]. However, disadvantages of autografts include loss of feeling and possible neuroma formation and pain at the donor site, insufficient donor tissue availability, secondary incisions, and less than optimal dimensions (diameter and/or length) of the donor nerve to span the injury site [18, 20].

Tissue engineered peripheral nerve

Tissue engineering has been defined as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ”. As it pertains to peripheral nerve surgery, tissue engineering efforts have primarily been devoted to the recapitulation and restoration of the distal nerve stump following peripheral nerve injury (Fig. 1). Traumatic peripheral nerve injury resulting in significant tissue loss at the injury zone necessitates the need for a bridge or scaffold for regenerating axons from the proximal stump to reach the distal stump, as direct surgical coaptation is not an option. Extensive research has been directed toward the development of a tissue engineered peripheral nerve substitute to act as a bridge for regenerating axons from the proximal nerve stump seeking the distal nerve. Ideally, this nerve substitute would consist of a *scaffold component* that mimics the extracellular matrix of the peripheral nerve and a *cellular component* that serves to stimulate and support regenerating peripheral nerve axons (Fig. 1). The current review briefly describes the components necessary to the development of a tissue engineered peripheral nerve substitute and reviews the literature devoted to this goal. We will not address the role of growth factor or drug delivery from a scaffold, which has been incorporated into the scope of modern tissue engineering, due to the extensive variables in

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modulating and optimizing drug delivery for peripheral nerve graft substitutes. The readers are directed to more specific reviews on the advances of drug delivery for peripheral nerve for more information [21].

Candidate scaffolds

Conduits

Lundborg et al. [24] initially considered using a tube or conduit to bridge a nerve defect. Besides creating an alternative to an autograft, they also desired a model to study peripheral nerve regeneration, where a tube would act as a viewing device for nerve regeneration across a defect. They first used a pseudosynovial neural sheath to bridge a nerve defect in the rat sciatic nerve [22, 23], which was shortly followed by silicone conduits. Both materials led to a convenient method to encapsulate tropic and trophic factors associated with nerve regeneration and evaluate the time course of nerve regeneration. These experiments provided the knowledge that axonal nerve sprouting into an empty conduit was supported by the migration of glial cells into the conduit and subsequent formation of a protein scaffold. They also demonstrated that nerves are capable of producing their own scaffold to support axonal regeneration over a short distance [22–25].

From this initial work, hollow tubes, conduits, or more commonly known as nerve guidance conduits (NGCs), have been studied extensively as a “off the shelf” alternative to nerve autografts for the treatment of nerve gaps. Early into the investigation it was determined that silicone conduits caused significant chronic nerve compression and irritation at the implantation site requiring removal [26–28]. The incompatibility of silicone and the long-term presence of a material surrounding the nerve were determined to be significant contributors to the chronic pathology and drove research into alternative materials that could act as temporary biodegradable conduits. A wide range of materials were evaluated for this purpose. Autologous blood vessels have been used as a biological substitute to nervous tissue because of their similarities to conduits, their natural biodegradation, and their general biocompatibility. However, their flimsy mechanical properties were a detriment to regeneration, where the thinness of the vessel wall often lead to luminal collapse and pressure on regenerating nerves [18]. To circumvent these limitations, conduits that can be synthesized or constructed with properties that are easily modulated, such as increasing mechanical strength, have been explored. The properties of numerous synthetic conduit materials such as poly-L-lactic acid [29], polylactic-co-glycolic acid copolymer [29], and poly (L-lactide–co-6-caprolactone) [30] have been explored to find an optimal mix of mechanical support and rate of conduit degradation. Polymer conduits are advantageous because their range of degradation, mechanical stability, and piezoelectric properties can be modulated to benefit nerve regeneration. Natural materials, such as collagen [31, 32], fibronectin [33], and fibrin [34], which are biodegradable, offer biocompatible advantages over polymers but the ability to adjust their mechanical and degenerative properties is limited. A more extensive review of conduit material properties is available by Pfister et al. (for review see [35]).

Clinically, conduits of material composition have been approved for the treatment of small diameter short gap (small volume) nerve defects in humans [36–39]. The significant history of clinical use and regulatory approval of conduits are desirable traits for the selection of a tissue engineering scaffold. However, their clinical use has also been associated with clinical morbidities that reveal the limitations of conduits [40]. Reports of clinical failures with significant patient morbidity have been made for conduits used with large diameter (large volume) nerve reconstruction [40, 41]. Overall, polymer or synthetic and natural material NGC generally support axonal regeneration for small defects (<3 cm) and fail to support regeneration to the level of an autograft [31, 42]. The absence of cellular support and

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luminal extracellular matrix at the time of repair is thought to be a contributing factor to the limited regeneration [43]. Thus, the use of a conduit as a tissue engineering scaffold would require the incorporation of a lumen substrate or extracellular matrix to encourage and promote SC and axonal migration into the conduit [44].

Initial research with silicone conduits demonstrated that extracellular matrix proteins accumulate within the lumen of the conduit allowing glial cell infiltration and axonal regeneration into the conduit [45]. Providing a matrix of natural proteins, such as laminin and collagen, for cellular migration would circumvent the need for the body to construct its own matrix before nerve regeneration. Inclusion of natural materials, such as collagen or laminin, into a conduit has been shown to enhance nerve regeneration [46, 47]. The luminal matrix assists SC migration into the conduits and leads to the increase in regeneration [48]. Fibrin gels or glue have also been employed as a matrix and have demonstrated positive regenerative effects [49–53].

Employment of a conduit as the scaffold component in a tissue engineered peripheral nerve graft has some advantages. They are currently used clinically and the materials used to fabricate conduits can be modulated to optimize the conduit properties for nerve regeneration. However, the clinically approved version of the conduits lack luminal structure to support the cellular component of the tissue engineered graft and axonal regeneration. The addition of a luminal structure or substance would require additional regulatory approval and would poorly mimic the organized endoneurial structure of peripheral nerve autografts. Therefore, other alternatives should be considered that may better mimic native nerve or autografts.

Biological grafts

The ideal substitute for peripheral nerve autografts would be a scaffold of basal lamina (longitudinally oriented) lined with fibronectin, laminin, and SCs. Nerve allografts, i.e., nerve tissue from organ donors, would be the perfect alternative to the autograft. Their use, however, requires immunosuppression to avoid rejection and regeneration failure [54]. The use of pharmacological immunosuppression is associated with significant clinical morbidity [55] and limits the use of allografts in peripheral nerve repair to only the most severe cases of nerve injury [56]. To avoid the morbidity associated with immunosuppression, tissue preparation methods have been employed to remove the cellular component and diminish the immunogenicity of the allografts. Acellular tissues, despite their lack of cells, maintain a highly organized extracellular matrix structure and this structural arrangement would provide an ideal scaffold component for a tissue engineered peripheral nerve. In addition to acellular nerve, acellular muscle is also a viable scaffold candidate.

Acellular muscle tissue offers an alternative to nerve as its basal lamina arrangement mimics the endoneurial tubes contained in peripheral nerve tissue and contains collagen type IV, fibronectin and laminin to promote nerve outgrowth [19, 57]. Promising results have been demonstrated with acellular muscle grafts compared to autografts in animal models [58–60]. However, the processing to produce decellularized muscle tissue can lead to a scaffold in which axons may grow out of the tissue construct with the potential for neuroma formation [19, 61]. Additionally, recruiting SCs to migrate within the muscle graft for large defects may be problematic to appropriate axonal regeneration [57].

Acellular nerve grafts are conceptually appealing because their physical, chemical, and mechanical properties are similar to those of a nerve autograft. They lack SCs, a vital component to nerve regeneration [8, 62, 63], but they do not induce an immune response [54, 64, 65]. Numerous methods have been used to remove cells and antigens from nerves, as reviewed recently by Szynkaruk et al. [63]. Examples of decellularizing techniques

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include: repeated freeze-thaw cycles, exposure to radiation, lyophilization, extended storage in cryopreservation solution, and decellularization with detergents [66–72]. The use of repeated freeze-thaw cycles, irradiation, and lyophilization are effective at eliminating living cells within cadaveric grafts, however these techniques fail to remove the remnants of cells from the graft resulting in delayed regeneration across the graft [67, 73, 74]. Additionally, repeated freeze-thaw cycles have been shown to damage basal laminae and structural proteins that support axonal growth [75, 76]. Extended storage in cryopreservation solution or the use of detergents to decellularize cadaveric nerve allografts effectively removes the remnants of cellular components of the graft [77–81]. Advantages of cold preservation of peripheral nerve grafts are that it produces a completely acellular graft with minimal damage to the basement architecture and elicits no host immune response [78, 81]. Despite these positive aspects of cold preservation, the duration to preserve the graft and render it completely acellular and nonantigenic is long (>7 weeks) requiring extensive coordination of preparation and use of the graft in a clinical setting.

Detergents have also been investigated for their ability to remove antigen presenting cells from peripheral nerves to render an acellular graft [77, 79]. Using the detergent Triton X-100 and a relatively short 4 day rinse protocol, Sondell et al. [79] produced an acellular graft that was shown to support outgrowth of axons and migration of host SCs without excessive signs of inflammation . However, the microstructure of basal laminae from grafts undergoing the Sondell et al. [79] detergent protocol is damaged and results in inferior regeneration [70]. Schmidt's lab developed a detergent protocol that can effectively decellularize a cadaveric nerve while preserving the internal structure of the native nerve [80]. A variation of this protocol was used in 2008 to develop the first commercially available acellular peripheral nerve allograft for clinical use. Produced by AxoGen, Inc. (Alachua, FL), nerve allografts are harvested from cadaveric human donors, processed to remove cells using a human variation of Hudson et al. [70] protocol and chondroitin-6-sulfate proteoglycan (a known inhibitor of axonal regeneration) [82–84], and then gamma irradiated for “off the shelf” use. This commercially acellular allograft was evaluated in a rat sciatic nerve model and it was determined that the grafts were nonimmunogenic, the endoneurial tubes remained intact, and laminin was present in the acellular grafts. While axonal regeneration was inferior to the autograft controls, the rat acellular nerve allograft (ANA) performed better than the most commonly used commercially available type I collagen nerve conduit at both 14 and 28 mm gap lengths (NeuraGen, Integra NeuroSciences, Inc.) [85]. Improved regeneration across the rat ANA in comparison to a nerve conduit was also found in a follow-up study by Moore et al. [86]. However, again the ANA did not have equal regeneration to the autograft controls. Although ANAs are now used clinically for peripheral nerve reconstruction, they still are unable to match the performance of the gold standard nerve autograft. The acellular nature is the primary reason for the deficiency in regeneration when compared to autografts. In fact, the addition of SCs to short length ANAs (14 mm) processed using Schmidt's detergent protocol produce axonal regeneration that is indistinguishable from that of autograft controls in a rodent model of nerve injury [87].

In summary, a candidate scaffold for the development of a tissue engineered peripheral nerve should be immunologically inert, have the mechanical properties of normal nerve, and have an luminal microstructure that is organized in close approximation of normal nerve. Currently, there is only one clinically available construct that meets all of those criteria and is, therefore, likely to be the best available candidate scaffold for tissue engineered peripheral nerve. Future research in the field of materials science should be devoted to fabricating synthetic peripheral nerve scaffolds that mimic the attributes of ANAs. However, the use of a perfectly processed ANA or synthetic nerve scaffold will still require the

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addition of a cellular component and/or growth factors to support axonal regeneration to a level similar to nerve autografts.

Cellular component of tissue engineered peripheral nerve

SCs are the primary intrinsic mediators of nerve regeneration in the peripheral nervous system. In contrast to the central nervous system, where glial cells lead to scarring and the persistence of myelin-based inhibitory proteins, SCs phagocytose myelin debris, and dedifferentiate into a migratory, proliferative phenotype after injury [88–90]. This dedifferentiated, proliferative phenotype [91–93] is characterized in part by the expression of proteins that support axonal regeneration. The protein expression of SCs supports axons through the deposition of basal lamina, excretion of trophic factors, and adhesion molecules that facilitate regeneration after nerve injury [94–98]. In fact, the absence of SCs following nerve injury and during regeneration severely limits the quality and extent of axonal regeneration [99, 100]. Consequently, the development of a tissue engineered construct for the injured peripheral nerve will likely require a cellular component that can mimic the multiple regenerative roles of the SC.

Primary SCs

The isolation and culture of primary SCs was first accomplished in the mid 1970's [101, 102] and was quickly identified as a potential cellular therapeutic for peripheral and central neuron diseases [103, 104]. Autologous transplantation of primary SCs has, therefore, been used to enhance nerve regeneration in animal models of peripheral nerve injury [71, 87, 105–112]. SCs isolated from nerve tissue and cultured in vitro retain the ability to express trophic factors to support axonal regeneration and to myelinate axons following regeneration [97, 108, 109, 113–116, 117–119]. Mitogens can be employed during the culture process to expand cultured SC populations and thus increase the number of cells needed for transplantation [120, 121]. The cultured cells can then be transplanted into acellularized tissues or biomaterial scaffolds to recapitulate peripheral nerve-like tissues. SCs in culture have also been shown to not transform or reach a proliferative limit that would alter their utility for later transplantation [122]. Most importantly, expanded human and rat SC populations have not demonstrated tumor formation when transplanted into rodents after mitogen removal [108, 123].

Clinical translation of SC transplantation requires surgical harvest of a peripheral nerve, effective isolation, and extended expansion time before transplantation is possible. Beyond the morbidity associated with harvesting a nerve to obtain cells, primary SCs culture is difficult. Harvested nerves contain a vast number of contaminating cells including fibroblasts, which replicate more readily in culture than the primary SCs. As a result, cultures that are not purely SCs can be overrun with fibroblasts and there is some evidence to suggest that transplantation of activated fibroblasts could actually harm peripheral nerve regeneration through the production of scar [124]. The difficulty associated with primary SC culture is evident by the numerous SC culture protocols that have been published to optimize the process [120, 125–130]. Even with optimization, the expansion of SCs requires 6–10 weeks from the time of harvest. Thus, the combined barrier of donor site morbidity and difficulty of effective SC isolation and culture has significantly impeded clinical translation despite decades of concerted interest and effort [71, 108, 109]. Only recently, after over 30 years of research, SCs were employed in a clinical trial as a treatment for spinal cord injury by the researchers of the Miami Project to Cure Paralysis. The safety associated with the use of SCs in this trial will likely have a significant impact on the use of SCs as a treatment for peripheral nerve injury.

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Stem cells

The difficulty associated with the isolation and culture of primary SCs has led researchers to search for an alternative source of cells to support axonal regeneration across a tissue engineered construct. Stem cells are a plausible choice because of their ability to differentiate into multiple cell types and self renew in culture. Stem cells are classified as either embryonic or adult stem cells. Embryonic stem (ES) cells isolated from the fertilized oocyte are defined as *totipotent stem cells* and ES cells taken from the blastocyst are called *pluripotent* as these cells appear to be forming the three germ layers during embryogenesis. Fully developed adult tissues and organs contain niches of *multipotent* adult stem cells; these cells have been isolated from a wide range of adult tissues such as brain, heart, lungs, kidney, adipose, dermis, and spleen. Stem cells of each type have been investigated in models of peripheral nerve injury to determine their ability to support axonal regeneration following injury.

Embryonic stem cell derived progenitors

The limitations associated with primary SCs culture, difficulty to isolate/purify, insufficient number, slow expansion, and donor site morbidity to patients, can be overcome by the use of ES cells. ES cells are readily attainable, can be expanded quickly and indefinitely in culture, and can be prepared in mass prior to clinical need [131, 132]. Compared to SCs, ES cells proliferate efficiently with doubling times for human and mouse ES cells taking 30–35 h and 12–15 h respectively [133]. However, transplantation of naive pluripotent ES cells to treat peripheral nerve injury is not a viable option due to the propensity for ES cells to excessively proliferate in vivo [134, 135]. To overcome this obstacle, ES cells can be induced in vitro using defined culture protocols to become progenitor cells that are still multipotent, but are more limited in their differentiation and proliferation potential.

Embryonic stem cell derived neural progenitor cells are identified by the expression of the intermediate neurofilament nestin and have the ability to differentiate into all neural cell subtypes [136]. Transplantation of these neural progenitor cells into conduits interposed between the proximal and distal stump of the transected sciatic nerve was shown to stimulate axonal regeneration more efficiently than conduit controls not containing cells. The transplanted cells differentiated into a SC phenotype in vivo, expressing the SC specific marker S100 and morphological colocalization with axons suggesting myelination [137]. The induction protocol used by Cui et al. [137] results in 70 % of the ES cells adopting a neural progenitor phenotype prior to transplantation [138]. In addition to ES cell derived neural progenitors, research efforts are also devoted to the development of induction protocols to direct ES cells to neural crest cells and ultimately SCs in culture [139, 140]. These induction protocols result in cell populations that are approximately 60 % positive for SC related markers and have demonstrated the ability to myelinate axons in neuronal coculture assays. However, they have yet to be evaluated for their impact on recovery in a peripheral nerve injury model. Another class of progenitor cells that has been shown to impact recovery following peripheral nerve injury is mesenchymal stem cells. Examples of peripheral nerve therapies using primary mesenchymal stem cells are discussed in depth in the next section, but ES cell derived mesenchymal stem cells have been used to treat nerve injury with some success [141].

Although ES cell transplantation might provide therapeutic benefit, the potential for uncontrolled proliferation following transplantation and the need for immunosuppression limit their translatability. Studies have shown that transplantation of cell population containing even small percentages of naive embryonic stem cells can result in the formation of teratomas [134, 142]. Additionally, transplantation of a heterogeneous neural stem cell population in a human clinical trial resulted in the formation of slow growing tumors [135].

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In all of the studies noted from the literature, the highest percent of cell induction of ES cells to a multipotent phenotype was 70 %, which leaves 30 % of the cells likely expressing markers of a pluripotent excessively naïve phenotype. The need to transplant a pure population of induced cells for therapeutic treatments is highlighted by numerous examples from the literature [134, 135, 142]. Compounding the dangers of excessive proliferation is the need for immunosuppression with ES cells to prevent immune rejection while the nerve regenerates [56]. Certain immunosuppressive compounds have been shown to improve axonal regeneration following nerve injury and could be seen as a positive side effect of ES cell therapy [143–158]. However, the barrier for the use of immunosuppressive therapies for nonlife threatening diseases, such as peripheral nerve injury, is high and will likely seriously impede the translation of ES cell derived therapies for peripheral nerve injury [55].

Mesenchymal stem cells

Mesenchymal stem cells (MSC) are self-renewing multipotent adult precursors that are a promising source of cells for tissue engineering [159]. MSC originate from the mesoderm germ layer and they give rise to connective tissue, skeletal muscle cells, and cells of the vascular system. Their multipotency, ease of isolation and expansion in vitro make them an attractive candidate as a component for tissue engineering applications. Under normal developmental paradigms and cellular environments, MSCs are able to differentiate into tissues of mesodermal origin, for example, muscle, bone, cartilage, fat, and tendon. However, recent data suggest that under specific cell culture conditions, MSCs have the potential to transdifferentiate into many cell lineages (other than mesodermal). With appropriate stimuli and environmental conditions, MSCs have been shown to differentiate into sweat glands [160], myocardium [161], endothelial cells [162], astrocytes [163], and neurons [164, 165]. Accumulating evidence has also demonstrated that MSCs can be directed under specific conditions to differentiate into myelinating SC-like cells.

In a preliminary study, Giorgio Terenghi's lab was the first to demonstrate that MSCs in culture could be directed into SCs like phenotype [166]. Exposing MSC to glial growth factor, a SC mitogen, which stimulates peripheral nerve regeneration and restricts neural crest cells to a glial fate [167], induced expression of S100 and glial fibrillary acidic protein (GFAP) in culture. These induced cells were then implanted into nerve conduits and inserted into a rat sciatic nerve defect. They demonstrated the ability to enhance axonal regeneration and myelination. Additionally, they evaluated the transplantation of MSC that were not exposed to glial growth factor and found that a portion of the transplanted cells expressed S100 following transplantation. These results suggested that the environment surrounding MSC has a significant impact on their differential cell fate. A series of subsequent studies have further evaluated the transdifferentiation of MSC to SCs and have demonstrated that under the correct culture conditions that they can express the SC markers S100, GFAP, and p75, express growth factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), express cell surface receptors erb3 and glial growth factor 2, and have the ability to myelinate axons in neuron coculture [168–174].

Despite these positive findings, there are causes for concern in the use of MSC as a SC substitute in tissue engineering constructs. The first reason is that the derivation of SCs from MSCs requires that the cells transdifferentiate. Transdifferentiation refers to the irreversible switch from differentiation along one cell lineage to another that is descendent of another germ line. In case of MSC transdifferentiation into SC, the switch would require cells of mesoderm lineage (MSCs) to differentiate into cells of ectoderm lineage (SCs), and the possibility of this process occurring in mammals is a point of significant debate in the literature that is beyond the scope of this review [175–177]. However, the instability of the

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MSC derived SC phenotype seems to, in part, support the conclusion that trandifferentiation of MSC is an artifact of the cell culture environment. As described above, it has been demonstrated that cocktails of growth factors and cytokines can foster a SC phenotype in cultured MSC. Subsequent studies have demonstrated, however, that removal of these in vitro signals results in reversion of the cultured cells to a myofibroblast phenotype that is consistent with the MSC germ line origin [178]. This would suggest that the switch is temporary and artificial, not allowing for long-term maintenance of nerve by SCs derived from MSCs. As with ES cells, there is an inherent danger in the transplantation of cells that are physiologically unstable and the instability of MSC derived SC phenotype likely will increase the barrier to translation of these cells. Before determining whether the in vivo peripheral nerve environment can stabilize the MSC derived SCs phenotype, it is necessary to determine the ultimate utility of these cells for peripheral nerve tissue engineering constructs.

Adipose derived stem cells

Adipose-derived stem cells (ASCs) were first isolated from the stromal vascular fraction of homogenized adipose tissue in rats [179], which led to their ultimate isolation in human tissue [180]. ASCs can be easily isolated from liposuction waste and can exhibit the potential for chondrogenic, osteogenic, adipogenic, and myogenic differentiation [181, 182]. They have many similarities to MSC; they arise from the same mesodermal germ layer, express an ~90 % similarity in cell surface markers, and have multipotent differentiation potential [182, 183]. ASCs, however, have the advantage of increased abundance in the body in comparison to MSCs and thus make them easier to isolate and culture [159, 184]. These factors make them an attractive therapeutic option for tissue engineering applications and their potential to adopt a SCs phenotype under controlled cell culture conditions makes them pertinent to the current discussion. Isolated ASCs, when treated with a mixture of glial growth factors (GGF-2, bFGF, PDGF and forskolin), adopt a spindle-like morphology similar to SCs. Analysis of the protein expression from these induced cells reveals expression of the glial markers, GFAP, S100 and p75, indicative of transdifferentiation into a SC like phenotype [185]. As with MSCs, an increasing number of studies have demonstrated the ability of ASC derived SCs to express neurogenic growth factors, to produce myelin, myelinate axons in vitro, and stimulate axonal regeneration in models of peripheral nerve injury [186–190].

SCs derived from ASCs also share the same concerns as those derived from MSCs. Their utility depends on the controversial process of transdifferentiation and is subject to the same instabilities in phenotype. Further, concerns about their ability to survive for extended periods following in vivo transplantation has cast doubt on the mechanism of regenerative benefit in many of the studies listed above [191]. Limited ability of ASCs to survive would suggest that prior regenerative benefit would be due to production of growth factors and not a functional replacement of lost SCs. As with MSCs, long-term studies tracking the survival and maintenance of the SCs phenotype are needed to validate the use of ASC derived SCs for tissue engineering constructs in peripheral nerve surgery.

Skin derived stem cells

The proliferation/immune concerns associated with ES cell derived cells, and the transdifferentiation/stability concerns associated with MSCs and ASCs are significant barriers to translation. Ideally, an autologous source of adult progenitor cells from the neural crest (ectoderm) lineage that can be readily expanded and induced to form true SCs would be optimal for the development of tissue engineered peripheral nerve. Neural crest progenitor cells have been identified in two locations in adult tissue; the gut [192] and the

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skin [193]. The gut is not a convenient source of autologous progenitor cells for tissue engineering. The skin, however, is readily accessible for clinical harvest. Isolation of stem cells from the dermis was done initially without full understanding of where the cells were located within the skin or from what lineage they were derived. Li et al. [194] demonstrated that the stem cells isolated from the dermis resided in a niche of the hair follicle. Further characterization of these cells revealed the cells to be of neural crest lineage and demonstrated their ability to differentiate into neural phenotypes [195–197]. Transplantation of these skin derived neural crest related progenitors (single-nucleotide polymorphism (SNP)) first demonstrated the capability of the cells to differentiate into SCs and myelinate regeneration axons [198]. These observations were followed by cell culture protocols demonstrating that SNPs responded to neural crest cues such as neuregulins to generate SCs like cells [199]. The SNP derived SCs express markers consistent with primary SCs (P0, S100, GFAP, and p75) and form myelin in the presence of axons. They have been shown to produce levels of neurotrophins (NGF, and NT-3) at rates greater than that of primary SCs [200]. These results suggest that the naïve phenotype of SNP derived SCs might make them a better candidate for tissue engineering constructs than primary SCs. These results, however, merit further investigation.

While SNP derived SCs are not limited by transdifferentiation, studies have demonstrated that following transplantation these cells have limited cell survival (less than 10 %) and that only 38 % of the transplanted cells maintain the SC phenotype [201]. The findings of this study demonstrate the need for increased focus on cell fate following transplantation. It is important to understand cell survival, proliferation and differentiation following transplantation to fully understand how the transplanted cell truly impacts the disease state. Methods can be employed to alter the transplantation environment to enhance cell survival and maintain differentiation [142, 202].

Genetically engineered cells

Despite recent advances in the understanding of the neurobiology related to nerve regeneration and refinement in surgical techniques, complete functional recovery after repair of a damaged nerve is rare. Growth factors are a major component of the regenerative process after peripheral nerve injury. Expression of growth factors after nerve injury is upregulated in the distal nerve stump and denervated muscles [203, 204]. The expression is higher in neuromuscular tissue distal to the site of injury, whereas minimal expression is observed in the proximal stump of the injured nerve [205, 206]. The increased production in the distal stump and denervated muscle following nerve injury occurs early after injury followed by a gradual decrease in the expression with increasing time [207–209]. The pattern of growth factor expression following injury creates a temporary concentration gradient that increases with distance distal to the site of injury [203]. The temporary distal upregulation and secretion of growth factors is a signal that promotes axonal regeneration. The duration of the signal is often shorter than the time necessary for the regenerating axons to reach the end organ target. The levels of growth factor in the distal nerve after chronic denervation (>4 weeks in the rat) are not sufficient to stimulate robust axonal regeneration from the proximal stump [210, 211]. Thus, before the nerve reaches its target the signal stimulating it is gone.

Exogenous administration of neurotrophic factors has often been studied as a route to augmenting peripheral nerve regeneration [212]. This approach has led to variable and scattered results on the regenerating peripheral nerve post injury secondary to poor in vivo diffusion and short windows of metabolic activity [213, 214]. Emerging research using genetically engineered cell systems has demonstrated that novel therapeutic gene transfer into the peripheral nervous system for local, long-term transgene expression as a new

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treatment approach to the injured peripheral nerve [215–217]. Though the use of genetically engineered cells has demonstrated success as a novel treatment modality for the injured peripheral nerve, certain draw backs have been noted—foremost being the so-called "*candy-store effect*", where elevated levels of neurotrophic factor 'trap' regenerating fibers, limiting neurite outgrowth, as neurites will not continue to regenerate against an unfavorable neurotrophic concentration gradient [87, 217–219]. To avoid the nerve trapping effects that have been previously demonstrated, engineered cells systems that allow for conditional expression of therapeutics need to be developed. In this way, physiologic levels of growth factor can be maintained in the areas of need in the distal stump for an appropriate time length and can be silenced after regenerating axons have transversed the defect.

Conclusions

The field of peripheral nerve surgery needs tissue engineers to create an equivalent alternative to the nerve autograft. The ideal replacement would consist of a scaffold and a cellular component that would support nerve regeneration across a nerve gap. Current research efforts would suggest that the tissue engineered construct will resemble an acellularized nerve allograft enhanced by the addition of SC-like cells. Skin-derived neural progenitor (SNPs) derived SCs would likely be the easiest and most clinically translatable cellular therapy due to their availability and safety, but further research is warranted. It should be noted that functional recovery as a result of even a perfect surgical repair using an autograft is not total and often results in only limited recovery [86, 220]. While we designate the autograft as the "gold standard" in actuality the results of functional recovery associated with the "gold standard" are frequently disappointing. For this reason, current clinical use of artificial constructs (i.e. ANA or nerve conduits) that do not support regeneration to the level of an autograft should be limited to small diameter noncritical sensory nerves with gap distances less than or equal to 4 cm [40, 43, 86, 221].

The field of tissue engineering should consider its challenge to not only meet the autograft "gold standard" but also to understand what drives and inhibits nerve regeneration in order to surpass the results of an autograft. To this end, especially in the case of large defects, genetically engineered cells that produce growth factors spatially and temporally in response to regenerating axons may be desirable. Although extensive biomedical research is needed to completely understand the efficacy of these methods ultimately, there is great promise in the future of tissue engineering as it pertains to peripheral nerve regeneration. Clinically, there is a large patient population with nerve injuries that will benefit from the ongoing research and scientific progress.

Acknowledgments

Funding from the National Institute of Health NINDS (5R01NS05170607, and 2R56NS03340618) supported the production of this manuscript. We would also like to thank Andrew Yee for supplying artwork for the manuscript figure.

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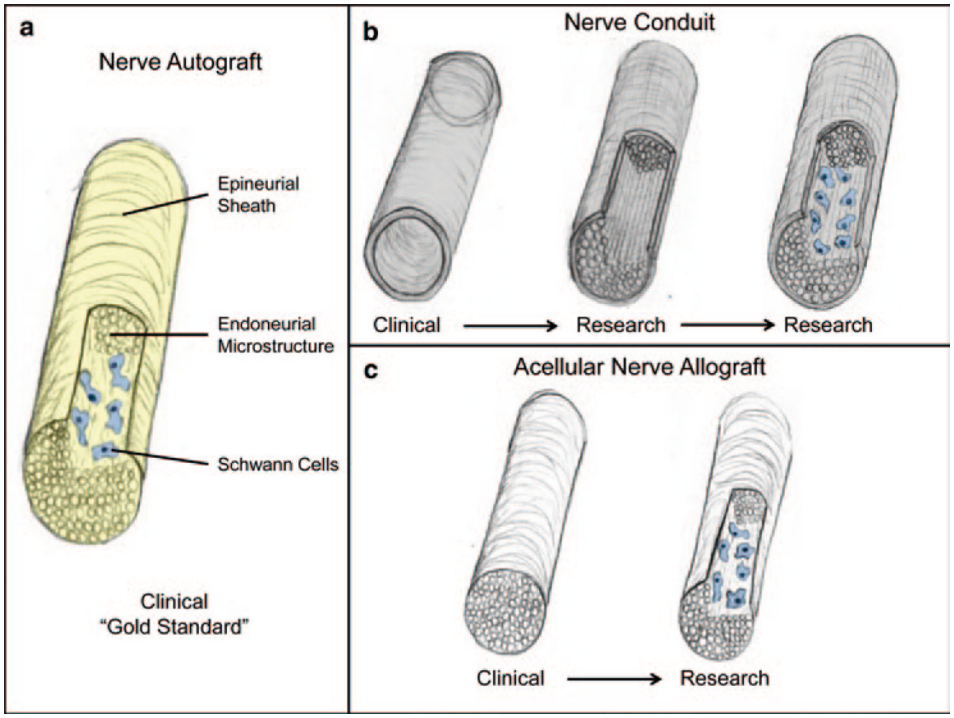

Fig. 1.

Tissue engineered peripheral nerve constructs. **a** The peripheral nerve autograft is used clinically and is the current "Gold Standard" for peripheral nerve reconstruction. An autograft provides three levels of regenerative support for regenerating axons of damaged peripheral nerves. The *epineurial sheath* provides gross guidance of axons from the proximal to the distal stump, *endoneurial microstructure* provides microscale support and guidance of axons, and *Schwann cells* are present that stimulate and support regeneration across the graft. **b** In contrast, nerve conduits used clinically lack *endoneurial microstructure* and *Schwann cells*. Tissue engineering research efforts are directed towards adding artificial *endoneurial microstructure* and *Schwann cells* to mimic the support of nerve autografts. **c** Similarly, acellularized nerve allografts (ANAs) that are used clinically lack *Schwann cells* and tissue engineering research is devoted towards the addition of *Schwann cells*. Both clinically available nerve conduits and ANAs can be used as candidate scaffolds for tissue engineered peripheral nerve constructs