World Journal of Stem Cells

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DOI: 10.4252/wjsc.v7.i1.51

World J Stem Cells 2015 January 26; 7(1): 51-64
ISSN 1948-0210 (online)

REVIEW

Current progress in use of adipose derived stem cells in peripheral nerve regeneration

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Telephone: +44-020-77940500
Received: July 28, 2014
Peer-review started: July 29, 2014
First decision: August 14, 2014
Revised: October 20, 2014
Accepted: October 28, 2014
Article in press: December 16, 2014
Published online: January 26, 2015

Abstract

Unlike central nervous system neurons; those in the peripheral nervous system have the potential for full regeneration after injury. Following injury, recovery is controlled by schwann cells which replicate and modulate the subsequent immune response. The level of nerve recovery is strongly linked to the severity of the initial injury despite the significant advancements in imaging and surgical techniques. Multiple experimental models have been used with varying successes to augment the natural regenerative processes which occur following nerve injury. Stem cell therapy in peripheral nerve injury may be an important future intervention to improve the best attainable clinical results. In particular adipose derived stem cells (ADSCs) are multipotent mesenchymal stem cells similar to bone marrow derived stem cells, which are thought to have neurotrophic properties and the ability to differentiate into multiple lineages. They are ubiquitous within adipose tissue; they can form many structures resembling the mature adult peripheral nervous system. Following early *in vitro* work; multiple small and large animal *in vivo* models have been used in conjunction with conduits, autografts and allografts to successfully bridge the peripheral nerve gap. Some of the ADSC related neuroprotective and regenerative properties have been elucidated however much work remains before a model can be used successfully in human peripheral nerve injury (PNI). This review aims to provide a detailed overview of progress made in the use of ADSC in PNI, with discussion on the role of a tissue engineered approach for PNI repair.

Key words: Peripheral nerve injury; Adipose derived stem cells; Cell based therapies; Stem cells

Core tip: Adipose derived stem cell differentiation is an area of important active research at present. Since adipose tissue is ubiquitous throughout the body it is an ideal source of cells for regeneration of damaged body parts. In the peripheral nervous system there are currently significant limitations in the methods of treatment and subsequent rehabilitation. Adipose stem cells can express proteins which are similar to schwann cells and are termed schwann like cells. In this review we provide an update on the current methods used in peripheral nerve reconstruction using adipose stem cells.

Zack-Williams SDL, Butler PE, Kalaskar DM. Current progress in use of adipose derived stem cells in peripheral nerve regeneration. *World J Stem Cells* 2015; 7(1): 51-64 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i1/51.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i1.51

INTRODUCTION

Background

The peripheral nervous system (PNS) consists of 3 principle cell types: axonal, glial and supporting stromal tissue[1] arranged in long efferent (motor) and afferent (sensory) fibres relaying information to the CNS. Peripheral nerve injury (PNI) is common occurring in 2.8%-5% of poly trauma patients[2,3] and may lead to long term functional deficiencies.

Traction related injuries are the most common pathophysiology seen during PNI[4]. The natural elasticity of the endoneurium and perineurium of the nerve permits stretching to occur within the planes of the collagen fibres. If a sufficient traction force is applied to the nerve a route avulsion may lead to disruption of anatomy and complete loss of function. The perineurium has much greater elastic strength than the endoneurium[4]. Therefore, elasticity is maintained within the nerve undergoing a stretching injury until the perineurium is devitalised. Peripheral nerve lacerations related to by penetrating trauma from knives and glass account for up to 7% of all brachial plexus lesions[5]. These injuries are usually partial injuries where a segment of the injured nerve remains in continuity. When a clean laceration occurs this is usually amenable to a primary repair[6]. Another common cause of peripheral nerve injuries is direct compression onto the nerve[4]. This pressure on the nerve results in both mechanical compression and ischaemia. Initial microvascular damage to the nerve is usually reversible if the injurious mechanism is discontinued within 8 h of initial injury. Early investigation of these nerves showed most of the early damage was occurring in the periphery of the nerve as opposed to the central segment were the greatest ischaemia occurred[4]. When these compressed tissues were viewed under electron microscopy the typical morphological features seen included: dislocations of the nodes of ranvier, as well as paranodal penetration and and subsequent invagination[7]. Figure 1 shows the common pathophysiological response to the damaging compression forces associated with nerve crush injuries.

Other less common causes of PNI can be attributed to thermal, electrocution, radiation, percussion and vibration injuries[3,8]. Irrespective of the cause of injury "the functional outcome of PNI depends on the severity of the injury"[9].

Classification of PNI

The severity of PNI was initially classified by Seddon and subsequently reclassified by Sunderland[10,11] as shown in Figure 2. Neurapraxia is the least severe form of nerve injury in the Seddon classification equating to grade I in Sunderland. Although the overall anatomy of the nerve remains the same, areas of segmental demyelination lead to reductions in the frequency of transmitted electrical signals[12]. Recovery from the primary injury is highly variable but most motor neuron lesions should resolve within 3 mo[13]. Axonotmesis and neurotmesis lie along a continuum and again are subcategorised by Sunderland. Sunderland grade Ⅱ is equivalent to axonotmesis with an intact endoneurium, grade Ⅲ occurs where the endoneurium is compromised but the perineurium remains and grade Ⅳ is where only the epineurium remains as demonstrated in Table 1. Sunderland grade Ⅴ or neurotmesis is associated with a complete transaction of the peripheral nerve. Neurotmesis is an indication for surgical exploration to perform: debridement to healthy tissue, tensionless nerve approximation, accurate fascicle alignment and atraumatic mechanical coaptation of the nerve ends[6]. The simplest nerve repair involves the approximation of the outermost layer of nerve tissue the epineurium. When 90% of the axonal tissue is lost, the remaining tissue heals by regeneration which is highly dependent upon the schwann cell (SC)[12].

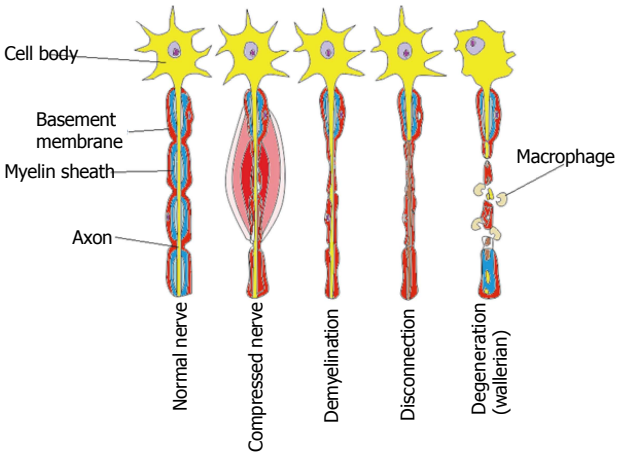

Figure 1 Changes occurring in a peripheral nerve following compression injuries adopted and modified from Ref[96].

Wallerian degeneration

Wallerian degeneration is a process that results following nerve injury, where the axon separated from the neuron's cell body degenerates distal to the injury. Wallerian degeneration occurs in a proximo-distal direction in the distal nerve stump associated with loss of integrity of the granular structures of the axonal cytoskeleton[14]. The cytoskeletal changes occurring were described in terms of a "cell body reaction"[15]. Multiple cellular changes occur within the injured nerve tissue including chromatolytic changes in neuronal cell body, eccentric positioning and change in nerve function to a nerve capable of growth and sprouting[9]. There is an influx of extracellular ions which cause macrophage influx and eventually apoptosis of the axon[1]. During the wallerian degeneration process SCs replicate whilst altering their morphology to produce mRNA to produce substances such as p75, nerve

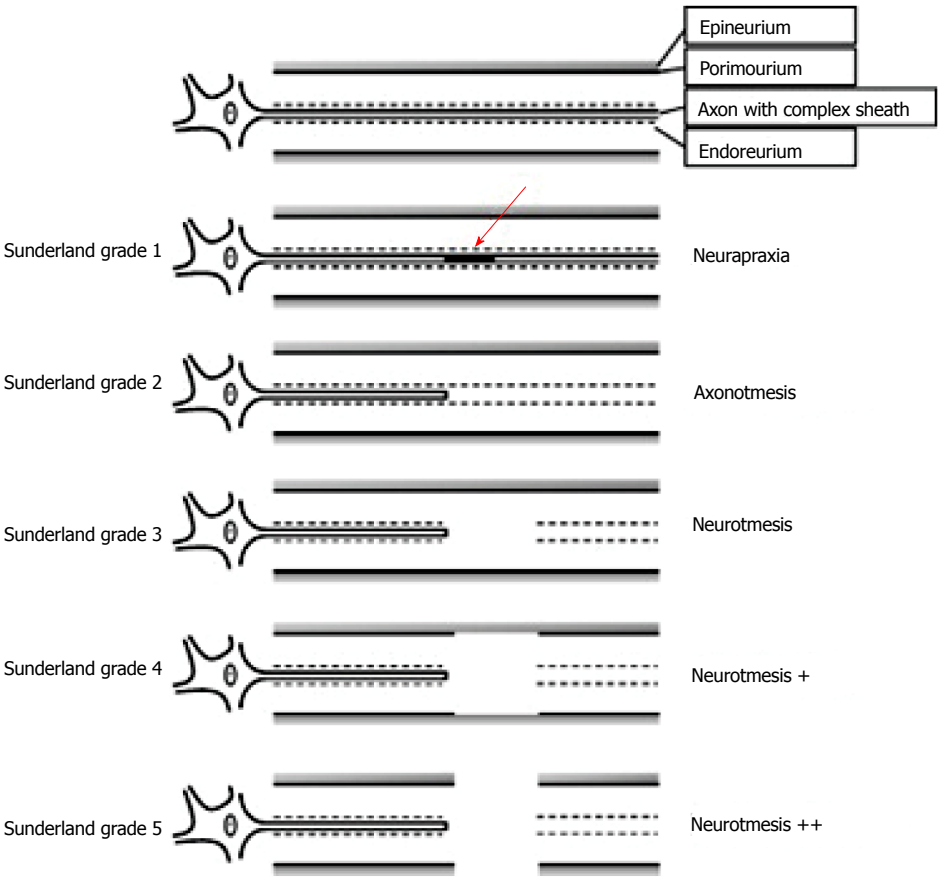

Figure 2 Sunderland classification of nerve injury adapted from Deumens *et al*[9], 2010.

growth factor and monocyte chemoattractant protein-1[16]. Reciprocal changes occur within the proximal nerve stump as SCs try to bridge the gap between the two nerve endings. Distal to proximal degeneration occurs within the proximal nerve ending. The degree of nerve tissue loss is dependent on the proximity of the nerve to the cell body in the spinal cord[1]. It has been shown that the survival rate of motor neurones close to the cell body following spinal nerve avulsion in the context of brachial plexus injuries is 10%-20%[17]. Lesions distal to the cell body cause axon death to occur up to the nearest node of ranvier in comparison to proximal lesions which may lead to apoptosis of the whole axon.

ADIPOSE DERIVED STEM CELL AND PERIPHERAL NERVE REPAIR

A key part of current peripheral nerve reconstruction involves the harbouring of SCs from autologous nerves. However, this technique is limited by the associated morbidity of sacrificing viable neurological tissue as well as the slow growth of SCs *in vitro*[18]. Adipose derived stem cells (ADSC) still remain a therapeutic option since they are multipotent and thus have an inherent ability to differentiate into mature adipocytes, chondrocytes, osteoblasts and muscle cells (Figure 3). Similar to the neural stem cell they are able to differentiate into specialised neurospheres under the action of growth factors[19-21]. This is combined with the benefit of being "easily obtained, rapidly expanded, showing low immunogenicity and can be differentiated into SCs *in vitro*"[22], thus making them potentially ideal for future peripheral nerve regenerative processes. The exact mechanism of how ADSCs interact with the regenerating nervous system is not known and theories are discussed in this review.

ADSC characterisation and harvesting

ADSC reside around pericytes structures within the mesodermally derived adipose tissue and are active during the first 50 d of the porcine embryo development and the 2nd trimester in humans[23]. They are multipotent cells, which are able to differentiate into multiple tissues of mesodermal origin including bone or skeletal muscle endodermal and ectodermal tissues such as neurone like tissue[20,24]. The ADSC express similar proteins and differentiating capabilities as cord and bone marrow mesenchymal stem cells (BMMSCs)[25,26]. They are the best characterised multipotent stem cell population[20] and form the basis of many of the early studies investigating the role of mesenchymal stem cells in nerve regeneration[27,28]. However, the ADSC have the advantages of minimally invasive harvesting and high cellular yield of between 0.25-0.375 $\times$ 10^6 cells per millilitre of liquid fat[29]. Since the early work of Rodbell *et al*[30] on adipocyte extraction; type I collagenase extraction has been a popular

Table 1 Summary of seddon and sunderland classifications following peripheral nerve injury adapted from[98]

Sunderland	Seddon	Injury	Neurosensory impairment	Recovery potential
I	Neuropraxia	Intrafascicular oedema, conduction block	Neuritis, paraesthesia	Ful (1-7 d)
		Segmental demyelination	Neuritis, paraesthesia	Full (1-2 mo)
II	Axonotmesis	Axons severed, endoneurial tube intact	Paraesthesia, episodic dysesthesia	Full (2-4 mo)
III		Endoneurial tube torn	Paraesthesia	Slow, Incomplete (12 mo)
IV		Only epineurium intact	Hypoaesthesia, dysaesthesia neuroma	Neuroma
V	Neurotmesis	Loss of continuity	Anaesthesia, extreme pain and neuroma formation	None
VI		As per V	As per V	Nil

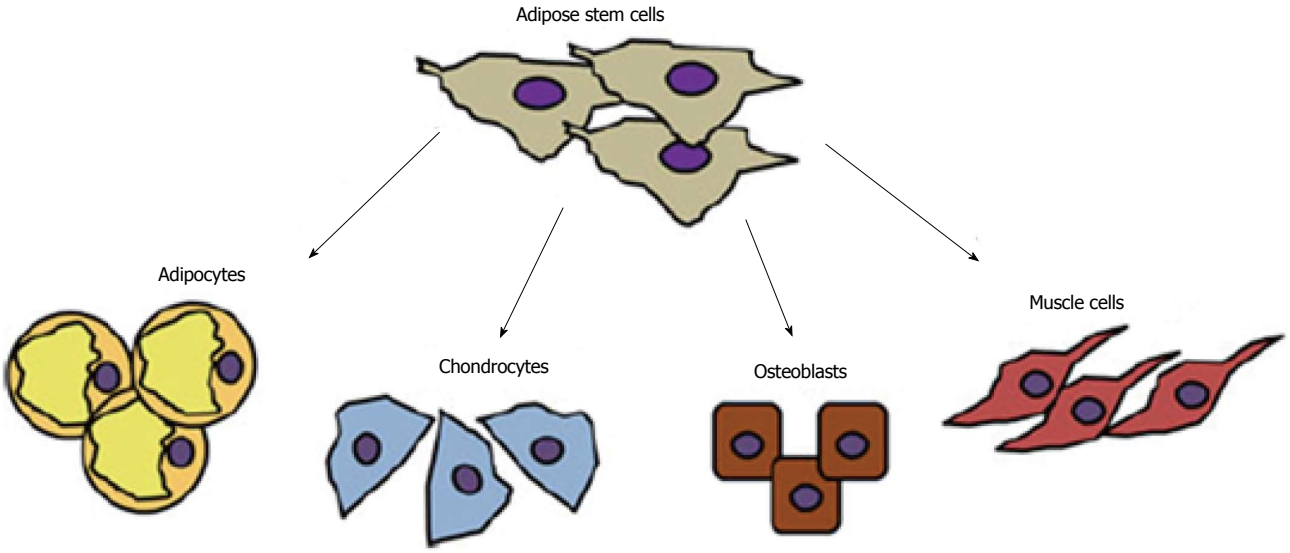

Figure 3 Differentiation of adipose derived stem cells into adipocytes, chondrocytes, osteoblasts and muscle cells adapted from Minteer *et al*[97].

method of adipocyte isolation. Following the discovery of ADSCs by Zuk's team in 2001[31], multiple techniques of ADSC isolation have been described[32]. The collagenase ADSC technique of isolation involves the gradual separation of the adipose tissue layers followed by numerous washes with PBS removing the contaminating RBCs, and incubation with type I A collagenase solution[33]. The infranatant is removed, followed by centrifugation to form the stromal vascular fraction (SVF) pellet. The SVF is acquired which contains the ADSCs which will be used for cell culturing and experimentation. The final processing of the SVF before culture is the repeated washing with PBS to remove the final adherent RBCs and adipocytes. Alternative, more economical methods of ADSC isolation including the use of trypsin and red blood cell lysis buffer solution have been described as an alternative to type I collagenase usage. Despite the relative expense and large number of steps required of the collagenase isolation method, it has remained the most popular method of ADSC isolation and results in up to 5 × more ADSCs available for experimental use[19,31-33].

The vast majority of studies in the literature for ADSC culture, involve the use of fetal bovine serum (FBS) as a standard ADSC culture condition[34]. FBS has multiple functions in tissue culture including a source of: growth factors, protein transport, fatty acid lipids as well as protease inhibitors[35]. Due to the approximately 1000 different chemicals within FBS, a serum free alternative has been difficult to manufacture with similar cellular efficacy to FBS. Serum free alternatives to FBS are limited in their availability and usefulness in the culturing of these and other types of MSCs[35]. Xenogenic and serum free growth factor media better comply with the good manufacturing practice (GMP) which is essential for any cell therapies which are to be considered in trials of animals or humans. Xenogenic and serum free alternatives have shown much promise thus far and the treated mesenchymal stem cells (MSCs) retain similar morphology, genetic expression, differentiation and proliferative capacities as those treated with xenogenic serum culture media[34,36]. Xenogenic serum free alternatives include human serum in the form of allogenic and autologous serum, allogeneic umbilical cord blood serum as well as human platelet derivatives[35].

Neurological differentiation of the ADSC

Early studies by Woodbury and colleagues provided evidence for the ability of MSCs to transdifferentiate into a neural phenotype[28]. They used human and rat BMMSCs under the stimulation of 1 mmol/L β-mercaptoethanol (BME). Following stimulation, these cells expressed neuron specific enolase (NSE); they were morphologically transformed so that they developed contracted cell bodies and terminal bulbs appearing similar to mature neurons.

Table 2 Table of various neuron markers their distributions and function adapted from[99]

Marker	Distribution	Protein function
GFAP	Mature cells of glial origin	Specifically found in astroglial cells
MAP 2	Mature marker of neurones	Thought to be involved in microtubule assembly during neurogenesis
Nestin	Developing nervous system, myogenic, endothelial and hepatic cells	Intermediate filament protein expressed in multiple tissues
NF-70	Most PNS neurones	Structural protein supporting large axons and synapses
NSE	Early neuronal marker	Three variants: α, β, γ with only the γ isoenzyme expressed in neuronal tissue
S100	Cytoplasm of SCs, astrocytes and astrogliosis	Non-specific cell marker involved in multiple cell processes including cell cycle progression and differentiation
Synaptophisin	Brain synaptic vesicles	Directs vesicle associated membrane protein-2 to intracellular compartments
TAU	Axons	βNeuronal microtubule associated protein stabilising microtubules and link to signalling pathways to cytoskeleton
βⅢ-tubulin	Central and peripheral nervous system axons	β tubulins heterodimerize to form microtubules involved in axon guidance

GFAP: Glial fibrillary acid protein; MAP2: Microtubule associated protein-2; NSE: Neuron specific enolase; PNS: Peripheral nervous system; SCs: Schwann cells.

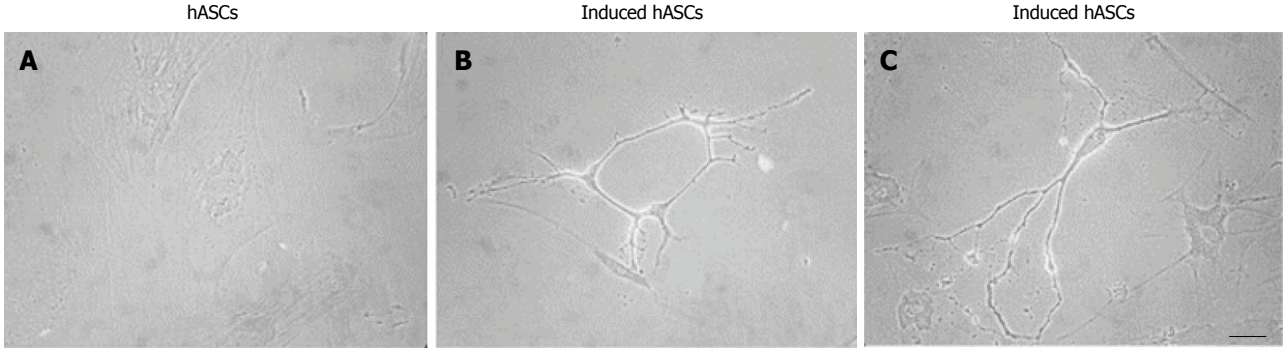

Figure 4 Morphological differences in stimulated *vs* unstimulated adipose derived stem cell. A: Unstimulated adipose derived stem cells (ADSCs) grown under normal conditions with fibroblast like morphology; B and C: Induced ADSC with a neuronal like phenotype reproduced from Cardozo *et al*[40] 2012. hASCs: Human adipose-derived stem cells.

Since ADSC share many characteristics with BMMSCs and have easier harvesting, many groups pursued ADSCs for the same reason[37,38]. Other groups followed on from this early work using a similar neurogenesis protocol to Woodbury, whilst Ashijan *et al* substituted BME for insulin, indomethacin and isobutylmethylxanthine (IBMX)[37,38]. Despite the modifications of previous work, their differentiated cells lacked the mature markers of neural development and were not tested for their ability to respond following electrophysiological stimulation[26]. Further concern was raised by other early reviewers Safford' s team addressed both of these inherent problems in their modified protocols of Zuk and Woodbury[39]. They used the combinations of butylated hydroxyanisole/KCL/valproic acid/forskolin/hydrocortisone/insulin. They also successfully demonstrated the expression of mature SC markers such as: glial fibrillary acid protein (GFAP), microtubule associated protein-2 (MAP2), β-Ⅲ tubulin; the presence of voltage-gated calcium channels and the ability to upregulate the glutamate receptor. A summary of neural markers and tissue they originate from is shown in Table 2.

Following the varying successes of early models to induce MSCs to a neural lineage some authors believe both genetic and immunocytochemical staining are necessary to confirm this transdifferentiation[26]. Cardozo *et al*[40] recently showed the cell morphological, genetic and immunocytochemical changes associated with the previous methods of chemical differentiation of ADSCs. A comparison between differentiated and undifferentiated ADSCs over a 14 d period was performed by real time polymerase chain reaction (RTPCR) showed a reduction in the levels of GFAP and nestin in the differentiated cells whilst there was an increase in β-Ⅲ-tubulin, NSE, TAU, MAP2 synaptophisin[40]. Figure 4 shows the classic morphological changes seen in ADSCs undergoing chemical differentiation under the action of BME.

Alternative methods of inducing chemical differentiation to a neural lineage involves the addition of growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) along with BME to ADSCs which again results in the changes in cellular morphology and expression of SC markers such as S100, GFAP, p75 and β-Ⅲ tubulin[41,42]. Using these protocols the differentiated ADSCs promoted significantly greater neurite outgrowth from the NG108-15 motor neurones than uncultured ADSCs suggesting a local stimulatory effect between the stem cell and the neuron. Further modifications of techniques have shown the potential of IBMX alone to differentiate ADSC into cells expressing ectodermal markers such as neurofilament 70 and cyclic nucleotide phosphodiesterase

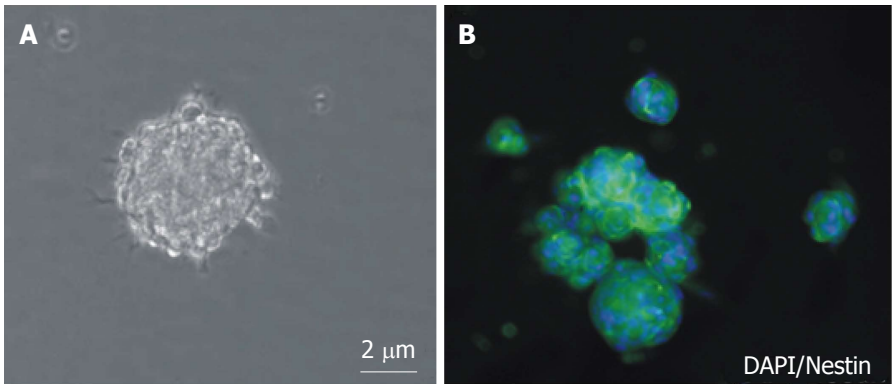

Figure 5 Appearance of neurospheres (A) Phase contrast image of free floating neurospheres; B: Fluorescence staining of nestin (Green) and nuclear stain (Blue) of neurosphere adapted from Radtke *et al*[43], 2009.

Table 3 Comparision the different methods of chemical differentiation of adipose derived stem cells

Nerve differentiation chemicals	Histochemical markers positive	Ref.
B27/bFGF/EGF	MAP2, β-Ⅲ tubulin, S-100, GFAP, Nestin,	[19,21,42-45,47]
IBMX/dexamethasone/insulin/indomethacin	S100, GFAP	[94]
β-mercaptoethanol/butylated hydroxanisole/retinoic acid/EGF/bFGF	GFAP, Nestin, β-Ⅲ tubulin, NSE, TAU, MAP2 and Synaptophisin	[40]
β-mercaptoethanol/retinoic acid/PDGF/bFGF/forskolin/GGF-2	GFAP, S100	[41,58,59,95]

GFAP: Glial fibrillary acid protein; MAP2: Microtubule associated protein-2; bFGF: Basic fibroblast growth factor; NSE: Neuron specific enolase; EGF: Epidermal growth factor; IBMX: Isobutylmethylxanthine; PDGF: Platelet derived growth factor; GGF-2 glial growth factor-2.

(CNPase); which are associated with oligodendrocytes from the CNS as well as SCs.

Alternatively, similar immunocytochemical markers are seen when ADSCs are cultured exclusively with bFGF and epidermal growth factor (EGF) to form neurospheres. Figure 5 demonstrates the morphological appearances of neurospheres staining positive for nestin as well as the non-specific nuclear stains. Neurospheres were originally found to develop from neural stem cells. However, these confluent groups of differentiated cells can be obtained from ADSCs and express the SC markers such as S100, P75 and GFAP[43]. A recent study compared the differentiation protocols of BME *vs* bFGF and EGF together[28,44,45]. The neurospheres produced from the latter protocol had higher cell viability and improved expression of neural markers when tested at weeks 1 and 3[45]. The higher cell viability of ADSCs in the absence of BME suggests an increased toxicity in the cells exposed to BME. This observation has been suggested in the literature previously using BMMSCs[27] and similar findings are reported by authors using dimethyl sulfoxide (DMSO) as the principle differentiation chemical[46]. Razavi *et al*[47] 2013 demonstrated greater neuron generation relative to glial cells when ADSCs were co-cultured with neurotrophic factor secreting ADSCs compared to control neurogenic and conditioned media. They showed the expression of MAP2 to be two times greater than GFAP in the control group. Neurotrophic ADSC containing media contained much higher levels of both brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF)[48].

Neuronal markers of differentiation

As shown in Table 3, there are a variety of markers which are expressed during the various neurological differentiation protocols of ADSCs within the literature. The molecular code model of ADSC differentiation has been mentioned elsewhere in the literature thus far[40]. In this model each marker is representative of neural tissues at different stages of neural differentiation and maturity. Each different neurological differentiation protocol results in the expression of a range of different cell surface proteins or markers. The markers expressed are dependent on various factors including cell passage, culturing media, as well as the differing types of induction media[49]. When the process of neurogenesis is compared *in vitro* ADSC differentiation model, the gradual changes in cell surface markers have been documented previous studies depending on the maturity of the tissue and are found to be time dependent[40,50].

THERAPEUTIC STRATEGIES AND EXPERIMENTAL MODELS FOR PNI REPAIR USING NERVE CONDUITS AND ADSCS

As discussed earlier, multiple *in vitro* studies have shown the transformation in the morphology of ADSCs under a range of stimuli to form tissues similar to neural tissue. Multiple animal studies have been described using ADSC to treat a range of canine and murine specific neurological conditions[51,52]. To bridge the gap in the damaged peripheral nerves some authors used conduits combined with ADSCs whilst others used ADSC therapy with nerve allografts. Since axons only grow a short distance beyond their own reparative matrix and an intact endoneurium is associated with better outcomes, there has been a strong research focus on bridging the gap *via* conduits as well as

reconstruction of the extracellular matrix[1,53].

Nerve conduits

Nerve conduits are used in peripheral nerve gaps of up to 3 cm in length[54]. Natural conduits include the autogenous vein nerve conduits (AVNCs), allografts and autogenous nerve harvesting[14]. A variety of synthetic conduits are also available for nerve repair, including Polyglycolic Acid (PGA), Poly-carprolactone (PCL), Collagen, Polyvinyl alcohol which have passed FDA testing and thus permitted for use in humans[55].

The rat sciatic nerve model remains a classic clinical model for investigating the effects of cells on peripheral nerve regeneration. PCL conduits have been tested over a 6mm gap model to compare the nerve regenerative effects of autografts as well as PCL conduits containing ADSCs with and without nerve guides and a negative control of an unrepaired nerve[56]. At 3 wk the cells containing the guides and ADSCs were associated with a significant improvement in the sciatic nerve index (SNI) compared to other groups (P = 0.010). However, this effect only lasted for 5-6 wk before the sciatic nerve function became similar to the other groups. The re-harvested sciatic nerves which were observed after cellular implantation of ADSCs were also thicker than those with the conduit alone. This suggests a possible interaction between the implanted ADSC and the conduit material, thus aiding the regeneration of the peripheral nerve. Similar results were found by other authors who used sciatic nerve defects of 13 mm[57]. In their study there were several experimental groups, which included rats receiving allografts from similar breeds as a positive control. The other experimental groups included rats receiving silastic conduits containing either differentiated or undifferentiated ADSCs; these both of these groups also either had or didn't have exchange of ADSC media on days 14 and 28. The groups of rats, which received allografts as well as those receiving the silastic conduit containing differentiated ADSCs and media exchange, had the highest Sciatic Functional Index (SFI) when measured at 3 and 4 mo. In this paper the authors also reviewed another motor function and found that the extensor postural thrust was highest in the allograft group as well as the differentiation and undifferentiated ADSCs within silastic conduits with media exchange. The findings from this study suggest a role for ADSC in peripheral nerve recovery and also stress the importance of maintaining the stem cell *via* supplementation with fresh nutrients. Despite the multifaceted improvements in motor function, the presence of ADSCs leads to a slower improvement than compared to sensory function. Similar to a previously mentioned[56] study, the ADSCs did not differentiate into SCs within the injury model.

When fibrin conduits have been used for 10 mm rat sciatic nerve models; it was associated with decreased growth associated protein (GAP-43) expression in the dorsal root ganglion relative to the spinal cord and could account for the difference in sensory and motor nerve regeneration seen in previous studies[58]. These findings may suggest ADSCs are better at regenerating motor neurones specifically[56]. Fibrin conduits have also been used in rat 10 mm sciatic nerve model and compared to SCs alone, ADSCS and BMMSCs[42] (Figure 6). SCs were able to regenerate over significantly greater distances than ADSCs and BMMSCs. However, there was no statistical significant difference between the ADSC and BMMSC. Poly-3-hydroxybutyrate conduits have also been used within a fibrin matrix conjugated with PDGF, bFGF and glial growth factor-2 for 10 mm sciatic nerve gap models[59]. The variables in this study included ADSCs with and without growth factors. Axonal regeneration was significantly improved in all of the models which contained ADSCS with P values < 0.05. The addition of these growth factors didn't result in a significant improvement in nerve regeneration. In this study ADSCs survived for a short period, which may be explained by immune consumption or an inability to form a niche in the requisite environment[18].

Alternatively, microporous conduits over a 15 mm nerve gap have been used to compare the effects of ADSCs, ADSCs co-cultured with SCs, SCs alone or dental pulp stem cell co-cultured with SCs[60]. ADSC and SC co-culture was associated with better motor recovery and nerve conduction velocity. ADSC and SC co-culture resulted in 50% increase of NGF release which is thought to be an important factor in SC development[61]. Table 4 provides a summary of the animal studies using ADSCs in peripheral nerve recovery.

Cell based therapy and xenogeneic acellular nerve matrices

Adipose cell transplantation without the use of a conduit is an option for reconstructing peripheral nerves. The cells may be administered systemically *via* intravenous, subcuticular or intramuscular routes as used for traditional drug therapy. When ADSCs have been introduced intravenously, they spread throughout the body and locate to damage tissue. In the context of traumatic brain injury, these cells spread *via* the reticuloendothelial system directly into the diseased brain tissue[62]. In this study, the observed benefits of human ADSC injection were largely dependent on the recipient rat's age. In the older rats, fewer cells transited though the spleen. This subsequently led to differences in cell distribution within injured parts of the rat's brains. In other studies when ADSCs were administered *via* the intravenous route they were also shown to improve neuropathic pain in rats which had the ill effects of chronic pain simulated[63].

In other studies when ADSCs were administered *via* the subcutaneous route, the cells were found to stay within the area of infiltration and did not translocate to distal sites such as the spleen or brain[64]. For up to 1 year the transplanted cells were found to slowly migrate from the upper to lower subcutaneous adipose layers. Many of the cells were found to differentiate into adipocytes following injection. Therefore, this route of administration is unlikely to be the adequate to allow a sufficient yield of stem cells to engraft within the

Table 4 Animal studies modelling adipose derived stem cells and conduits for peripheral regeneration

Animal models	Details	Measured outcomes	Ref.
Rat	Polycaprolactone conduit for 6 mm nerve gap	SNI, nerve thickness	[56]
Rat	Fibrin conduit 10 mm nerve gap	Axon regeneration	[42]
Rat	Polyhydroxybutyrate conduit 10 mm nerve gap	Axon regeneration	[59]
Mouse	Nerve crush model and local administration ADSC ± matrigel	Axon regeneration	[48]
Rat	Decellularised vascular conduit v autogenous nerve and vascular conduits containing ADSC	Axon regeneration, nerve conduction velocity	[90]
Rat	Silastic conduit for 13 mm nerve gap	SNI, EPT, sensory nerve function	[57]
Rat	Silicon conduit ± collagen or ADSCs	Axon regeneration, nerve conduction velocity	[100]
Rat	Nerve crush model with systemic injection of ADSC	Axon regeneration, motor recovery	[68]
Rat	Genipin-Gelatin-Tricalcium phosphate conduit 10 mm nerve gap	SNI	[92]
Rat	ADSC injected into repaired chronic denervated nerve	Axon regeneration, PFI	[89]
Rat	Microporous conduit for 15 mm nerve gap	Motor recovery	[60]
Rat	Fibrin conduit for 10 mm nerve gap	Motor recovery, GAP-43 expression in dorsal root ganglion	[58]
Dog	Polytetrafluoroethylene tube and alginate hydrogel l 7 mm nerve gap	Axon regeneration	[51]

ADSC: Adipose derived stem cell; SNI: Sciatic nerve index; GAP-43: Growth associated protein.

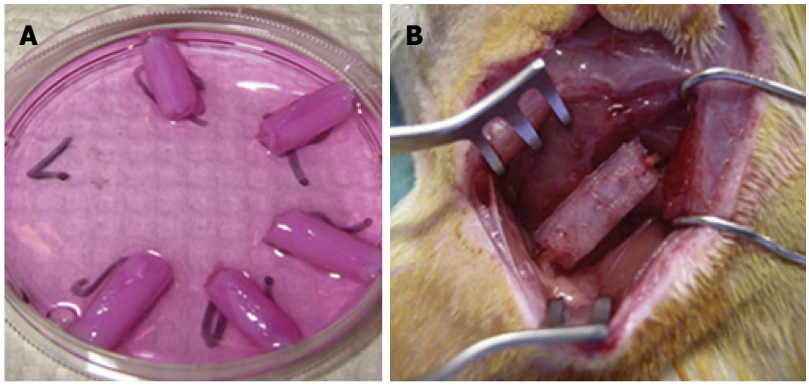

Figure 6 Fibrin nerve conduits. A: Fibrin conduits placed in DMEM prior to seeding with ADSCs; B: Fibrin conduit being inserted surgically to bridge a rat sciatic nerve injury[42]. ADSCs: Adipose derived stem cells; DMEM: Dulbecco's modified eagle medium.

injured neural tissue.

Conduits are currently not used in humans for nerve gaps greater than 3 cm nor are they used on large nerves[65]. Combinations of bridging acellular nerve matrices and rate ADSCs have been used in 15 mm sciatic nerve gaps in rats[66]. Similar to conduits studies motor response and nerve conduction velocities are regularly measured. The implanted ADSCs and nerve graft were associated with improved walking track analysis, nerve conduction and increased number of myelinated fibres within the grafted tissue. When human ADSCs were injected into an acellular nerve graft as therapeutic cells, similar improvements in rat motor peripheral nerve function were obtained[67].

In a sciatic crush nerve model, infiltration of ADSCs was associated with significant improvements in SFI score and axonal regeneration which was marked by increased GAP-43 expression in the recovering nerves[68]. The allogenic ADSCs also reduced the immune response to the nerve injury and were able to migrate through the reticuloendothelial system to home to the zone of injury. Using ADSC therapy the authors were able to maintain the levels of glial cell line derived neurotrophic factor (GDNF) which has been proven to be important in increasing the number of myelinated axons in previous sciatic nerve injury models[69]. The effects of transforming growth factor beta-1 (TGFβ-1) on the survival of ADSCs injected into xenogeneic acellular nerve matrices (XANM) has been reviewed in multiple animals[52]. In a 50 mm nerve defect, the combination of XANM-ADSC-TGFβ-1 led to significantly greater areas of axonal regeneration compared to XANM-ADSC or XANM- TGFβ-1; but the level was similar to that seen when an autograft was implanted. Significantly greater proportions of cells from the XANM-ADSC-TGFβ-1 compared to the XANM-ADSC survived over a 28 d time period (0.46 ± 0.16) *vs* (0.2 ± 0.13). TGFβ-1 lead to increased expression of genes for collagen Ⅰ, collagen Ⅲ and Laminin which are key structures in developing neurones[2,70]. TGFβ-1 also led to a 7x increase in the release of vascular endothelial growth factor (VEGF) which was thought to be neuroprotective. When the ranges of growth factors secreted by ADSCs was tested, IGF-1 was found to be the most important in the prevention of caspase 3 and akt mediated apoptosis in rat cerebellar granule neurone[71]. The theoretical interactions between ADSCs and the regenerating nerves are shown in Figure 7.

Electrical stimulation techniques

Electrical stimulation has been shown experimentally to improve neurite outgrowth particularly towards the negatively charged cathode electrode and axonal

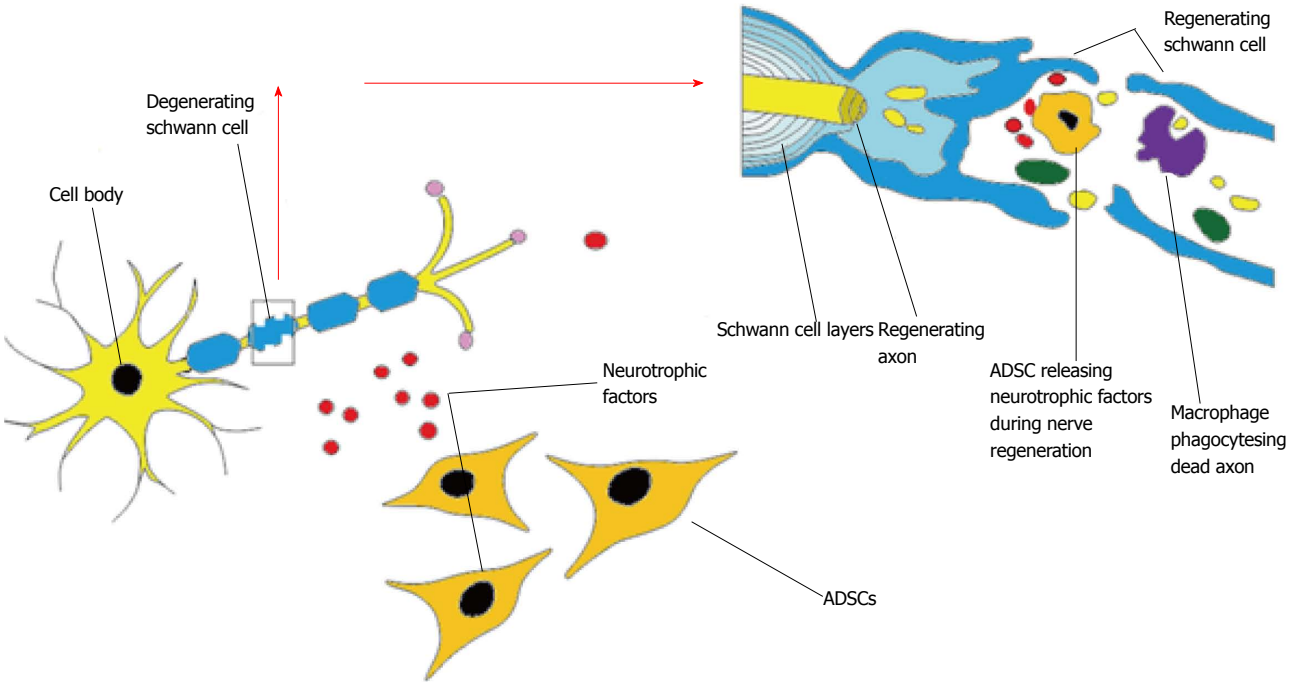

Figure 7 Potential interaction of adipose derived stem cells and the effects on peripheral nerve regeneration. ADSCs: Adipose derived stem cells.

regeneration[72,73]. Electrical stimulation of ADSCs has been used in a number of studies for various potential clinical applications[74-78]. BMMSC have been shown to respond in different ways to various form of electrical stimulation including capacitive coupling (CC), pulsed electromagnetic field, degenerate wave (DW) as well as the direct current (DC)[79]. When these transformed cells were tested in assays those which had CC and DW stimulation showed greater invasive propensity to invade a collagen type I matrix *vs* DC treated cells which showed the greatest galvanotaxic effects. These differences in the behaviour of these cells could be used in an engineered approach for regenerating nerves. Adipose stem cells have also been shown to have improved galvanotaxis following DC stimulation were they preferentially migrate to the cathode electrode; assemble perpendicular to the applied electric field, disassemble gap junctions and modify the expression of multiple genes[74,75]. The ability of adipose stem cells to respond to electrical stimulus is explained by findings of multiple ion channels found within this heterogenous group of cells[80]. The galvanotaxis effect has been found to be dose dependent and involves a number of cellular pathways including is mediated through MAPK, PI3K and its downstream target Akt as well as ROCK pathway and transient increases of intracellular calcium[74]. More recently electrical stimulation of mesenchymal stem cells (MSC) has been combined with surfaces which have high conductivity to improve the cues for necessary to obtain neural differentiation[81]. A range of different conductivities of Polyaniline (PANI) was combined with standardised electrical stimulation of MSCs. The higher conducting polymers of PANI were associated with greater expression of neural phenotypes Nestin and β-Ⅲ-tubulin confirmed by RTPCR and immunocytochemistry. These cells also underwent morphological changes to appear as neuron like cells although their functionality was not confirmed. On the surface of highest conductivity the cellular processes extended further than those surfaces which were less conductive.

DISCUSSION

ADSC based therapy remains an area of strong scientific research; they have been shown to improve the neurological recovery in murine nervous system ischaemic models[71,82]. ADSCs can differentiate into a range of supporting structures such as Schwann like cells which carry many of the markers of mature PNS cells using chemical or neurosphere differentiation[37,38,83]. These cells also have a morphological phenotype similar to mature SCs. Scepticism exists within the literature as to the functionality and safety of these differentiated cells with many believing the *in vitro* morphological and cytological changes to be secondary to the toxicity of the chemical differentiating protocols[27]. The regeneration of diseased tissue occurs through the 3 processes of cell engraftment and differentiation, release of neurotrophic factors and immunosuppression[84]. The cell engraftment is necessary for the ADSCs to form a cell niche, whilst differentiation is required for these cells to transdifferentiate from mesodermal to ectodermal lineages. Cell engraftment occurs following administration of these cells into a host environment. The ADSCs release a range of neurotrophic factors such as EGF, TGFβ-1, VEGF, bFGF, HGF, IGF-I, and BDNF secreted at different phases of tissue regeneration which is also thought to aid the healing of the damaged peripheral nerve[48,84-86]. In particular TGFβ-1/3

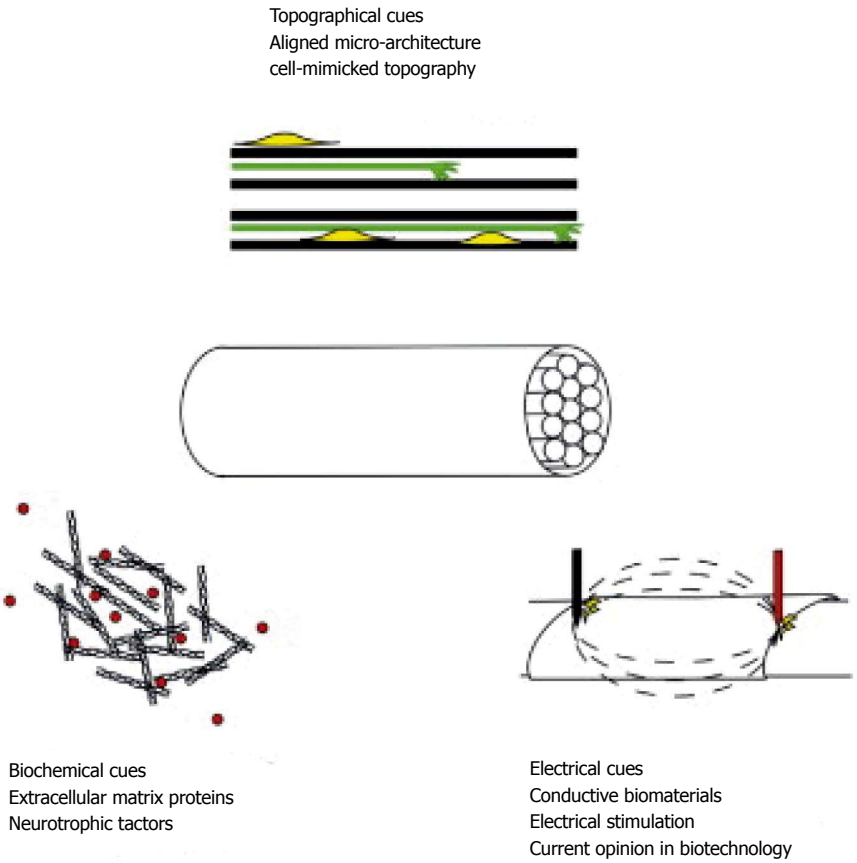

Figure 8 Interactions of topography, biochemical and electrical cues in engineering nerve tissues taken from[72].

are found in high concentrations amongst undifferentiated ADSC may have future immunomodulatory roles which are greater than that of BMMSC[87,88]. The combination of greater immunomodulatory effects and easier harvesting further justifies the greater emphasis of ADSC as opposed to BMMSCs. The question of whether ADSC can regenerate PNS neurons remains contentious. In smaller murine sciatic nerve models, ADSC neurotrophism has been shown to benefit both motor and sensory functions[57,60]. The sensory recovery appears to be faster than that of motor recovery[57]. The reason for this is currently unknown. Despite the numerous improvements in motor and sensory indices discussed in multiple studies following ADSC placement; there is yet to be a unified opinion regarding the precise mechanism of ADSC engraftment and differentiation at the nerve injury site[56,57,89,90]. This uncertainty is confounded by a lack of convincing evidence of ADSC transformation to neural tissue at the repair site[56,57]. It could be the improvements in the neurological recovery of injured animals was secondary to ADSCs providing a surge of growth factors which stimulate the remaining SCs still active within the nerve[60]. The authors believe the improvements in the multiple facets of neuron recovery in murine models may be related to their ability to stimulate the remaining surviving SCs through angiogenesis and the release of the numerous growth factors mentioned previously[52,91]. This would therefore suggest that ADSCs were partially able to regenerate a damaged neuron by stimulating the remaining functional cells within the neuron.

The murine model for nerve defects ranging from 1-2 cm in size remains the standard model for investigating ADSCs and nerve regeneration[42,57-60,92]. Studies have since been scaled up to a canine model to observe ADSC roles in larger animals more comparable to human sized defects[51,52]. Similar results were found to murine models were the ADSCs regenerated nerves to higher functionality in terms of faster nerve conduction velocities than those without ADSC application. Future research should be translated to larger animals in the longer term since multiple murine models have been trialled with variable successes. ADSCs may be placed *in vivo* in pre-differentiated or in undifferentiated states. Studies have shown the cells have neurotrophic effects in both states; however differentiated cells retain their regenerative capacity for longer[57]. Future challenges will involve the supplementation of ADSCs to enable them to maintain their regenerative properties for the time required *in vivo*.

Conduits remain an option for nerve reconstruction but are limited to defects less than 3 cm in size[93]. The conduits which have been used are a combination of natural and synthetic materials produced by a range of manufacturing processes. As discussed by Marquadt and Sakiyama Elber 2013 the ideal conduit would contain a combination of biochemical, topographical and electric cues to help determine the fate of the regenerative cells within the construct[72] (Figure 8). The combination of all 3 of these factors would possibly yield an improvement on the results seen with the current conduit engineering techniques. Concern exists with the stability of cells

treated with high concentrations of glial growth factors. There are many studies in the literature where ADSCs are given large doses of glial growth factors and are forced to become either neurone or schwann like cells[40-42,58,59,94,95]. The long term stability of these cells is largely unknown which may limit their clinical translatability into a peripheral nerve lesion human model. Alternative and potentially safer techniques using combinations of electrical stimulation and highly conductive topographical materials with MSCs have been used to obtain neural lineage absence of augmenting glial growth factors[81]. This growth factor negative technique of producing neural like cells may be useful and a safer option whilst becoming a platform regenerative medical technology using ADSC for peripheral nerve regeneration. These transformed cells could be co-cultured with SCs taken at the time of surgery and these schwann cells used to maintain the neural phenotype of these cells over a long time period.

Cell therapy with Xenogenic Acellular Nerve Matrices (XANMs) is a viable option where the natural extracellular matrix of the PNS is replaced. When injected systemically ADSCs had a homing response to the injured neurological tissues and passed through multiple cell systems to get to their target organs[68]. Porcine ANM have been used in rat models and were found to be well tolerated by the rat immune system despite the infiltration and interactions with host cell fibroblasts[70]. Therefore, it appears that if ANM are sufficiently decellularised and/or immunosuppressant therapy is used ADSC and XANM may be a therapeutic alternative to autografts alone. ANM may be suitable for long nerve gaps where a like for like tissue is required. This could potentially be supplemented with ADSC which could help regenerate the nerve as well as provide immunosuppressive cues to limit the amount of immunosuppressant required to prevent rejection of the xenogenic material.

CONCLUSION

Peripheral nerve injury recovery remains a problem despite advances in the surgical and medical management of this debilitating illness. There are multiple studies using ADSCs in a variety of animal models to aid the recovery. At present the role of the ADSC in PN injury is still being investigated and the optimum timings and methods of insertion of ADSC has yet to be determined. For translation into humans; ADSC therapy will need to build upon the knowledge already acquired from *in vitro* and *in vivo* small animal studies. The preliminary results so far suggest a future role in peripheral nerve regeneration, but future research is need towards further optimisation and safety. The literature is saturated with the use of growth factors and other inducing chemicals such as BME and future studies should use less toxic methods of differentiating the stem cells. Electrical stimulus could provide a useful alternative method of reducing the number and concentrations of growth factors required to obtaining a neural lineage from the ADSCs. The authors also feel greater focus should be given to surface modifications of nanomaterials to aid stem cell differentiation. Modifications of surface chemistry and nanotopography can be further adapted to support the expression of neural proteins within ADSCs. Material surface modifications along with electrical stimulation and essential glial growth factors could provide the stimulus required to obtain the neural lineages shown in previous studies, whilst reducing the risks of long term malignant transformation. The transplanted cells also need to be observed into larger animals than murine, as well as monitored for longer periods of time. This long term active surveillance would help to determine the malignant transformation potential of the ADSCs exposed to supraphysiologic levels of growth factors.

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