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New insights into peripheral nerve regeneration: The role of secretomes

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ABSTRACT

Neurons of the peripheral nervous system retain the intrinsic capability of regenerate their axons after injury, by triggering a complex activation response. This genetic switch is dependent of signals from the injured axon. Schwann cells (SCs) in the distal stump of an injured nerve also play an active role in the local regulation of axonal programs, by using cell-to-cell contacts but also secreted signals, the so-called secretome. Secretome contains all the proteins (cytokines, growth factors and others) secreted by the cell and includes extracellular vesicles. The released vesicles can transport signaling proteins and both coding and regulatory RNAs, thus facilitating multilevel communication. It is nowadays clear that secretome of SCs is fundamental to both orchestrate Wallerian degeneration and to sustain axonal regeneration.

Therefore, the use of secretome has emerged as an alternative to cell therapy in the field of tissue regeneration. In fact, separate components of SC secretome have been extensively used in experimental models to enhance peripheral nerve regeneration after injury. However, the most used secretome in neural therapies has been the one derived from mesenchymal (MSC) or other derived stem cells. In fact, the effects of cell therapy with MSCs have been mainly associated with the secretion of bioactive molecules and extracellular vesicles, which constitute their secretome.

In this review, we first describe the role of SC and macrophage secretomes on Wallerian degeneration and axonal regeneration after peripheral nerve injury. Then, we review the different works reported in the literature that have used secretomes of SCs or MSCs in the treatment of peripheral nerve injuries in experimental models, to highlight the use of secretomes as a promising cell-free therapeutic approach, that reduces some of the risks associated with the use of cells, such as tumor formation or rejection.

In contrast to most neurons in the central nervous system, mature neurons of the peripheral nervous system are able to regenerate after axotomy. Peripheral neurons retain intrinsic growth capability after injury, triggering a complex activation response in both the soma and the axon of the injured neuron. At the neuronal body, injury regulates a gene expression program, that mainly consist in downregulating genes related with neural activity and neurotransmission, whereas up-regulating some transcriptional factors related with growth and cyto-skeleton elements (He and Jin, 2016). Therefore, the neuron switches from a transmission state to a pro-regenerative state. Different transcription factors have been implicated in this switch, like c-Jun, Jun D, ATF3, sox11 and STAT 3 (Allodi et al., 2012; Raivich and Makwana,

Abbreviations: ATF3, Activating Transcription Factor 3; ASC, Adipose-derived MSCs; bFGF, Basic fibroblast growth factor; BMSC, Bone marrow derived MSC; BDNF, Brain derived neurotrophic factor; CNTF, Ciliary neurotrophic factor; CM, Conditioned media; DPSC, Dental pulp stem cells; DNA, Deoxyribonucleic acid; DRG, Dorsal root ganglia; EGF, Epidermal growth factor; ECM, Extracellular matrix; EV, Extracellular vesicles; GDNF, Glia derived neurotrophic factor; GAP43,, Growth associated protein 43; HGF, Hepatocite growht factor; SHED, Human exfoliated deciduous teeth; IGF-1, Insulin growth factor 1; IL-10, Interleukin-10; IL-1α, Interleukin-1α; IL-1β, Interleukin-1β; IL-6, Interleukin-6; JAKs, Janus kinases; LIF, Leukemia inhibitory factor; mTOR, Mammalian target of Rapamycin; MSCs, Mesenchymal stromal/stem cells; miRNAs, MicroRNAs; EV, Microvesicles; MCP-1, or chemokine C—C motif ligand 2, CCL2, Macrophage inflammatory protein 1αMIP-1α, Monocyte chemoattractant protein-1; NGF, Nerve growth factor; GTPase, Nucleotide guanosine triphosphate hydrolase; OECs, Olfactory ensheathing cells; PAP-III, Pancreatitis-associated protein III; PTEN, Phosphatase and tensin homolog; PLA2, Phospholipase 2; PDGF, Platelet-derived growth factor; PDGF**α**, Platelet-derived growth factor alpha; PTN, Pleiotrophin; qRT-PCR, Real-time quantitative reverse transcription- Polymerase chain reaction; RNAs, Ribonucleic acids; SCs, Schwann cells; sSiglec-9, Secreted ectodomain of sialic acid-binding Ig-like lectin-9; STATs, Signal transducer and activator of transcription proteins; SLN, Superior laryngeal nerve; TNF-α, Tumor necrosis factor α; TNFβ, Tumor necrosis factor β; VEGF, Vascular endothelial growth factor; VEGFα, VEGF alpha.

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2007). Activation of the JACK/STAT pathway through mammalian target of Rapamycin (mTOR) is also a key element to promote the intrinsic growth ability of neurons. In fact, central neurons do not activate mTOR after injury, leading to failure of regeneration, whereas forced activation of mTOR (Liu et al., 2010; Park et al., 2008) promotes growth of corticospinal and retinal ganglion axons.

The genetic switch at the neuronal body is dependent of signals from the injured axon. At the lesion site, plasma membrane of the affected axons is disrupted, leading to a massive calcium entrance and disorganization of the cytoskeleton. This calcium input triggers signaling cascades that spread to the cell body. If the injured neuron has the intrinsic ability to regenerate, the tip of the injured axon will be rearranged to become a growth cone (He and Jin, 2016). Moreover, part of the intrinsic ability of neurons to grow is localized at the same axon, since local mRNA translation and protein synthesis at the axonal level is fundamental for a successful regeneration (Gumy et al., 2010; Terenzio et al., 2018; Verma, 2005). In fact, mTOR, a key protein to enhance mRNA translation and protein synthesis (Saxton and Sabatini, 2017), is activated in injured axons. Activated mTOR would regulate both its own translation as well as local translation of other factors, such as importin β1 and STAT3 (Terenzio et al., 2018). These molecules retrogradely signal injury and thus, contribute to the activation of the intrinsic growth program of neurons (Abe and Cavalli, 2008).

However, regeneration is not just a neuron-key event. At the distal level, a permissive milieu for axonal regeneration has to be created. Therefore, the distal nerve stump undergoes Wallerian degeneration, a process that leads to the lysis and elimination of axon and myelin debris. Schwann cells (SCs) are the glia of peripheral nerves and in the mature state can be myelinating (the ones that ensheath and myelinate a single axon) or Remak SCs (the ones that ensheath a group of unmyelinated axons). During Wallerian degeneration, SCs switch to a repair phenotype. The activation of the transcription factor c-jun triggers this conversion from a mature to a repair state (Arthur-Farraj et al., 2012), activating an injury-specific program that partially involves non-coding ribonucleic acids (RNAs) and deoxyribonucleic acid (DNA) methylation (Arthur-Farraj et al., 2017). Activation of c-jun allows SC to become a cell specialized to support regeneration by increasing their proliferative capability and the expression of neurotrophic and chemotactic factors (Arthur-Farraj et al., 2012). These factors recruit hematogenous macrophages that together with SCs (Catenaccio et al., 2017), contribute to the phagocytose of myelin and axonal debris (Brück et al., 1996). Moreover, repair SCs have the ability to elongate (Gomez-Sanchez et al., 2017) and align inside the endoneurial tubes, forming the bands of Büngner, that will create a regenerative pathway for the growing axons.

1. SC secretome

SCs in the distal stump of an injured nerve also play an active role in the local regulation of axonal programs, including axonal extension and local proteins synthesis (Court et al., 2008; Court and Álvarez, 2005). The axon-glia interaction is mediated by cell-to-cell contacts but also by secreted signals, the so called secretome. Secretome contains all the proteins (cytokines, growth factors, and other proteins) secreted by the cell and includes extracellular vesicles (EV) (Fig. 1). It is nowadays clear that the secretome of SCs is fundamental to both orchestrate Wallerian degeneration and to sustain axonal regeneration. In fact, the decreased ability of SCs to maintain the secretion of trophic factors during long periods of time can explain the failure of regeneration through chronically denervated nerves (Höke et al., 2002; Sulaiman and Gordon, 2000).

1.1. Cytokines and chemokines

Denervated SCs overexpress several trophic factors and cytokines, with a specific time course of expression for each factor (Boyd and Gordon, 2003). These secreted factors have been implicated in neuronal

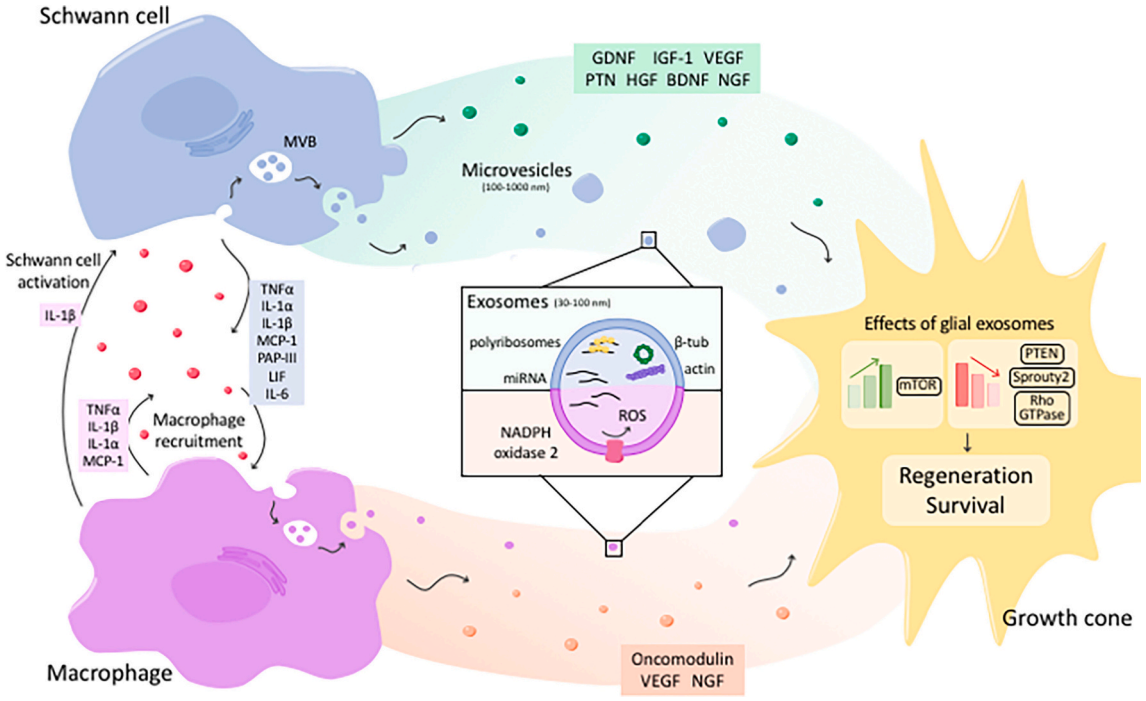

Fig. 1. Effects of glial and macrophage secretomes in nerve regeneration. Schwann cells (SCs) and macrophages secrete growth factors, cytokines and extracellular vesicles that enhance axonal regeneration either directly or indirectly. Regenerating axons receive several neurotrophic factors (such as GDNF, IGF-1, or PTN), microvesicles and EV (containing several proteins, miRNA and mRNA) from SCs and macrophages. These signals, particularly those from the EV, induce an upregulation of mTOR and a downregulation of regeneration inhibiting factors (PTEN, Strouty2, Rho GTPase), thus promoting axon regeneration and neuronal survival. Some factors from SCs and macrophages also contribute to regeneration indirectly by recruiting more hematogenous monocytes and activating SCs, which create a permissive environment to axonal regrowth.

survival, axonal growth, cell differentiation and axon remyelination (Chen et al., 2007) and also in the immune response of the distal nerve stump, contributing to recruitment of immune cells (Chen et al., 2015).

Cytokines and chemokines secreted by SCs are fundamental for macrophage chemoattraction and regulation that lead to myelin clearance in Wallerian degeneration. The most important ones are interleukin-1α (IL-1α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1, or chemokine C—C motif ligand 2, CCL2), macrophage inflammatory protein 1α (MIP-1α), pancreatitis-associated protein III (PAP-III), leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) (Perrin, 2005). The chemokines MCP-1 and MIP-1a probably contribute to higher extent to recruitment of macrophages compared to IL-1β, since blocking antibodies against the two chemokines have stronger impact on macrophage recruitment (Perrin, 2005). Denervated SCs upregulate pro-inflammatory molecules such as tumor necrosis factor α (TNF-α), Il-1α and β (Shamash et al., 2002) within hours. Besides its role in macrophage recruitment, Il-1 β contributes to SC proliferation (Conti et al., 2002; Perrin, 2005). Some of these cytokines, such as IL-1β, TNFα and MCP-1 also modulate the phagocytic ability of the macrophages in injured nerves (Perrin, 2005; Shamash et al., 2002), probably by stimulating phospholipase 2 (PLA2). Breakdown of compact myelin is an early event in the degenerating nerve, and it is mediated by early PLA2 secretion by SCs prior to the entry of hematogenous macrophages to the nerve (Martini et al., 2008).

The cytokines LIF and IL-6 are also secreted by SCs and upregulated within hours after nerve injury (Banner and Patterson, 1994; Kurek et al., 1996), suggesting that they are early factors of injury (Ito et al., 1998). LIF acts on macrophages but is also retrogradely transported by axons to promote the neuronal regenerative response (Curtis et al., 1994). In contrast, IL-6 secreted at the injury is not retrogradely transported (Kurek et al., 1996) and has a role in the local inflammatory response. Interestingly, mRNA of anti-inflammatory cytokines interleukin-10 (IL-10) and tumor necrosis factor β (TNFβ) are parallelly upregulated to those of pro-inflammatory cytokines, and probably contribute to modulation of pro-inflammatory changes in the injured nerve to guarantee a safe immune response (Perrin, 2005).

1.2. Neurotrophic factors

Besides these cytokines and chemokines, denervated SCs secrete a plethora of neurotrophic factors, that are key to promote axonal growth (Boyd and Gordon, 2003). This ability is dependent on the creation of a dynamic gradient. SCs secrete these factors while denervated, but when they regain contact with the regenerating axons, the expression of neurotrophic factors and their receptors is suppressed, and thus, trophic factor secretion is limited to SCs located distally to the regenerative front in the distal nerve. Expression of these factors follows a specific time course but for a limited period of time (Höke et al., 2002). Failure of chronically denervated SCs to maintain sufficient levels of trophic factors accounts for poor axonal regeneration from 2 months after denervation (Sulaiman and Gordon, 2000). Interestingly, chronically denervated SCs can be reactivated by administering the cytokine TGFβ (Sulaiman and Gordon, 2002), secreted by proliferating SCs and macrophages during Wallerian degeneration.

The mRNA of the neurotrophin nerve growth factor (NGF) shows a biphasic upregulation in the injured nerve, with peaks first at 6 h and later at 3 days, that last for 14 days (Heumann et al., 1987). The second peak correlates with macrophage invasion in the degenerating nerve and is dependent on IL-1β secreted by macrophages (Lindholm et al., 1987). Upregulation of mRNA of brain derived neurotrophic factor (BDNF) and glia derived neurotrophic factor (GDNF) follow a slower course. There is a continuous slow increase of BDNF mRNA starting 3 days after injury that reaches a plateau at 21–28 days (Meyer et al., 1992). GDNF mRNA also shows a slow increase that peaks at 7 days and last for at least one more week (Naveilhan et al., 1997). A distinctive profile of secreted neurotropic factors by SCs has been proposed dependent on the type of injured nerve analyzed. Thus, GDNF is markedly increased in nerve roots whereas insulin growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) expression is limited to peripheral nerves. Denervated nerves containing motor axons expressed higher levels of pleiotrophin (PTN) than the ones containing sensory axons, whereas hepatocyte growth factor (HGF), BDNF and NGF were markedly upregulated in cutaneous nerves and dorsal roots (Brushart et al., 2013; Höke et al., 2006). Although it is clear that SC derived trophic factors are key molecules to promote axonal regeneration after nerve injury (see reviews by (Allodi et al., 2012; Boyd and Gordon, 2003; Raivich and Makwana, 2007; Terenghi, 1999), there are not consistent evidences in the literature that related the different neurotrophic profiles of motor and sensory SCs with specificity of motor and sensory axon regeneration respectively (Bolívar et al., 2020). As an example, BDNF has been extensively used to promote regeneration of motor axons (Boyd and Gordon, 2001; Vögelin et al., 2006). On the other hand, although initial studies pointed that PTN could enhance motor axons regeneration (Chu et al., 2009) a recent study showed that this factor attracts sensory axons when confronted to a combination of GDNF and BDNF, a mixture that promotes motor regeneration in a Y tube repair model (Anand et al., 2017).

1.2.1. Other proteins

SCs also secrete several molecules of the extracellular matrix (ECM), such as laminin, a major component of the basal lamina and an abundant component of the ECM of peripheral nerves (Gonzalez-Perez et al., 2013). Laminin is considered one of most important ECM molecules to sustain axonal regeneration and its expression increases after nerve injury (Wallquist et al., 2002). Laminin gives the regenerative promoting capability to basal lamina scaffolds after nerve injury, and the use of anti-laminin antibodies dramatically reduces the ability of axons to grow through basal lamina scaffolds (Wang et al., 1992).

1.3. Extracellular vesicles

EV are vesicles secreted by cells that contain cytosol sealed by a lipid bilayer membrane and allow cells to exert paracrine effects by interchanging information through their selected cargos (proteins, lipids, RNAs and DNAs). Some authors divide these EV, that are quite heterogeneous, into exosomes or microvesicles (MV) (Bruno et al., 2019). However, these terms have not a deep consensus in the literature, and some authors classify exosomes and MV on the basis of differential centrifugation (EV that sediment when centrifugation at 7000–10,000 $\times g$ are exosomes, whereas when sedimenting at 10,000 g are microvesicles). Others prefer a biogenetic definition, being MV larger and the ones originate from the plasma membrane itself, whereas exosomes are smaller and originating from multi-vesicular bodies, that can fuse to lysosomes to be degraded. When fused with the plasma membrane, exosomes can be extracellularly released. Finally, some authors use MV or exosomes indistinctly, to refer to EV (Gould and Raposo, 2013). In fact, the exosome term is quite extended in the literature as a synonym of EV. Therefore, in this work, we will use the most general term EV, following the International Society for Extracellular Vesicles recomendations (Witwer and Théry, 2019), independently of the term used by the authors of the cited studies.

In fact, both MV and exosomes mediate cell-to-cell communication (Raposo and Stoorvogel, 2013; Simons and Raposo, 2009). The released vesicles can transport signaling proteins and both coding (mRNAs, that can translate proteins) and regulatory (microRNAs -miRNAs- that can suppress protein production) RNAs, thus facilitating multilevel communication (Rajendran et al., 2014) and modulating the phenotype of the target cells.

In the nerves, transfer of molecular cargos between glial cells and neurons through EV has been described (Lopez-Verrilli et al., 2013; Lopez-Verrilli and Court, 2012). In fact, some works have pointed that EV from different sources can mediate regeneration by targeting the

mTOR pathway (Tang, 2018). As pointed above, the ability of this pathway to locally enhance messenger RNA (mRNA) translation and protein synthesis is a key event for axonal regeneration (Terenzio et al., 2018). EV of SCs can be internalized by axons, and transfer polyribosomes and genetic material to the axon after axonal damage and during regeneration (Court et al., 2008). These EV can act at a local level, by enhancing neurite growth in vitro and increasing axon growth in vivo. The effects seem mediated by the ability of glial EV to suppress the inhibitory activity of Rho GTPase on the growth cone (Lopez-Leal and Court, 2016; Lopez-Verrilli and Court, 2013).

MicroRNAs are non-coding RNAs that impact on protein expression at a post-transcriptional level and can regulate about 60% of mammalian genes (Friedman et al., 2008). An important amount of these miRNAs has been detected in axons. Although miRNA can be transported from the neuronal soma (Kosik, 2006), recent findings of the ability of EV to transfer non-coding RNA to other cells (Valadi et al., 2007) support a direct role of SCs on local transfer of miRNA. In this sense, SC modify their EV cargo when switching to a pro-regenerative phenotype after nerve injury, allowing these EV to promote neurite growth. This growth ability is dependent on the increased expression of miRNA-21 (López-Leal et al., 2020). Together with miRNA-21, miRNA-222 has also been found in SC EV (Ching et al., 2018; López-Leal et al., 2020) and, by downregulating different growth inhibitors in axons, as PTEN (López-Leal et al., 2020; Zhou et al., 2012) and Sprouty2 expression (Strickland et al., 2011), both miRNAs enhance neurite growth of dorsal root ganglia (DRG) neurons (Strickland et al., 2011; Zhou et al., 2012). Therefore, the interchange of miRNA through EV at the injury site can mediate multidirectional transfer of information from the main cells implicated in the regenerative response after nerve injury.

Interestingly, miRNAs are more abundant in the axons compared to the neuronal soma (Natera-Naranjo et al., 2010). By locally regulating gene expression in the axon, miRNAs can affect axonal protein synthesis, local energy metabolism, and the modulation of axonal outgrowth and branching (Kaplan et al., 2013). These miRNAs may have a compartmentalized and differentiated action at the soma, axons and growth cones (Iyer et al., 2014). It has been reported that nerve injury induces a set of 22 miRNAs that coordinate SC differentiation and dedifferentiation through combinatorial modulation of positive and negative gene regulators in the acute phase injury (Adilakshmi et al., 2012).

EV from SCs have also mRNA of proteins related with regeneration (Ching et al., 2018), such as Growth associated protein 43 (GAP43), a neural growth-associated protein related with growth cone guidance, and Tau protein, that stabilizes microtubules. EV also contain two members of the Rho GTPase family, Rac1 and RhoA (Ching et al., 2018). Rac1 is a regulator of cytoskeletal dynamics and plays a critical role in axon growth and guidance (Hua et al., 2015), whereas RhoA is an inhibitor of regeneration, and local translation of this molecule mediates growth cone collapse (Wu et al., 2005).

2. Secretome of macrophages

During Wallerian degeneration, activation of SCs is accompanied by recruitment of immune cells into the lesion site, including neutrophils, macrophages and lymphocytes. Macrophages play a crucial role removing myelin debris and promoting SC activation, and also contribute to the creation of a permissive environment for axonal regeneration in the distal stump, by releasing different pro-regenerative factors, including cytokines and chemokines, growth factors and ECM molecules (Chen et al., 2015; Gaudet et al., 2011). Recent work also strengthens the important role of macrophage derived endosomes on Wallerian degeneration and axonal regeneration (Hervera et al., 2018).

Peripheral nerves have resident macrophages, that can induce inflammatory response in the acute phase by secreting different pro-inflammatory cytokines, like IL-13 and IL-1β (De Francesco-Lisowitz et al., 2015). Resident macrophages proliferate and contribute to phagocytosis of myelin debris already at 2 days post-injury (Mueller et al., 2001). At 4 days, an important infiltration of circulating monocytes can be observed in the injured nerve (Mueller et al., 2003). These monocytes, which will be differentiated into macrophages in the tissue, are recruited from the blood by cytokines and chemokines secreted by SCs (Perrin, 2005; Tofaris et al., 2002); see above). Some of these factors, such as CCL2, TNF-α, IL-1α and IL-1β, can also be produced by macrophages (Kiguchi et al., 2013; Shamash et al., 2002), thus reinforcing the recruitment of more monocytes. Hematogenous macrophages are the main cells contributing to remove myelin and axonal debris during Wallerian degeneration. The phagocytic ability of macrophages is regulated by MCP-1 and IL-1β; neutralization of these molecules leads to an important reduction of the number of phagocytic macrophages in the injured nerve (Perrin, 2005). Macrophages can also secrete the anti-inflammatory cytokine IL-10 during Wallerian degeneration (George et al., 2004) and thus it is proposed that they could regulate themselves to switch off their pro-inflammatory responses (Martini et al., 2008).

On the other hand, macrophages produce a wide range of factors, such as proteases and growth-promoting factors/cytokines, and stimulate ECM remodeling to promote peripheral nerve regeneration (Gaudet et al., 2011). In fact, macrophage-conditioned medium improves neurite outgrowth in vitro (Luk et al., 2003). One of the proposed candidates for the pro-regenerative effects of macrophage secretome is oncomodulin (Zigmond and Echevarria, 2019). In addition, macrophages also secrete factors that affect SCs, as for example IL-1 (Gaudet et al., 2011). The hypoxic environment induced in the bridge of a cut injured nerve attracts macrophages, that secrete VEGF-A, polarize endothelial cells and mediate the migration of SCs through the bridge that unites the two nerve stumps, facilitating axonal regeneration (Cattin et al., 2015).

Finally, macrophages can also contribute to axonal regeneration through release of EV (Qing et al., 2018). miR-223 contained in macrophage derived EVs promotes SC migration and proliferation both in vitro and in vivo, and their secretion of NGF and laminin in vitro (Zhan et al., 2015). A recent study has described that macrophage derived EV contain enzymes that produce reactive oxygen species. When endocytosed by axons, these enzymes are retrogradely transported to the soma, where they induce the inactivation of PTEN and the consequent enhancement of the intrinsic neuronal growth (Hervera et al., 2018).

3. Secretome: an emerging alternative to cell therapy to promote axonal regeneration

Cell-free treatment is an emerging alternative to cell therapy in the field of tissue regeneration, and therefore, the use of the secretome instead of the cell itself has been postulated as a promising therapeutical tool. Moreover, pre-conditioning the cells of interest can alter the composition of the secretome (Ferreira et al., 2018), thus becoming an interesting strategy to increase their pro-regenerative potential. Finally, EV can also be used as a therapeutic delivery vehicle (Ha et al., 2016), with the advantage of its lack of immunogenicity. Studies using secretomes to promote nerve regeneration are sumarized in Table 1 (in vivo) and Table 2 (in vitro).

3.1. Use of SC secretome in peripheral nerve injury

Separate components of the SC secretome have been widely used in experimental models to enhance peripheral nerve regeneration after injury. In fact, addition of trophic factors, normally secreted by repair SCs, to the injured nerve promotes neuronal growth. Pioneer work from Lindsay demonstrated that NGF and BDNF applied on DRG cultures promoted neurite growth, but were not needed for neuronal survival (Lindsay, 1988). Plenty of works have posteriorly evaluated the effects of trophic factors secreted by repair Schwann cells on neuronal growth in vitro and in vivo (see reviews by (Allodi et al., 2012; Boyd and Gordon, 2003; Raivich and Makwana, 2007; Terenghi, 1999). Initial works

Table 1
In vivo experimental studies evaluating therapeutical role of MSC and SC secretomes in nerve injury models.

Reference	Nerve model	Therapeutical approach	Outcome
Rich et al., 1989	Sciatic nerve section	Silicone chamber fiiled with NGF	Increased number of regenerating myelinated axons
Hollowell et al., 1990	Sciatic nerve transection (8 mm gap)	Silicone chamber filled with NGF	No increased number of regenerating motor and sensory axons through the chamber
Derby et al., 1993	Sciatic nerve transection (10–15 mm gap)	Silastic or semipermeable chambers filled with NGF	Enhanced presence of axons and non-neuronal cells in the chamber at early periods Increased regenerated unmyelinated and myelinated axons in the chamber at early time points
Vögelin et al. 2006	Sciatic nerve transection (20 mm gap)	Fascia tubes with capsules to continuously release BDNF	Faster growth of axons into the tube Decreased neuropathic pain
Boyd and Gordon, 2001	Sciatic nerve section and delayed repair (chronically denervated)	Continuous administration of BDNF	Increased number of motor neurons regenerating into the distal stump No effect in acute repair (fresh distal nerve)
Fine et al., 2002	Sciatic nerve transection (15 mm gap)	Artificial guide delivering GDNF	Increased myelinated axons into the conduit Increased number of motor and sensory neurons regenerating
Boyd and Gordon, 2003	Sciatic nerve section and delayed repair (chronically denervated)	Continuous administration of GDNF	Increased number of motor neurons regenerating into the distal stump
Leong et al. 1999	Section and repair of gastrocnemius nerve	Slow release of LIF	Improved muscle mass and function
Emel et al., 2011	Sciatic nerve crush injury	IGF-1 on the crush site	Improved functional recovery
Lopez-Verrilli and Court, 2013	Sciatic nerve crush in rats	Intraneural injection of SC derived MV	Increased axonal regeneration Improved functional recovery
Marconi et al. 2012	Sciatic nerve crush in mice	Intravenous MSC (ASC)	Increased axon sprouting Reduced inflammatory response in the nerve
Sun et al., 2019	Sciatic nerve transection (10 mm gap) in rat, repaired with an engineered conduit	Enclosed MSC in the conduit to favor release of their secretome	Improved functional recovery Increased myelination of regenerating axons
Zhang et al., 2020	Sciatic nerve transection (10 mm) in rat, repaired with a chitosan tube	OEC plus suspension of MV from MSC	Marginal synergistic effect, with improved motor and sensory recovery
Sugimura-Wakayama et al., 2015	Sciatic nerve transection (10 mm) in rat, repaired with a silicone tube	SHED-CM	Improved reinnervation of target organs
Kano et al., 2017	Facial nerve section repaired with a collagen graft	Graft soaked with SHED-CM	Increased number of regenerating axons Improved functional recovery
Tsuruta et al., 2018	Supra-laringeal nerve injury	Intravenous SHED-CM	Improved recovery of swallow function Increased density of regenerating axons

focused on the role of NGF on nerve regeneration. Addition of NGF in a silicone chamber used to repair a sciatic nerve section improved number of regenerating axons (Rich et al., 1989). The same approach used to repair a nerve gap was (Derby et al., 1993) or was not (Hollowell et al., 1990) effective in enhancing axon growth into the tubes. On the other hand, application of NGF after axotomy delays the onset of regeneration (Gold, 1996), probably by attenuating the response of neurons to injury (Mohiuddin et al., 1999). Local infusion of BDNF improved nerve regeneration in neural conduits (Vögelin et al., 2006), but not when applied acutely after cut and suture of the sciatic nerve (Boyd and Gordon, 2001)). In contrast, the same authors described pro-regenerative effects of BDNF when applied in a chronically denervated nerve, that usually has a poorer ability to sustain regeneration (Boyd and Gordon, 2003).

Neural guides that delivery GDFN used to repair a nerve gap enhanced regeneration (Fine et al., 2002). Similar to BDNF, GDNF applied to the proximal stump of chronically denervated nerves increased regeneration of motor neurons (Boyd and Gordon, 2003). On the other hand, application of IGF-1 into a crush site improved functional recovery (Emel et al., 2011), whereas slow release of LIF after a nerve section increased muscle mass and function (Tham et al., 1997). Trophic factors secreted by SC, either alone or combined, have also been used in experimental studies to selectively potentiate regeneration of motor and sensory axons in vitro (Allodi et al., 2011) and in vivo (Anand et al., 2017; Lotfi et al., 2011).

Besides the amount of evidence of the potential role of trophic factors to enhance axonal regeneration, it is nowadays accepted that any effective strategy to significantly improve functional recovery after nerve injury has to contemplate multiple neurotrophic and neurotropic factors acting synergistically and in a defined sequence. Moreover, the administration of these factors has to be limited in time and create a correct gradient to promote long distance regeneration. In fact, long lasting expression of GDNF by transfecting SCs with viral vectors trapped regenerating axons in the site of application in a nerve transection model (Tannemaat et al., 2008), thus limiting the extent of regeneration.

In recent years, the discovery that SCs also secrete EV has pointed to new therapies to mimic the positive effects of SCs on axonal regeneration avoiding the limitations of cell therapy. In this line, it was shown that SC EV enhanced neurite growth in vitro. The effect was specific, since fibroblast EV did not have any effect on that study (Lopez-Verrilli et al., 2013). In an in vivo model, daily injections of EV into the nerve distal to a crush injury produced two times longer regenerating axons and a positive response to the pinch test at a longer distance from the crush site, indicating that EV greatly enhance the rate of regeneration after nerve injury (Lopez-Verrilli et al., 2013). This work demonstrated the valuable cargo of SC EV for axonal regeneration and also pointed to its potential therapeutical role.

Nevertheless, cell therapy with SCs has some limitations in humans, mainly related with the sacrifice of a healthy nerve to obtain autologous cells. Therefore, use of mesenchymal stromal cells (MSCs) has been an alternative widely explored in the field to improve axonal regeneration and functional recovery (see below). Probably this is the reason that the use of MSC derived secretome has also received more attention (Fig. 2).

Table 2
In vitro studies evaluating the pro-regenerative effects of MSC and SC secretome on neurons.

References	In vitro model	Therapeutical approach	Outcome	Proposed mechanisme
Lindsay, 1988	DRG primary cultures	NGF and BDNF	Enhanced neurite growth	Factors not needed for neuronal survival
Lopez-Verrilli et al., 2013	DRG primary explants	SC MV	Enhanced neurite growth	Intact proteins on the MV surface, as well as MVcontent
Ching et al., 2018	NG108–15 neuron culture	CM or concentrated of EV from ASC differenciated towards SC-like	Enhanced neurite growth	Proregenerative mRNA and miRNA from MV
Sugimura-Wakayama et al., 2015	DRG primary cultures	SHED-CM	Neuritogenesis	NGF, BDNF, NT3, CNTF, GDNF, VEGF, HGF contained in the CM

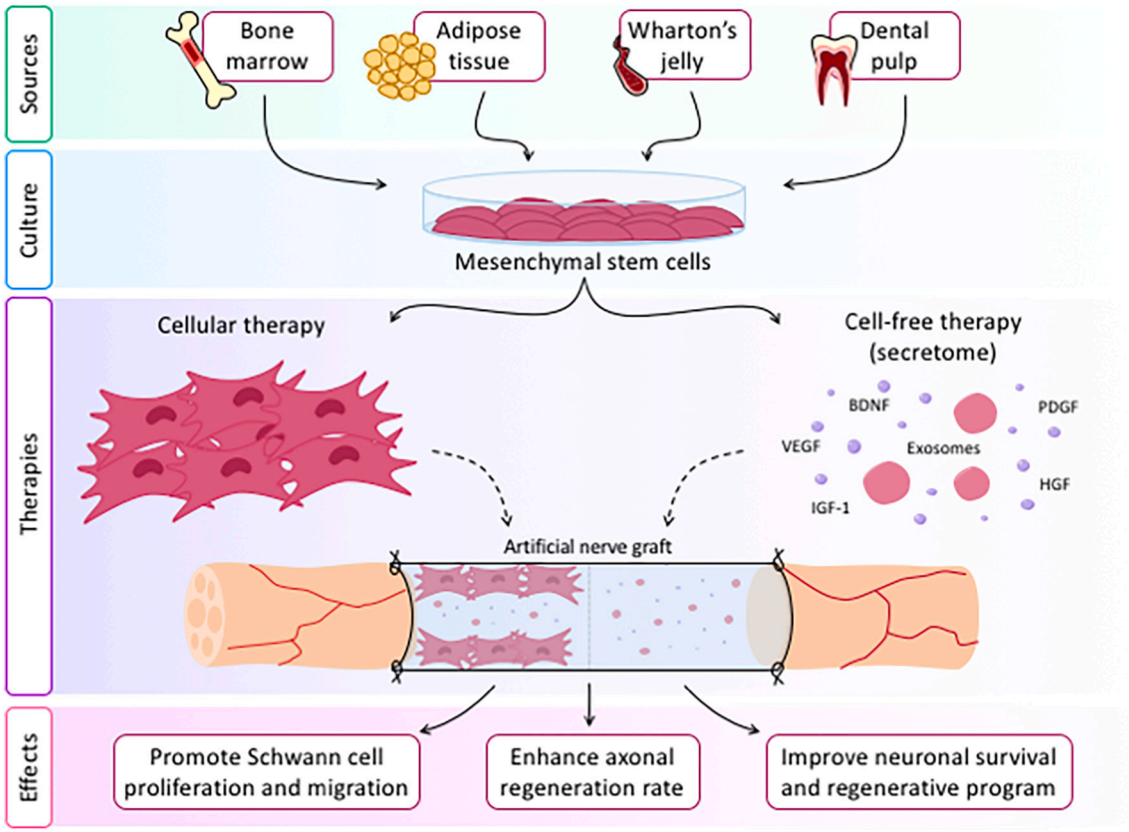

Fig. 2. Therapies based on mesenchymal stem cells (MSCs) to improve nerve regeneration. MSCs are relatively easy to isolate from bone marrow, adipose tissue, Wharton's jelly or dental pulp. These cells have been widely used as regenerative therapy for the damaged nervous system. A promising option to enhance axonal regeneration after severe nerve lesions that need surgical repair consists of using artificial nerve grafts filled with different factors to bridge the nerve stumps. The use of MSCs in the nerve conduits as well as the secretome of these cells (conditioned medium) has been proved to have beneficial effects in axonal regeneration after nerve injuries.

In fact, a recent paper used conditioned medium from primary cultures of SCs, that contained EV, to promote neurite growth in vitro, and found similar effect when using a conditioned medium from cultures of adipose stem cells that had been differentiated into a SC-like phenotype (Ching et al., 2018).

3.2. Use of MSC secretome in peripheral nerve injury

MSCs are adult multipotent progenitor cells found in many organs and tissue types. Due to their relative ease of isolation and expansion in culture, MSCs have been used as a multi-purpose cell-based therapy, in which transplanted MSCs would reach damaged tissues and help to reduce neuroinflammation and even differentiate in specific cell types (Wu et al., 2020). Over the last decades, substantial work has assessed the impact of MSCs as regenerative therapy on nervous system damage (Volkman and Offen, 2017). Thus, the intraspinal injection of a suspension of MSCs promoted axonal regeneration and neuronal survival after spinal root injury (Torres-Espín et al., 2013) and spinal cord injury (Ruzicka et al., 2017; Sykova et al., 2021; Torres-Espín et al., 2014). However, transplanted MSCs did not sustain these positive effects for long periods of time due to their poor survival when grafted within the neural tissue (Torres-Espín et al., 2014). Although in these studies cell transplants moderately improved neuronal survival and axonal regeneration, they presented disadvantages, such as the high number of cells needed for clinical application and the need of autologous source or alternatively immunosuppressive therapy (Torres-Espín et al., 2015).

Considering the reduced time that the cells survive within the host tissue after transplantation, it is mostly considered that the therapeutic effects of MSCs, as well as other derived stem cells, are mainly associated with the secretion of bioactive molecules and extracellular vesicles, which constitute their secretome (Wu et al., 2020). Thus, cells secretome might be used as a cell-free therapeutic approach, avoiding risks, such as tumor formation or rejection, associated with the use of cells. The proteomic analysis of MSC secretomes derived from different sources has

revealed the existence of several trophic factors, such as VEGF, IGF-1, platelet-derived growth factor (PDGF) and HGF, as well as anti-inflammatory cytokines (Shin et al., 2021). However, the secretome composition is not definitively characterized and varies significantly between cultures dependent on subject donor, original tissue source and culture conditions used (Lv et al., 2020; Rizk et al., 2016). Moreover, studies revealed the existence of MSC subpopulations that co-express neurotrophins and other neuro-regulatory molecules, which may differentially contribute to MSC effects on neuronal survival and axonal regeneration (Crigler et al., 2006). Nevertheless, it has to be noted that the composition of stem cells secretome is dependent on the microenvironment in which the cells are placed, making it possible to precondition them to improve specific profiles most adequate to the injured tissue (Ferreira et al., 2018).

Marconi et al. (2012) found that an intravenous administration of adipose-derived MSCs (ASC) 1 week after sciatic nerve crush injury in mice, produced presence of a restricted number of undifferentiated ASC together with an increase of axonal sprouting and reduction of inflammatory infiltrates in the injured nerves up to 3 weeks. They also showed that ASCs produced in culture neuroprotective factors such IGF-1, BDNF or basic fibroblast growth factor (bFGF), that may contribute to peripheral nerve regeneration. Moreover, ASCs escape immune system surveillance, because they possess cell surface antigens that are poorly recognized by T cells; therefore, MSC can be transplanted as an autograft, allograft, and even xenograft (Bai et al., 2009; Marconi et al., 2012).

Interestingly, application of conditioned media (CM) or concentrated extracellular vesicles derived from adipose-derived stem cells differentiated towards a SC-like phenotype significantly enhanced in vitro neurite growth of NG108–15 neurons, similarly to media from primary SCs. qRT-PCR demonstrated that the obtained EV contained mRNAs and miRNAs known to play a role in nerve regeneration and these molecules were upregulated by the SC differentiation protocol (Ching et al., 2018). However, when exploring the cargo of these EV, the authors found differences depending on their origin. Thus, EV from differentiated adipose cells expressed higher amount of GAP43 and Tau mRNA than SC EV, but lower amounts of Rac and RhoA mRNA. Moreover, EV of adipose stem cells and SCs contained similar levels of miR-21 and miR-222mi, whereas R-18a and miR-182 expression was higher in differentiated adipose derived EV. Several studies have demonstrated that EVl miRNAs (including miR-199b, miR-218, miR-148a, miR-135b and miR-221) isolated from MSC cultures can influence neuronal differentiation and axonal outgrowth (Qing et al., 2018).

Addition of CM obtained from cultured MSCs as well as from Neural Crest precursor cells increased viability of primary sensory neurons of the DRG after oxygen-glucose deprivation, and enhanced neurite growth (Shi et al., 2019). MSC-CM also promoted survival and proliferation of cultured SCs, and increased the expression of NGF, BDNF and bFGF by the cultured SCs (Yang et al., 2009). These effects were related to the contents of trophic factors in the CM, including epidermal growth factor (EGF), platelet-derived growth factor alpha (PDGF**α**), ciliary neurotrophic factor (CNTF) and VEGF alpha (VEGF**α**). In addition, MSCs play an immunomodulatory role regulating immune cells through direct cellular contact and the release of cytokines (Brini et al., 2017). Indeed, MSCs obtained from adipose tissue and from bone marrow were found to express a similar profile of neurotrophic factors secretion (Hsiao et al., 2012), and most of the trophic factors detected in those cells secretome are known to promote axonal regeneration from in vitro and in vivo studies (Allodi et al., 2011). A comparative study of MSCs derived from human adult bone marrow, adipose tissue and Wharton's jelly, focusing on gene expression and secretome content and neurotrophic properties, revealed that, despite the differences in growth factor secretion, the MSC secretome derived from all cell sources had significant potential to stimulate neurite outgrowth of DRG neurons and to reduce oxidative cell death (Petrenko et al., 2020).

The development of artificial nerve grafts, composed of a biocompatible nerve conduit filled with a neurotropic matrix and seeded with competent pro-regenerative cells has been pursued for decades, as a promising option to enhance nerve regeneration and become an alternative to the classical autologous nerve graft for repairing long gap injuries. Mostly SCs but also MSCs have been used embedded in a hydrogel filling a variety of conduits with significant success in enhancing nerve regeneration and allowing regeneration over critical long gaps (for reviews see (Deumens et al., 2010; Sarker et al., 2018). However, the need of an autologous cell source (Dezawa et al., 2004; Rodríguez et al., 2000) or the administration of immunosuppression (Udina et al., 2004) have been reported as needed for optimizing the effect of the cell graft to reach the functional outcome of autologous graft repair.

Using the conduit-supported approach Sun et al. (2019) enclosed MSCs in the wall of conduits made of *co*-spun polycaprolactone and gelatin methacrylate. This increased cell local preservation, for up to 4 weeks, while when injected within a matrix into the conduit lumen the cell abundance started to decline after just 1 week. By spatially restricting cell enclosure, the released secretome allowed a directional chemokine gradient of endogenous SCs in nerve conduits. This resulted in significantly enhanced functional recovery and axonal myelination compared with non-cell or lumen-cell conditions after repair of a 10 mm gap in the rat sciatic nerve. This study offers proof that controlled cell seeding to produce neurotrophic gradients within an engineered nerve conduit is a potential strategy for secretome application in peripheral nerve regeneration.

Zhang et al. (2020) added human umbilical cord MSCs-derived EV to olfactory ensheathing cells (OECs) in culture and observed that EV promoted survival and migration of OECs in hypoxic conditions, and effectively increased BDNF secretion. Using a 10 mm sciatic nerve defect rat model repaired with a chitosan-collagen conduit, they reported that prefilling the conduit with an OECs plus EV suspension synergistically promoted motor and sensory recovery of the injured sciatic nerve, although differences between groups were marginal.

Sugimura-Wakayama et al. (2015) investigated the effects of CM derived from stem cells obtained from human exfoliated deciduous teeth (SHED). In vitro, SHED-CM stimulated neuritogenesis of DRG. In vivo they evaluated a 10-mm gap in the rat sciatic nerve repaired with silicone conduits containing SHED-CM or control medium. The group with SHED-CM showed improved reinnervation of target muscles and functional recovery, and higher number of regenerate axons after 12 weeks of nerve transection. The SHEDs medium contained NGF, BDNF, NT-3, CNTF, GDNF, VEGF and HGF that enhance peripheral nerve regeneration. On the other hand, the authors showed that factors secreted from SHED may also influence SCs proliferation and production of extracellular matrix components. However, it should be considered that the effect of a fluid medium inside the conduit had likely a short time persistence. In order to maintain a longer release, (Kano et al., 2017) implanted a collagen graft soaked with SHED-CM within the nerve gap created by transection of the facial nerve in rats. The combination of anti-inflammatory M2 macrophage inducers, MCP-1 and secreted ectodomain of sialic acid-binding Ig-like lectin-9 (sSiglec-9) was found essential for SHED-CM mediated functional recovery after facial nerve injury. Notably, MCP-1/sSiglec-9 induced the polarization of M2 macrophages, which antagonized the pro-inflammatory M1 conditions associated with nerve injury, promoted proliferation and migration of endogenous SCs, and enhanced extension of the peripheral nerve. On the other hand, (Tsuruta et al., 2018) established a novel model for superior laryngeal nerve (SLN) injury following trauma, that causes delay in the onset of the swallowing reflex and gain of laryngeal residue in the pharynx. Systemic intravenous administration of 1 ml SHED-CM in rats after a SLN injury improved swallowing function and increased the density of regenerated myelinated fibers, associated to M2 macrophage polarization and neovascularization, suggesting that SHED-CM may provide therapeutic benefits for patients with such injury. The potential of stem cells from dental sources was pointed out by the comparative study of the secretome composition of MSCs from dental apical papilla

(SCAPs) and from bone marrow; it was found that SCAPs secreted significantly larger amounts of chemokines and neurotrophins than bone marrow derived MSCs (BMSCs; (Yu et al., 2016).

3.3. Use of MSC secretome in neuropathic pain

The therapeutic potential of MSC-derived CM has been also investigated in models of neuropathic pain induced by sciatic nerve ligation.

Injured mice were treated by endovenous route with bone marrow-derived MSCs or their CM. As early as 12 h after injection, neuropathic mice treated with MSCs and with CM showed a clear antinociceptive effect that was maintained throughout the evaluation period of 60 days. In contrast, gabapentin used as control treatment induced only short-lasting antinociception. The effects could be related with changes in cytokine levels; IL-1β, TNF-α, and IL-6 were reduced, and IL-10 was increased in nerve and spinal cord by treatment with CM and MSCs (Gama et al., 2018). Interestingly, it has been reported that conditioned medium (CM) collected from painful human schwannomatosis tumor cells, but not that from nonpainful ones, was able to sensitize DRG neurons in culture, causing increased sensitivity to depolarization and also upregulated the expression of pain-associated genes. Multiple cytokines were also detected at higher levels in CM from painful tumors, underlying the pain-promotion action (Ostrow et al., 2019).

4. Conclusions

New insights into the mechanisms that allow cell-to-cell communication have highlighted the important role of the secretome, specially EV, in the local control of axon growth and degeneration by SCs and macrophages, and have also pointed to the potential therapeutical role of secretomes as an alternative to cell therapy to improve axonal regeneration. Both trophic factors and EV from SC promotes axonal regeneration when locally applied at the injury site. However, these strategies have a limited impact on functional recovery after nerve injury. Secretomes can also be used as a part of a repair strategy to improve the outcome of artificial neural guides, similar to laminin and SC derived trophic factors that have been frequently used in the design of these guides, although currently there are no competitive alternatives to the gold standard autograft. Secretome of MSCs has also been extensively studied since transplantation of these cells in the nervous system has potent effects even when their survival is limited in time and conditioned by the immune response. Similar to SC secretomes, MSC secretomes improve axonal regeneration after nerve injury, but with a limited impact on functional recovery. Innovative strategies using specific secretomes to create gradients that favor directed axonal regeneration and new ways of administration with clinical translational perspective would increase the impact of secretomes as therapeutical tool in the field of nerve regeneration.

Declarations of interest

None.

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