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Regulation of Schwann Cell Differentiation and Proliferation by the Pax-3 Transcription Factor

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Abstract

Pax-3 is a paired domain transcription factor that plays many roles during vertebrate development. In the Schwann cell lineage, Pax-3 is expressed at an early stage in Schwann cells precursors of the embryonic nerve, is maintained in the nonmyelinating cells of the adult nerve, and is upregulated in Schwann cells after peripheral nerve injury. Consistent with this expression pattern, Pax-3 has previously been shown to play a role in repressing the expression of the myelin basic protein gene in Schwann cells. We have studied the role of Pax-3 in Schwann cells and have found that it controls not only the regulation of cell differentiation but also the survival and proliferation of Schwann cells. Pax-3 expression blocks both the induction of Oct-6 and Krox-20 (K20) by cyclic AMP and completely inhibits the ability of K20, the physiological regulator of myelination in the peripheral nervous system, to induce myelin gene expression in Schwann cells. In contrast to other inhibitors of myelination, we find that Pax-3 represses myelin gene expression in a c-Jun-independent manner. In addition to this, we find that Pax-3 expression alone is sufficient to inhibit the induction of apoptosis by TGF β 1 in Schwann cells. Expression of Pax-3 is also sufficient to induce the proliferation of Schwann cells in the absence of added growth factors and to reverse K20-induced exit from the cell cycle. These findings indicate new roles for the Pax-3 transcription factor in controlling the differentiation and proliferation of Schwann cells during development and after peripheral nerve injury.

Keywords

Schwann; Pax-3; myelination; Krox-20; proliferation

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Introduction

The Pax family of transcription factors consists of nine members, which play pivotal roles in the development of many different cell lineages (Lang et al., 2007). The paired-homeodomain domain transcription factor Pax-3, through the analysis of the *Spotch* and *Spotch-delayed* Pax-3 mutant alleles in mice, has been shown to play a key role in limb muscle development, and lack of functional Pax-3 leads to abnormalities in the formation of derivatives of the neural crest such as melanocytes, spinal ganglia, Schwann cells, and cardiac structures (Bober et al., 1994; Epstein, 1996; Franz, 1990; Goulding and Paquette, 1994). For the Schwann cells of the peripheral nervous system, *Spotch* mutants, which die at embryonic day (E) 13.5, have a complete lack of Schwann cells, whereas *Spotch-delayed* embryos show reduced numbers of Schwann cells at birth (Franz, 1990). More recently, examination of Pax-3 mRNA expression using either *in situ* hybridization or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis has shown expression in both rats and mice from embryonic Schwann cell precursors [E12 mouse/E14 rat] through until birth and the early postnatal period (Blanchard et al., 1996; Kiousi et al., 1995). An examination of later postnatal stages found that Pax-3 expression remained in nonmyelinating Schwann cells of the adult sciatic nerve and nonmyelinated cervical sympathetic trunk (Blanchard et al., 1996; Kiousi et al., 1995). In addition, it was also shown that Pax-3 expression inhibited the induction of myelin basic protein (MBP) and the activation of an MBP promoter construct by cyclic AMP in Schwann cells (Kiousi et al., 1995).

We have performed experiments in Schwann cells to observe the interplay between Pax-3 and Krox-20 (K20; Egr2), the physiological regulator of myelination *in vivo* (Topilko et al., 1994; Zorick et al., 1999), and to find that Pax-3 completely opposes the ability of K20 to both induce myelin gene expression and to cause withdrawal from the cell cycle (Parkinson et al., 2004, 2008). Furthermore, we find that Pax-3 expression alone is sufficient to induce the proliferation of Schwann cells in culture and to inhibit the apoptosis of Schwann cells in response to TGF β *in vitro*. Our previous work has defined a role for the transcription factor c-Jun in the inhibition of myelin gene expression and in promoting the dedifferentiation of Schwann cells following nerve injury (Parkinson et al., 2008). Experiments in c-Jun null cells show that Pax-3 represses K20-driven myelin gene expression in a c-Jun-independent manner, thereby revealing a novel mechanism for this inhibition of myelination. These findings further characterize the possible roles of Pax-3 in controlling Schwann cell proliferation and development in the peripheral nervous system.

Materials and Methods

Materials

The LacZ/GFP control and Pax-3/GFP-expressing adenoviruses were gifts from Paul Hamel (Toronto, Canada; Wiggan and Hamel, 2002). Control adenovirus and adenovirus-expressing CRE recombinase were gifts from Axel Behrens (Cancer Research UK, London). GFP control and K20/GFP-expressing adenoviruses were gifts from Jeff Milbrandt (Nagarajan et al., 2001). The c-Jun N-terminal kinase (JNK) inhibitor SP600125 (Bennett et al., 2001) was obtained from Affiniti Research (Exeter, UK). The p38 MAP kinase inhibitors SB202190

and SB203580 were obtained from Calbiochem (Nottingham, UK). Reverse transcriptase, random hexamers, and Taq DNA polymerase were obtained from Promega (Southampton, UK). Sox-10 antibodies were gifts from Michael Wegner (University of Erlangen, Germany) and James Briscoe (National Institute for Medical Research, London, UK). Antibodies to ErbB2, ErbB3, Oct-6, p21, and NGFI-A/Egr-binding co-repressor 2 (NAB2) were obtained from Santa Cruz (London, UK); monoclonal antibody to P-zero (P_0) was a gift from Juan Archelos (Archelos et al., 1993); antibody to periaxin was a gift from Peter Brophy (University of Edinburgh, Scotland; Gillespie et al., 1994); and antibody to JNK-interacting protein (JIP-1) was a gift from Roger Davis (Howard Hughes Medical Institute, MA; Yasuda et al., 1999). Antibody to c-Jun was obtained from BD Transduction Laboratories (Oxford, UK), and antibodies to serine-63 phospho-cJun, phospho-JNK, phospho-Akt phospho-p38, and PDGFR- β were obtained from New England Biolabs (Hitchin, UK). Antibody to K20 was obtained from Cambridge Bioscience (Cambridge, UK); antibody to bromodeoxyuridine (BrdU) from Roche (Welwyn Garden City, UK); and antibody to S100 β from Dako (Ely, UK). Antibody to β -tubulin was obtained from Sigma (Poole, UK), and antibody to Ki67 from Abcam (Cambridge, UK). Plasmids expressing the KRAB-Pax-3 and KRAB(DV)-Pax-3 proteins were obtained from Frank Rauscher III (Wistar Institute, Philadelphia, PA; Fredericks et al., 2000). Recombinant human EGF domain of neuregulin-1 (NRG-1) was purchased from R&D Systems (Abingdon, UK). Sources of other reagents have been detailed elsewhere (Archelos et al., 1993; Morgan et al., 1991, 1994; Parkinson et al., 2004, 2008).

Culture, Transfection, and Adenoviral Infection of Schwann Cells

Schwann cells were purified from sciatic nerve and brachial plexus from newborn or postnatal Day 3 rats as previously described (Dong et al., 1999; Morgan et al., 1991). Schwann cells from c-Jun^{fl/fl} mice (Behrens et al., 2002) were prepared using serum purification (Brookes et al., 1979). Cells were cultured, unless otherwise stated, in defined medium (Jessen et al., 1994) containing 0.5% fetal calf serum and 10^{-6} M insulin, referred to as DM. Transfection and adenoviral infection of Schwann cells were performed as described previously (Parkinson et al., 2001, 2003, 2004).

Immunocytochemistry and Western Blotting

Immunolabeling with BrdU and P_0 antibodies was performed as described previously (Morgan et al., 1991; Stewart et al., 1993, 1996). For all other antibodies, cells were fixed in 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) (pH 7.5) for 10 min. Cells were then permeabilized and blocked in antibody diluting solution (ADS: PBS containing 10% calf serum, 0.1 M lysine, and 0.02% azide), supplemented with 0.2% Triton X-100, for 30 min. Primary and secondary antibodies were diluted in ADS, with the secondary antibody conjugated to FITC, Cy3, or Cy5. Western blotting was performed as previously described (Parkinson et al., 2004).

Scanning Electron Microscopy (SEM)

For scanning electron microscopy, rat Schwann cells were cultured on glass cover slips and infected with Pax-3/GFP-expressing adenovirus as above. Forty-eight hours after the addition of adenovirus, cover slips were fixed in 2.5% w/v glutaraldehyde, diluted in 0.2 M

sodium cacodylate buffer, at room temperature for 2 h and then washed twice in 0.2 M sodium cacodylate buffer. Cover slips were washed in 30, 50, 70, 90, and 100% absolute ethanol for 15 min each at room temperature. Samples then underwent critical point drying through CO₂ in a K850 Critical Point Dryer (Emitech, Ashford, UK), and cover slips were mounted on support stubs and gold coated (nominal thickness = 10 nm) in a K550 Sputter Coater (Emitech). Samples were imaged on a JSM-5600LV scanning electron microscope (JEOL (UK) Ltd., Welwyn Garden City, UK) operated at 15 kV, with an 11-mm working distance and at 1100× magnification.

Semiquantitative RNA Analysis

Total RNA was extracted using an RNeasy purification kit (Qiagen UK Ltd., Crawley, UK) from rat Schwann cells infected with either control LacZ or Pax-3-expressing adenoviruses 48 h after infection. cDNA was synthesized using reverse transcriptase and amplified using specific primers to measure levels of c-Jun or Pax-3 mRNAs using 18S rRNA as an internal control. The following primer sequences were used: c-Jun: forward 5'-gatggaaacgaccttctacgac-3' and reverse 5'-agcgtattctggc tatgcagtt-3' (product size = 369 base pairs); Pax-3: forward 5'-caatcagctcggaggagtatt-3' and reverse 5'-taaa catgcctgggttctct-3' (product size = 275 base pairs); and 18S rRNA: forward 5'-cctcgaaagagtctctgta-3' and reverse 5'-gggaacgcgtgcattat-3' (product size = 341 base pairs). All measurements of c-Jun and Pax-3 mRNA levels were made in triplicate from independent RNA samples from control LacZ and Pax-3-expressing Schwann cells.

Results

Pax-3 Inhibits K20-Mediated Expression of P₀ and Periaxin in Schwann Cells

Previous findings have shown that Pax-3 is expressed in embryonic and nonmyelinating Schwann cells and acts to inhibit the cyclic AMP induction of MBP in Schwann cells (Kioussi et al., 1995). Therefore, we tested whether Pax-3 would inhibit regulation of myelin genes by the physiological inducer of myelination, the zinc finger transcription factor K20 (also known as Egr2). Using either a control LacZ/GFP-expressing adenovirus (LacZ) or a Flag-tagged-Pax-3/GFP (Pax-3)-expressing virus (Wiggin et al., 2002), we performed coinfections with K20 and measured the effects of Pax-3 expression on the K20-dependent induction of P₀, periaxin, NAB2, and JIP-1 proteins in Schwann cells. Pax-3 expression almost completely inhibits the induction of P₀ and periaxin by K20 (Fig. 1A and H-L). Analysis of the K20 target, the MAP kinase scaffold protein JIP-1 (Parkinson et al., 2004), showed that Pax-3 also inhibited the induction of this protein by K20 (Fig. 1A).

In addition, using *in situ* hybridization, we observed that Pax-3 inhibited the induction of P₀ mRNA by K20 (Fig. 1B,C). Immunolabeling and Western blotting of cells co-infected with K20 and Pax-3 show that K20 is still strongly expressed and localized to the nucleus of the cells, but is unable to drive P₀ and periaxin expression (data not shown; Fig. 1A). Rat Schwann cells in culture express basal levels of P₀ protein, which is suppressed in nonmyelinating Schwann cells. This basal level of P₀ protein expression may be detected by immunocytochemistry using a P₀ polyclonal antibody (Lee et al., 1997). Immunolabeling of Schwann cells infected with LacZ/GFP control virus shows basal levels of P₀ protein (D and

E), whereas in Schwann cells infected with Pax-3 virus, P₀ protein is undetectable (F and G). NAB2 is a target of K20 in Schwann cells (Srinivasan et al., 2007); however, unlike P₀, periaxin, and JIP-1, NAB2 induction by K20 was unaffected by Pax-3 coexpression.

Having observed that Pax-3 prevents the induction of periaxin by K20, we next tested whether inhibition of endogenous Pax-3 activity would enhance the ability of K20 to induce periaxin in Schwann cells. Previously, expression of a construct consisting of the DNA-binding region of Pax-3 fused to a KRAB domain has been used to inhibit endogenous Pax-3 transcriptional activity in cells and to inhibit the malignant phenotype of tumor cells transformed by a Pax3-FHCR fusion protein (Fredericks et al., 2000). We performed transfections of Schwann cells with a low concentration of K20/GFP-expressing plasmid [pBABEG-K20 (Parkinson et al., 2003); 0.2 µg plasmid per cover slip], which is sufficient to only weakly induce periaxin in 40.9% ± 6.2% of transfected cells. Co-transfection of K20 with the Pax3-KRAB construct gave much higher levels of periaxin protein in 68.6% ± 8.8% transfected cells ($P < 0.001$; Fig. 1M–Q). Co-transfection of K20 with the Pax3-KRAB(DV) construct, in which two amino acids of the KRAB domain have been mutated to alanine to create an inactive KRAB domain (Fredericks et al., 2000), does not enhance K20 induction of periaxin (Fig. 1Q).

Induction of Oct-6 and K20 by Cyclic AMP Is Blocked by Pax-3

Previous work has shown that activity of the JNK pathway or the expression of c-Jun is sufficient to block K20, but not Oct-6 induction in Schwann cells in response to cyclic AMP (Parkinson et al., 2008). Therefore, we next tested whether Pax-3 overexpression would also block induction of Oct-6 or K20. Immunolabeling and Western blotting showed that Pax-3 strongly blocks induction of both Oct-6 and K20 by cyclic AMP in Schwann cells (Fig. 2A–G). The induction of both Oct-6 and K20 is controlled by Sox-10 in Schwann cells (Finzsch et al., 2010; Ghislain and Charnay, 2006; Jagalur et al., 2011; Reiprich et al., 2010), and therefore, we tested the possibility that repression of Sox-10 by Pax-3 caused the inhibition of Oct-6 and K20 induction. By both immunolabeling and Western blotting, we found that levels of Sox-10 protein were slightly reduced by Pax-3 expression (Fig. 2H–L). Immunolabeling with antibody to the Schwann cell-specific marker S100b confirmed that Pax-3-expressing cells are still S100-positive (Fig. 2M,N).

Expression of Pax-3 Is Sufficient to Induce Proliferation in Schwann Cells

The expression pattern of Pax-3, together with our observations that there appeared to be an increase in cell number after Pax-3 expression, led us to test whether Pax-3 expression was sufficient to induce cell proliferation in Schwann cells. BrdU labeling of Schwann cells showed that, in defined medium alone, Pax-3 expression was sufficient to induce DNA synthesis. The proliferation of Pax-3-expressing cells was further induced by the addition of β-NRG-1, NRG-1 (20 ng/ml; Fig. 3A–C). Proliferation of Schwann cells induced by Pax-3 expression was also confirmed using the proliferation marker Ki67 (Fig. 3I–K). Although proliferative, Pax-3-expressing cells did not overgrow one another and always demonstrated contact inhibition of growth in these assays (Fig. 3G,H). Pax-3-induced proliferation, measured using either BrdU or Ki67, was seen only in areas of lower cell density and not in fully confluent cells. We next tested the requirement for MAP kinase signaling for Pax-3-

induced proliferation in cells. Previous findings have identified a requirement for the extracellular signal-regulated kinase (MEK1/2-ERK1/2), JNK, and phospho-inositide 3-kinase (PI3-K) pathways for Schwann cell proliferation in response to NRG-1 (Arthur-Farraj et al., 2006; Maurel and Salzer, 2000; Monje et al., 2006; Parkinson et al., 2004). We examined activation of these MAP kinase pathways in Schwann cells and found increased ERK1/2 phosphorylation in Pax-3-expressing cells. Phosphorylation of Akt (as a measure of PI3-kinase activity) was also slightly increased, whereas both JNK and p38 phosphorylation were detectable but slightly decreased (Fig. 3D). We found that inhibition of the MEK/ERK, JNK, or PI3-K signaling pathways strongly reduced Pax-3-induced proliferation in cells, both in the absence and presence of added NRG-1 (Fig. 3F). We next examined whether activity of the p38 MAP kinase pathway was required for Pax-3-induced proliferation. In other experiments, addition of the p38 inhibitor completely prevented NRG-1-induced proliferation of Schwann cells (data not shown). Inhibition of the p38 MAP kinase pathway, using either the inhibitors SB202190 or SB203580, had no effect on the rate of proliferation in Pax-3-expressing cells in defined medium alone or Pax-3-induced proliferation in the presence of NRG-1 (Fig. 3F). Increased activity of the ERK1/2 pathway has been shown to elevate levels of the cell cycle inhibitor p21 in Schwann cells (Lloyd et al., 1997), and Western blotting (Fig. 3E) shows that Pax-3 increases p21 expression in Schwann cells at 48 and 72 h after adenoviral infection; no increase was seen in GFP/LacZ control cells. The levels of ErbB2 and ErbB3 receptors were unchanged by Pax-3 expression (Fig. 3E). The levels of nuclear p21 protein increase after peripheral nerve injury, and analysis of p21 null nerves has shown that it acts to limit the injury-induced proliferation of Schwann cells (Atanasoski et al., 2006). We observed a time-dependent increase in the number of Pax-3-infected cells with nuclear expression of p21, with $9.3\% \pm 2.8\%$ at 48 h, $13.1\% \pm 3.9\%$ at 72 h, and $26.9\% \pm 2.0\%$ at 96 h after infection with Pax-3 adenovirus. Double immunolabeling for p21 and Ki67 72 h after infection shows that Pax-3-infected Schwann cells with nuclear p21 stain are Ki67-negative (Fig. 3I-K).

Pax-3 Reverses K20-Induced Cell Cycle Arrest

We have previously demonstrated that expression of K20 inhibits NRG-1-dependent proliferation of Schwann cells in culture (Parkinson et al., 2004). We next tested whether expression of Pax-3 was sufficient to overcome the block in proliferation imposed by K20. Rat Schwann cells, retrovirally infected with either control (BP2) or K20-expressing constructs, were infected with either GFP/LacZ control or GFP/Pax-3-expressing adenoviruses and cell proliferation monitored by BrdU incorporation. As would be expected, NRG-1 induces proliferation in the control (BP2) cells infected with control GFP/LacZ adenovirus (BP2/LacZ), but NRG-1-induced proliferation is reduced in K20-expressing cells infected with GFP/LacZ virus (K20/LacZ). To our surprise, expression of Pax-3, either in the absence or presence of NRG-1, is sufficient to strongly induce proliferation in K20-expressing cells (Fig. 4). Immunolabeling of K20-expressing cells infected with Pax-3 virus showed that Pax-3 expression also downregulated periaxin and P₀ expression under these conditions, demonstrating that Pax-3 may also reverse the induction of these myelinating Schwann cell markers despite ongoing K20 expression (data not shown). These experiments clearly show that Pax-3 can not only inhibit the K20-dependent induction of myelin gene expression when both transcription factors are co-expressed at the same time (Fig. 1), but

also, more impressively, inhibit and reverse K20-dependent established myelin gene expression in Schwann cells.

Pax-3 Expression Reduces c-Jun Levels and Promotes Schwann Cell Survival

Having observed the effects of Pax-3 on cell proliferation, we next tested the effect of Pax-3 expression on Schwann cell survival *in vitro*. Signaling by the growth factor TGF β regulates Schwann cell survival both *in vitro* and *in vivo* (D'Antonio et al., 2006; Parkinson et al., 2001), and we have previously shown that K20 expression in Schwann cells prevents apoptosis induced by TGF β 1 (Parkinson et al., 2004). Using an *in vitro* assay and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) to measure apoptotic cells (Parkinson et al., 2001, 2004), we measured the ability of TGF β 1 to induce apoptosis in control (LacZ) or Pax-3-expressing cells. Pax-3 expression in Schwann cells significantly ($P < 0.001$) increased Schwann cell survival both in the absence and presence of 20 ng/ml TGF β 1 (Fig. 5G). TGF β -induced apoptosis requires c-Jun activity (Parkinson et al., 2001), and therefore, we next measured c-Jun levels in Pax-3-expressing cells when compared with control LacZ-infected cells. By both immunolabeling and Western blot (Fig. 5A–E), we observed a downregulation of both phospho-cJun and c-Jun protein levels in Pax-3-expressing cells when compared with control cells. Measurement of mRNA levels by PCR showed no significant repression of c-Jun mRNA by Pax-3 (Fig. 5F). Pax-3 has also been shown to activate the promoter of the antiapoptotic protein Bcl-X_L (Margue et al., 2000); however by both Western blot and immunolabeling, we have not observed any significant upregulation of Bcl-X_L in Pax-3 overexpressing Schwann cells (data not shown).

Pax-3 Inhibits K20-Driven Myelin Gene Expression in a c-Jun-Independent Manner

We have previously shown that the c-Jun transcription factor inhibits both K20-driven myelin gene expression and plays a role in promoting the dedifferentiation of Schwann cells after peripheral nerve injury (Parkinson et al., 2008). We next tested whether the repression of myelin gene expression by Pax-3 required c-Jun in Schwann cells. Schwann cells were purified from mice carrying a floxed c-Jun allele (c-Jun^{fl/fl}; Behrens et al., 2002) and infected *in vitro* with adenoviruses either expressing CRE recombinase to remove c-Jun or GFP as a control. Immunolabeling with c-Jun antibody confirmed that c-Jun was excised in greater than 99% of cells (data not shown). Next, we performed co-infections with either K20 plus GFP/LacZ control (K20/LacZ) or K20 plus GFP/Pax-3 (K20/Pax3) into c-Jun control or c-Jun null cells and measured induction of periaxin by immunocytochemistry. As previously reported (Parkinson et al., 2008), we observed a stronger induction of periaxin by K20 (K20/LacZ) in the c-Jun null cells when compared with the c-Jun control cells (Fig. 6). However, we found almost complete repression of periaxin by Pax-3 even in c-Jun null cells, demonstrating that Pax-3 does not require c-Jun for the repression of myelin gene expression in cells. Similarly for the K20 induction of P₀ protein, Pax-3 could repress P₀ expression in a c-Jun-independent manner (data not shown). Consistent with this finding, we did not observe re-expression of either c-Jun or the HMG transcription factor Sox-2 (Le et al., 2005; Parkinson et al., 2008) in control K20/Pax-3 coexpressing cells (data not shown).

Discussion

The loss of Pax-3 function during development in the natural mouse mutants *Spotch* and *Spotch Delayed* has identified the importance for this transcription factor in both muscle development and the formation of derivatives of the neural crest. In the Schwann cell lineage, the analysis of Pax-3 mutants revealed either a complete lack of Schwann cell precursors in the *Spotch* mutant or greatly reduced numbers of Schwann cells at birth in the *Spotch-Delayed* mutant (Franz, 1993). It is unclear whether the lack of Schwann cells in the *Spotch-Delayed* mutant was due to the lack of differentiation of Schwann cell precursors from the neural crest or a reduction in the survival or the proliferation of Schwann cell precursors in this mutant. In this regard, our findings that Pax-3 expression both enhances the survival and is sufficient alone to induce the proliferation of Schwann cells are of interest. Previous analysis of the *Spotch* mutant has identified increased apoptosis associated with the neural tube defects in these embryos (Phelan et al., 1997), and later work has shown that breeding the *Spotch* mutant onto a p53 null background rescues the neural tube defect in these animals (Pani et al., 2002). We show that Pax-3 expression downregulated c-Jun expression in Schwann cells and blocked the induction of apoptosis by TGF β 1 *in vitro*, a growth factor we have shown to regulate cell death both during development and after peripheral nerve injury (D'Antonio et al., 2006; Parkinson et al., 2001). We do not find, however, that Pax-3 reduces levels of c-Jun mRNA in Schwann cells, but levels of both phospho-cJun and c-Jun are also regulated by several ubiquitin ligases and consequent degradation by the proteasomal pathway in cells (Babaei-Jadidi et al., 2011; Nateri et al., 2004), and we did find that addition of the proteasomal inhibitor MG-132 prevented the downregulation of c-Jun protein by Pax-3 in Schwann cells (data not shown). Previous data have identified the prosurvival gene *Bcl-X_L* as target of Pax-3 in both normal and neoplastic myocytes and to activate the *Bcl-X_L* promoter in transfection assays, and this has been speculated to underlie the antiapoptotic effect of both Pax-3 and the Pax-3/Forkhead transcription factor fusion protein (Pax-3/FHFR), the latter of which is associated with rhabdomyosarcoma tumors (Margue et al., 2000). By both immunolabeling and Western blot, however, we did not observe an increase in Bcl-X_L protein levels in Pax-3-expressing Schwann cells.

The regulation of cell proliferation by Pax-3 is also intriguing. Semiquantitative RT-PCR analysis demonstrated a peak of Pax-3 mRNA expression at E17 in the mouse, which corresponds to the peak in proliferation at E19 in the rat Schwann cell lineage (Stewart et al., 1993). The induction of proliferation by Pax-3 is similar to that seen by the expression of the Notch intracellular domain (NICD; Li et al., 2004; Woodhoo et al., 2009), in that expression of each protein is sufficient in growth factor free conditions to induce cell division in Schwann cells. Another similarity is that for both Pax-3 and NICD, the induced proliferation is MEK1/2 and JNK dependent, but is independent of p38 MAP kinase activity in Schwann cells (Woodhoo et al., 2009), whereas proliferation induced by NRG-1 alone appears p38 dependent, possibly indicating a separate and distinct mechanism for Pax-3-inducing proliferation in Schwann cells. However, we did see that addition of the ErbB2 tyrosine kinase inhibitors GW2974 or AG1478 both inhibited induction of proliferation induced by Pax-3 alone (data not shown), indicating that signaling through an ErbB2-dependent

pathway may be involved. In control experiments, both ErbB2 inhibitors (at the same concentrations) also blocked NRG-1-induced cell proliferation, validating the efficacy of these inhibitors (data not shown). Although proliferative, Pax-3-expressing cells do not appear to be transformed in any way, they remain contact inhibited in their proliferation, and do not display the elevated levels of ErbB2, ErbB3, or PDGF- β receptors (data not shown; Fig. 3) that are seen for instance in schwannoma cell tumors (Ammoun et al., 2008; Lallemand et al., 2009).

The increase in both proliferation and levels of the p21 cell cycle inhibitor by Pax-3 in Schwann cells does seem paradoxical. The earliest induction of proliferation by Pax-3 is seen ~24 h after adenoviral infection, whereas increasing levels of p21 are not seen in cells until 48 h increasing up to 96 h (see Results section). One model to reconcile these observations would be that Pax-3 expression induces a self-limiting burst of proliferation, for instance, after nerve injury, which is then inhibited by increasing accumulation of p21 in cells. *In vivo* analysis of conditional Pax-3 null Schwann cells after peripheral nerve injury would be required to fully test this hypothesis.

In muscle satellite cells, Pax-3 levels are increased by the expression of activated Notch-1 and suppressed by the Notch antagonist Numb (Conboy and Rando, 2002); however, whether this is also the case in Schwann cells remains to be tested. However, Pax-3 cannot account for all the actions of NICD as overexpression of NICD also raises levels of ErbB2 receptor in Schwann cells (Woodhoo et al., 2009) and we observe no changes in ErbB2 or ErbB3 protein levels in Pax-3 overexpressing cells. However, both NICD and Pax-3 expression do reduce the levels of Sox-10 protein in Schwann cells (Li et al., 2004), although our *in vitro* experiments show that NICD expression appears more effective than Pax-3 at reducing Sox-10 protein levels (data not shown). Although Pax-3 does reduce Sox-10 levels in Schwann cells, it appears unlikely that this is the only mechanism for the observed inhibition of Oct-6 and K20 that we observe in Pax-3-expressing cells. Pax-3 also induces activation of the ERK1/2 pathway, activation of which alone has also been shown to inhibit both Oct-6 and K20 induction (Harrisingh et al., 2004).

Recent studies, both *in vitro* and *in vivo*, have identified a number of different signaling pathways and transcription factors that inhibit both the developmental regulation of myelination and control the formation of a denervated Schwann cell that is specialized to promote nerve repair after peripheral nerve injury. An involvement of ERK1/2, JNK, and p38 MAP and PI-3 kinase pathways has been shown to be important, as well as signaling through the Notch pathway and the transcription factors c-Jun and Sox-2, all presumably playing their part in controlling the timely onset of myelination in conjunction with promyelinating transcription factors such as Oct-6 and K20 or in driving the dedifferentiation of Schwann cells after loss of axonal contact (Guertin et al., 2005; Haines et al., 2008; Harrisingh et al., 2004; Le et al., 2005; Maurel and Salzer, 2000; Mirsky et al., 2008; Ogata et al., 2004; Parkinson et al., 2004, 2008; Svaren and Meijer, 2008; Woodhoo et al., 2009), although how these mechanisms are coordinated is by no means clear at the moment. Our data point to a likely additional role for the Pax-3 transcription factor in this process in regulating both Schwann cell proliferation and myelination.

Acknowledgments

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Fig. 1. Pax-3 inhibits Krox-20 induction of P₀, periaxin, and JIP-1, but not NAB2, expression in Schwann cells.

A: Western blot of Schwann cells co-infected with GFP/LacZ control adenoviruses (GFP/LacZ), Krox-20 plus GFP/LacZ (K20/LacZ), and Krox-20 plus GFP/Pax-3 (K20/Pax3) expressing adenoviruses. Note that Pax-3 expression almost completely inhibits the induction of P₀, periaxin, and JIP-1 by Krox-20, whereas induction of NAB2 by Krox-20 is seemingly unaffected by Pax-3 expression. **B** and **C**: Co-infection of Pax-3 with Krox-20 (K20/Pax3; Panel C) inhibits the induction of P₀ mRNA, as visualized by *in situ*

hybridization, seen in Krox-20-infected Schwann cells (K20/LacZ; Panel B). **D–G**: Pax-3 expression inhibits basal P₀ protein expression in Schwann cells. Schwann cells infected with control GFP/LacZ virus (D and E) or GFP/Pax3 virus (F and G) were immunolabeled with P₀ polyclonal antibody to detect basal levels of P₀ expression (E and G). **H–L**: Pax-3 expression inhibits Krox-20-induced periaxin induction in Schwann cells. **H–K**: Co-expression of Pax-3 with Krox-20 (K20/Pax3; Panels I and K) inhibits the induction of periaxin seen in Krox-20-infected cells (K20/LacZ; Panels H and J). **L**: Graph showing quantification of percentage periaxin/GFP-positive cells in Krox-20/LacZ control (K20/LacZ) and Krox-20/Pax-3 (K20/Pax3) co-expressing cells. **M–Q**: Inhibition of endogenous Pax-3 activity enhances Krox-20 induction of periaxin. **M–P**: Schwann cells transfected with low amounts (0.2 µg) of pBABEG/K20 plasmid co-expressing GFP and Krox-20 together with 0.4 µg of either control empty vector (K20/EV) or plasmid-expressing Pax3-KRAB construct (K20/Pax3-KRAB). Note increased levels of periaxin expression in K20/Pax3-KRAB-transfected cells (O and P) as compared to K20/EV transfected cells (M and N). **Q**: Graph showing quantification of percentage periaxin/GFP-positive cells for K20/EV, K20/Pax3-KRAB, and K20/Pax3-KRAB(DV)-transfected cells. Error bars represent one standard deviation of the mean; scale bars = 15 µm.

Fig. 2. Pax-3 expression prevents the induction of Oct-6 and Krox-20 by cAMP and reduces the levels of Sox-10 in Schwann cells.

A–D: Immunolabeling of control GFP/LacZ (LacZ; A and B) and GFP/Pax-3-infected Schwann cells (Pax3; C and D) treated for 24 h with 1 mM cAMP. Note lack of induction of nuclear Oct-6 in Pax-3-expressing Schwann cells (D). Scale bar = 15 μ m. **E:** Western blot of control (–) and cyclic AMP (+)-treated GFP/LacZ and GFP/Pax-3-expressing Schwann cells showing lack of induction of Oct-6 in Pax-3-expressing cells. **F and G:** Graphs showing percentage Oct-6 (F) and Krox-20 (G) positive control GFP/LacZ or GFP/Pax-3-expressing

Schwann cells following addition of cAMP for 24 h. Note significant ($P < 0.02$) inhibition of both Oct-6 and Krox-20 induction by Pax-3 expression. Error bars represent one standard deviation of the mean. **H–L**: Reduction of Sox-10 protein levels by Pax-3. **H–K**: Immunolabeling of control GFP/LacZ (H and I) and GFP/Pax-3-infected Schwann cells (J and K) with Sox-10 antibody. Note that GFP/Pax-3-infected cells show a slightly lower level of Sox-10 expression. **L**: Western blot showing reduced levels of Sox-10 in Pax3-expressing Schwann cells. **M** and **N**: Immunolabeling of control GFP/LacZ (M) and GFP/Pax-3-infected Schwann cells (N) showing that both control and Pax-3-expressing cells are positive for the Schwann cell marker S100 β . Scale bars = 15 μ m.

Fig. 3. Pax-3 expression induces DNA synthesis in Schwann cells.

A and B: Immunolabeling of LacZ control (A) and Pax-3-infected Schwann cells (B) cultured in the absence of β -neuregulin-1 with anti-BrdU antibody. Arrowheads indicate BrdU-positive nuclei. **C:** Graph showing percentage of BrdU-positive-infected (GFP-positive) Schwann cells in GFP/LacZ control and GFP/Pax-3-infected cultures in the absence (-NRG-1) and presence (+NRG-1) of β -neuregulin-1 (20 ng/ml). Note that Pax-3 induces DNA synthesis even in the absence of added β -neuregulin-1. **D:** Measurement of MAP kinase activity by Western blotting in control LacZ and Pax-3-expressing cells at 48 h

time point. **E**: Western blot of LacZ and Pax-3-expressing Schwann cells 48 and 72 h after infection showing increasing levels of ERK1/2 phosphorylation and p21 protein in Pax-3-expressing cells. **F**: Induction of DNA synthesis by Pax-3 requires the MEK/ERK and JNK and PI3-kinase pathways, but is independent of the p38 MAP kinase pathway. Graph of percentage BrdU-positive GFP/Pax-3-infected cells in the absence (–NRG-1) and presence of β -neuregulin-1 (+NRG-1) cultured in defined medium alone (Cont.) or in medium containing the JNK inhibitor SP600125 (SP6; 15 μ M), MEK1/2 inhibitor UO126 (UO1; 10 μ M), the PI3 kinase inhibitor LY294002 (LY; 10 μ M), or the p38 inhibitor SB202190 (SB; 10 μ M). **G** and **H**: High magnification images of GFP/Pax-3-infected cells showing either GFP fluorescence (G) or SEM (H). Note that Pax-3-expressing cells do not overgrow one another at high density. **I–K**: Immunolabeling of Pax-3-infected Schwann cells with Ki67 and p21 antibodies. Arrowheads indicate Ki67-negative nuclei (J) that show nuclear p21 expression (K). Scale bars = 15 μ m.

Fig. 4. Pax-3 expression in Schwann cells reverses the Krox-20-induced inhibition of proliferation in response to β -neuregulin-1.

Schwann cells retrovirally infected with either control empty pBABE-puro vector (BP2) or pBABEpuro vector expressing Krox-20 (K20) were infected with either GFP/LacZ control or GFP/Pax-3-expressing adenoviruses. Cells were then cultured in the absence (–NRG-1) or presence (+NRG-1) of β -neuregulin-1 (20 ng/ml), and DNA synthesis was measured by BrdU incorporation. Error bars represent one standard deviation of the mean.

Fig. 5. Regulation of Schwann cell survival by Pax-3.

A–D: Pax-3 reduces levels of c-Jun protein in Schwann cells. Immunolabeling of control LacZ/GFP (A and B) and Pax-3/GFP-infected cells (C and D) with c-Jun antibody (B and D) showing reduced levels of c-Jun protein in Pax-3-expressing cells. Scale bar = 15 μ m. **E:** Western blot analysis of c-Jun, Serine 63-phosphorylated c-Jun (P-cJun) in LacZ control, and Pax-3-expressing Schwann cells 48 and 72 h after infection. **F:** Measurement of c-Jun and Pax-3 mRNA levels by semiquantitative PCR in control LacZ and Pax-3-expressing Schwann cells. Amplification of 18S rRNA used as loading control. **G:** Graph showing

inhibition of TGF β 1-induced apoptosis by Pax-3. The percentage of TUNEL-positive apoptotic-infected GFP-positive cells in GFP/LacZ control and GFP/Pax-3-infected cells cultured in the absence (–) or presence (+) of TGF β 1 (20 ng/mL) is shown. Error bars represent one standard deviation of the mean.

Fig. 6. Pax-3 represses Krox-20 induction of periaxin in a c-Jun-independent manner.

c-Jun control and c-Jun null cells were co-infected with Krox-20 and GFP/LacZ control (K20/LacZ) or Krox-20 and GFP/Pax-3 (K20/Pax-3)-expressing adenoviruses and then immunolabeled with periaxin antibody. The percentage of periaxin-positive cells that are also GFP-positive is shown. Note that Pax-3 suppresses periaxin induction by Krox-20 even in c-Jun null cells. Error bars show one standard deviation of the mean.