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SRY-BOX CONTAINING GENE 11 (Sox11) TRANSCRIPTION FACTOR IS REQUIRED FOR NEURON SURVIVAL AND NEURITE GROWTH

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Abstract—The transcription factor Sox11 is expressed at high levels in developing sensory neurons and injured adult neurons but little is known about its transcriptional targets and function. In this study we examined the role of Sox11 using Neuro2a neuroblastoma cells and cultured mouse dorsal root ganglia (DRG) neurons. Results show Sox11 has an essential role in regulation of neuron survival and neurite outgrowth in Neuro2a cells and primary sensory neurons. Neuro2a cells increase expression of Sox11 as they differentiate in culture. Following addition of 20 μM retinoic acid (RA), a stimulus for differentiation that enhances neurite growth and differentiation, Sox11 level rises. RNAi-mediated knockdown of Sox11 in RA-differentiated Neuro2a cells caused a decrease in neurite growth and an increase in the percent of apoptotic cells. RNA expression analysis showed that Sox11 knockdown modulated the level of mRNAs encoding several genes related to cell survival and death. Further validation in the Neuro2a model showed Sox11 knockdown increased expression of the pro-apoptotic gene BNIP3 (*Bcl*II interacting protein 1 NIP3) and decreased expression of the anti-apoptotic gene TANK (TNF receptor-associated factor family member-associated NFκB activator). Cultured primary DRG neurons also express Sox11 and treatment with Sox11 small interfering RNA (siRNA) caused a significant decrease in neurite growth and branching and a decrease in mRNA encoding actin-related protein complex 3 (Arpc3), an actin organizing protein that may be involved in axon growth. The percent of apoptotic neurons also increased in cultures of DRG neurons treated with Sox11 siRNA. Similar to Neuro2a cells, a decrease in TANK gene expression occurred, suggesting at least some overlap in Sox11 transcriptional targets in Neuro2a and DRG neurons. These data are consistent with a central role for Sox11 in regulating events that promote neurite growth and neuron survival. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neurite, apoptosis, RNAi, Penetratin, axon growth, regeneration.

Members of the *S*RY b*ox*-containing (Sox) family of transcription factors are emerging as important transcriptional regulators whose activity underlies the development and differentiation of multiple organ systems. Twenty Sox genes have been identified in the mouse and human genomes and all contain a DNA-binding high mobility group (HMG) domain and protein specific domains implicated in activation and repression of gene transcription (Kamachi et al., 2000; Schepers et al., 2002). Several Sox genes are expressed in the developing CNS and peripheral nervous system and appear to regulate differentiation and cell fate in cell type specific manners. For example, in mouse, Sox10 is expressed in neural crest precursors and has a role in establishing glial cell populations (Paratore et al., 2001). Sox 1, 2 and 3 are expressed in spinal neurons of developing chick embryos and downregulation of these factors is requisite for expression of the neuronal markers NeuroM, NeuN and Tuj1, suggesting an important role in regulating commitment to the neuronal phenotype (Bylund et al., 2003). In *in vitro* models, Sox6, which is also expressed in developing brain, was found to be essential for differentiation and neurite outgrowth of the embryonic carcinoma (EC) cell line P19 following retinoic acid (RA) stimulation (Hamada-Kanazawa et al., 2004). Sox6 was also critical for survival of RA-stimulated P19 cells as evidenced by the increased apoptotic death that ensued upon inhibition of Sox6 expression. In human EC cells similarly induced to differentiate into a neuronal phenotype with RA, Sox3 expression was up-regulated as Sox2 expression declined, suggesting regulatory roles for Sox2 and Sox3 in human EC differentiation (Stevanovic, 2003).

In the peripheral nervous system, the Sox11 transcription factor has been of interest because of its high level of expression in developing trigeminal and dorsal root ganglia (DRG) (Uwanogho et al., 1995; Hargrave et al., 1997) and potential role in the development of gliomas (Weigle et al., 2005). In sensory neurons the high level of embryonic Sox11 expression is markedly reduced at birth (Hargrave et al., 1997; Tanabe et al., 2003; Jankowski et al., 2004), suggesting tight control of its transcriptional activity during postnatal neuron maturation. Toward identification of the targets and mechanism of Sox11 action, mice that lack a functional Sox11 gene were recently isolated (Sock et al., 2004). Newborn Sox11$^{-/-}$ mice exhibited widespread de-

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Abbreviations: Arpc3, actin-related protein complex (subunit 3) gene; Blk, B lymphoid kinase; BNIP3, *Bcl*II interacting protein1, NIP3; casp3*, activated caspase 3; DRG, dorsal root ganglia; EC, embryonic carcinoma; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HMG, high mobility group; MEM, minimal essential medium; MEMS, minimal essential medium containing 10% fetal bovine serum; MEMS-RA, minimal essential medium containing 20 μM retinoic acid; PBS, phosphate buffered saline; RA, retinoic acid; RISC, RNA-induced silencing complex; RT, room temperature; RT-PCR, reverse transcriptase–polymerase chain reaction; siRNA, small interfering RNA; Sox, *S*RY b*ox*-containing; TANK, TNF receptor-associated factor family member-associated NFκB activator; TRAF, TNF receptor-associated factor.

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velopmental abnormalities in the heart, lung, stomach, pancreas and skeletal systems and died soon after birth due to the severe defects in organ development. How knockout of Sox11 affected sensory neuron survival is still unclear, although neonatal $Sox^{-/-}$ mice did respond to tactile stimuli suggesting that at least some sensory neurons were present.

We and others have provided evidence that Sox11 transcription is markedly elevated in adult sensory neurons following nerve cut injury (Tanabe et al., 2003; Jankowski et al., 2004). Sox11 may therefore have an important role in developing neuron growth and survival as well as recovery of adult neurons following injury. To better understand the role of Sox11 and the genes it may modulate, we examined its function in regulating differentiation and survival using the mouse neuroblastoma cell line Neuro2a model system and primary sensory neurons of the adult mouse. Similar to EC cell lines, Neuro2a cells can be stimulated to differentiate and project long neurites by exposure to RA, a potent modulator of neuronal specification and axonal outgrowth (Shea et al., 1985; Maden, 2001). Using small interfering RNA (siRNA) -mediated knockdown, we asked if Sox11 was essential for survival and neurite extension in Neuro2a cells following RA-induced differentiation and in axotomized DRG neurons grown in culture. Results indicate that Sox11 is required for optimal neurite growth and that it transcriptionally regulates genes associated with cell survival and growth in Neuro2a and primary adult DRG neurons.

EXPERIMENTAL PROCEDURES

Cell culture

The mouse neuroblastoma cell line Neuro2a (ATCC clone number CCL-131, Manassas, VA, USA) (Olmsted et al., 1970) was maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (MEMS) and 1% penicillin/streptomycin in an incubator set at 37 °C and 5% CO_2. Cells used were passaged no more than three times. For all experiments, cells were plated into 12- or 24-well plates at a concentration of 10,000 and 5000 cells/well, respectively, or plated into two- or four-well chamber slides. Cells were grown in MEMS to 50% confluence (18–24 h) and then treated with 20 μM RA (Sigma, St. Louis, MO, USA), siRNAs or both depending on the experiment. The time of RA addition was considered the 0 h time point. Cells were incubated according to experimental design and at appropriate times RNA was isolated or cultures were fixed for immunocytochemistry. Experiments to determine the effect of siRNA treatment at various times after RA addition were done by setting up cultures as described above and then adding siRNA at various times (0 h, 6 h, 12 h or 24 h) after RA addition. Transfected cultures were harvested for RNA or morphological analysis at 24 h after siRNA addition.

To culture adult sensory neurons, 6–8 wk-old male C57/Bl6 mice were deeply anesthetized with 2.5% Avertin anesthesia, intracardially perfused with Hanks' balanced salt solution (HBSS) and their DRG collected into cold HBSS from all spinal cord levels. Ganglia were incubated in 60 U of papain (Worthington, Lakewood, NJ, USA) in a solution of cysteine (1 mg/3 ml HBSS) and $NaHCO_3$ for 10 min at 37 °C. The solution was removed and 12 mg collagenase Type II (Worthington) in 3 ml HBSS was added for 10 min at 37 °C. Collagenase was removed, cells were washed in F12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% Pen/Strep, gently triturated with fire-polished glass Pasteur pipettes and plated onto laminin (0.1 mg/ml) and poly-D-lysine (5 mg) -coated glass coverslips. Cells were incubated for 1–2 h at 37 °C in F12 medium with 10% FBS and 50 ng/ml NGF (Harlan Bioproducts, Madison, WI, USA) and then treated with complete medium containing Penetratin-linked siRNAs.

siRNA treatment protocol

Neuro2a cells. Two hours prior to siRNA transfection fresh medium was added to cultures. Cells were then treated with either 20 μm RA alone or in combination with 10 nM of Sox11 Smart-Pool siRNAs or 10 nM non-targeting siRNA (Dharmacon, Lafayette, CO, USA). For transfection, TRANSIT-TKO transfection reagent (Mirus Corporation, Madison, WI, USA) was used; 2–4 μl of the transfection reagent was added to 50 or 100 μl of serum-free MEM, respectively. The solution was mixed, incubated at room temperature (RT) for 5–20 min and the appropriate volume of 1 μM siRNAs added to obtain a final concentration of 10 nM siRNA per culture well. Solutions were further mixed by pipetting, incubated at RT for 5–20 min and then added to the cultures.

Primary DRG neurons. Four Sox11 Smart-Pool siRNAs (Dharmacon) were tested for their ability to knock down Sox11 expression. Two of the four siRNA sequences were able to individually produce an 80–90% knockdown of Sox11 expression in Neuro2a cells. The oligo sequence that showed the best knockdown (90%) was chosen for linkage to the peptide Penetratin-1 (Q-Biogene, Carlsbad, CA, USA) (Davidson et al., 2004). Linkage was done using nontargeting and Sox11 siRNAs that were synthesized with 5′ thiol groups on the sense strand and then 2′ deprotected and HPLC purified. Thiol-coupled siRNAs were resuspended in 1× siRNA buffer (Dharmacon) and an equal concentration of Penetratin 1 added. The mixture was heated to 65 °C for 15 min and then incubated at 37 °C for 1 h. Cultures were flooded with 1 ml of F12 media containing 50 ng/ml NGF and Penetratin-conjugated siRNAs at a final concentration of 80 nM/well; 80 nM was used instead of 10 nM (used for Neuro2a cells) based on Davidson et al. (2004) and our own siRNA concentration testing.

Cell counting, morphology and differentiation measures

Neuro2a cells. Cells grown on chamber slides were fixed 5 min with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and coverslipped with glycerol for light microscopic analysis on an Olympus BH-2 microscope equipped with a differential interference contrast filter (Olympus, Tokyo, Japan). Differentiated Neuro2a cells were quantified using criteria similar to those described in Munch et al. (2003). Cells with at least two neurites extending from the soma or one neurite longer than the diameter of the soma were considered differentiated. Cells undergoing apoptosis were identified by shrunken cell membranes, reduced size or disrupted nuclei (Hoshi et al., 1998; Sakurai et al., 2003). Cells are reported as a percent of total cells counted within gridlines of four randomly selected squares each 600 μm^2 using a photoetched gridded coverslip (Electron Microscopy Sciences, Hatfield, PA, USA) at 10× magnification ($n=3$ per condition). The percent of cleaved caspase 3(casp3*) -labeled cells was calculated by counting the number of casp3* positive cells in four randomly selected fields equal to the size of the photoetched grids used for morphological quantification. For each condition, n equaled 3. Statistical significance between conditions was determined by P-values$\leq$0.05.

Primary DRG neurons. DRG neurons were treated with siRNAs 1–2 h after plating and the amount of neurite growth and branching was analyzed at 24 h and 4 days using anti-NF200 or PGP9.5 labeling. For each condition, 10–30 PGP 9.5-labeled cells were analyzed. Cells for analysis were chosen randomly by

selecting two 600 μm^2 squares on opposite ends of a photoetched gridded coverslip (Electron Microscopy Sciences). Neurite growth measures were done at 20× magnification ($n=3$ per condition; 387 total cells) using NIH Image software. Growth was quantified by measuring the length of all primary neurites, calculating an average length and an average maximum length per neuron. The number of branch points per neuron was also determined. Branching index was calculated as the number of branch points divided by the number of primary neurites on each neuron.

Cell immunolabeling. After fixation and rinsing, cells were blocked in 0.25% Triton X-100 with 5% normal goat serum in PBS for 30 min and incubated in either goat anti-Sox11 (1:100; Santa Cruz Biotech, Santa Cruz, CA, USA) or rabbit anti-casp3* (1:100; Cell Signaling Technologies, Danvers, MA, USA) overnight at RT. Cells were washed in PBS and incubated 2 h in 1:500 dilutions of FITC-conjugated donkey anti-rabbit antibody or CY2-conjugated donkey anti-goat (Jackson ImmunoResearch, West Grove, PA, USA). Slides were rinsed in PBS, coverslipped in glycerol and images captured using a Leica fluorescence microscope. Neurites in DRG cultures were visualized by immunolabeling using monoclonal anti-neurofilament 200 antibody (clone N52, 1:400; Chemicon, Temecula, CA, USA) followed by a goat anti-mouse Cy2-conjugated secondary antibody or by immunolabeling with the general neuronal marker, protein gene product 9.5 (PGP9.5, 1:500; Ultraclone). Anti-PGP binding was detected using donkey anti-goat IgG conjugated to HRP followed by a diaminobenzidine reaction.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Culture medium from triplicate cultures was removed and plates were washed three times with PBS. Cells were lysed with RLT buffer (Qiagen, Valencia, CA, USA) with beta-mercaptoethanol and RNA was isolated using the Qiagen RNeasy protocol for animal cells. After RNA isolation, samples were treated with DNase I (Invitrogen) for 15 min at RT and 1 μg of DNased RNA reverse transcribed using Superscript II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). Samples were stored at −80 °C until used in PCR reactions. For real-time PCR, samples of cDNA were added to a SYBR Green MasterMix (Applied Biosystems, Foster City, CA, USA), run in triplicate on an Applied Biosystems Imager. Ct values (threshold cycles) were normalized to GAPDH by subtracting the target gene from the GAPDH control. A ΔΔCt value was then calculated by subtracting the normalized experimental Ct from the normalized control condition with fold change reported as $2^{\Delta\Delta Ct}$. Primer sequences used were: Sox11 5′ATCAAGCGGCCCATGAAC3′, 5′TGCCCAGCCTCTTGGAGAT3′; TANK 5′GTTTCCGCCTATGGACAATGAC3′, 5′CGGTCCTGGCACACTGTCT3′; Bcl10 5′AGCACGGCTCCCTTCTTCTC3′, 5′TCGAGGAAGAGTGGCTGAAGAG3′; Blk 5′GTTGACTCTGCCCTGTGTGAAC3′, 5′ACCCAAGTTTCCGGACCAA3′; BNIP3 5′GGTTTTCCTTCCATCTCTGTTACTG3′, 5′GTTGTCAGACGCCTTCCAATG3′; Arpc3 5′GAGACCAAAGACACGGACATTGTG3′, 5′CCACTTGCTGGGTTTATCACTCTG3′.

SuperArray filter hybridization. Focused DNA microarrays (GEArray Q Series Mouse Apoptosis Gene Array; MM-002) were used to identify genes involved in Neuro2a cell survival and death that may be regulated by Sox11 (SuperArray Bioscience, Frederick, MD, USA). Parallel cultures were treated with 20 μM RA or 20 μM RA plus 10 nM Sox11 siRNAs. Total RNA (3 μg) was isolated at either 12 h or 24 h post-siRNA addition using the Qiagen RNeasy Mini-Kit protocol. RNA was reverse transcribed into ^{32}P-dCTP-labeled probes according to the manufacturer's protocol and annealed with primers at 70 °C for 3 min followed by incubation at 37 °C for 10 min. A cocktail containing RNase inhibitors and reverse transcriptase was added to the annealing reactions at equal volume and incubated at 37 °C for 25 min. RNA was hydrolyzed at 85 °C for 5 min, reactions were placed on ice, and then linear polymerase reactions containing primers specific to the apoptosis array were performed using ^{32}P-dCTP spiked reactions according to PCR parameters provided. Probes were denatured at 94 °C for 2 min prior to hybridization. Filters were prehybridized 2 h at 60 °C in GEAHyb solution containing 100 μg/ml sheared salmon sperm DNA (GEAPrehyb). Denatured probe in 0.75 ml of GEAprehyb solution was incubated with filters overnight at 60 °C with gentle agitation (5–10 r.p.m.). Filters were washed twice in 2× SSC, 1% SDS at 60 °C for 15 min with agitation, twice in 0.1× SSC, 0.5% SDS at 60 °C for 15 min and then covered with plastic wrap and exposed to a phosphoimaging screen for an empirically determined amount of time. Phosphoimaging screens were read on a BioRad phosphoimager and values obtained by subtracting filter background intensity and normalizing to GAPDH controls on the filter.

Sciatic nerve axotomy. Male Swiss Webster mice (Hilltop, Scottdale, PA, USA) approximately 4–6 weeks of age were anesthetized using a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). The animal was closely shaved on the left side and Betadine was applied prior to the incision. An incision was made at lumbar level and a mini-Goldstein retractor (Fine Science Tools, Foster City, CA, USA) was inserted into the incision and opened, providing an area in which to work. Once identified, the sciatic nerve was tightly ligated with 6.0 silk sutures and transected distal to the suture. The muscles were then sutured and the wound was closed with microclips (Roboz, Gaithersburg, MD, USA). DRGs were analyzed for RNA levels 0, 4, 7 and 14 days after axotomy. Animals were anesthetized again with ketamine/xylazine and intracardially perfused with ice cold saline prior to DRG dissection.

Statistics

Statistical analysis was determined using Student's *t*-test with P values <0.05 as statistical significance.

RESULTS

Sox11 is expressed in Neuro2a neuroblastoma cells

Neuro2a cells provide an excellent model system to study transcriptional regulation of neuronal differentiation. They are easily propagated, they can be stimulated to differentiate by addition of 20 μM RA and upon differentiation stop dividing and extend neurites (Shea et al., 1985; Sajithlal et al., 2002; Munch et al., 2003; Noguchi et al., 2003). Under culture conditions without RA, Neuro2a cells appear as amoeboid neuroblasts that begin to extend neurites after 1–3 days in culture. To assess if Sox11 might have a role in regulating genes important for the transition to the differentiated state, we assayed Sox11 mRNA levels at times prior to and coinciding with the initiation of neurite growth (6 h, 1 day, 2 days and 3 days post-plating). RT-PCR assays showed that at 6 h post-plating, replicating cells with minimal neurite growth had a relatively low level of Sox11 mRNA (Fig. 1A, also see Fig. 3A). At 1 day and 2 days post-plating, a steady increase in Sox11 mRNA was found that correlated with an increasing number of neurite containing cells. By 3 days in culture, Sox11 had increased 280% relative to the 6 h time point level. Thus, a steady increase in Sox11 paralleled the increase in the number of cells exhibiting neurites and undergoing differentiation.

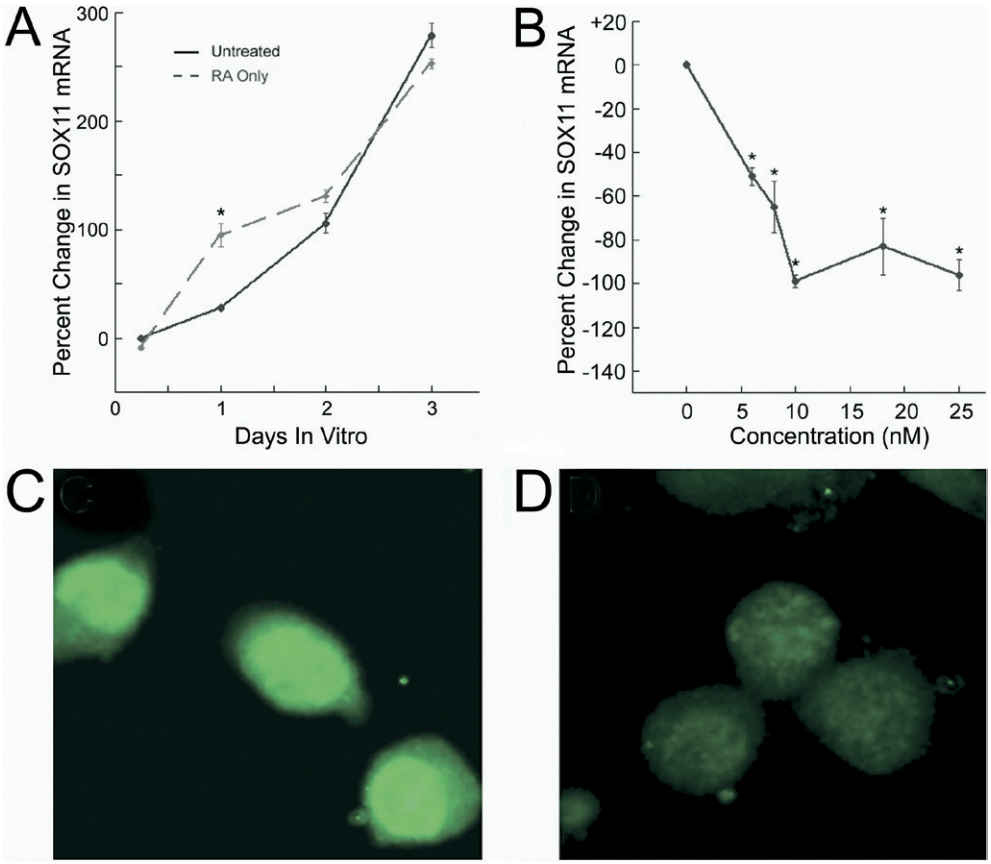

Fig. 1. (A) Change in Sox11 mRNA in Neuro2a cells grown with or without RA. (A) Sox11 mRNA increases gradually in cultures grown without RA (solid line). Addition of 20 μM RA enhances Sox11 at 24 h after cell plating (dashed line) but at later times levels are equivalent to cultures grown without RA. $N=3$ at each time point; asterisk indicates $P<0.001$. (B) Transfection of Sox11 siRNAs reduces Sox11 mRNA level in Neuro2a cells. Measures were done 24 h after addition of four Smart pool siRNAs against Sox11. Asterisk indicates $P<0.001$. Levels are normalized to cells treated with transfection reagent alone. (C) Untreated Neuro2a cells show nuclear immunoreactivity for Sox11 protein in comparison to Sox11 siRNA-treated Neuro2a cells (D), which lack nuclear staining for Sox11. Images are of cells 24 h after siRNA treatment.

It has previously been shown that Neuro2a cells undergo accelerated differentiation when grown in minimal essential medium containing 20 μM retinoic acid (MEMS-RA) (Shea et al., 1985). To determine if Sox11 expression was modulated by this RA-induced differentiation, cells were treated with RA and the level of Sox11 mRNA measured (Fig. 1A). Neuro2a cells grown in MEMS-RA showed a 100% increase in Sox11 mRNA at 1 day, in comparison with the 29% increase in cells grown without RA (Fig. 1A). Sox11 expression continued to increase in RA medium and was increased 250% at 3 days, a value not significantly different from that measured in cultures grown without RA. The transient increase in Sox11 mRNA in 1 day-old RA-treated cultures versus untreated cultures suggested Sox11 has a role in regulating genes important for the early stages of RA-induced Neuro2a differentiation. We therefore chose to examine the role of Sox11 during this RA-mediated transition phase.

siRNA-mediated knockdown of Sox11 occurs on translational and transcriptional levels

To test the role of Sox11 in early Neuro2a differentiation, we reduced the cellular level of Sox11 by transfecting cells with siRNAs targeted to Sox11. Initial studies to test transfection efficiency using Cy3-labeled nontargeting siRNAs showed a high efficiency (93%) of transfection (not shown). We then confirmed Sox11 knockdown on the transcriptional level using RT-PCR assays. Knockdown occurred in a dose dependent manner (Fig. 1B) with 10 nM Sox11 siRNAs producing a maximal 98% knockdown in Sox11 mRNA. Translational level knockdown of Sox11 was also confirmed using Sox11 antibody immunolabeling of transfected cells. In untransfected cultures or ones transfected with non-targeting siRNAs, cells with Sox11-labeling localized to the nucleus were easily detected (Fig. 1C). In contrast, Sox11-positive cells were rarely seen in cultures transfected with Sox11 siRNA (Fig. 1D).

The specificity of the Sox11 siRNAs was confirmed using various types of non-targeting siRNAs (Fig. 2A, B). All nontargeting siRNAs tested had no effect on Sox11 expression level assayed 24 h after transfection (Fig. 2A, B). Cultures treated with nontargeting siRNAs used as controls in this study (Fig. 1A) had a 1.35$\pm$0.6-fold increase in Sox11 mRNA at 24 h post-transfection ($n=3$; $P<0.05$) compared with the 1.29$\pm$0.03-fold increase

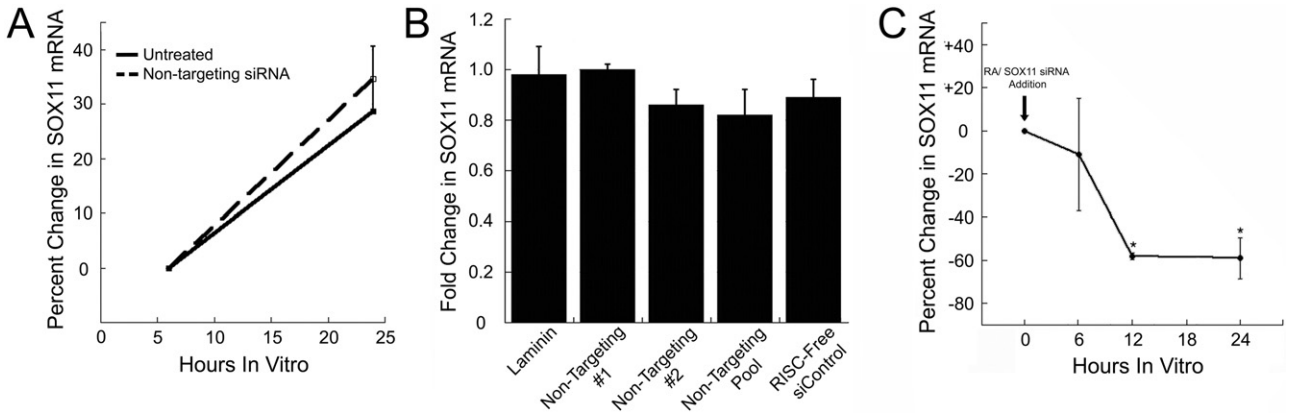

Fig. 2. Sox11 expression is knocked down in Neuro2a cells cotreated with RA and Sox11 siRNAs. (A) Transfection of 10 nM nontargeting control siRNA did not change the level of Sox11 mRNA (broken line) in comparison to untreated (solid line) cultures. (B) Transfection of various siRNAs did not change the level of Sox11 expression. Non-targeting #1 and #2 siRNAs and the RISC-free siControl comprise the Non-Targeting pool. Fold-change values are calculated at 24 h post-transfection and are relative to cultures treated with transfection reagent alone. (C) 10 nM Sox11 siRNAs blocked the RA-induced increase in Sox11 mRNA within 12 h of addition. Knockdown was maintained for up to 24 h at which time a 59% decrease was measured. $N=3$ for each time point. Asterisks indicate $P<0.001$ relative to transfection reagent only control.

seen in untreated cultures (Figs. 1A, 2A). Thus, the knockdown in Sox11 mRNA caused by transfection of Sox11 siRNAs appears to be specific and not due to off-target effects.

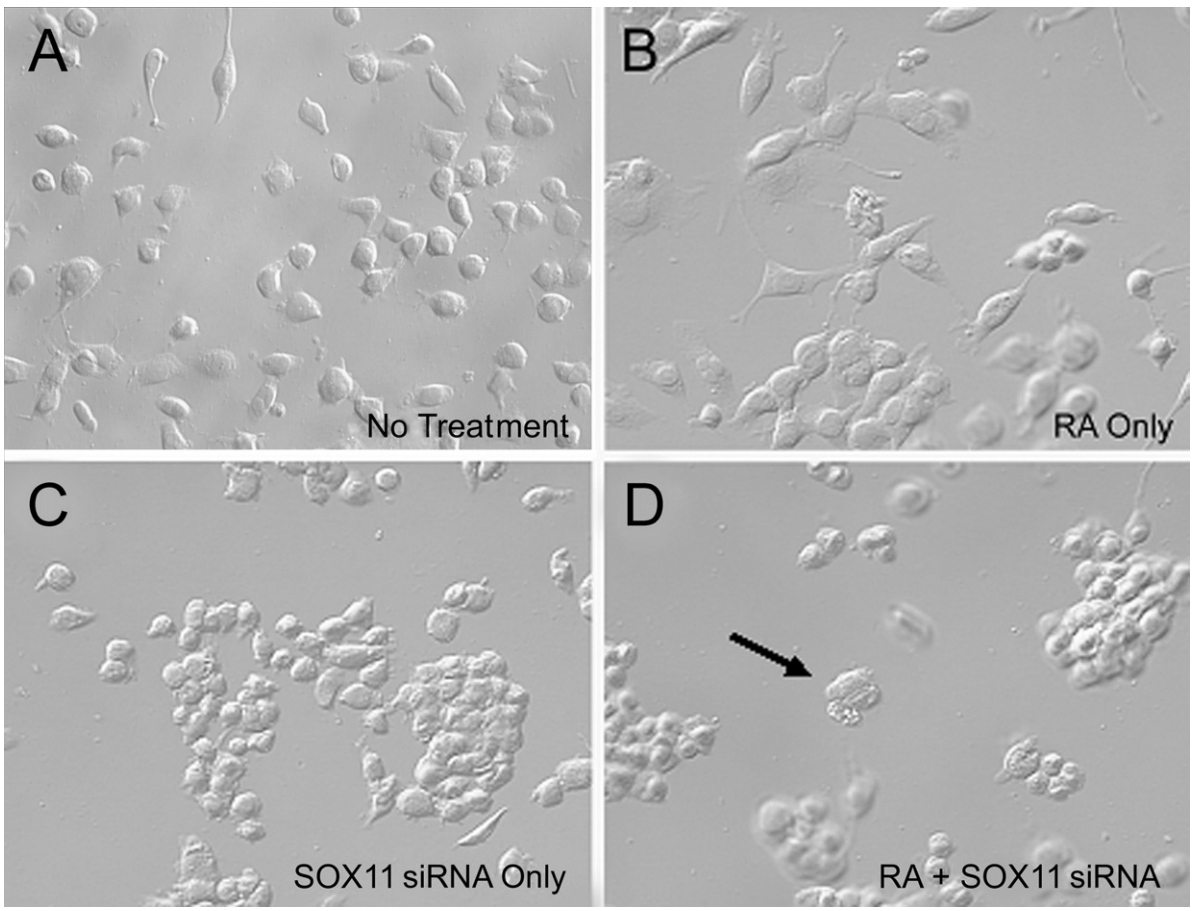

Fig. 3. Morphology of Neuro2a cells treated with RA and Sox11 siRNAs. (A) Neuro2a cells grown in MEMS media for 24 h show typical amoeboid morphology with some cells displaying short neurite projections. (B) Many cells in cultures treated with RA for 24 h had enhanced neurite projections. (C) Cultures treated with Sox11 siRNA without RA have few neurite-containing cells. (D) Cultures treated with RA plus Sox11 siRNAs rarely display neurites and more appear apoptotic (arrow). See morphological quantification in Fig. 4. All images were taken at 24 h post-treatment.

Increased expression of Sox11 is required for survival and neurite growth following RA-stimulation of Neuro2a cells

To determine if Sox11 was required for RA-induced differentiation and neurite growth, Neuro2a cells were treated with a combination of RA and either 10 nM nontargeting control (non-targeting #1) or Sox11 siRNAs. Whereas nontargeting siRNAs did not affect Sox11 mRNA levels (see below), transfection of Sox11 siRNAs into RA-treated cultures caused a 59% decrease ($n=3$; $P<0.05$) in Sox11 mRNA by 12 h that was maintained out to 24 h post-RA/siRNA treatment (Fig. 2C).

Knockdown of Sox11 in RA-treated Neuro2a cultures caused morphological changes as well. Compared with cultures treated with RA-only (Fig. 3B), cells treated with RA plus Sox11 siRNAs had fewer neurites and many appeared shrunken and vacuolated 24 h post-treatment (Fig. 3D). Cells treated only with Sox11 siRNA appeared shrunken as well (Fig. 3C), though less so compared with RA plus Sox11 siRNA-treated cells (Fig. 3D). Cells treated with transfection reagent alone or nontargeting siRNAs did not appear markedly different from untreated cultures (not shown). To quantify the observed morphological changes, we determined the percent of differentiating cells with neurites, the percent of cells exhibiting features of apoptotic cell death (shrunken cell membranes, reduced size, disrupted nuclei) and the percent of cells expressing the apoptotic cell marker, activated caspase 3 (casp3*) (Figs. 4, 5). All measures were done at 24 h after treatment. In cultures grown in RA-only, less than 5% of the cell population analyzed had morphological features of apoptotic cells or were casp3*-positive (Fig. 4). Differentiating cells with neurite extensions were prominent and comprised 22% of the population. Cultures treated with control nontargeting siRNA were not significantly different from untreated cells. Cultures treated with Sox11 siRNAs and no RA showed no change in the percent of differentiated cells relative to untreated cells but did exhibit an increase ($8.9\pm0.4\%$ vs. $3.2\pm0.1\%$; $P<0.01$) in the percent of cells with apoptotic morphology (though had no change in casp3*-positive cells) (Fig. 4). In contrast, cultures treated with RA plus Sox11 siRNAs had a much greater increase in the percent of cells displaying apoptotic morphologies (18%) and casp3*-immunoreactivity (21%; $P<0.0001$) (Figs. 4, 5C). In addition, only 8% of these cells showed a differentiated morphology, which was significantly less than the percent of differentiated cells in RA only treated cultures. Values from cultures treated with RA plus nontargeting control siRNAs were not significantly different compared with RA-only-treated cultures. Collectively, these results indicate that knockdown of Sox11 expression in unstimulated Neuro2a cells can cause a moderate decrease in cell survival. This effect is greatly amplified however, in cultures treated with RA and Sox11 siRNAs where significantly less neurite growth occurs and a far greater level of apoptotic morphologies and casp3* expression is found.

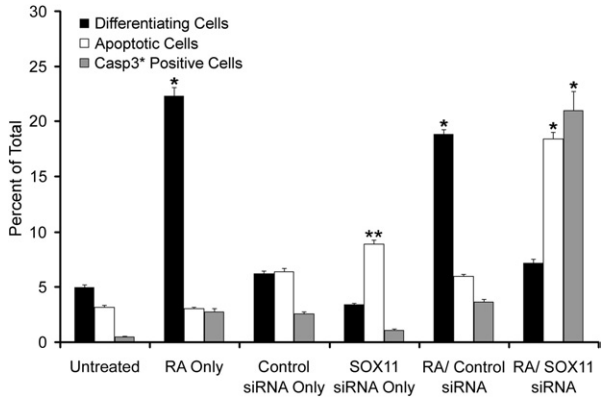

Fig. 4. Combined treatment of RA and Sox11 siRNA enhances Neuro2a cell death. Chart shows quantification of differentiation (neurite growth) and apoptosis in cultures of Neuro2a cells 24 h after treatment with RA only, control (nontargeting) siRNA only, Sox11 siRNA only, RA plus control siRNA and RA plus Sox11 siRNA. Untreated cultures have few differentiating cells with neurites (black bar), few apoptotic cells (white bar) and virtually no cells immunopositive for casp3* (gray bar). RA-only treated cultures had a significant increase in the percent of differentiating cells relative to untreated cultures. Neurite growth and apoptosis was not significantly different in cultures treated with control siRNAs. Sox11 siRNA slightly increased the percent of morphologically apoptotic cells. Co-treatment with RA and control siRNA caused an increase in neurite containing cells, as expected. RA plus Sox11 siRNA treatment reduced neurite growth and significantly increased the percent of apoptotic and casp3* immunoreactive cells. The number of cells analyzed per group were: untreated, 1674 cells; RA only, 1275; Control siRNA only, 1857; Sox11 siRNA only, 981; RA plus control siRNA, 1398; RA plus Sox11 siRNA, 1292. Values on chart are a percent of the total cells analyzed. * $P<0.0001$, ** $P<0.01$, both relative to untreated controls.

Knockdown of Sox11 and its effects in RA-treated Neuro2a cells is dependent on the time of siRNA transfection

To determine whether the level of Sox11 is important for later stages of RA-induced differentiation, we attempted knockdown of Sox11 at various times after RA treatment. Cultures were treated with 20 μm RA (0 h time point) and then with 10 μM siRNAs added at 0 h (as above in Fig. 4), 6 h, 12 h or 24 h post-RA addition (Fig. 5). The relative level of Sox11 mRNA (Fig. 5E) and changes in morphology and casp3* expression (Fig. 5A–D) were then assessed at 24 h post-siRNA addition. Interestingly, the level of Sox11 mRNA was reduced only in cultures in which siRNA transfection was done at the same time of RA addition (0 h, Fig. 5E). No reduction of Sox11 mRNA occurred in cultures transfected at 6 h, 12 h or 24 h after RA addition. The morphology of cells treated with Sox11 siRNAs 24 h after RA addition was consistent with a lack of Sox11 knockdown, i.e. many cells had neurite extensions and differentiated morphology (Fig. 5B). Cells in parallel cultures treated with Sox11 siRNA at the time of RA addition (0 h) displayed few neurites and many had apoptotic morphologies (Fig. 5A), as previously observed (Fig. 3D). Few if any casp3*-labeled cells were visible in cultures treated with Sox11 siRNA 24 h after RA (Fig. 5D), consistent with the prominent neurite growth in these cultures (Fig. 5B). This was in contrast to the many casp3* cells in cultures

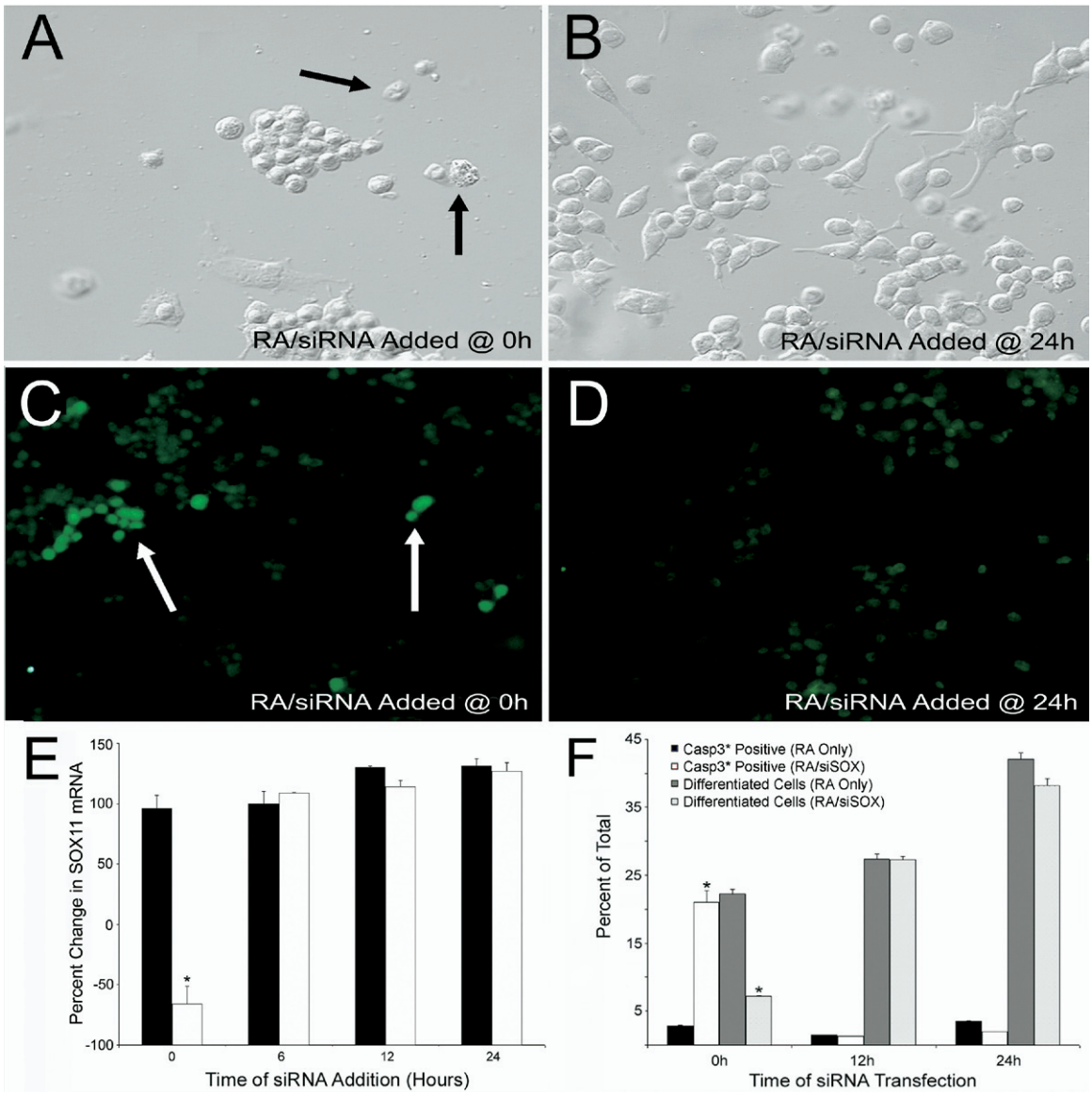

Fig. 5. Sox11 expression is required at the early phase of RA-induced Neuro2a differentiation. Differential interference contrast (DIC) images of Neuro2a cultures treated with Sox11 siRNAs at the time of RA treatment (0 h) (A) and 24 h after addition of RA (B). Note prevalence of cells with neurites in B but not A. (C) casp3*-Labeling (arrows) appears in many cells co-treated with RA and Sox11 siRNAs at the 0 h time point. (D) Cells grown in RA for 24 h and then treated with Sox11 siRNAs do not exhibit casp3* labeling. (E) Knockdown in Sox11 mRNA occurs only when Sox11 siRNAs are added simultaneously with RA at the 0 h time point. For all time points, the level of Sox11 mRNA was assayed 24 h post-siRNA treatment. (F) Plot of differentiating, apoptotic and casp3*-positive cells in cultures treated with RA and Sox siRNAs at various times after RA stimulation. Cultures in which RA and Sox11 siRNAs are added together (0 h) have more casp3* positive cells (white box) and fewer differentiating cells (tan box) compared with RA-only treated cells (black and gray boxes). Sox11 siRNA transfected at 12 h or 24 h after RA addition does not increase the number of casp3*-positive cells or decrease the number of differentiating cells normally seen following RA addition. All samples are $n=3$. The number of cells analyzed in each group was: At 0 h, RA-only, 2351 cells; RA/siRNA, 2419 cells. At 12 h, RA-only, 2185 cells; RA/siRNA, 2061 cells. At 24 h, RA-only, 2552 cells; RA/siRNA, 3744 cells. Asterisks indicate $P<0.001$ relative to RA-only control values.

treated with Sox11 siRNA at the time of RA addition (Fig. 5C).

To evaluate these effects quantitatively, we assayed morphology and casp3* across treatment groups in which siRNA was added at 0 h, 12 h or 24 h after RA (Fig. 5F). All measures were done at 24 h post-siRNA addition. Cells treated concurrently with RA and siRNAs (0 h time point) showed a high percentage of casp3*-positive cells (Fig. 5F, open box) and low percentage of differentiated cells (Fig. 5F, light gray box), similar to results from previous assays (Fig. 4). However, in cultures in which Sox11 siRNAs were added at 6 h (not shown) or 12 h (Fig. 5F) after RA treatment, the percent of differentiated and casp3*-positive cells was unchanged from RA-only treated cells. For 12 h cultures, 27% of cells showed a differentiated phenotype in both RA-only and RA plus Sox11 siRNA groups and a very low percent of casp3*-positive cells. Similar results were found for cells treated with RA and

transfected 24 h later with siRNAs to Sox11 (Fig. 5F). Co-treated cultures had 38% differentiated cells, which is similar to the 42% percent of cells in the RA-only group. Thus, siRNA knockdown of Sox11 and its resulting effect on Neuro2a differentiation and apoptosis was only possible if it was done at the time RA was added to cultures.

Knockdown of Sox11 in Neuro2a cells alters expression of genes associated with apoptosis

To begin to identify how Sox11 knockdown contributes to RA-induced Neuro2a cell death, we assayed gene expression following knockdown using tailored gene arrays spotted with cDNAs encoding genes linked to cell death pathways. RNA isolated from Neuro2a cultures cotreated with RA and Sox11 siRNAs was analyzed at 12 h or 24 h after transfection. Analysis of gene arrays processed in parallel showed several genes were differentially expressed following Sox11 knockdown (Table 1). For several genes, the change in transcript abundance was different at 12 h versus 24 h post-transfection suggesting a dynamic change in gene expression occurs over this time period.

It is difficult to know whether these changes in transcript abundance are directly related to the reduction in Sox11 or whether they represent secondary effects of other transcriptional events. To address this issue and further validate some of the changes indicated by the gene arrays, four genes that contain one to four Sox factor HMG binding consensus domains (5′-(A/T)(A/T)CAA(A/T)G-3′) within 1–2 kb of their start codons were further analyzed using real time RT-PCR assays (Fig. 6A, B). The genes chosen and results of this analysis are: 1) TRAF (TNF receptor-associated factor) family member-associated NFkB activator (TANK), which was decreased 31% at 24 h in the RA-Sox11 siRNA treated cultures, 2) *Bcl*II interacting protein1, NIP3 (BNIP3), which was increased 35% at 24 h, 3) B lymphoid kinase (Blk), which was statistically unchanged, though a trend toward increased expression was found, and 4) B-cell lymphoma 2-like 10 (Bcl2l10), which was unchanged by Sox11 inhibition on both the gene array and by RT-PCR analysis. The RT-PCR analysis of gene expression (Fig. 6A) was done in triplicate and was most consistent with the 24 h time point array data listed in Table 1. This may reflect a more dynamic state in gene expression at the 12 h time point or the greater accuracy of the real time RT-PCR assays versus the filter arrays. Taken together, these data suggest TANK and BNIP3 are targets of Sox11 transcriptional regulation that have importance in mediating the RA-induced program of differentiation and survival. In addition, the positive and negative regulation of gene expression suggests Sox11 can function as both an activator and repressor of gene transcription.

Table 1. Change in mRNA level of apoptotio-associated genes in RA-treated Neuro2a cells at 12 h and 24 h post-Sox11 siRNA-mediated knockdown

Gene	Accession number	Direction of change 12 h	Direction of change 24 h
Casp8ap2	NM_011997	Increased	Increased
BNIP3	NM_009760	Increased	Increased
Blk	NM_007549	Decreased	Increased
TANK	NM_011529	Increased	Decreased
Tnfrsf21	NM_178589	Increased	Decreased
Fas	NM_007987	Decreased	Decreased
Casp2	NM_007610	Increased	Decreased
Casp3	NM_009810	Increased	Decreased
Casp9	NM_015733	Unchanged	Decreased
Bcl2a1d	NM_007536	Increased	Decreased
Bok	NM_016778	Increased	Decreased
Biklk	NM_007546	Decreased	Decreased
Birc6	NM_007566	Decreased	Decreased
Mcl1	NM_008562	Decreased	Decreased
Bcl2l10	NM_013479	Unchanged	Unchanged

Change is relative to cells treated with RA alone. Bcl2a1d, B cell leukemia/lymphoma 2 related protein A1d; Bcl2l10, Bcl2-like 10; Biklk, Bcl2-interacting killer-like; Birc6, Bacloviral IAP repeat-containing 6; Bok, Bcl2 related ovarian killer protein; Casp2, caspase 2; Casp3, caspase 3; Casp8ap2, Casp8 associated protein 2; Casp9, caspase 9; Fas, TNF receptor superfamily member; Mcl1, Myeloid cell leukemia sequence 1; Tnfrsf21, TNF receptor superfamily, member 21.

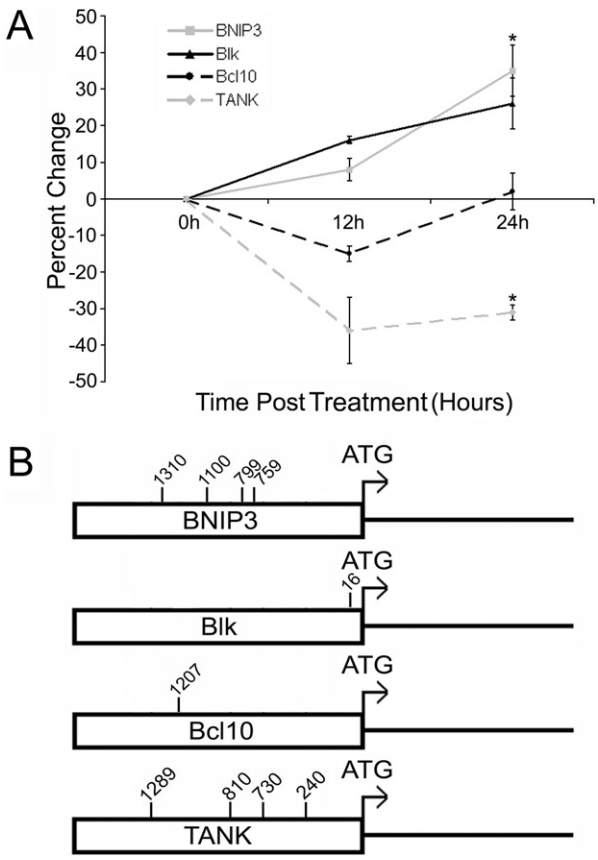

Fig. 6. Knockdown of Sox11 mRNA alters expression of genes involved in cell death pathways. RNA isolated from Neuro2a cells treated with RA only or RA plus Sox11 siRNAs for either 12 h or 24 h were analyzed using gene array filters. Plot shows RT-PCR validation analysis for four genes: TANK, Bcl10, Blk and BNIP3. All except Bcl10 were changed following knockdown of Sox11. All contain at least one Sox transcription factor consensus site in their promoter regions which are indicated by numbers. $N=3$ for each time point. Asterisk indicates $P<0.05$.

Sox11 expression is increased in cultured adult DRG sensory neurons

Expression of the Sox11 transcription factor is prominent in developing sensory neurons of the dorsal root and trigeminal ganglia (Hargrave et al., 1997; Jankowski et al., 2004). At late embryonic and postnatal times this expression is significantly decreased. We were interested in whether Sox11 expression might be modulated by nerve injury and, using real time PCR analysis, found that Sox11 mRNA rises significantly in adult DRG neurons following axotomy of the sciatic nerve (Fig. 7A). This finding is in agreement with recent microarray data (Tanabe et al., 2003) that showed a similar increase in Sox11 mRNA following sciatic nerve cut. The increase in Sox11 following nerve injury (up to 13-fold at day 7) suggests that Sox11 is important for transcriptional changes that occur in response to adult neuron injury. Neurons plated in culture, which in many ways models axotomy, would therefore be expected to express increased levels of Sox11. To test this we assayed Sox11 mRNA in mouse DRG sensory neurons that were enzymatically and physically dissociated and cultured *in vitro* (Fig. 7B). Cultured neurons had an immediate increase in Sox11 expression, with a 2.9-fold increase by 24 h and 6.8-fold rise at 7 days. The steady increase in Sox11 in neurons placed in culture suggests parallel mechanisms of gene activation occur following *in vitro* and *in vivo* axotomy. Cultured DRG neurons therefore provide a model system in which to identify how Sox11 expression is regulated and its affect on sensory neuron survival and neurite growth following injury.

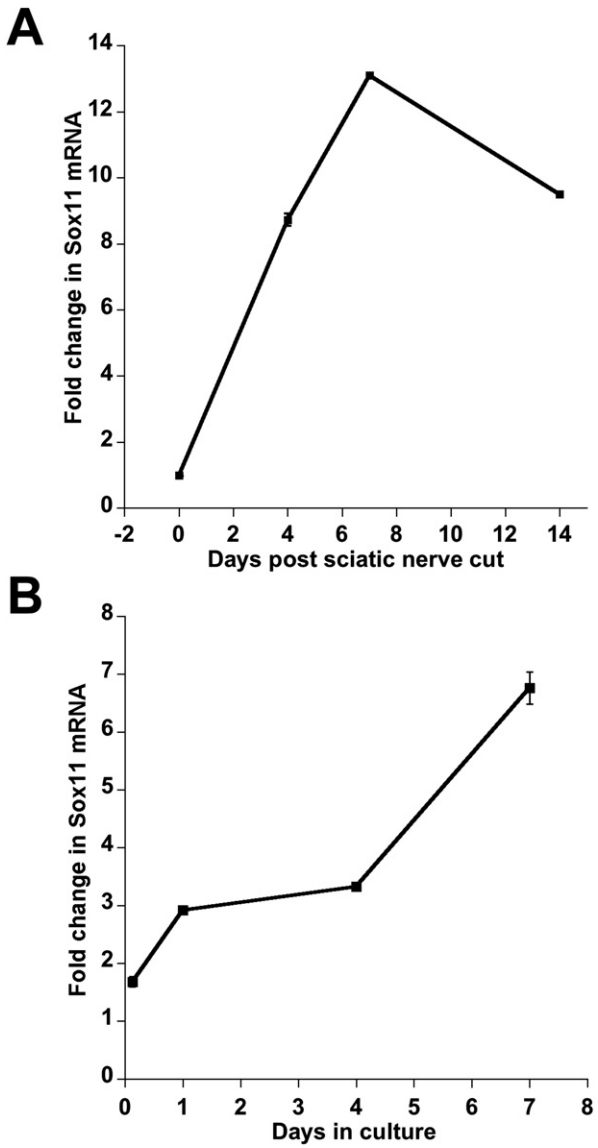

Fig. 7. Sox11 mRNA is increased following injury of DRG neurons. (A) Relative expression of Sox11 mRNA increases in lumbar DRG following sciatic nerve axotomy. Values were normalized to GAPDH. $N=3$ animals per time point. (B) Sox11 mRNA increases in primary DRG neurons following dissociation and culture *in vitro*. $N=3$ per time point. Values were normalized to the neuron specific transcript neuron specific enolase (NSE) to avoid contribution from contaminating glial cells. SEMs were too low for visualization except at the day 7 time point.

Knockdown of Sox11 mRNA inhibits neurite growth and survival of cultured primary DRG neurons

To assess if Sox11 was required for DRG neuron survival and neurite growth, we followed the paradigm used for Neuro2a cells and knocked down Sox11 expression by transfecting cultures with either control nontargeting siRNAs or Sox11 siRNAs. Because adult DRG neurons are extremely difficult to transfect, we used a newly described procedure that provides a high (99%) transfection efficiency in cortical neurons (Davidson et al. 2004). In this method, Sox11 siRNAs are thiol conjugated to Penetratin, a 16-amino acid thiol containing membrane permeable protein that translocates across the lipid membrane of mammalian cells (Hallbrink et al., 2001; Muratovska and Eccles, 2004). Once inside the cell the Penetratin peptide is removed from the siRNA through reduction of the thiol bond. Transfection of a Cy3-conjugated siRNA into adult DRG cultures showed a 90–95% transfection rate (Fig. 8A, B), similar to that seen in primary cortical neurons (Davidson et al. 2004). We therefore transfected primary DRG neurons using the Penetratin method. To verify that Sox11 siRNAs reduced the expression of Sox11 mRNA in DRG neurons, RT-PCR was used to determine the relative amount of Sox11 mRNA in control and Sox11 siRNA treated cultures (Fig. 8C). Treatment of cultures at 1 h post-plating caused knockdown of Sox11 at 3 h (34% decrease) and 24 h (25% decrease) post-transfection that was restored by 4 days post-treatment. Antibody labeling for Sox11 in treated cultures indicated this knockdown occurs at the protein level as well, since few siRNA-transfected neurons exhibited Sox11 nuclear labeling at 24 h (not shown). In addition, immunolabeling indicated that Sox11 reactivity is confined to DRG neurons and not in contaminating glial cells (Fig. 8D–F). The lack of Sox11 expression in glia suggests that any effect of Sox11 knockdown is DRG neuron specific.

The lack of a more significant Sox11 knockdown in DRG cultures, as seen in undifferentiated Neuro2a cells where a 92% knockdown occurred at 24 h, may reflect the

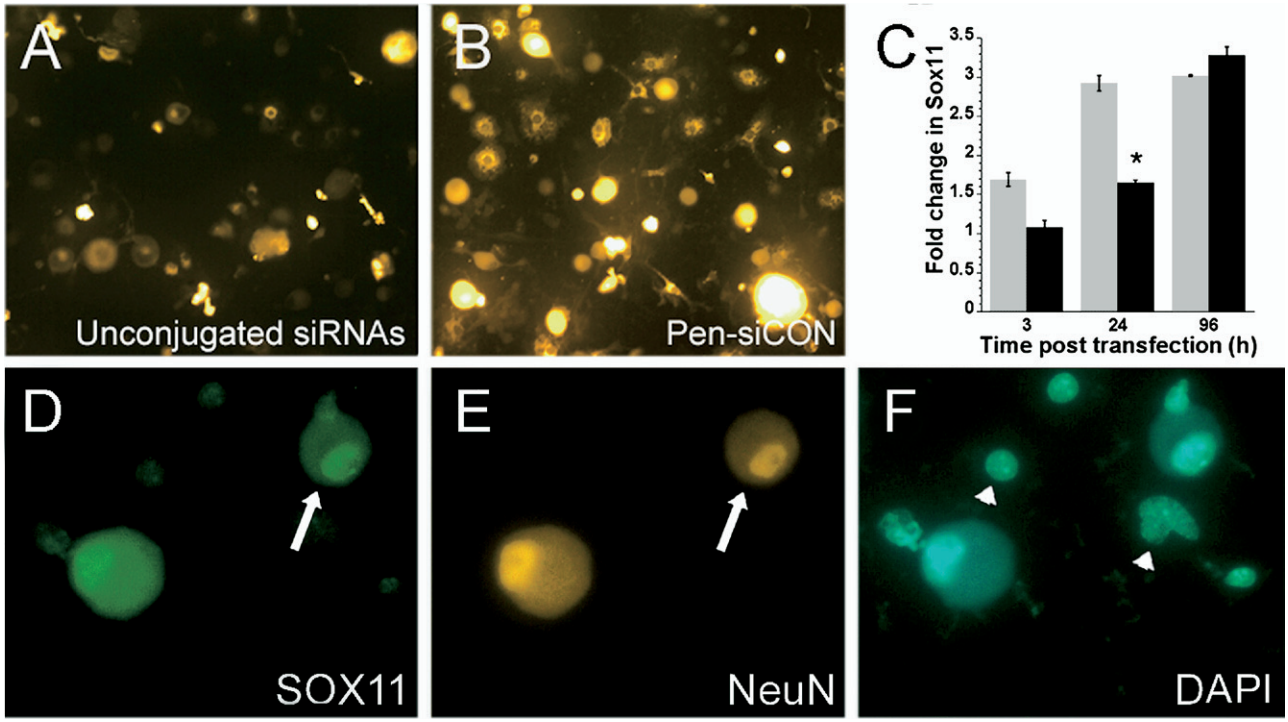

Fig. 8. Transfection of Penetratin-linked Sox11 siRNAs reduces Sox11 expression in cultured DRG neurons. (A) Cultured DRG neurons 24 h after addition of non-targeting Cy3 labeled siRNA not linked to Penetratin. Few of the large, round neurons are brightly labeled. (B) Parallel cultures incubated with non-targeting Cy3 labeled siRNA conjugated to Penetratin show a significant number of Cy3-labeled neurons. (C) Addition of Penetratin-linked Sox11 siRNAs reduces Sox11 mRNA level measured using RT-PCR ($N=3$ per group). Asterisk indicates $P<0.05$. DRG neurons are immunopositive for SOX11 (panel D; arrow) and NeuN (panel E; arrow). Non-neuronal cells present in primary DRG cultures stained for DAPI are not SOX11 or NeuN positive (panel F; arrowheads).

strong induction of endogenous Sox11 mRNA in DRG neurons following plating (see Fig. 7). This induction effect may also explain the reduced knockdown (59%) measured in Neuro2a cultures stimulated with RA to differentiate and express Sox11.

Having established reduction in Sox11 using the Penetratin transfection procedure, we tested the effect of Sox11 knockdown on DRG neuron survival and neurite growth. DRG cultures incubated with Penetratin-linked nontargeting siRNAs (Fig. 9A, C) appeared healthy with significant axon growth at 4 days post-siRNA transfection. In contrast, cultures treated with Penetratin-linked Sox11 siRNA had a significant reduction in neurite length and branching and appeared to have a smaller average somal size suggesting they were under stress (Fig. 9B, D). We therefore assessed neuron survival and growth by measuring neurite length, the number of neurite branch points and the percent of neurons positive for casp3* at 24 h and 4 days post-RNAi treatment (Table 2). These results indicate that the modest knockdown in Sox11 expression at the time of plating caused a significant decrease in neurite length (at 4 days) and neurite branching. In addition, an increase in the number of apoptotic cells, as defined by casp3*-reactivity, was also measured at the 24 h and 4 day time points (Table 2). Thus, the early knockdown in Sox11 at the time of plating is likely to cause a continual loss of neurons through apoptotic mechanisms.

Possible transcriptional targets of Sox11 in cultured DRG neurons

Neuro2a cell analysis suggested that a transcriptional target of Sox11 is TANK, a gene whose downregulation following Sox11 knockdown could contribute to the increase in apoptotic cells in Neuro2a cultures. We therefore assayed changes in TANK expression in cultures of DRG neurons. At 3 h following plating cultures treated with nontargeting siRNAs showed no significant change in TANK expression when compared with levels in untreated cultures (Table 3). A significant increase in TANK from baseline levels was however measured at 24 h post-plating (1.69-fold increase; Table 3). Cultures treated with Sox11 siRNAs showed a trend toward reduction in TANK mRNA ($P=0.07$) at 3 h and a nonsignificant change at 24 h post-transfection. Thus, whether TANK and the NFkB signaling pathway is associated with Sox11 level in DRG neurons is unclear.

The impact of Sox11 knockdown on neurite growth in Neuro2a and primary DRG neurons suggests Sox11 regulates genes that are involved in neurite projections. To identify genes dependent on Sox11 expression that may influence neurite growth in DRG neurons, we identified genes that have Sox consensus binding domains in their proximal promoter region and whose expression was increased in developing sensory ganglia of transgenic animals that overexpress neurotrophic factors in

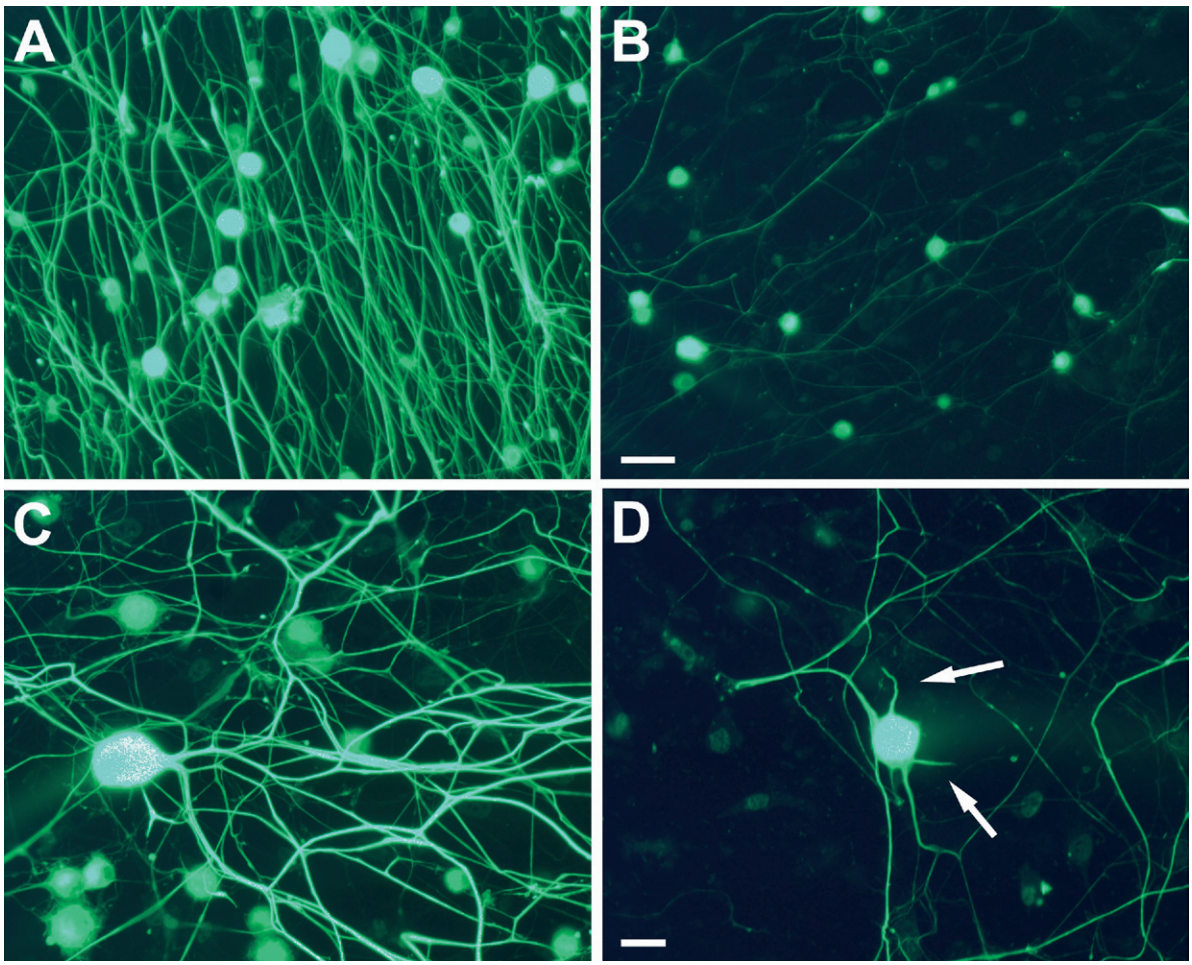

Fig. 9. Knockdown of Sox11 in cultured DRG neurons inhibits neurite growth and increases neuron death. Nontargeting (A, C) and Sox11 targeted siRNAs (B, D) were added to cultures of mouse DRG neurons 1 h after plating. At 4 days, cells were fixed in 4% paraformaldehyde and immunolabeled with anti-NF200 neurofilament antibody followed by a Cy2-conjugated IgG secondary to visualize neurite projections. NF-200 labeling in figure shows neurons treated with Sox11 siRNAs had smaller somas and neurites. Arrows in D indicate short truncated neurites commonly seen on neurons in Sox11 siRNA treated cultures. Panels A and B are 10× mag; panels C and D are 20×. See Table 2 for quantification of data.

the skin (Albers et al., 1994; Zwick et al., 2002). These overexpresser mice have enhanced axon growth and are known to express increased levels of Sox11 in developing sensory neurons (M.P. Jankowski and K.M. Albers, unpublished observations). Using these criteria we assayed the actin-related protein complex (subunit 3) gene (Arpc3). Arpc3 is involved with actin filament reorganization (Mullins et al., 1998; Cooper et al., 2001) and could therefore underlie, in part, cytoskeletal changes required for the enhanced axon growth exhibited by the OE animals and neurite growth of cultured neurons. We found that treatment of DRG neuron cultures with Sox11 siRNA caused a significant reduction in the level of Arpc3 mRNA at 24 h following Sox11 knockdown (Table 3). These data support a role for Arpc3 in early events of neurite growth and a role for Sox11 as a critical regulator of early transcriptional signaling events important for axon growth following injury.

Table 2. Reduction of Sox11 inhibits neurite growth and branching and increases apoptosis

Treatment	Avg neurite length (μm)		Branching index		% Casp3* neurons	
	24 h	4 days	24 h	4 days	24 h	4 days
Nontargeting siRNA	82.1±12.1	112.9±7.7	1.78±18	2.21±0.32	11%	10%
Sox11 siRNA	73.4±10.7	80.2±8.7*	1.01±0.14*	0.92±0.12*	20%*	20%*

All values except the 24 h neurite length comparison showed significant change in Sox11 siRNA-treated cultures.
* $P<0.05$ relative to non-targeting siRNA value at either 24 h or 4 days.

Table 3. Reduction of Sox11 in mouse DRG neurons inhibits expression of mRNAs encoding TANK and Arpc3

Gene assayed	Fold change following Sox11 knockdown	
	3 h	24 h
PenCon-TANK	−1.32±0.03	1.69±0.14
PenSox-TANK	−2.31±0.003	−1.07±0.08
PenCon-Arpc3	1.21±0.01	2.23±0.03
PenSox-Arpc3	1.14±0.01	1.28±0.04*

RT-PCR analysis of mRNA expression in DRG neurons treated with either nontargeting- (PenCon) or Sox11-targeted (PenSox) siRNAs that were linked to Penetratin peptide. Values are relative to those obtained from neurons that were dissociated and plated but not Penetratin treated. Knockdown in Sox11 caused a trend in reduction in TANK mRNA at 3 h post siRNA addition ($P=0.07$, $n=3$). RNA encoding the actin reorganization protein Arpc3 was unchanged in Sox11 siRNA-treated cultures at 3 h but was significantly lower in treated cultures at 24 h post-knockdown. Asterisk indicates $P<0.05$.

DISCUSSION

The identification of Sox11 as a developmentally expressed transcription factor that is rapidly elevated in adult neurons following axotomy prompted us to explore how this transcription factor regulates gene expression in injured adult sensory neurons of the DRG. To begin this analysis we used primary cultures of DRG neurons and the neuronal cell line Neuro2a to determine if Sox11 affects cell survival and neurite extension and if so, what transcriptional targets Sox11 might regulate. Using the Neuro2a and DRG model systems we show that the transcription factor Sox11 is indeed a critical regulator of cell survival and neurite growth. We found that Sox11 is normally expressed in the Neuro2a neuroblastoma cell line and that its expression is significantly increased as these cells differentiate and exhibit neuronal morphologies. Sox11 expression in primary cultures of DRG neurons was also found to rise substantially over time in culture, similar to the rise seen in the DRG following *in vivo* axotomy. These findings suggest that Sox11 is an essential regulator of transcriptional signaling in neuronal cell types that are undergoing differentiation and recovering from injury.

Sox11 expression is required for Neuro2a cell differentiation and survival

In Neuro2a cells grown without RA, Sox11 knockdown had little or no affect on either the number of differentiated or casp3*-positive cells present at 24 h after siRNA treatment. However, stimulation of differentiation using RA enhanced expression of Sox11 during early phases of Neuro2a cell differentiation. If the increased level of Sox11 was reduced at the time of RA stimulation using RNAi, an increase in apoptotic cells and reduction in neurite growth occurred in Neuro2a cells. These changes only occurred if Sox11 knockdown coincided with RA addition.

That Sox11 knockdown in RA-treated cultures increased apoptosis suggests that Sox11 is a critical factor that regulates early transcriptional events induced by RA that promote entry into differentiation and survival pathways. This finding agrees with several lines of evidence that suggest differentiated cells are more susceptible to apoptosis then undifferentiated cells if a critical factor is inhibited. As an example, differentiated neuroectoderm requires the presence of the mitochondrial matrix protein frataxin, and deficiency of frataxin induces differentiated P19 cells to undergo apoptosis (Santos et al., 2001). In a similar manner, the orphan receptor TR4 has been shown in P19 cells to be necessary for RA-induced differentiation and inhibition of TR4 causes differentiated but not undifferentiated cells to undergo apoptosis (Lee et al., 2004).

The requirement for Sox11 in Neuro2a differentiation and survival following RA stimulation parallels findings from other studies of Sox factors in cell lines induced to differentiate using RA stimulation. As mentioned, Sox6 was important for differentiation and neurite outgrowth in the RA-stimulated EC cell line P19 (Hamada-Kanazawa et al., 2004). Similarly, human EC cells induced to differentiate with RA showed regulation of Sox2 and Sox3 expression (Stevanovic, 2003). How each of the different Sox factors regulates gene expression and entry into differentiation programs following RA-stimulation is yet to be defined but these studies suggest a central regulatory role for this family of transcriptional regulators.

The effect of Sox11 knockdown is time dependent

The reduced number of differentiated cells and increased number of apoptotic cells that were found when RA and Sox11 siRNAs were added together (at 0 h) were not seen if siRNAs were added at 6 h, 12 h or 24 h following RA stimulation. The level of Sox11 mRNA was unchanged as well. Why such a short window for effective Sox11 knockdown occurs in Neuro2a and DRG cultures is unclear. One possibility is that the increasing level of Sox11 mRNA that was induced by RA addition or DRG axotomy was too great to be effectively knocked down by a single siRNA treatment. Another possibility is that RA-induced differentiation and the DRG response to injury reduced the efficiency of Sox11 siRNA action. The entry and processing of transfected siRNAs in RNA-induced silencing complexes (RISCs) may have changed following RA stimulation or axotomy and as a result, inhibition of siRNA processing occurred. Precedence for inhibition of RISC activity is well documented in neuronal cells of *Caenorhabditis elegans*, which are refractory to RNA interference and resistant to siRNA modulation (Timmons, 2004). A similar mechanism of inhibition may underlie the resistance to Sox11 RNAi modulation observed in these neural culture systems.

Genes related to cell survival and apoptosis may be transcriptionally regulated by Sox11

To begin to identify target genes of Sox11 involved in Neuro2a survival and differentiation, tailored gene arrays containing cDNAs involved in apoptosis and survival were used. Total RNA from cultures treated with RA alone were compared with RNA isolated from cultures treated with both RA and siRNAs against Sox11. Comparison of probe binding intensities showed that expression of several genes changed in response to Sox11 knockdown. RT-PCR validation of can-

didate Sox11 modulated genes, defined by having at least one Sox11 binding site within two kb 5′ of the start codon, showed results generally consistent with the array analysis. Genes of interest were TANK, BNIP3, Blk and Bcl10, all of which have the potential to modulate entry into apoptosis. TANK, which was decreased following Sox11 knockdown, can have pro-survival activity through its binding to NEMO (NF-κB essential modulator) (Chariot et al., 2002) and subsequent activation of the transcriptional regulator NF-κB (Bonif et al., 2006). TANK works with the co-inducer TRAF2 to increase NF-κB activity and this increase in activity generally induces genes favoring neuron survival (Mattson and Meffert, 2006). The reduction in TANK mRNA level associated with the siRNA-mediated Sox11 knockdown may therefore contribute to the increased number of apoptotic cells in the Sox11-deficient cells.

In contrast to TANK, the pro-apoptotic BNIP3 gene was increased following Sox11 knockdown, suggesting Sox11 may negatively regulate BNIP3. BNIP3 is a mitochondrial protein whose expression is regulated by nitric oxide signaling (Yook et al., 2004). BNIP3 expression is significantly increased in cortical neurons following ischemia and its transfection into MCF-7 and Rat-1 cells induces a rapid onset of apoptosis (Chen et al., 1999; Althaus et al., 2006). Conversely, siRNA knockdown of BNIP-3 blocked cell death (Manka et al., 2005). Thus, the increased expression of BNIP3 in Neuro2a cells treated with Sox11 siRNAs and RA is consistent with the enhanced cell death measured in these cultures.

TANK and BNIP-3 expression were both significantly changed after knockdown of Sox11 in RA-treated Neuro2a cells suggesting that Sox11 normally acts to transcriptionally regulate these genes in early phases of RA-induced differentiation. Consistent with this possibility is that the 5′ regulatory regions of TANK and BNIP3 contain four Sox binding domains, compared with only one domain in the Blk and Bcl10 genes (Fig. 6B). Blk, a pro-apoptotic, mitochondrial membrane-associated protein that interacts with the anti-apoptotic protein *Bcl*II (Hegde et al., 1998), was increased on the gene array at 24 h (though decreased at the 12 h time point), and RT-PCR validation showed only a trend toward increased expression. It seems unlikely therefore, that changes in Blk transcription are required for the Sox11 knockdown-induced cell death observed in the Neuro2a cultures.

Sox11 is a regulator of survival and neurite growth in DRG sensory neurons

The effect of Sox11 knockdown on cultured DRG neurons was assessed by transfection of Penetratin linked Sox11-siRNAs into cultured mouse DRG neurons. Knockdown of Sox11 significantly increased the number of casp3*-positive neurons and decreased the length and branching of growing neurites. Thus, similar to Neuro2a cells, DRG neurons are also dependent on Sox11 for cell survival and neurite growth, in this case following the stress and injury associated with enzymatic dissociation and cell culture. This finding parallels recent *in vivo* analysis of Sox11 that showed it was rapidly elevated in DRG neurons following nerve cut (Tanabe et al., 2003; Jankowski et al., 2004). Given the high level of Sox11 expression during sensory neuron development, this injury-associated rise in Sox11 raises the possibility that transcriptional targets of Sox11 in embryonic neurons are reactivated in injured adult neurons to promote survival and regeneration of damaged afferents. One possible transcriptional target of Sox11 that may be involved in neurite extension and branching is Arpc3, a protein that localizes to membrane ruffles and dynamic regions of actin assembly which is thought to contribute to actin filament nucleation (Mullins et al., 1998). Although further confirmation is required, the reduction in Arpc3 following Sox11 knockdown suggests a new means of regulating axon growth.

The finding that the developmentally expressed Sox11 protein has a critical role in regulating genes important in nerve regeneration is in line with the idea that following peripheral axotomy of adult sensory neurons, which is permissive for axon regeneration, surrounding environmental cues allow neurons to 'dedifferentiate,' access embryonic growth programs and express regeneration-associated genes (Aubert et al., 1995). Consistent with this possibility is the increased expression of the developmentally important growth factors NGF, GDNF, BDNF, NT3 and NT4 in satellite cells of the DRG and Schwann cells on the distal end of the cut nerve (Verge et al., 1996; Terenghi, 1999). In particular, increased expression of BDNF in injured neurons occurs, with 83% expressing BDNF immunoreactivity (Karchewski et al., 2002). By 1 week and up to 3 weeks later, expression is still apparent in 66% of injured neurons. These data, in combination with the finding that Sox11 mRNA is increased in developing sensory ganglia of transgenic mice that overexpress neurotrophic factors, support the possibility that Sox11 is a trophic factor-modulated transcriptional regulator during development and in the adult following injury.

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