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Adipose-Derived Stem Cells in Peripheral Nerve Regeneration

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Abstract
Purpose of the Review Peripheral nerve injuries are common, debilitating, and costly. The human body's innate regenerative capacity is slow, and nerves are often misguided. The purpose of this article is to review a specific cellular, regenerative engineering technique that holds promise for the treatment of peripheral nerve injuries.
Recent Findings Over the past several decades, research has focused on the utilization of stem cells for peripheral nerve repair. More recently, stem cells collected from adipose tissue (adipose-derived stem cells or ADSCs) have gained traction due to their relative ease of collection and differentiation potential. Both undifferentiated and Schwann cell-like differentiated ADSCs have been used to seed conduits with variable results.
Summary Technical and ethical issues surrounding stem cells' self-expansion potential and genetic makeup exist. Ultimately, randomized control trials and FDA approval will be required before widespread clinical translation in the US is realized.

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Introduction

Peripheral nerve injuries (PNI) are common with 1.4 million injuries occurring per year in the United States [1], and they commonly lead to significant functional impairment. While many diverse pathologies can lead to PNI, trauma is one of the most common causes. It is estimated that approximately 2.8–5% of all trauma patients sustain such an injury [2, 3]. PNI is a significant public health issue, with over 20 million traumatic injury discharges from the US hospital per decade [4] affecting both adults and children. Unlike the central nervous system, the peripheral nervous system (PNS) harbors some intrinsic regenerative capacity, although recovery is slow and often incomplete. Muscle fibers atrophy quickly after denervation with a 60–80% volume reduction is seen at 4 months [5]. Injury mechanism dictates treatment strategy with direct nerve repair or grafting often indicated. Despite advances in treatment of PNI, poor outcomes are still commonplace encouraging novel treatment strategies. Recent developments in biomaterials and insight into stem cell biology may hold great promise for peripheral nerve injury treatment.

Pathophysiology and Classification

The PNS relays sensory and motor impulses between the central nervous system to targets throughout the body. It is composed of neurons, glial cells (including Schwann cells), and supporting stroma. Subsequent to injury, neurons

undergo changes in genetic expression that leads to the release of neurotrophic factors and upregulation of corresponding receptors. These factors support axonal elongation from the proximal injured nerve stump. Damaged axons in the distal nerve fragment regress through a process termed Wallerian degeneration [6]. Schwann cells (SC) and infiltrating macrophages support this process by clearing myelin debris and secreting neurotrophic factors [7]. In addition, macrophages support angiogenesis and form a connective tissue bridge in the nerve gap [8•]. The un-innervated Schwann cells form endoneurial tubes called bands of Bungner, which serve as guides for axonal regeneration initiated from growth cones (usually located at nodes of Ranvier) (Fig. 1). As axons regenerate, they are frequently misdirected and do not reach the intended target [9]. The degree to which recovery is possible directly relates to the extent of injury.

Classification of PNI was first undertaken by Seddon [10] and further defined by Sunderland [11], based on extent of injury. According to Sunderland, a first-degree injury is the result of a conduction block and is also referred to as neuropraxia. Recovery is complete and usually takes place over the course of days to months. Second-degree injuries result from axonal disruption and are called axonotmesis in the Seddon classification, while third-degree injuries affect both axons and endoneurium. Recovery is spontaneous and nerves regenerate at about 1 inch/month, with second-degree injuries recovering completely, but only partial recovery can be expected in third-degree injuries. Sunderland fourth- and fifth-degree (neurotmesis in the Seddon classification) injuries have disruption of the perineurium, and perineurium and epineurium, respectively (Fig. 2). This category includes nerve avulsion and transections, which require surgical repair.

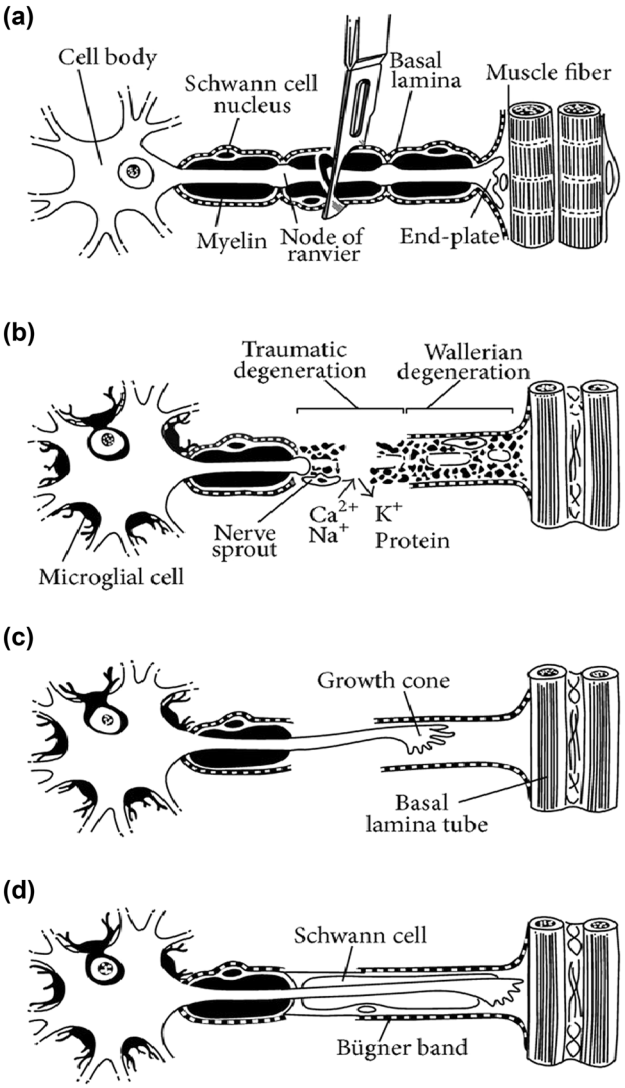

Fig. 1 Degeneration and regeneration after PNI. [5] (Reproduced with permission from Hindawi Publishing Corporation)

Current Treatment

Most commonly, nerve injuries are attributed to traction, compression, or laceration. Laceration may require surgical repair to restore sensory or motor function. Surgical management typically includes direct repair or nerve grafting. In complete nerve transections, direct repair is considered the gold standard [12]. The proximal and distal ends need to be debrided, opposed, and coapted in a tension-free manner, with most repairs just re-approximating the epineurium. Other direct repairs include the grouped fascicular repair, in which individual fascicles are coapted to each other. However, none consistently show better outcomes than epineural repair [13].

When tensionless coaption cannot be performed, repair relies on nerve grafting. Immediately after coaption, the nerve graft undergoes Wallerian degeneration, thereby serving more like a scaffold instead of an immediately functional replacement. Autologous nerve grafts are considered standard of care; unfortunately, this approach has many limitations [3]. Cutaneous nerves such as the sural nerve are frequently used; however, the cross-sectional area and length are often inadequate. As autologous grafting necessitates a healthy nerve to be procured from the patient, it results in variable donor site morbidity. As such, motor nerves are not typically used as grafts. Vascular grafts have also been described, but veins have thin walls which are easily compressed by surrounding scar tissue [14] which may not lead to optimal results. This has led to interest in biomaterials and the design of conduits to bridge nerve gaps [15, 16].

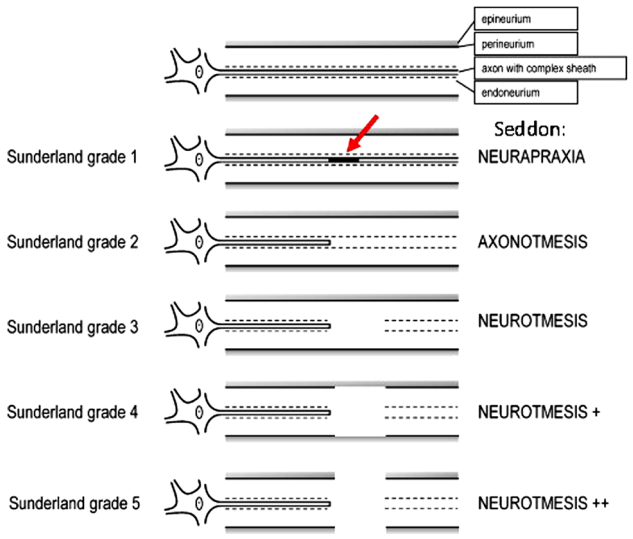

Fig. 2 Schematic representation of the five degrees of nerve injury. Grade 1: conduction block indicated by *red arrow* (neuropraxia), Grade 2: transection of axon with an intact endoneurium (axonotmesis), Grade 3: transection of the nerve fiber (axon and endoneurium) within an intact perineurium (neurotmesis), Grade 4: transection of funiculi, epineural tissue maintains nerve trunk continuity (neurotmesis +), Grade 5: transection of the whole nerve trunk (neurotmesis ++). [20] (Reproduced with permission from Elsevier)

Conduits encase the proximal and distal nerve ends providing a guide for nerve regeneration. Historically, various materials have been used including decalcified bone, polytetrafluoroethylene (Gore-Tex), and silicone. More recently, modern biomaterials such as polyglycolic acid (Vicryl), collagen, and polycaprolactone have been developed [16]. The first collagen nerve conduit, Integra NeuraGen®, was approved in 2001. These conduits are made of purified collagen and therefore may minimize immunologic concerns [17]. Integra conduits are very expensive, and cannot be used in patients with a bovine allergy [16, 18]. Despite advances in conduit technology, elongation of axons past the two sites of neurorrhaphy is often misdirected leading to sparse reinnervation of target organs and variable functional outcomes, with sensory recovery more frequent than motor [18–20].

Many studies have obtained good results with injury gaps <3 cm, but poor and variable results are seen with larger gaps [21]. The explanation for the 3 cm limit is still debated; however, it is recognized that axonal restoration in hollow conduits is limited by the matrix and cellular migration phases [22]. The regenerative process in guidance conduits comprises five phases: (1) fluid phase, (2) matrix phase, (3) cellular migration phase, (4) axonal phase, and (5) myelination phase (Fig. 3). In phase one, there is an influx of plasma, containing neurotrophic factors and extracellular matrix (ECM), from both the proximal and distal nerve stumps which peak at 3–6 h. Next, an acellular fibrin cable is formed between nerve stumps, which begins within 1 week. In the third phase, SCs migrate along the fibrin cable from the nerve stumps forming the glial bands of Bunger. During the axonal phase, nerve sprouts are guided by bands of Bunger from the proximal to distal nerve stump and reach their target after approximately 2–4 weeks. Finally, in the fifth phase, SCs convert to the myelinating phenotype to form myelinated axons 6–16 weeks after the initial repair. As the fibrin cable degrades approximately 2 weeks following repair, any gaps that cannot be traversed by SCs in this period will persist, resulting in a 3–4 cm critical limit. Several studies have shown that the addition of SCs to acellular nerve grafts improves functional recovery [23, 24•]. Acellular conduits effectively serve only as scaffolds, while SC-impregnated conduits are thought to directly impact nerve regeneration biology through the continual release of neurotrophic factors and signals. Although SCs are vital to regeneration, they have limited applicability due to unavailability without a nerve biopsy and a considerable time requirement to obtain the requisite quantity for conduit seeding [25]. Therefore, stem cells may represent an ideal starting material for SC differentiation.

Translational Research

Mesenchymal Stem Cells

Stem cells can be categorized into four distinct groups based on origin: embryonic, fetal, adult, and induced pluripotent stem cells [26]. Adult stem cells can be found throughout the body including the brain, fat, skeletal muscle, liver, retina, skin, and bone marrow [27]. Mesenchymal stem cells (MSCs) isolated from bone marrow (BM) were the first to be utilized to address clinical pathologies. Limitations of utilizing a BM donor source include an invasive and painful harvest which only yields a limited volume of stem cells. In addition, the life span and differentiation ability of BM-derived stem cells (BMSC) decline with increasing age [28]. Despite these limitations, there are multiple studies utilizing BMSCs to improve peripheral nerve repairs [29–31]. In culture, varying cytokine combinations have differentiated BMSCs into a Schwann cell phenotype capable of expressing S100 protein, GFAP, and p75 [32, 33]. These are all markers of a glial lineage, including SCs. S100 proteins are present in cells originating from the neural crest, and GFAP is an intermediate filament protein expressed in the central nervous system. The p75 receptor binds neurotrophins critical for the development, maintenance, survival, and death of the

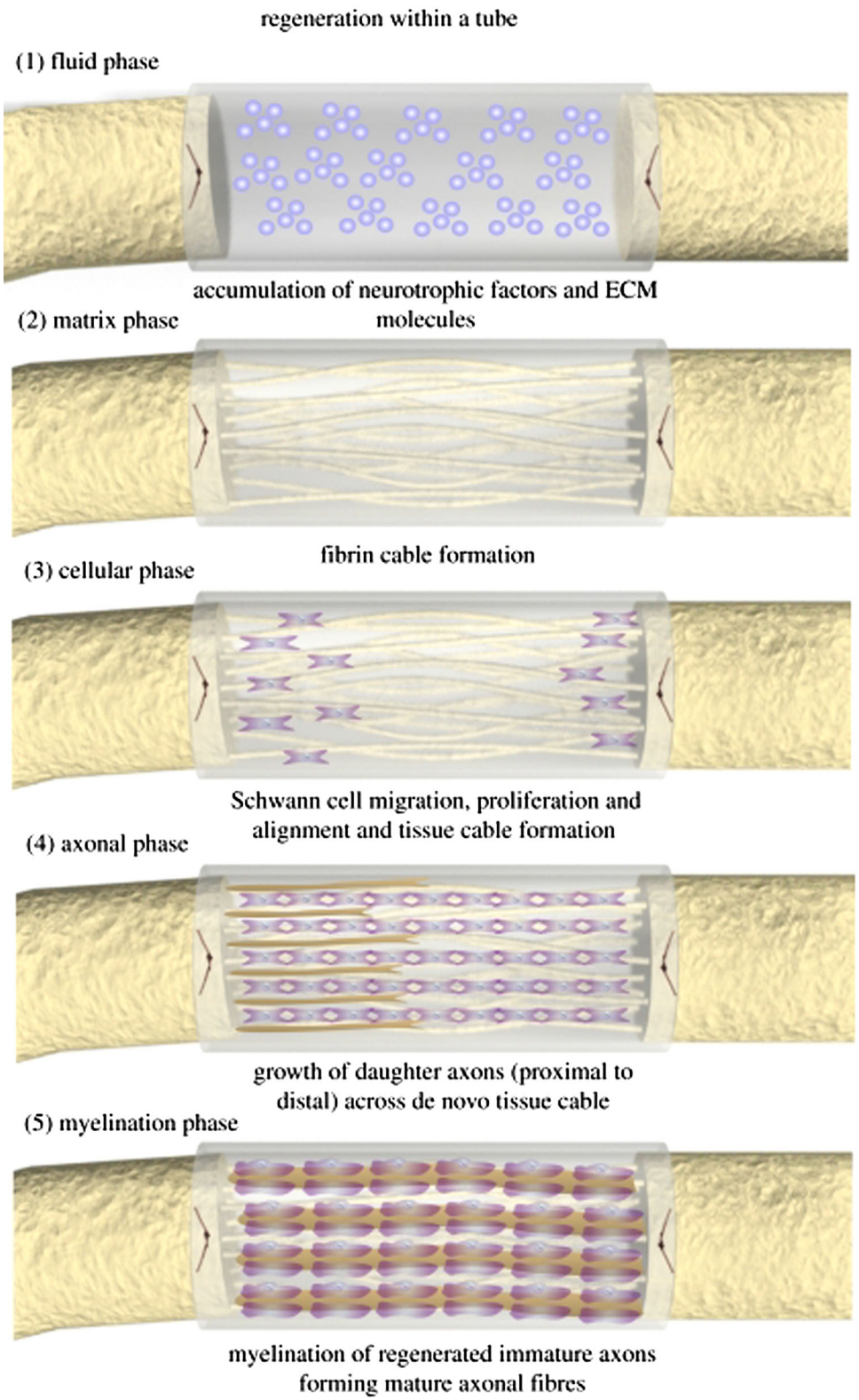

Fig. 3 The regenerative process can be divided into five main phases: (1) the fluid phase; (2) the matrix phase; (3) the cellular migration phase; (4) the axonal phase; and (5) the myelination phase. [22] (Reproduced with permission from The Royal Society)

nervous system. Graft incorporation of these differentiated cells has yielded improvements in repair electrophysiology and morphology in models of PNI, in vivo [34]. Given the potential of MSCs in tissue engineering, alternative sources which are less invasive and more yielding are continually being investigated.

Adipose-Derived Stem Cells

Over the past two decades, it has been recognized that adipose tissue is more than just an energy reservoir. In 2001, Zuk et al. [35] isolated mesenchymal stem cells from lipoaspirate, which they termed adipose-derived stem cells (ADSCs). Since then, ADSCs have been broadly investigated for regenerative applications including being differentiated into both mesenchymal and non-mesenchymal cellular lineages. Adipose has increased in popularity for stem cell retrieval as ADSCs are present in large quantities in both intraabdominal and subcutaneous depots [35, 36]. Compared to bone marrow donor sites, adipose tissue offers up to a fivefold increase in stem cell yield [37] per unit volume while allowing for longer culture periods and faster growth rates than BMSCs [38].

Neural Differentiation of ADSCs

After excised adipose tissue is minced, rinsed, digested with collagenase, and centrifuged, the stromal vascular fraction (SVF) is obtained. The SVF includes ADSCs, leukocytes, endothelial cells, and pericytes. ADSCs can be isolated based on surface markers such as CD10, CD13, CD29, CD34, CD44, CD49e, CD59, CD73, CD90, CD105, and CD166 [39, 40•]. Either isolated ADSCs or the complete SVF can be plated and used for (Fig. 4) directed neuronal differentiation. Multiple studies have documented neuronal differentiation from ADSCs, and recently advanced techniques have allowed for increased conversion rates [41–43]. Jang and colleagues [41] showed that using bFGF and forskolin, ADSCs can be differentiated to neuron-like cells and confirmed by using neuron-specific markers such as Nestin, NSE, NeuN, NEFL, and Synaptophysin [42]. In our lab, we use commercially available media (HyClone and Promocell) for differentiation. Of note, both SC- and neuron-like cells undergo similar development in that they are both embryologically derived from neural crest cells (Fig. 5) [44]. These names are interchangeably used in the literature and as differentiation media contain similar components, it is likely that ADSCs are being converted into both SCs and neurons. Following differentiation, engineered cells can be incorporated into a conduit scaffold.

Strategies for Incorporating ADSCs in Peripheral Nerve Regeneration

Several research paradigms have emerged focusing solely on ADSC-based therapies in peripheral nerve regeneration. Following PNI, if axonal contact with the distal site is not quickly reestablished, support cells and the microenvironment are not maintained [34]. Adjunct ADSC therapy attempts to address this through three distinct approaches: (1) culture and differentiation of ADSCs into a SC-like phenotype, (2) the use of undifferentiated ADSCs, or (3) utilizing the SVF. It is still unclear if tissue regeneration is a direct result of differentiated stem cells or by undifferentiated stem cell influence on surrounding tissue through paracrine signaling mechanisms [45••]. Schwann cells may hold significant promise for nerve regeneration strategies if the aforementioned limitations are addressed. [25].

Utilizing differentiated SCs versus undifferentiated ADSCs or SVF has two potential benefits: (1) reduced risk of undesired differentiation, such as teratomas and (2) potential for incorporation of engineered SCs into the ongoing regeneration and myelination process [46]. Several methods differentiating ADSCs into cells expressing SC markers, such as GFAP and S100, have been described [47]. In addition, multiple in vivo studies have established the increased ability of differentiated Schwann cells to support nerve regeneration. Di Summa et al. [48] implanted fibrin conduits seeded with ADSC-derived Schwann cells in a 1 cm Sprague–Dawley nerve gap model which after two weeks showed a reduction in muscle atrophy. In a follow-up study, an increase in the size and number of myelinated fibers was seen after 16 weeks [49]. Another group seeded nerve grafts with SCs derived from BM or ADSCs and evaluated axonal regeneration distance. The rats with SC-seeded grafts were found to have greater axonal regeneration distance compared to grafts alone [50]. Liu et al. [51] similarly found that ADSC-seeded conduits had increased nerve fibers and myelination when compared to conduits alone.

Others have investigated the incorporation of undifferentiated ADSCs into models of peripheral nerve injury (both crush and gap). Santiago et al. [52] seeded undifferentiated ADSCs in polycaprolactone synthetic conduits and used these to repair 6 mm unilateral sciatic nerve defects in athymic rats. This model showed efficacy in regaining innervation to and preventing atrophy of the target muscle. It was also demonstrated that ADSCs survived transplantation for up to 12 weeks. The transplanted ADSCs did not differentiate into a neural lineage (absence of colocalization with immunostaining for GFAP and anti-human lamin A/C), and some cells differentiated to adipocytes while many remained undifferentiated. The authors concluded that the random fates of these ADSCs in vivo may lead to varied results in terms of nerve recovery. Shen et al. [53] created a genipin-cross-linked gelatin annexed with tricalcium phosphate ceramic particle (GGT) biodegradable nerve conduit which showed nerve morphology and distributions in the ADSC/GGT group superior to GGT alone. More recently, Xu et al. [54] combined both ADSCs and SCs into a silk fibroin/collagen scaffold which showed nerve regeneration superior to scaffolds

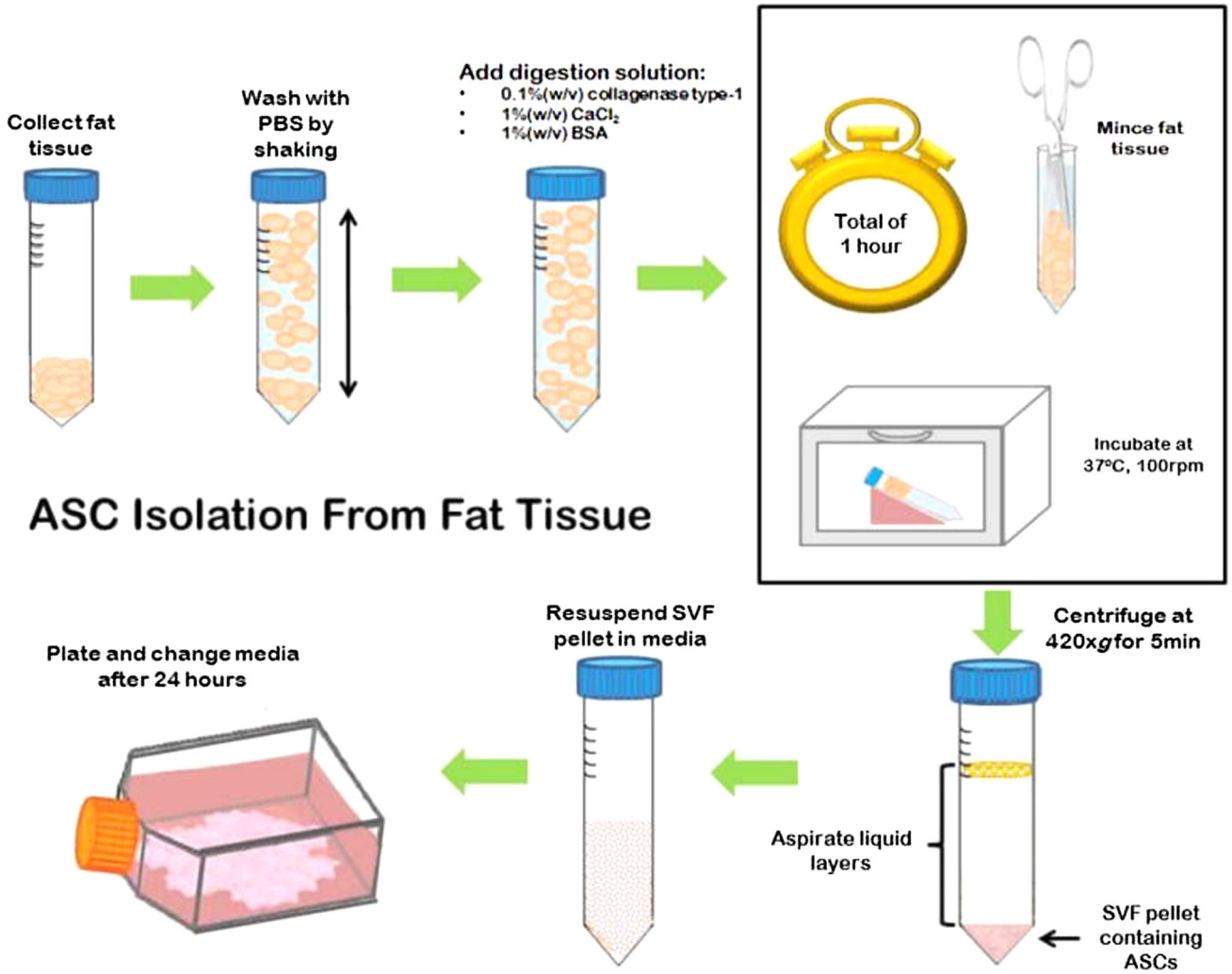

Fig. 4 ADSC isolation from fat tissue. ADSCs are isolated from fat tissue after digestion, isolation of SVF, and plating. [66] (Reproduced with permission from Springer)

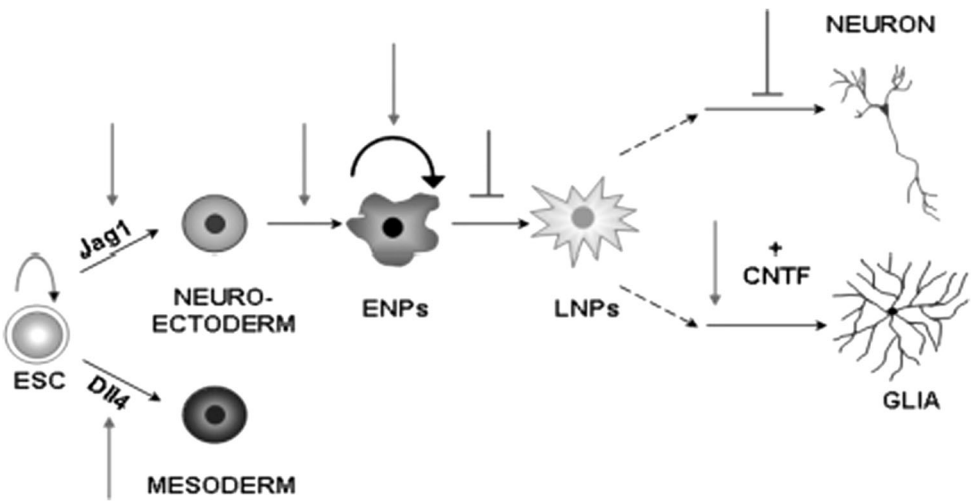

Fig. 5 Embryologic origin of neurons and glial cells. Both neurons and Schwann cells (glial origin) are derived from the same embryologic precursor. [44] (Reproduced with permission from Springer)

alone; however, functional recovery was unsatisfactory. Another approach has been the incorporation of ADSCs into fibrin glue with injection around a primary epineural suture repair [55].

Additional steps are required to separate ADSCs from the SVF, and it can take several weeks to differentiate subpopulations of cells from ADSCs. Therefore, some researchers have used the SVF itself, which is a multicellular collection of ADSCs, endothelial cells, pericytes, and leukocytes. Advantages include less cell manipulation without the need for a multistep differentiation process, quicker processing times, and no need for specialized equipment. All of these may lead to easier FDA approval ultimately. However, the amount of ADSCs may be variable and only a few studies have been performed. Matsumine et al. [56•] showed that incorporating the SVF in acellular conduits improved nerve regeneration when compared to conduits alone in a facial nerve rat model, but function was not studied. Another study compared SVF-filled chitosan grafts to chitosan grafts alone in a sciatic nerve rat model and showed increased nerve fibers as well as target muscle mass [57]. The same group also showed an improvement of regeneration with SVF-filled inside-out vein grafts [58]. Despite progress, the majority of studies did not show functional improvement and have only been performed in animal models.

Limitations of Bioengineered Neurons

Cost

The cost of personalized regenerative medicine applications is currently unknown. Liposuction and lipectomy are two methods used for fat harvest. Liposuction performed in an outpatient setting versus inpatient costs $1500 and $5000, respectively, whereas inpatient lipectomy costs approximately $10,000 [59]. ADSC isolation, culture, and neuron differentiation cost approximately $1000–1500 per 500 g of adipose tissue which yields approximately 10 million stem cells. However, overall cost will also be dependent on graft length requirement and cell density. For comparison, 3 cm-long Integra Neuragen® nerve conduits cost approximately $1500 each. However, as with any new developments, costs will likely fall as demand increases. The estimated cost for procedures to repair PNI in the US is $1.31–1.93 billion dollars per year [60]. This does not account for the cost of disability, missed work, and lost income for afflicted individuals. Therefore, there is tremendous potential for advanced technologies to reduce the cost of PNI treatment in the future while improving functional outcomes.

Ethics

Developments in the use of cell and biomaterial technologies are quickly advancing the fields of tissue engineering and regenerative medicine. Ethical concerns surrounding in vitro cell culture research has been disputed since the 1950s when the HeLa cell line was created without the consent of the patient or her family [61]. Stem cells can undergo self-expansion and differentiation into multiple cell lineages infinitely, which raises concerns about the loss of patient control of their cell line. In addition, with each passage in culture, the genomic stability of the cellular material may be compromised leading to fears of cancer transformation [62]. Widespread clinical application will likely require commercial development and associated financial pressures which may lead to unscrupulous behavior.

FDA Concerns

The FDA has created the term "tissue engineered medical product (TEMP)" which is defined as "a medical product that repairs, modifies, or regenerates the recipients cells, tissues, and organs or their structure and function, or both" [63]. Clinical application of ADSCs and differentiated neurons will likely include harvesting tissue and maintaining it in vitro prior to use in peripheral nerve repair/regeneration. Regenerative medicine which utilizes human cells and tissue components falls under "Current Good Tissue Practice for Human Cell, Tissue, and Cellular and Tissue-Based Product Establishments; Inspection and Enforcement" (69 FR 68,612) published in 2004 by the FDA [64]. As these technologies advance rapidly, standards must continuously change leading to a lack of consensus on the utilization of ADSCs in regenerative medicine. Recently, it was described in Belgium that autologous ADSC use in humans can be considered a safe tool for clinical implications as documented by genomic stability, contamination, and in vivo adverse events after transplantation of two autologous ADSC-derived products [65]. However, randomized control trials will be required to create FDA standards for the use of human tissues, prior to widespread clinical translation in the US.

Conclusion

Peripheral nerve injuries are common and lead to significant patient morbidity. Current strategies seeking to limit functional impairment includes spanning the axonal gap of the injured peripheral nerve with conduits or autologous interposition nerve grafts. The field of personalized regenerative medicine holds promise in improving

outcomes in patients with peripheral nerve injury; however, several issues require resolution before clinical application can be achieved. Improvements in stem cell technology coupled with advances in biocompatible material will likely be the direction forward as clinicians seek to treat larger nerve gaps (>3 cm) and improve the efficacy of treatment in smaller injuries.

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Compliance with Ethical Guidelines

Conflict of interest Drs. Leberfinger, Ravnic, Payne, Rizk, Koduru, and Hazard declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
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