Title

Repair Schwann cell update: adaptive reprogramming, EMT and stemness in regenerating nerves

Running title: Repair cell update

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Abstract

Schwann cells respond to nerve injury by cellular reprogramming that generates cells specialized for promoting regeneration and repair. These repair cells clear redundant myelin, attract macrophages, support survival of damaged neurons, encourage axonal growth and guide axons back to their targets. There are interesting parallels between this response and that found in other tissues. At the cellular level, many other tissues also react to injury by cellular reprogramming, generating cells specialised to promote tissue homeostasis and repair. And at the molecular level, a common feature possessed by Schwann cells and many other cells is the injury-induced activation of genes associated with epithelial-mesenchymal transitions and stemness, differentiation states that are linked to cellular plasticity and that help injury-induced tissue remodelling. The number of signalling systems regulating Schwann cell plasticity is rapidly increasing. Importantly, this includes mechanisms that are crucial for the generation of functional repair Schwann cells and nerve regeneration, although they have no or a minor role elsewhere in the Schwann cell lineage. This encourages the view that selective tools can be developed to control these particular cells, amplify their repair supportive functions and prevent their deterioration. In this review we discuss the emerging similarities between the injury response seen in nerves and in other tissues and survey the transcription factors, epigenetic mechanisms and signalling cascades that control repair Schwann cells, with emphasis on systems that selectively regulate the Schwann cell injury response.

Keywords

Schwann cells, repair cells, nerve regeneration, nerve injury, adaptive reprogramming, epithelial-mesenchymal transition, c-Jun

Main points

Nerve injury triggers reprogramming of myelin and Remak Schwann cells to generate cells specialized for promoting repair. This shows many similarities with injury responses of other tissues, including the process of adaptive programming and activation of epithelial mesenchymal transitions/stemness genes. The generation of repair Schwann cells is controlled by dedicated signalling mechanisms, which should facilitate the development of molecular tools for boosting the function of these cells to improve the clinical outcome of nerve injuries.

Introduction: the changing view on the Schwann cell injury response

It has long been recognized that after injury to a peripheral nerve, Schwann cells undergo a radical change in identity by converting to denervated Schwann cells, which unlike the Schwann cells of uninjured nerves provide powerful support for regeneration (reviewed in Chen & Strickland, 2007: Zochodne, 2012; Glenn and Talbot, 2013; Scheib & Hőke, 2013; Brosius Lutz & Barres, 2014; Faroni et al., 2015; Jessen et al., 2005; 2008, 2015b, 2016; Cattin & Lloyd 2016; Boerboom et al.,2017). Although this Schwann cell injury response is central to nerve repair, the nature of this this process has been uncertain and disputed (Fig.1).

A recent contribution to this debate, namely the identification of partial epithelial mesenchymal transition (EMT) in injured nerves (Arthur-Farraj et al., 2017; Clemets et al., 2017), highlights how our understanding of the Schwann cell injury response has progressed by degrees, with each step providing new insights into this complex process.

Early studies on injured nerves focussed on the obvious structural changes distal to injury, in particular the fragmentation for the myelin sheath following axonal death, rearrangement of Schwann cells into cellular columns, Bungner Bands, and invasion of macrophages, events traditionally referred to as Wallerian degeneration (e.g. Lubinska, 1977; Stoll et al., 1989).

With the identification of molecular profile of myelin and Remak cells, and of their developmental ancestors in the Schwann cell lineage, it became clear that the generation of denervated cells after injury was accompanied by a striking down-regulation of the molecules that characterized myelin cells. These denervated cells therefore shared one prominent feature, namely the absence of high levels of myelin gene expression, with immature Schwann cells of developing nerves prior to myelination. This reversal of myelin differentiation was instrumental in generating a view of the entire Schwann cell injury response essentially as a developmental regression to an earlier developmental stage, or de-differentiation (e.g. Chen & Strickland 2007; Jessen and Mirsky 2008).

The picture changed again when it was found that the generation of denervated cells fully capable of supporting repair depended not only on myelin de-differentiation, but also on the activation of transcriptional mechanisms that were not significantly involved in Schwann cell develpoment. Two transcriptional controls, c-Jun and chromatin modifications involving H3K27 demethylation, H3K27 de-acetylation and H3K4 methylation, were found to be important for the normal execution of the Schwann cell injury response, although these

mechanisms did not regulate neonatal Schwann cells or myelination (Arthur-Farraj et al., 2012; Hung et al., 2015; reviewed in Ma and Svaren 2018). Additional control mechanisms that are selectively important in denervated cells have now been identified, including Merlin and STAT3 (Benito et al 2017; Mindos e al., 2107). Denervated adult Schwann cells have also been shown to express de novo molecular markers that are low/absent during development, including Sonic Hedgehog (Shh), and Olig1 (Lu et al., 2000; Bosse et al., 2006; Arthur-Farraj et al., 2012; Fontana et al., 2012; Lin et al., 2015; reviewed in Jessen & Mirsky 2016). Again in contrast to developing cells, denervated cells express high levels of cytokines, recruit macrophages, and activate myelin autophagy to clear myelin (Gomez-Sanchez et al., 2015; reviewed in Martini et al., 2008; Rotshenker 2011; Stratton and Shah 2016), Denervated adult cells also adopt a striking elongated and often branched morphology, which is very different from that of Schwann cells in developing nerves, and which enables them to form regeneration tracks, which guide re-growing axons (Gomez-Sanchez et al., 2017).

In view of these substantive differences between developing and denervated Schwann cells, it is no longer useful to view the Schwann cell injury response simply as a developmental regression or de-differentiation. Notably, most of the properties of denervated cells that are distinctive and differentiate them from developing cells, also make these cells exceptionally well suited for supporting nerve regeneration, leading to the suggestion that these cells are best understood as cells specialized for supporting this process, repair Schwann cells (reviewed in Jessen and Mirsky 2016). This concept recognizes that the Schwann cell injury response represents a dual change: loss of myelin differentiation coupled with the activation of a set of features that support regeneration, a repair programme. As outlined in the subsequent section, this adaptive response shows similarities to injury-induced cell conversions in other tissues, which are typically referred to as direct (or lineage) reprogramming. We have therefore referred to it as adaptive cellular reprogramming (Jessen et al., 2015a).

The most recent addition to the changing picture of the Schwann cell injury response,namely the finding that it includes up-regulation of the genes associated with epithelial-mesenchymal transitions (EMT) and stemness, bring Schwann cells into line with several other systems in which injury also activates EMT/stemness genes (Arthur-Farraj et al., 2017; Clements et al., 2017). This adds important components to our understanding of the repair program, and makes biological sense because EMT-like changes typically provide cells with increased motility and morphological flexibility. In Schwann cells, this would facilitate the extensive tissue re-modelling that takes place when the nerve trunk distal to the injury converts into a

collection of regeneration tracks, and when a tissue bridge forms a across the injury site in severed nerves (Fig.1 & 2). Further, increase in stemness generally shifts cells to a state associated with a change of identity. This is consistent with the view that injury reprograms Remak and myelin cells to adopt the distinct alternative identity of a repair cell.

We will now discuss the general concept of adaptive reprogramming and how this notion relates to the Schwann cell response to injury.

Adaptive cellular reprogramming

Peripheral nerves provide a striking example of how mammalian tissues can regenerate and recover function after injury. Thus, some 3-4 weeks after crush of the sciatic nerve in rodents, which results in the destruction of axons and myelin and loss of both sensory and motor function in the hind limb, the nerve has regenerated, myelin sheaths have reformed and the limb is again fully functional.

The radical change in the differentiation state of myelin and Remak Schwann cells to a repair-supportive penotype, which is a key mechanism behind this impressive regenerative capacity, shows interesting similarities with the cellular response to damage in a number of other tissues, including the pancreas, ear, liver eye and skin. In common, these systems respond to injury, not by developmental regression, but by cellular reprogramming to new differentiation states, which restore tissue homeostasis.

Several tissues show this response, which works in one of two ways (reviewed in Jessen et al., 2015a). In some systems, the surviving cells convert to cells similar to those that were lost during injury. Such generation of replacement cells takes place for instance in the murine pancreatic isles. Following destruction of the insulin secreting β-cells, the remaining glucagon secreting α-cells replace the loss by converting to β cells in a process that combines the down-regulation of glucagon expression with activation of insulin secretion. The new replacement cells are functional and capable of partially restoring glycaemic control to the animal (Thorel et al., 2010; reviewed in Chera & Herrera 2016). Formation of replacement cells is also seen the vestibular and cochlear epithelium of the ear. Here, damage to hair cells, involved in balance and hearing, triggers the direct conversion of neighbouring glial-like supportive cells to new hair cells. (Forge et al., 1998; Mizutari et al., 2013; Bramhall et al., 2014; Cox et al., 2014; Richardson & Atkinson 2015). In the liver also, damage to biliary epithelial cells sparks the re-programming of surrounding hepatocytes to form new biliary epithelial cells (Yanger et al., 2013; reviewed in Eberhard & Tosh 2008).The classical non-mammalian example of this type of cell conversion is the transformation of

pigmented epithelial cells of the dorsal iris to transparent lens cells, illustrating the combination of partial de-differentiation (loss of the pigment phenotype), with activation of alternative differentiation (up-regulation of lens cell features, such as crystalline expression).

Illustration of the other form of adaptive reprogramming, the generation of repair cells, is provided by myofibroblasts in skin and heart injuries (Davis et al., 2012; reviewed in Hinz et al., 2012). Myofibrolblasts are absent from normal uninjured tissue. But after injury, myofibroblasts are generated from fibroblast and function to accelerate wound healing by secretion of extracellular matrix and restoring tension to the damaged tissue. Blocking the fibroblast-myofibroblast conversion results in defective skin and heart repair, demonstrating the adaptive nature of this reprogramming event.

In peripheral nerves, neurons respond to injury by activating gene programmes that temporarily shift their function from that of cell-cell communication to that of regenerating axons, a response classically referred to as the signalling to growth mode switch or the cell body response (Allodi et al. 2012; Blesch et al. 2012; reviewed in Fu and Gordon 1997; Doron-Mandel et al. 2015). In many ways, this response is comparable to the events discussed hare as adaptive reprogramming.

As detailed in the following section, Schwann cells provide a further example of adaptive cellular reprogramming. In this case also, cells with a repair-supportive phenotype, which are normally absent, are generated directly from resident differentiated cells of the injured tissue (Gomez-Sanchez et al., 2017) and function to restore tissue homeostasis.

The generation of repair Schwann cells

The Schwann cell injury response has been reviewed elsewhere (Chen & Strickland 2007; Jessen & Mirsky 2008; 2016; Monk et al., 2015; Boerboom et al., 2017). Briefly, it has two principal components. One is the reversal of myelin differentiation. This involves transcriptional down-regulation of the pro-myelin transcription factor Egr2 (Krox20), the enzymes of cholesterol synthesis, and a number of structural and membrane associated myelin related proteins. At the same time, the cells re-express molecules that were suppressed when immature Schwann cells started to myelinate during development, including L1, NCAM, p75NTR and glial fibrillary acidic protein (GFAP) and many others.

The other component of the injury response is the sequential activation of a diverse features all of which support repair: (l) Up-regulation of proteins that support neuronal survival and promote axonal regeneration, such as GDNF, artemin, BDNF, NT3, NGF, VEGF, erythropoietin, pleiotrophin, p75NTR and N-cadherin (Fontana et al. 2012; Brushart et al.

2013; reviewed in Boyd & Gordon, 2003; Chen & Strickland 2007; Scheib & Höke, 2013; Wood & Mackinnon, 2015). (II) Activation of an innate immune response, comprising the up-regulation of cytokines including TNFα, LIF Il-1α, Il-1β, LIF and MCP-1 (reviewed in Martini et al., 2008; Rotshenker, 2011). This recruits macrophages, which help Schwann cells to clear redundant myelin, stimulate the formation of blood vessels and axon growth (Hirota et al. 1996; Cafferty et al. 2001; Barrette et al. 2008; Niemi et al. 2013; Cattin et al. 2015; reviewed in Hirata & Kawabuchi, 2002; Bauer et al. 2007). (III) Structural re-organization, generating extremely elongated, cells, which are about three fold longer than myelin and Remak cells. Lineage tracing has shown that these cells are generated directly from myelin and Remak cells, and that they can convert directly to myelin cells following regeneration (Gomez-Sanchez et al. 2017). These cells often carry long, parallel processes, which together with their length, allows repair cells to partly overlap and promotes the formation of the compact cellular columns (Bungner bands - Fig. 1 & 2), which are obligatory regeneration tracks for regenerating axons (Gomez-Sanchez et al. 2017). (IV) Activation of myelinophagy by which myelin cells initiate the breakdown of their own myelin sheath (Gomez-Sanchez et al., 2015, Suzuki et al. 2015; Jang et al., 2016; Brosius Lutz et al., 2017).

The phenotype of repair Schwann cells, like other cells in the lineage, depends on environmental signals. Thus, as regeneration proceeds and repair cells become redundant, they obey axonal signals to return to the myelin and Remak phenotypes. The repair cells are therefore transient, produced on demand and present as long as needed (reviewed in Jessen and Mirsky 2016).

Timing and activation of the repair program

The changes that constitute the Schwann cell injury response, and outlined above, do not all take place simultaneously. Rather, nerve injury starts the execution of a repair programme, which is a temporal sequence of changes in cellular gene expression, structure and function. Thus, expression of cytokines such as Il-1β and TNFα reach peak levels within one day after injury (reviewed in Rotshenker 2011), myelin autophagy is maximally activated around day five (Gomez-Sanchez et al., 2017), GDNF protein levels peak around day seven, while BDNF protein peaks at two to three weeks (Eggers et al., 2010). c-Jun is activated rapidly, but c-Jun protein levels continue to increase at least until seven to ten days after injury (J. Gomez-Sanchez, K.R.Jessen and R.Mirsky unpublished), and full cellular elongation is not achieved until about four weeks after nerve injury or later (Gomez-Sanchez et al., 2017). The

phenotype of the repair cell therefore undergoes an orderly series of changes, which collectively promote nerve regeneration.

The identification of the cell-extrinsic signals that initiate these changes after injury is an important area of future work. The first signs of the injury response can be detected surprisingly early (5h-24h) after nerve cut. This includes the elevation of phospholipase A2 immunoreactivity, expression of mRNA for LIF, c-Jun and several other AP1-family members, and of c-Jun, IL1 Il-1β and TNFα protein, phosphorylation of ErbB2 neuregulin receptor, p38 MAPK activation and actin ploymerization (Banner & Patterson 1994; Kurek et al., 1996; Dea et al., 2003; Parkinson et al., 2008; Jung et al 2011; Yang et al., 2012; Guertin et al 2015; Arthur-Farraj et al 2017; reviewed in Martini et al., 2008; Rotschenker 2011; Wong et al 2017). These Schwann cell responses are seen before appreciable structural axonal degeneration and substantial macrophage influx. They are therefore likely to be triggered mainly by signals from transected/crushed axons. These signals remain largely unidentified, although the early ErbB2 phosphorylation raises the possibility of neuregulin involvement. There is evidence that purines, which have a role in many other injury responses, play a part through activation of Schwann cell protein kinase C or ERK1/2 (Xu et al., 2013; Negro et al., 2016). Neurone-derived hydrogen peroxide has also been implicated in early Schwann cell activation involving Annexin proteins (Duregotti et al., 2015; Negro et al., 2018), and induction of injury-related cytoskeletal changes (Reynolds and Woolf 1992; Son et al.,1996; Gomez-Sanchez et al.,2017).

Signals that control repair cells

Below is an outline of the molecular signalling that controls the generation and function of repair cells (Fig.3). In addition, we summarize studies that describe a nerve injury phenotype in vivo following conditional gene inactivation in Schwann cells (Table 1). Many of the factors which regulate the Schwann cell injury response also control other aspects of Schwann cell development and myelination. Except for re-myelination after injury, these other functions will not be discussed in detail, nor will signals that control Schwann cell proliferation.

Transcription Factors

The transcription factor c-Jun is a key regulator of the Schwann cell injury response. After injury, it is rapidly up-regulated in Schwann cells where it negatively regulates the myelin program, and promotes expression of the repair program. This includes increase in trophic support for neurons, acceleration of myelin breakdown by autophagy, promotion of Schwann cell elongation and formation of the bands of Bungner. As a result, regeneration of motor and sensory axons, and the survival of sensory neurons and facial motorneurons are

severely compromised if Schwann cell c-Jun is inactivated. Conversely, in situations where normal regeneration is impaired, such as after chronic denervation and in aging animals, enhanced expression of Schwann cell c-Jun promotes axonal regeneration (Parkinson et al., 2008; Arthur-Farraj et al., 2012; Fontana et al., 2012; Fazal et al., 2017; Wagstaff et al., 2017; Figlia 2018). Interestingly, macrophage recruitment, and Schwann cell numbers, at least at early time points after injury, are less affected in the absence of c-Jun than other aspects of the injury response (Arthur-Farraj et al., 2012). A microarray screening shows that c-Jun controls 172 genes, including directly regulating several neurotrophic factors such as GDNF, BDAF, Shh and Artemin (Arthur-Farraj et al., 2012; Fontana et al., 2012). However, after nerve injury microarray and RNA-Seq analysis demonstrate at least 4000-5000 differentially expressed RNAs, showing that c-Jun only controls a subset of injury induced genes (Nagarajan et al., 2002; Bosse et al., 2006; Barrette et al., 2010; Arthur-Farraj et al., 2012, 2017; Chang 2013).

The extracellular signals that activate Schwann cell c-Jun after injury are obscure. Nevertheless, a number of intracellular pathways that potentially regulate c-Jun levels have been identified. This includes the Rac1-MKK7-JNK pathway and mTORC, which elevate c-Jun and have been implicated as upstream activators of c-Jun after nerve injury, and the cAMP pathway that suppresses c-Jun (Monje et al., 2010; Arthur-Farraj et al., 2011; Shin et al., 2013; Norrmén et al., 2018). Histone deacetylase 2 (HDAC2), which is activated in injured nerves, also down-regulates c-Jun (Brügger et al., 2017; reviewed in Jacob 2017). The transcription factors Krox20 and Oct6 also antagonize c-Jun expression (Parkinson et al., 2008; Brügger et al., 2017). Since Krox20 is suppressed but Oct6 elevated after injury, these factors modulate c-Jun levels in opposing directions in damaged nerves.

Sox2 as well as other negative regulators of myelination including Id2 and Pax-3 are also induced in repair cells, and are likely to take part in suppressing myelin genes after injury although this has not yet been shown directly *in vivo*. (Kioussi et al., 1995; Le et al., 2005 Doddrell et al., 2012; Florio et al., 2018; revived in Jessen and Mirsky 2008; Stolt & Wegner 2016). Sox2 activation in Schwann cells has a role in the tissue bridge between the proximal and distal stumps where it mediates ephrin-B/EphB2 signalling between fibroblasts and Schwann cells, which promotes the clustering of migrating Schwann cells and ordered axon growth in the bridge (Parinello et al., 2010). Enforced expression of Sox2 in vivo suppresses myelination both after injury and during development, but does not control the number of regenerated axons in injured nerves (Roberts et al., 2017).

The Notch pathway is activated in Schwann cells upon nerve injury and inactivation of Notch signalling or constitutive Notch activation specifically in Schwann cells, retards or accelerates demyelination, respectively (Woodhoo et al., 2009). Notch suppresses myelin genes, and other signals including the transcription factor Zeb2 and let-7miRNA, which promotes myelination and myelin gene expression, are thought to act in part by inhibiting Notch (Pereira 2010; Gőkbuget et al., 2015; Quintes et al., 2016; Wu et al., 2016). Enforced activation of Notch alone, even in uninjured nerves, is sufficient to induce myelin breakdown (Woodhoo et al., 2009). Whether the Notch-mediated promotion of myelin clearance after injury affects regeneration or whether Notch has broader effect on repair cells is yet to be studied.

Zeb2 levels are elevated in Schwann cells of injured nerves. This does not appear to regulate the expression of c-Jun and Sox2 at early time points after injury suggesting that Zeb2 is relatively unimportant for the initial generation of repair cells. However, during re-myelination, Zeb2 contributes to the suppression of negative regulators such as Sox2 and Id2 and the Notch pathway effector Hey2, and is therefore needed for normal generation of myelin in regenerating nerves, a function that mimics the role of this transcription factor during developmental myelination (Quintes et al., 2016; Wu et al., 2016).

Importantly, genome wide mapping of active enhancers in injured rat nerves with acetylated histone H3K27 CHIP-Seq found that the most abundant enriched motifs, in addition to c-Jun/AP-1, were for Runx2 and the Ets family of transcription factors (Hung et al., 2015). Ets transcription factors have previously been implicated in the support of Schwann cell survival after injury (Parkinson et al., 2002). It will be important to define the role of these proteins in the Schwann cell injury response.

Axons take a long time, up to years in humans, to reach their targets after injury. Even for very distal injuries such as in the wrist, axons can take months to re-innervate the finger tips. For successful regeneration therefore, repair cell generation needs to be followed by long-term maintenance of the repair cell population. Because failure of repair cell maintenance is thought to be a significant reason for poor regeneration outcomes in humans, it is important to identify transcription factors and other signals the sustain repair cells. The first known transcription factor with this function is STAT3. This protein is not required for the initial generation of repair Schwann cells, but during chronic denervation, STAT3 is important both for the long-term survival of these cells and for promoting the expression of c-Jun and other repair program genes (Benito et al., 2017).

Intracellular signalling pathways

Although myelin breakdown is an important component in the conversion of myelin cells to repair cells, the mechanism by which Schwann cells get rid of their redundant myelin sheaths has long been debated. However, a Schwann cell specific knockout of *ATG7*, a key protein in the autophagy process has now demonstrated that Schwann cells digest their own myelin through a process termed, myelinophagy, a type of macro-autophagy (Gomez-Sanchez et al., 2015; Jang et al., 2016; Brosius Lutz et al., 2017). In addition to myelinophagy, Schwann cells also phagocytose smaller amounts of myelin debris via TAM receptor phagocytosis (Brosius Lutz et al., 2017). Interestingly, *ATG7* null Schwann cells demonstrated impaired upregulation of other repair program genes such as *GDNF*, *Olig1* and *Shh,* suggesting that disrupting myelinophagy impairs generation of the repair cell phenotype (Gomez-Sanchez et al., 2015). It remains to be tested, whether delaying myelinophagy ultimately affects axonal regeneration and functional nerve repair.

Another early function of repair Schwann cells, in addition to myelinophagy, is to recruit macrophages into injured nerve. These cells make an important contribution to myelin clearance by phagocytosing myelin debris, in addition to stimulating vascularization and likely promoting axonal re-growth by direct signalling to neurons (Hirota et al., 1996; Cafferty et al., 2001; Barrette et al., 2008; Cattin et al., 2015; reviewed in Bauer et al., 2007; Martini et al., 2008). Schwann cell recruitment of macrophages is under strong regulation by the Raf-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. ERK1/2 phosphorylation is rapidly induced in Schwann cells after injury, and implicated in the up-regulation of the major macrophage recruitment signal MCP-1 (Sheu et al., 2000; Fischer et al., 2008). Pharmacological inhibition of ERK1/2 activation, or genetic inactivation of CEMIP, a protein that stimulates ERK1/2 activity, delays myelin clearance after injury (Harrisingh et al., 2004; Napoli et al., 2012; reviewed in Boerboom et al., 2017). Conversely, strong activation of ERK1/2, whether by constitutively active Raf or MEK1, promotes macrophage recruitment, myelin breakdown and suppression of myelin genes. This is seen even in uninjured nerves in experiments using Raf (Napoli et al., 2012), but only after injury in studies using a constitutively active MEK1 variant to stimulate ERK1/2 (Cervellini et al., 2017). It still remains controversial whether Raf-MEK-ERK1/2 signalling has a role in activating c-Jun in Schwann cells (Monje et al., 2010; Syed et al., 2010; Shin et al., 2013).

The function of ERK1/2 in Schwann cells is, however complex, and not restricted to promoting the injury response, because ERK1/2 is also essential for developmental myelination (Newbern et al., 2011). In agreement with that, enhanced ERK1/2 activation in Schwann cells during early development results in increased myelin thickness (Ishi et al., 2013; Sheean et al., 2014). Notably, during re-myelination of regenerating nerves, enhanced

Schwann cell ERK1/2 activation has a different outcome. In this case, there is no effect on myelin thickness, but myelin compaction, Cajal bands, internodal length and axonal regeneration are compromised (Cervellini et al., 2018).This indicates that repair cells differ from immature Schwann cells with respect to the intracellular signalling pathways that control myelination. The role of neuregulin in myelination also differs between these two Schwann cell types (see below).

The c-Jun N terminal kinase (JNK) pathway is rapidly activated within minutes to hours after nerve injury, although c-Jun protein levels continue to rise for several days after injury (Parkinson et al., 2008; Yang et al., 2012; J Gomez-Sanchez, KR Jessen and R Mirsky unpublished). While pharmacological experiments indicate that the Rac1-MKK7-JNK pathway is important for c-Jun up-regulation (Monje et al., 2010; Shin et al., 2013), the requirement for JNK and c-Jun phosphorylation after nerve injury has not yet been tested through genetic inactivation. Although c-Jun is a major phosphorylation target of JNK, JNK phosphorylates a number of other proteins, and at least in developing Schwann cells, c-Jun suppresses myelin genes without phosphorylation (Parkinson et al., 2008). The relationship between JNK and the functions of Schwann cell c-Jun in injured nerves is therefore unclear.

The above findings suggest that the Raf-MEK-ERK1/2 and the Rac1-MKK7-JNK signalling pathways carry out distinct functions in repair cells (reviewed in Lee et al., 2014). The Raf-MEK-ERK1/2 pathway, is particularly implicated in cytokine expression, macrophage recruitment and myelin breakdown, while its role in c-Jun activation is not clear. The Rac1-MKK7-JNK pathway activates c-Jun and regeneration-associated genes, including GDNF and P75NTR, down-regulates the pro-myelin transcription factor Krox20, and promotes myelinophagy. In line with this, there does not appear to be significant overlap in the genes, particularly cytokines, dysregulated by Raf activation and those by c-Jun inactivation in Schwann cells (Arthur-Farraj et al., 2012; Napoli et al., 2012).

Although the p38 mitogen-activated protein kinase (p38MAPK) pathway is strongly and rapidly activated in Schwann cells after injury, a Schwann cell specific knockout of the major p38 isoform, p38α, which abolished p38MAPK activity after nerve injury, demonstrated only slightly slower clearance of myelin proteins, and no effect on macrophage influx, axonal regeneration or functional recovery (Roberts et al., 2017a).

Merlin, is a tumour suppressor protein which has a significant role in repair Schwann cells (Hilton & Hahnemann, 2014; Mindos et al., 2017). In mice with Schwann cell specific knockout of Merlin, axonal regeneration is severely impaired and there is noticeable loss of regenerated large myelinated fibres. Re-myelination is suppressed and there is a large

expansion in endoneurial size, likely due to increased extracellular matrix production. The hippo pathway effector YAP is overexpressed in Merlin null nerves, and genetic inactivation YAP almost completely restores the regenerated nerve to normal (Mindos et al., 2017). A comparable, albeit much milder, regeneration phenotype is seen following overexpression of a dominant negative Merlin construct in Schwann cells, possibly due to incomplete inhibition (Truong et al., 2018). The molecular mechanism of how Merlin regulates the phenotype of repair Schwann cells and axonal regeneration is unclear, although both c-Jun and ERK1/2 activation are dysregulated in Merlin null Schwann cells (Mindos et al., 2017).

The mTORC1 pathway is activated in Schwann cells by injury. This is required for normal c-Jun activation likely because TORC1 promotes c-Jun translation. Genetic inactivation of the TORC1 pathway results in subdued activation of c-Jun and other repair cell genes, including *Olig1, Shh and Runx2*. While macrophage recruitment is unaffected, the capacity of repair cells to clear myelin is impaired and re-myelination is delayed (Norrmén et al., 2018).

Extracellular ligands, receptors and cytokines

Among the growth factors and extracellular signalling molecules implicated in regulating the phenotype of repair Schwann cells, the neuregulin-1 signalling pathway is probably the most studied. Neuregulin-1 is dispensable for myelin maintenance in adult uninjured nerves (Atanasoski et al., 2006; Birchmeier & Nave 2008). Although Schwann cells elevate expression of both ErbB2/3 receptors and neuregulin-1 I/II isoforms after injury, neuregulin-1 appears not to be not involved in injury-induced Schwann cell proliferation, macrophage recruitment and myelin breakdown (Carroll et al., 1997; Kwon et al., 1997; Atanasoski et al., 2006; Fricker et al., 2013; Ronchi et al., 2013; Stassart et al., 2013). Nerves with selective inactivation of neuregulin-1 in Schwann cells also re-myelinate after injury, although myelination is significantly impaired (Stassart et al., 2013). Even after removal of neuregulin-1 from both Schwann cells and axons, re-myelination is eventually normal after a substantial delay (Fricker et al 2013; Stassart et al., 2013). Axonal regeneration is also slower in mice without neuregulin-1 in axons and Schwann cells, suggesting that endogenous neuregulin signalling through ErbB2/3 receptors in repair cells promotes the repair phenotype and the capacity of these cells to support axon growth (Fricker et al., 2011; 2013). Direct evidence for this mechanism of action is missing, however. Nevertheless, artificially enhancing neuregulin signalling might serve as a tool for promoting nerve repair, because enforced Schwann cell ErbB2 expression, and exogenously applied neuregulin increase axonal regeneration *in vivo* (Gambarotta et al., 2013; Han et al., 2017). Another factor that also

potentially binds to ErbB receptors, betacellulin, is up-regulated in Schwann cells after injury, but the involvement of betacellullin in injured nerves are unclear (Vallières et al., 2017).

The G-protein coupled receptor GPR126 in Schwann cells is essential for both developmental myelination and re-myelination (Mogha et al.,2016a,b). This protein is not required for the maintenance of myelin in uninjured nerves, but inactivation of Gpr126 in repair Schwann cells results in reduced axonal regeneration, reduced expression of Tnf and a restricted group of chemokines, and reduced recruitment of macrophages, cells which potentially promote axonal regeneration (Barrrette et al., 2008).On the other hand, Gpr126 is dispensable for injury-induced c-Jun elevation in Schwann cells, Schwann cell proliferation and myelin clearance (Mogha et al., 2016b). In developing nerves, GPR126 binds to laminin 211 to elevate cyclic AMP levels and promote myelination (Monk et al., 2009; Petersen et al., 2015). After nerve injury, the identity of the GPR126 ligand is unclear, though it may be laminin since removal of *laminin γ1* in Schwann cells impairs axon regeneration (reviewed in Chen and Strickland, 2007).

Full knockouts of Toll-like receptor (TLR) 2 and 4 or their downstream effector MyD88 significantly impair early induction of cytokines, including MCP-1, IL-1β in injured nerves, and retard macrophage influx and myelin clearance. Conditional inactivation of TLRs is, however, required to investigate their specific role in repair Schwann cells (Boivin et al., 2007).

TGF-β1 is upregulated after nerve injury in Schwann cells and endoneurial fibroblasts and is also expressed by macrophages (Scherer et al., 1993; Sulaiman & Nguyen 2016). *In vitro*, TGF-β1 shifts Schwann cells from a myelin towards a repair phenotype, since it suppresses myelin proteins and up-regulates the adhesion molecules L1 and NCAM, both of which are elevated after nerve injury (Stewart et al., 1995; Morgan et al., 1994). TGF-β1 treatment of Schwann cells is also reported to improve axonal regeneration *in vivo* (Sulaiman & Nguyen 2016). Removal of the TGF-β receptor II in Schwann cells shows that TGF-β has a role specifically at the site of nerve injury for promoting Schwann cell migration from the proximal stump, across a bridge region to reconnect with the distal stump. Disruption of this Schwann cell migration leads to mis-targetting of axons at the injury site, although long-term axonal regeneration and functional recovery were normal in the absence of Schwann cell TGF-β signalling (Parinello et al., 2010; Caittin et al., 2015; Clements et al., 2017).

Epigenetic Processes

Several epigenetic processes regulate the repair Schwann cell phenotype (reviewed in Jacob 2017; Ma & Svaren 2018). Injury sets in train chromatin remodelling involving, first, the loss of the active histone mark H3K27 acetylation on enhancers of a number of myelin genes. Second, a proportion of injury induced genes (around 20%), which are normally marked by the polycomb PRC2 complex with the repressive histone mark H3K27 trimethylation at their promoter region, are demethylated. Simultaneously, they gain the active H3K4 methylation promoter mark and their expression is de-repressed. It is likely that the histone demethylase JMJD3, which is up-regulated by injury, mediates the demethylation of histone H3K27 in repair Schwann cells, because inhibition of this enzyme in nerve explants blocks upregulation of a number of repair program genes such as, *Fgf5*, *Shh* and *Runx2* (Gomez-Sanchez et al., 2013; Hung et al., 2015; Ma et al., 2015; reviewed in Ma & Svaren 2016). JMJD3 also mediates promoter H3K27demethylation of *p19Arf* and *p16Ink4a* to prevent Schwann cell over-proliferation (Gomez-Sanchez et al., 2013).

Histone deacetylase 1 and 2 (HDAC1/2) are up-regulated in Schwann cells after injury. They suppress c-Jun and delay the conversion to repair cells. Accordingly, axonal regeneration is faster if Schwann cell HDAC1/2 is inactivated, but re-myelination is impaired (Brügger et al., 2017). HDAC4 also suppresses c-Jun and elevates myelin gene expression (Gomis-Coloma et al. 2018). In contrast, HDAC3 up-regulates negative regulators of myelination including Id2 and Sox2, and the Notch and Hippo signalling pathways. Myelination is therefore enhanced if Schwann cell HDAC3 is inactivated (He et al., 2018).

MicroRNAs (miRNAs) are 21-24 nucleotide regulatory RNAs. After nerve injury, there is a significant change in expression of hundreds of miRNAs (Viader et al., 2011; Adilakshmi et al., 2012; Arthur-Farraj et al., 2017). Disruption of miRNA processing through deletion of the enzyme DICER impairs myelination during development and after injury (Pereira 2010; Viader et al., 2011). Deletion of another component of miRNA processing, *Dgcr8*, similarly causes defective myelination, but results also in upregulation of a number of genes that are upregulated after injury, including *Sox2,* and repair program genes, such as *Shh*, *Gdnf*, *c-Jun* and *Olig1* (Lin et al., 2015). MiR-9 in particular has been implicated in negative regulation of the injury response (Zhou et al., 2014). Taken together, this suggests that miRNAs repress the repair program and promote myelin gene expression. Interestingly, c-Jun controls expression of a number of miRNAs, including miR21 and miR34 (Arthur-Farraj et al., 2017).

Two other epigenetic mechansims that may have a role in repair Schwann cells are long non-coding RNAs (LncRNAs) and DNA methylation. LncRNAs, are RNA molecules greater

than 200 base pairs in length with no coding potential. There is a significant change in LncRNA expression after nerve injury, but the function of these molecules in repair Schwann cells is currently unknown (Arthur-Farraj et al., 2017). In contrast, there are only limited changes in genome-wide CpGDNA methylation after nerve injury, which argues against global regulation of gene expression through this mechanism in nerve repair (Arthur-Farraj et al., 2017). DNA methylation has been shown to regulate developmental myelination but it is not known whether it also regulates re-myelination after injury (Varela-Rey et al., 2014).

Distinctive control mechanisms in repair cells

In a number of cases, the molecular signals that control the Schwann cell injury response are not important for Schwann cell development, which is consistent with the view that injury generates a distinct repair-supportive Schwann cell. This includes c-Jun and Merlin. While these proteins are essential for the generation of functional repair cells as discussed above, c-Jun does not have a significant role in early Schwann cells, and Schwann cell development shows only minor impairments in mice without Merlin (Arthur-Farraj et al., 2012; Mindos et al., 2017). Similarly, developmental myelination is not controlled by the chromatin cross talk that in repair cells promotes silencing of myelin genes and activation of injury genes, and involves H3K27 demethylation coupled with H3K27 acetylation and H3K4 methylation (Hung et al., 2015; reviewed Ma & Svaren 2018). STAT3 likewise is not involved in Schwann cell development although this factor is important for long-term repair cell maintenance (Benito et al., 2017). Further, the proliferation of adult Schwann cells that is triggered by injury and associated with the generation of repair cells requires cyclin D1, although this protein is not involved in the proliferation of developing Schwan cells (Kim et al., 2000). Other differences between repair and developing cells concern neuregulin-1 and ERK1/2 as outlined earlier. Thus repair cells, but not developing Schwann cells, activate an autocrine neuregulin signalling loop that supports re-myelination, while axon-associated neuregulin-1 acts only as a timer for repair cell re-myelination, although it is essential for developmental myelination (Fricker et al., 2013; Stassart et al., 2013). Further, enhanced ERK1/2 increases myelin thickness during development, but not during re-myelination (Ishi et al., 2013; Cervellini et al., 2018).

These data show that the reprogramming of myelin and Remak cells to repair cells involves dedicated injury-associated control systems that are not important in developing or undamaged, normal nerves. Interestingly, Schwan cell reprogramming has now been found to involve another set of gene changes that are not connected to Schwann cell development, namely that associated with epithelial mesenchymal transition (EMT). EMT is also

associated with tissue damage in many other systems. We will now outline the general association between EMT and injury, and the role of EMT in the Schwann cell injury response.

Activation of EMT and stemness genes after tissue injury

EMT represents a process of cellular reprogramming that was initially conceived as a transition between two states, involving the wholesale conversion of epithelial cells to a motile mesenchymal phenotype (reviewed in Yang & Weinberg 2008; Thiery et al., 2009; Forte et al., 2017; Kim et al., 2017; Skrypek et al., 2017). EMT has been intensively studied in development and cancer, and is now known to be controlled by multiple signals, including secreted signals such as the TGFβ family, transcription factors including the Snail, Slug, Zeb and Twist families and others, in addition to miRNAs, e.g. the miR-200 family. EMT is characteristically associated with down-regulation of molecules that promote cell-cell adhesion, increased capacity for migration and matrix invasion, increased morphological plasticity including loss of cell polarity, and proliferation. Recently, three important developments have modified the classical view of EMT.

First, it has become clear that rather than an all-or-none response, EMT-like changes are often graded, or partial. Evidence for partial EMT comes from developing systems, cancer, fibrosis and injury (reviewed in Grigore et al., 2016; Nieto et al., 2016). This work has demonstrated the existence of a heterogenous group of intermediate cellular states, which share both epithelial and mesenchymal features, showing a graded decrease in cell polarity and cell-cell adhesion often without resulting in frank cellular dispersion or loss of lineage identity.

Second, partial EMT has now been established as a normal physiological response to tissue injury, serving as a component of the healing mechanism. This is unsurprising because many of the features originally identified as characteristic of EMT in developing systems and listed above, are also important for an effective response of adult tissues to injury. Injury-induced EMT occurs in many different systems (reviewed in Weber et al., 2012; Saw & Martin 2016; Nieto et al., 2016), in addition to peripheral nerve, as discussed further below. This includes, zebrafish heart, basal cells in the airway, ovarian epithelium, CNS meninges, skin wounds and kidney tubules. In skin wounds, for instance, partial EMT is triggered in the cells at the wound boundary. This involves the down-regulation of adherens and tight junctions, which promotes healing by allowing previously immobile epithelial cells to move and re-epithelialize the damaged area, in a process regulated by the classical EMT driver Slug (Hudson et al., 2009; Nunan et al., 2015; reviewed in Saw & Martin 2016). In the

kidney, obstruction or chemical injury induces partial EMT in the epithelial cells of the renal tubules, which also depends on Slug activation. The cells lose morphological differentiation and polarity markers, and express cytokines to attract macrophages, yet remain in the wall of the renal tubule and retain contact with the basal lamina they contacted before the injury (Grande et al., 2015; Lovisa et al., 2015). This response is reminiscent of the reprogramming events that generate repair Schwann cell in the distal nerve stump after injury, as discussed in a previous section.

Third, activation of EMT in injury and many other situations is frequently associated with activation of genes typical of stem cell states, and increased stemness (reviewed in Fabergat et al., 2016; Liao & Yang 2016). Stemness is associated with loosened phenotypic restrictions and enhanced plasticity. Therefore, the strong correlations between stemness and EMT, in particular intermediate or partial EMT (Nieto et al., 2016; Forte et al., 2017), is in line with the notion that EMT represents cellular reprogramming and a change of differentiation state. The EMT/stemness association is shown for instance by the findings that typical inducers of EMT, such as Snail, Slug or TGFβ, also activate stem cell properties (Mani et al., 2008; Morel et al., 2008). A stem-cell like phenotype is also induced by other EMT inducers including Zeb and Twist (reviewed in Fabregat et al., 2016). In line with this, invasive tumour cells express both EMT and stemness characteristics (Rhim et al., 2012; reviewed in Jordan et al., 2011).

Although the implications of the intriguing association between ETM and stemness has been the subject of much speculation, particularly in cancer, the significance may be clearer in the context of tissue injury, This is because in many cases, the injury response requires not only the classical EMT attributes of motility, morphological flexibility and proliferation, but also enhanced plasticity as surviving cells change differentiation states to acquire phenotypes adapted to promote healing, as discussed in the previous section on adaptive reprogramming. The combined activation of EMT/stemness programmes in injured tissues therefore appears strongly adaptive and biologically useful. As discussed below, this mechanism has now been established in peripheral nerves.

Repair Schwann cells undergo partial EMT.

Two recent papers show the involvement of an EMT-like process in Schwann cells of injured nerves (Arthur-Farraj et al 2017; Clements et al. 2017). RNA-Seq performed on seven day injured whole nerves demonstrates enrichment for EMT mRNAs and miRNAs. This involves down-regulation of RNAs associated with mesenchymal-epithelial transition (MET), including *E-cadherin*, *Wt1*, *Fgf1*,*Ndrg1*, mir30, mir33 andmir137, and up-regulation of EMT associated

RNAs, including *Tgf-β1*, *Met*, Hmga2, mir21,mir221 and mir222. c-Jun target genes are also enriched for an EMT signature (Arthur-Farraj et al., 2017). Similarly, a study involving FACs sorting of td-tomato labelled Schwann cells from the tissue bridge and from the distal stump of injured nerves shows enrichment for EMT genes (Clements et al.2017). In line with the close relationship between EMT and stemness (Mani et al. 2008; Morel et al. 2008), this study also showed activation of the Myc stemness and Core pluripotency modules and suppression of polycomb-related factors.

In the distal stump, tissue remodelling involves converting myelin and Remak cells into repair cells in Bungner bands (reviewed in Jessen and Mirsky 2016), while in the bridge it involves the formation of new tissue by migrating Schwann cells, together with fibroblasts and immune cells (reviewed in Cattin & Lloyd 2016). Interestingly, EMT/stemness changes in Schwann cells were even more prominent in the bridge, where they were associated with localized signalling by TGFβ, a typical inducer of EMT and stem cell properties (Clements et al., 2017).

Conclusions

Recent work has underlined the complexity of the Schwann cell injury response, and established the involvement of novel epigenetic mechanisms and morphogenetic transformations in this process. Significant issues have also been brought into focus. One of these is the close similarity between the way nerves and other tissues respond to injury. For instance, Schwann cells activate EMT/stemness-like programmes during injury-induced tissue remodelling, as seen after damage in many other systems. Further, like cells in a number of other tissues, Schwann cells adopt an alternative differentiation state after injury, which is adapted to meet the particular needs that arise after damage. On the other hand, the distinctive nature of the Schwann cell injury response has also emerged, since it is now clear that it is controlled by molecular mechanisms a number of which have no or little role elsewhere in the Schwann cell lineage. The observation that repair Schwann cells are regulated by dedicated mechanisms, and the large number of novel signals that have recently been implicated in this process, suggest that pharmacological or genetic tools can be developed to manipulate these particular cells. This is a useful development from the translation point of view, because there is a strong clinical need to learn how to amplify the repair-supportive functions of denervated Schwann cells, and how to prevent their deterioration in older age and during the prolonged periods required for axonal regeneration in human nerves.

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Figure legends

Figure 1. Major cell components of regenerating nerves

The diagram shows the proximal and distal stumps and the bridge that connects them. (a) regeneration unit, (b) blood vessel, (c) Schwann cells migrating from the distal stump, (d) macrophages and fibroblasts, (e) Bungner bands. The basal lamina that covers the Schwann cells in the proximal stump and the Bungner bands in the distal stump is not shown. In crushed nerves, this basal lamina and surrounding connective tissue remain uninterrupted, allowing axons to regenerate within their original basal lamina tubes from the proximal nerve and along the distal stump. In cut nerves, axons accompanied by Schwann cells must traverse the bridge and successfully associate with a regeneration track for regeneration and target re-innervation to be possible. The length of the bridge depends on the extent of the injury and the type of surgical intervention.

Figure 2. Regeneration tracks (Bungner bands)

Electron micrograph showing a transverse section through the distal stump of the mouse tibial nerve 4 weeks after transection (regeneration prevented). Several Bungners bands (example indicated at B) are visible, one of which shows a repair cell nucleus (N). The number of cell profiles differs considerably between the bands, each of which is surrounded by a basal lamina (examples arrowed) and collagen-rich endoneurial connective tissue. This represents the terrain along which axons grow during the often extended period of nerve repair in larger animals.

Figure 3. Signals that regulate the Schwann cell injury response and re-myelination

The diagram shows the repair Schwann cell in the context of other cells in the Schwann cell lineage, and indicates the major molecular systems implicated in any aspect of repair cell generation (left of double vertical arrows) and myelination/re-myelination (right of double vertical arrows). ΔH3K27/H3K4: loss of H3K27 trimethylation and gain of H3K4 methylation; TGFβR: TGFβ receptors; NRG: neuregulin-1; for other abbreviations and references see text. The developmental options of Schwann cell precursors are also indicated (for references see Jessen et al 2015b).

Table 1. Summary of in vivo phenotypes caused by genetic modifications in Schwann cells

SC: Schwann cell; BNB : Blood nerve barrier

Figures

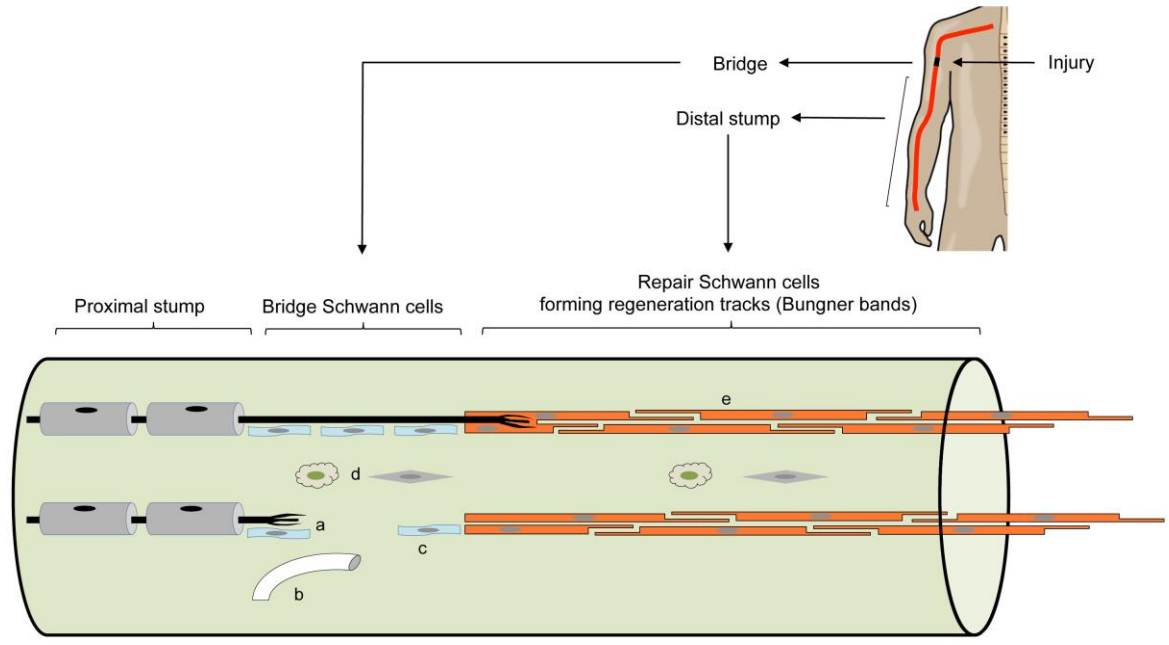

Figure 1

Figure 1

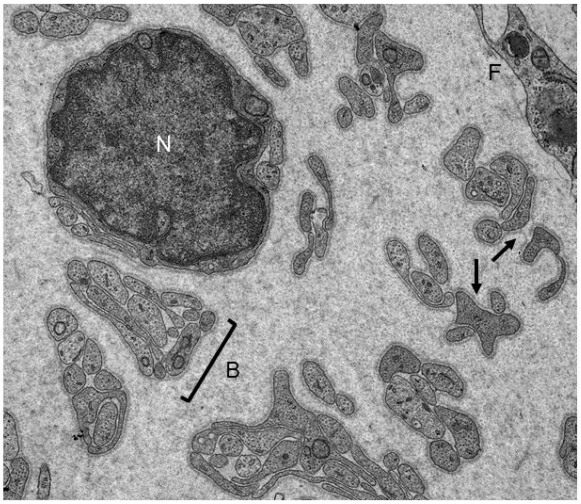

Figure 2

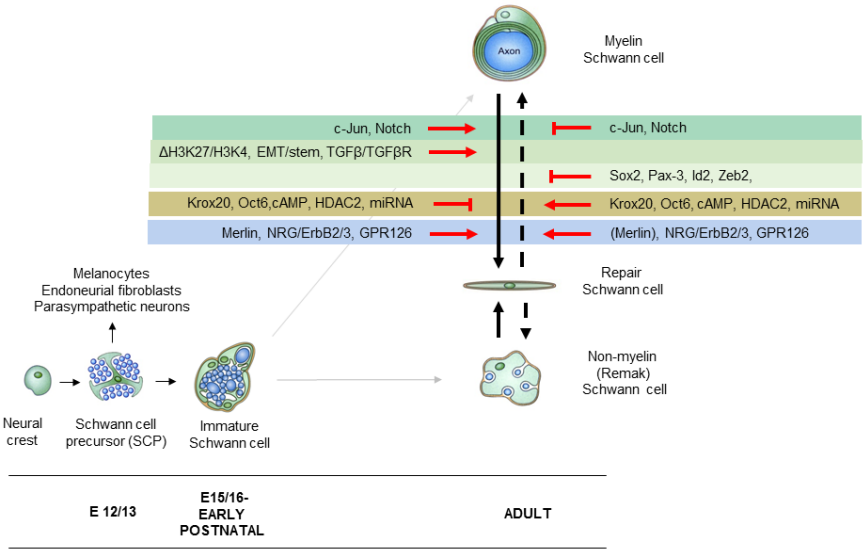

Figure 3