

Akt Increases Sox2 Expression in Adult Hippocampal Neural Progenitor Cells, but Increased Sox2 Does Not Promote Proliferation

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Multiple extracellular factors have been shown to modulate adult hippocampal neural progenitor cell (NPC) proliferation and self-renewal, and we have previously shown that Akt is an important mediator of the effects of these extracellular factors on NPC proliferation and differentiation. However, very little work has investigated how and whether Akt is involved in maintaining the multipotency of these cells. Here we demonstrate that Akt promotes expression of Sox2, a core transcription factor important for the self-renewal of NPCs. Retroviral-mediated overexpression of wild-type Akt increased Sox2 protein expression, particularly under conditions that promote cell differentiation, whereas Akt inhibition decreased Sox2. Similarly, quantitative reverse transcription (RT)-PCR in differentiating cultures indicated that Akt rescued Sox2 mRNA to levels present under conditions that promote cell proliferation. Additionally, pharmacological inhibition of Akt did not affect Sox2 protein levels in cells constitutively expressing Sox2 from a retroviral vector, indicating that Akt does not affect Sox2 protein stability. Further, in contrast to Akt overexpression, Sox2 overexpression does not increase NPC viable cell number or proliferation yet does inhibit differentiation. Collectively, these results indicate that Akt promotes cell proliferation and maintenance of a multipotent state via two downstream paths.

Introduction

NEURAL PROGENITOR CELLS (NPCs) from the adult hippocampus have the potential to maintain their population, a process called self-renewal, as well as to undergo lineage commitment and differentiation into the three major cell types of the mammalian brain: neurons, astrocytes, and oligodendrocytes [1]. The regulation of these processes is central to adult neurogenesis [2,3], which in turn may be important for learning, memory, and mood regulation [4–10]. A number of extracellular factors have been shown to modulate NPC proliferation and self-renewal, including basic fibroblast growth factor (FGF-2) [11], epidermal growth factor [12], and Sonic hedgehog [13]. However, the intracellular signaling cascades that functionally mediate these extracellular signals in NPCs have only recently been explored. The importance of Akt has been demonstrated in many stem cell types, including mouse embryonic stem (ES) cells [14,15], primate ES cells [16], rabbit ES cells [17], mesenchymal stem cells [18], and hematopoietic stem cells [19,20]. Additionally, we have previously reported that Akt is important for NPC proliferation and inhibition of differentiation [21]. Others have shown the importance of signaling events that lie

downstream of Akt to NPC maintenance, including mTOR [22], FoxO [23], and GSK-3 [24]. However, very little work has investigated whether and how this signaling pathway may be involved in maintaining the multipotency of these cells.

The SRY-related HMG-box 2 (Sox2) transcription factor is important in the self-renewal of ES cells [25] and is a critical factor that can contribute to reprogramming and generation of induced pluripotent stem (iPS) cells [26]. Within the central nervous system, lineage tracing studies have shown that Sox2-positive cells in the hippocampus can self-renew and generate differentiated progeny [27]. It is also involved in NPC maintenance [28] and has increasingly been utilized as an NPC marker [29]. Further, in chick embryos, Sox2 overexpression prevents differentiation [30], and it is also known to repress the transcription of glial fibrillary acidic protein (GFAP), an important astrocytic marker [31], consistent with its promotion of an immature cell state.

Control of Sox2 expression and activity in NPCs is not well understood, though some Sox2 control mechanisms have been studied in other cell types. Post-translational modifications, including sumoylation [32], phosphorylation [33], and poly(ADP-ribosylation) [34], as well as heterodimer formation with Oct4 [35], can regulate Sox2

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activity. Transcriptional control of Sox2 is promoted by the Sox2 regulatory region 2, a Sox2 enhancer known to regulate its expression in the telencephalon [36]. Despite these advances in our understanding of Sox2 regulation, little is known about the signaling pathways that drive Sox2 expression in general and particularly in NPCs. The importance of both Akt and Sox2 to pluripotency [14–17] and multipotency [18–20] raises the question whether Sox2 expression is linked to Akt activity.

Here we extend our previous work with Akt [21] to demonstrate that it both enhances cell proliferation via a Sox2-independent mechanism, as well as promotes the expression of the transcription factor Sox2 to support multipotency. Retroviral-mediated overexpression reveals that Akt activity promotes increased Sox2 expression. We also find that increased Sox2 protein levels, while inhibiting differentiation, do not increase proliferation. These results indicate that Akt serves as an important master regulator in NPC maintenance by independently promoting downstream cell proliferation and Sox2-dependent self-renewal.

Materials and Methods

Cell culture

Adult NPCs isolated from the hippocampi of 6-week-old female Fischer 344 rats as described [37] were cultured on tissue culture polystyrene coated with poly-ornithine and 5 µg/mL of laminin (Invitrogen). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) high-glucose medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/mL recombinant human FGF-2 (Peprotech).

Mutant cell lines

Progenitor cells constitutively expressing wild-type Akt or Sox2 were generated by retroviral infection. Wild-type murine Akt1 cDNA (Akt) was a kind gift from S. Ferguson (Robarts Research Institute, London, ON, Canada), and wild-type murine Sox2 cDNA was obtained from Stemgent. Neither cDNA included untranslated regions. Both cDNAs were subcloned into the MLV retroviral vector CLGPIT, a variant of CLPIT [38] in which the puromycin resistance gene puromycin *N*-acetyl transferase is replaced with a gene encoding a GFP-puromycin *N*-acetyl transferase fusion [39] (a kind gift from M. McVoy, Governor's School for Government and International Studies, Richmond, VA). An empty control vector was also produced, and correct products were confirmed by sequencing. Retroviral vectors were packaged using CMV gag-pol and CMV VSV-G envelope helper plasmids by calcium phosphate transfection as described [38]. Vectors were harvested, concentrated by ultracentrifugation, and titered on HEK 293Ts. Progenitor cells were infected at a multiplicity of infection of 1 IU/cell and subsequently selected with 0.6 µg/mL puromycin (Sigma) for 4 days.

WST-1 assay

NPCs overexpressing wild-type Sox2 or an empty vector control were plated at 1,000 cells/well on 96-well poly-ornithine/laminin-coated tissue culture plates with either 0 or 1 ng/mL FGF-2 in DMEM/F-12 + N-2 medium. Each condition was cultured in biological quintuplicate, and 50%

medium changes were conducted daily. After 5 days in culture, cell number was quantified using the WST-1 assay following the manufacturer's instructions (Roche Applied Science) and utilizing a standard curve generated with known cell numbers, according to hemocytometer.

Bromodeoxyuridine proliferation assay

NPCs transduced with retrovirus to overexpress wild-type Sox2 or the corresponding empty vector control were seeded in triplicate at 15,000 cells/well onto Falcon 8-well chamber slides coated with poly-ornithine and 10 µg/mL laminin. Cells were cultured overnight in either 0 or 1 ng/mL FGF-2, pulsed with 10 µM bromodeoxyuridine (BrdU) for 12 or 24 h, and then fixed with 4% paraformaldehyde for 10 min. Cells were rinsed with phosphate-buffered saline (PBS) 3 times and then incubated in 2 N HCl at 37°C for 30 min to expose the BrdU antigen. The HCl was removed, and residual HCl was neutralized with 0.1 M borate buffer followed by 3 PBS washes. Cells were then blocked for 1 h in PBS containing 5% goat serum + 0.3% Triton X-100 followed by staining with rat anti-BrdU (Abcam, ab6326, 1:250) in blocking buffer overnight at 4°C. After 3 PBS washes, slides were stained with Cy3-conjugated, goat anti-rat secondary antibody (Jackson ImmunoResearch, 1:250) for 2 h in blocking buffer. Slides were washed 3 more times, and the final wash contained 5 µg/mL of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, D21490). Slides were mounted with Cytoseal 60 (Fisher Scientific), and the percentage of cells staining positive for BrdU was quantified manually.

Quantitative reverse transcription-PCR

NPCs were seeded at 200,000 cells/well in 6-well poly-ornithine/laminin-coated culture plates in DMEM/F-12 + N-2 medium containing either 1 ng/mL FGF-2 to sustain multipotency or 1% fetal bovine serum (FBS) plus 1 µM retinoic acid (RA) (Biomol) to induce differentiation. Eight replicates were performed for each condition. The medium was replenished daily, and on day 5 RNA was isolated by TRIzol (Invitrogen) according to manufacturer's instructions. cDNA was then generated using Invitrogen's ThermoScript RT-PCR kit according to manufacturer's instructions. Using a BioRad iCycler, TaqMan probe QPCR was performed for the astrocytic marker GFAP, the neuronal marker β-tubulin III, or Sox2 with the 18S ribosomal subunit as an internal control. Quantitative RT-PCR for GFAP and β-tubulin III has been shown to be a reliable measure of NPC differentiation [40]. GFAP, β-tubulin III, and Sox2 probes from Biosearch Technologies contained the FAM490 fluorophore with Black Hole Quencher, whereas the 18S rRNA probe contained the CAL610 fluorophore with Black Hole Quencher. Table 1 lists primer and probe sequences.

Western blotting

NPCs were cultured in 6-well poly-ornithine/laminin-coated plates for 5 days, unless otherwise indicated. Cells were lysed by adding lysis solution directly to the culture plate. Lysis solution contained IGEPAL (1%; Sigma), sodium dodecyl sulfate (SDS) (0.1%), phenylmethanesulfonyl-fluoride (0.1 mg/mL; Sigma), aprotinin (0.03 mg/mL; Sigma), and sodium orthovanadate (1 mM; Sigma) in PBS. Lysate

TABLE 1. PRIMER AND PROBE SEQUENCES FOR QUANTITATIVE RT-PCR

		<i>Sequence</i>
β-Tubulin III		
Forward primer		5'-GCATGGATGAGATGGAGTT CACC-3'
Reverse primer		5'-CGACTCCTCGTCGTCATCTT CATAAC-3'
Probe		FAM490-TGAACGACCTGGTGT CTGAG-BHQ
GFAP		
Forward primer		5'-GACCTGCGACCTTGAGTCCT-3'
Reverse primer		5'-TCTCCTCCTTGAGGCTTTGG-3'
Probe		FAM490-TCCTTGGAGAGGCAA ATGCCG-BHQ
Sox2		
Forward primer		5'-CGAGTGGAAACTTTTGTCGG AGAC-3'
Reverse primer		5'-CGGGAAGCGTGACTTATCC TTCTT-3'
Probe		FAM490-CTCTGCACATGAAGGA GCACC-BHQ
18S		
Forward primer		5'-GTAACCCGTTGAACCCCA TTC-3'
Reverse primer		5'-CCATCCAATCGGTAGTAGC GA-3'
Probe		CAL610-AAGTGCGGGTCATAAG CTTGCG-BHQ

BHQ, Black Hole Quencher; GFAP, glial fibrillary acidic protein.

protein concentrations were quantified by BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Equal amounts of protein from each lysate were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). In cases where blots were stripped and re-probed, phosphorylated epitopes were probed first, as previously described [41]. Primary antibodies included rabbit anti-Sox2 (Abcam, ab15830, 1:2000), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, ab9485, 1:2000), rabbit anti-phosphoT308 Akt (Cell Signaling, 4056, 1:1,000), rabbit anti-phosphoS473 Akt (Cell Signaling, 9271, 1:1,000), and rabbit anti-total Akt (Cell Signaling, 9272, 1:2,000). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce, 31460, 1:10,000) was used to develop the blots.

Immunofluorescence

NPCs were seeded onto Falcon 8-well chamber slides coated with poly-ornithine and 10 μg/mL laminin. Cultures grown in 1 ng/mL FGF-2 were seeded with 20,000 cells/well, whereas cultures grown with 1% FBS + 1 μM RA or with 1 μM API-2/triciribine (Akt inhibitor, dissolved in DMSO; EMD Biosciences) were seeded with 40,000 cells/well. Cells were grown for 5 days with 50% media replenishment daily then fixed for 10 min with 4% paraformaldehyde. After 4 PBS washes, cells were blocked/permeabilized for 2 h with blocking buffer containing 0.3% Triton X-100 + 5% donkey serum in PBS. Cells were then stained with goat anti-Sox2 (Santa Cruz, sc-17320, 1:200) or IgG isotype control in

blocking buffer for ~72 h at 4°C. After 4 PBS washes, slides were stained with Cy3-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, 705-165-147, 1:250) for 2 h in blocking buffer. Slides were washed 4 more times, and the final wash contained 5 μg/mL of the nuclear stain DAPI (Invitrogen, D21490). Slides were mounted with Cytoseal 60 (Fisher Scientific). Four random fields from each well were used for quantification. Using the freely available image analysis software CellProfiler [42], primary objects/nuclei were identified using the DAPI stain, and the pixel intensity of Sox2 staining within each nucleus was then measured. In all cases, Sox2 staining remained localized to the nucleus. The average intensity of the control condition was arbitrarily set to unity, and all other experimental conditions were normalized accordingly. At least 400 cells were quantified for each experimental condition.

Results

Sox2 expression and Akt activation increase under proliferating conditions

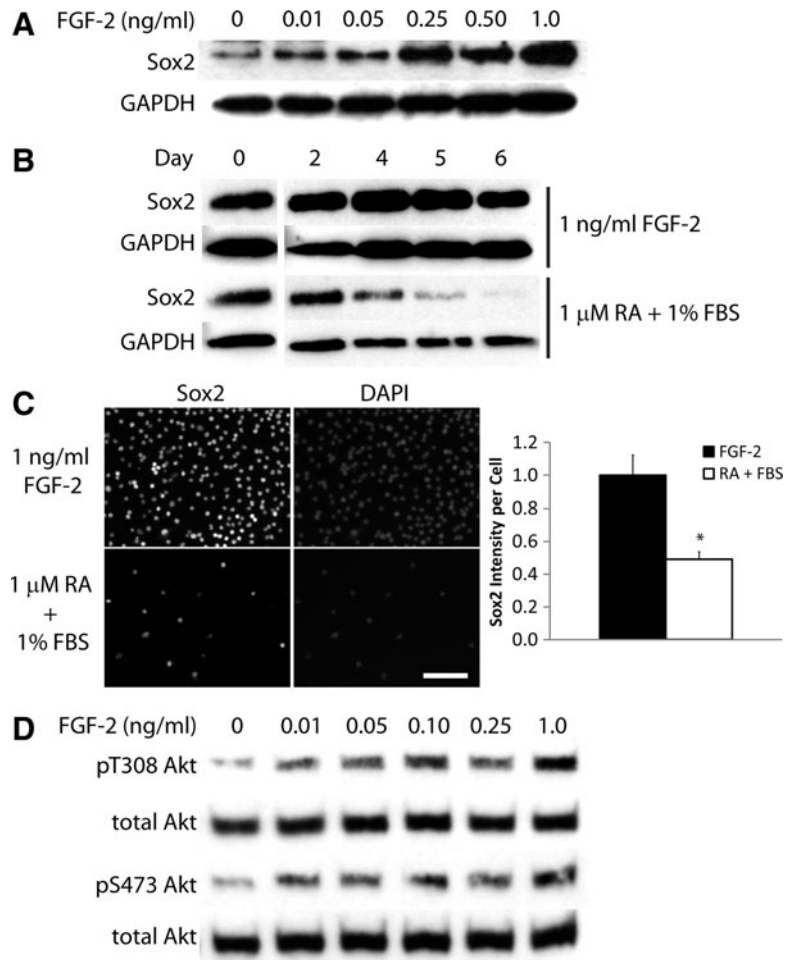
Given the importance of Sox2 in numerous stem cell processes, we sought to determine how its expression is regulated in NPCs. We first analyzed Sox2 protein levels under culture conditions known to support NPC self-renewal and proliferation. Specifically, we cultured NPCs with varying FGF-2 concentrations for 5 days, lysed the cultures, and probed for Sox2 via immunoblotting (Fig. 1A). Sox2 expression increases with increasing FGF-2 concentration. We also performed the converse experiment in which cells were differentiated for 6 days with 1 μM RA + 1% FBS, which also halts proliferation, and Sox2 protein expression was measured by immunoblotting (Fig. 1B). Consistent with its role as a NPC marker, Sox2 expression decreases upon differentiation, a result confirmed quantitatively by immunostaining (Fig. 1C).

Our previous results showed that Akt stimulates NPC proliferation and inhibits differentiation [21]; therefore, we sought to determine whether Akt activation and Sox2 expression acted in concert. To analyze Akt activation as a function of FGF-2 dosage, analogous to the experiment with Sox2 (Fig. 1A), NPCs were starved of FGF-2 overnight, stimulated with various FGF-2 concentrations, lysed, and probed for phosphorylated, active Akt (Fig. 1D). Increasing FGF-2 concentrations resulted in higher Akt phosphorylation at both the S473 and T308 sites, which are critical for full activation of the molecule. These data indicate a correlation between Akt activation and Sox2 expression within NPCs.

Akt promotes Sox2 expression

To begin to analyze whether the parallel between Akt activity and Sox2 expression is correlative or causal, we overexpressed wild-type Akt in NPCs via retroviral transduction. Cells were cultured under either proliferative (1 ng/mL FGF-2) or differentiation (1 μM RA + 1% FBS) conditions. After 5 days in culture, the intensity of Sox2 expression was measured via immunofluorescence and compared with empty vector infected control cells and control cells cultured with the Akt inhibitor API-2/triciribine (1 μM) (Fig. 2A, B). Cells overexpressing Akt had only slightly increased Sox2 expression under proliferation conditions,

FIG. 1. Sox2 expression and Akt activation both increase under proliferating conditions. **(A)** Cells were cultured in various fibroblast growth factor 2 (FGF-2) concentrations. Lysates were probed for Sox2, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. **(B)** Cells were cultured for the indicated number of days in media containing either 1 ng/mL FGF-2 or 1 μ M retinoic acid (RA) + 1% fetal bovine serum (FBS). All bands are from the same blot; however, intervening lanes have been removed for clarity. **(C)** *Left panel:* cells were cultured in the indicated media and stained for Sox2. Counterstain: DAPI. Scale bar: 200 μ m. *Right panel:* quantification of images. Error bars are 95% confidence intervals, and $*P < 0.05$ according to Student's *t*-test. **(D)** Cells were FGF-2-starved overnight, then stimulated with indicated concentrations of FGF-2 for 10 min. Lysates were probed for Akt phosphorylated at S473 and T308. Loading control: total Akt.

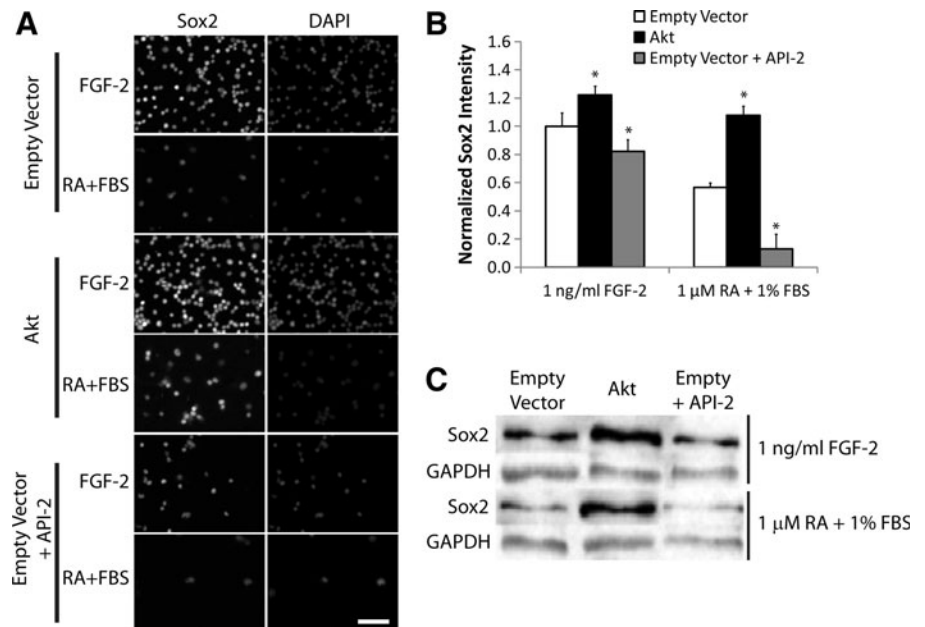


likely due to the already strong mitogenic signal provided by the FGF-2 present in the culture. However, despite the finding that differentiation induces a considerable loss of Sox2 expression (Fig. 1B), cells overexpressing Akt cultured under differentiation conditions maintained Sox2 expression

levels comparable to control cells cultured in proliferation conditions, indicating a complete rescue of Sox2 expression.

Additionally, empty vector control cells cultured with API-2 had decreased Sox2 expression under proliferation conditions. This partial but not complete elimination of Sox2

FIG. 2. Akt promotes Sox2 expression. **(A)** Cells overexpressing Akt, vector control cells, or control cells cultured with Akt inhibitor (1 μ M API-2/triciribine) were seeded in media containing either 1 ng/mL FGF-2 or 1 μ M RA + 1% FBS. Cells were stained for Sox2. Counterstain: DAPI. Scale bar: 200 μ m. **(B)** Quantification of images. Results are normalized to the vector control + FGF-2 condition. Error bars are 95% confidence intervals, and $*P < 0.05$ according to Student's *t*-test. **(C)** Same cells and conditions as above. Lysates were probed for Sox2, using GAPDH as loading control.



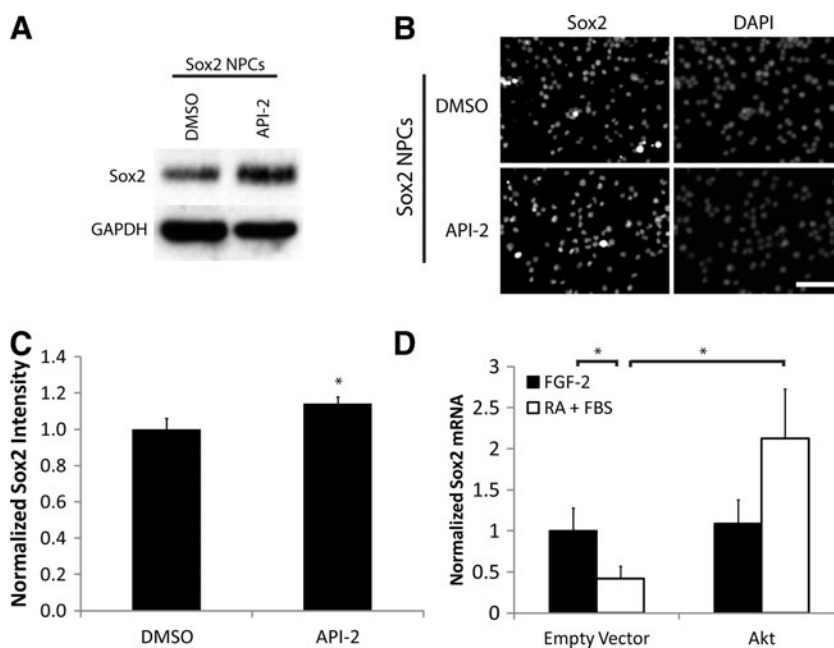


FIG. 3. (A) Sox2-overexpressing neural progenitor cells (NPCs) were cultured with API-2 (Akt inhibitor) or dimethyl sulfoxide (DMSO) carrier control. Lysates were analyzed by immunoblotting for Sox2, using GAPDH as a loading control. (B) Similar to above, Sox2-overexpressing cells were cultured with API-2 or DMSO and stained for Sox2, with DAPI counterstain. Scale bar: 200 μ m. (C) Quantification of images. (D) Akt-overexpressing NPCs along with control were cultured with either 1 ng/mL FGF-2 or 1 μ M RA + 1% FBS. Quantitative RT-PCR was performed to detect Sox2. Samples were normalized to the 18S ribosomal subunit as an internal control. Error bars are 95% confidence intervals, and * $P < 0.05$ according to Student's *t*-test.

expression in the presence of API-2 indicates that Akt is important but likely not the sole mediator of FGF-2 upregulation of Sox2 expression. That said, Akt inhibition under differentiation conditions almost completely eliminated Sox2 expression. To further confirm the immunofluorescence results, we repeated the experiment and probed cell lysates for Sox2 levels via Western blotting (Fig. 2C). Akt overexpression again increased Sox2 protein expression, whereas Akt inhibition decreased expression, particularly under differentiation conditions.

Akt effects on Sox2 protein stability and mRNA concentration

Akt could modulate Sox2 levels by acting at the protein and/or mRNA level. To analyze whether Akt promotes Sox2 protein stability, we stably overexpressed wild-type Sox2 in NPCs using a retroviral vector, where Sox2 is constitutively transcribed and expressed. These cells were cultured for 5 days with 1 ng/mL FGF-2 and 1 μ M API-2 and analyzed for Sox2 protein expression by western blot (Fig. 3A) and immunofluorescence (Fig. 3B, C). Compared with carrier control (DMSO), API-2 did not decrease Sox2 protein concentration. In fact, immunofluorescence quantification revealed a slight but statistically significant increase in Sox2 intensity per cell when cultured with the Akt inhibitor (Fig. 3C). However, the fact that Akt inhibition did not decrease Sox2 protein in cells constitutively expressing Sox2 indicates that Akt is unlikely to promote Sox2 protein stability.

To assess alternatively whether Akt increases Sox2 protein concentration by modulating mRNA levels, we again cultured Akt-overexpressing NPCs and empty vector control cells for 5 days under proliferation or differentiation conditions. RNA was isolated, and quantitative RT-PCR for Sox2 was performed on the resulting cDNA (Fig. 3D). As expected, Sox2 transcript concentration decreased upon differentiation of the control cells; however, Sox2 mRNA did not decrease in Akt-overexpressing cells under the same

conditions, demonstrating rescue of Sox2 by Akt, similar to the rescue of Sox2 protein expression (Fig. 2). Taken together, these results indicate that Akt may drive Sox2 expression by increasing the levels of its mRNA, rather than promoting Sox2 protein stability; however, more study is required to elucidate the precise mechanism.

Sox2 inhibits differentiation but does not promote proliferation of NPCs

We have shown that Akt upregulates Sox2 expression (Fig. 2), as well as promotes cell proliferation and inhibits differentiation [21], but it is unclear whether Sox2 promotes proliferation, maintains NPC multipotency, or both. We again used the Sox2-overexpressing NPCs, which were confirmed by Western blot analysis (Fig. 4A). Both the Sox2 cell line and an empty vector control cell line were infected in parallel and differentiated with 1 μ M RA + 1% FBS, a condition that promotes the generation of both neurons and astrocytes. After 5 days, quantitative RT-PCR of lineage markers was used to analyze cell differentiation (Fig. 4B). We have previously shown this technique to be a reliable measure of NPC differentiation [40], and it has been used in other work [21,40,43–46]. Compared with cells infected with the control vector, Sox2-overexpressing NPCs had significantly decreased expression of both the neuronal marker β -tubulin III and the astrocytic marker GFAP; however, GFAP expression was more strongly inhibited than β -tubulin III, very similar to previous results in cells overexpressing wild-type Akt [21].

We have also previously shown that Akt strongly upregulates NPC proliferation [21]. To determine whether Sox2 has a similar effect, we cultured the cells in both a WST-1 assay, measuring live cells, and a BrdU proliferation assay (Fig. 4C, D). In the WST-1 assay, Sox2 overexpression surprisingly had no effect on the number of live NPCs in the absence of FGF-2. Further, it moderately decreased viable NPCs in the presence of the growth factor. Similarly, Sox2 overexpression had no effect on overall NPC proliferation, or

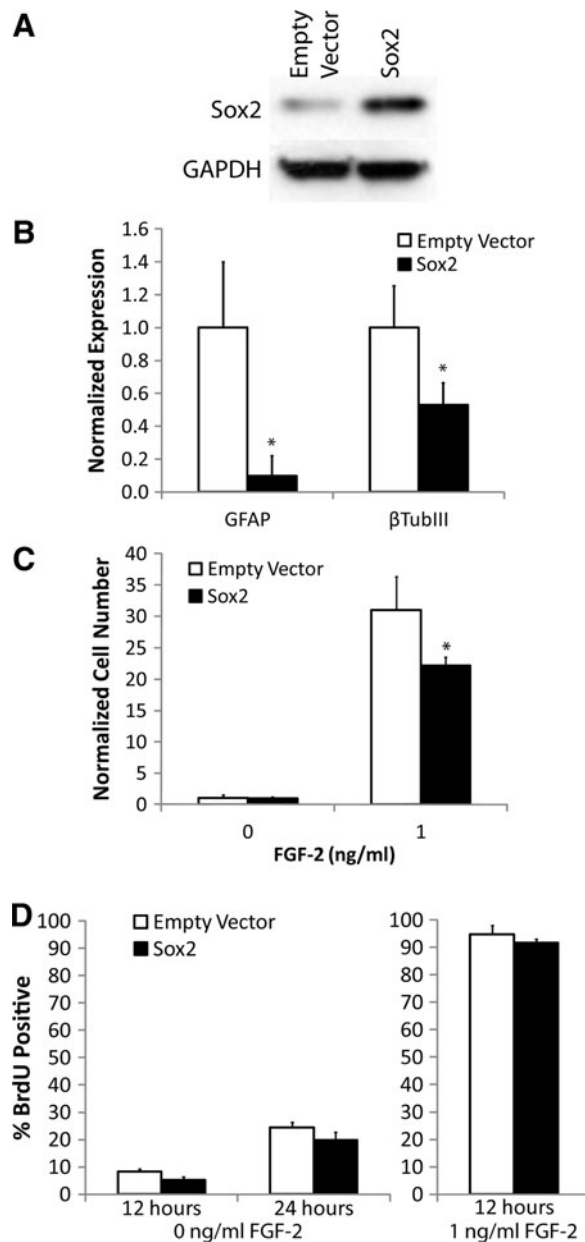


FIG. 4. Sox2 inhibits differentiation without affecting proliferation of NPCs. **(A)** Lysates from cells overexpressing Sox2 or vector control were probed to confirm Sox2 overexpression. Loading control: GAPDH. **(B)** Cells overexpressing Sox2 or vector control were cultured under differentiating conditions (1% FBS + 1 μ M RA). Quantitative RT-PCR was performed for glial fibrillary acidic protein (GFAP) and β -tubulin III, and samples were normalized to the 18S ribosomal subunit as an internal control. **(C)** Sox2-overexpressing cells and control cells were cultured with and without FGF-2. Viable cell number was quantified by WST-1 and normalized to the control sample. **(D)** Cells cultured with 0 or 1 ng/mL FGF-2 were pulsed with bromodeoxyuridine (BrdU) for the indicated time period, and the percentage of BrdU-positive cells was quantified. Error bars are 95% confidence intervals, and $*P < 0.05$ according to Student's *t*-test.

more specifically DNA synthesis, as determined in the BrdU assay. Taken together, these results indicate that Sox2 inhibits NPC differentiation, consistent with its known role in self-renewal [27,28], but does not promote NPCs proliferation.

Discussion

Sox2 is an important regulator of ES cell and NPC self-renewal [27–30], and it is regulated as a part of the core transcriptional circuitry controlling ES cell self-renewal [35,47,48]. However, very little is known about how it is regulated by upstream signaling pathways. One upstream pathway important for the maintenance of numerous stem cell populations is the PI3K/Akt pathway [14–20]; however, it is unknown how or even whether these two critical elements of stem cell regulatory machinery interact. Here we demonstrate that Akt promotes Sox2 protein expression; however, increased Sox2 expression did not increase NPC proliferation. Given our previous work showing that Akt drives NPC proliferation and inhibits differentiation [21], this indicates that Akt is an important regulator of both proliferation and self-renewal in NPCs, but via different downstream pathways.

Akt overexpression completely rescued differentiation-induced loss of Sox2 expression (Fig. 2). Additionally, Akt slightly increased Sox2 expression in cells cultured under proliferative conditions, though this was not a large effect, likely because the cells are already exposed to a strong, FGF-2-mediated mitogenic signal. Interestingly, though Akt inhibition led to a statistically significant reduction in Sox2 expression in cells cultured with FGF-2, the effect was not strong, indicating that Akt is not the only mediator of FGF-2-induced Sox2 expression. There is some evidence that Wnt/ β -catenin signaling is important for NPC self-renewal [49,50]; however, a link between the Wnt signal and Sox2 expression was not established in either of these studies. Additional studies may reveal interesting links between Sox2 and other signaling pathways.

It is well known that Akt promotes protein translation by activating its downstream effector mTORC1 [51]. However, one interesting result of our work is that Akt rescues Sox2 transcript levels in cells cultured under differentiating conditions (Fig. 3D). Additionally, Akt inhibition did not affect Sox2 protein levels in a constitutively expressing Sox2 mutant (Fig. 3A–C), where the exogenous Sox2 cDNA did not contain untranslated regions, indicating that Akt inhibition did not modify protein levels by increasing protein degradation. Overall, these results indicate that Akt activation may modulate *sox2* transcription. One potential mechanism for this is through stabilization of c-Myc [52,53], a transcription factor shown to modulate Sox2 expression in mouse ES cells [48]. Further experimentation is required to determine the precise effects of Akt on Sox2 transcription and translation in addition to any potential intermediate steps between Akt activity and Sox2 expression.

In addition to its role in NPCs, Akt is also known to promote the proliferation and self-renewal of ES cells [14–17]; therefore, investigating the effects of Akt on Sox2 expression in ES cells could have important implications for the development of more efficient ES cell culture systems and perhaps eventually ES cell-based therapies. Further, enhancement of Akt signaling may conceivably improve the

efficiency of reprogramming and the generation of iPSC cells (for a review of other modulators of reprogramming, see [54]). Recent work has demonstrated that pharmacological inhibition of ERK and GSK3 β can generate iPSC cells from neural stem cells transduced with only two of the four Yamanaka factors [55]. Other studies have used GSK3 β inhibition along with ERK and ALK5 inhibition to promote reprogramming [56]. Because GSK3 β activity is directly inhibited by Akt, these findings, taken together with our results, may indicate that modulation of the Akt pathway could potentially improve reprogramming efficiencies by upregulating expression of endogenous Sox2.

Our Sox2-overexpressing NPCs had a decreased ability to differentiate as measured by quantitative RT-PCR (Fig. 4B). In particular, upon exposing cells to conditions strongly favoring astrocytic and neuronal differentiation, Sox2-overexpressing cells had $\sim 10\times$ lower expression of the astrocytic marker GFAP and $\sim 2\times$ lower expression of the neuronal marker β -tubulin III. These results, similar to those seen in NPCs overexpressing Akt [21], further support observations in chick embryos constitutively expressing Sox2, which also experienced impaired neuronal differentiation [28]. Notably, however, Sox2 is still required for proper neuronal differentiation and development. Cells derived from Sox2 hypomorphic mice and cultured in vitro generated abundant β -tubulin III-positive cells, but those cells failed to mature [31]. Therefore, a minimal threshold amount of Sox2 appears to be required for proper neuronal development.

Although Sox2 overexpression inhibited differentiation, it did not increase the number of viable NPCs or their proliferation (Fig. 4C, D). This is consistent with the observation that Sox2-positive cells within the subgranular zone of the hippocampus are able to remain quiescent for extended periods [27]. Additionally, it indicates that although Sox2 is important for NPC self-renewal, it is not sufficient for their proliferation. This is not the case in other cell types. Sox2 promotes proliferation of tracheal and airway stem cells [57], and as an oncogene it is necessary for the proliferation and transformation of lung or esophageal squamous cell carcinomas [58].

In summary, this work demonstrates that in addition to being important for NPC proliferation [21], Akt is also an important promoter of Sox2 expression, thereby maintaining NPC multipotency. Importantly, however, Sox2 itself does not promote adult NPC proliferation, but it does inhibit neuronal and glial differentiation. Therefore, Akt is a key, parallel regulator of NPC proliferation and self-renewal. It may also play these roles in maintaining or even generating other stem cell types, such as ES and iPSC cells.

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Author Disclosure Statement

No competing financial interests exist.

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