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Caspases and p53 Modulate FOXO3A/Id1 Signaling During Mouse Neural Stem Cell Differentiation

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ABSTRACT

Neural stem cells (NSCs) differentiate into neurons and glia, and a large percentage undergoes apoptosis. The engagement and activity of apoptotic pathways may favor either cell death or differentiation. In addition, Akt represses differentiation by up-regulating the inhibitor of differentiation 1 (Id1), through phosphorylation of its repressor FOXO3A. The aim of this study was to investigate the potential cross-talk between apoptosis and proliferation during mouse NSC differentiation. We determined the time of neurogenesis and gliogenesis using neuronal β-III tubulin and astroglial GFAP to confirm that both processes occurred at ~3 and 8 days, respectively. p-Akt, p-FOXO3A, and Id1 were significantly reduced throughout differentiation. Caspase-3 processing, p53 phosphorylation, and p53 transcriptional activation increased at 3 days of differentiation, with no evidence of apoptosis. Importantly, in cells exposed to the pancaspase inhibitor z-VAD.fmk, p-FOXO3A and Id1 were no longer down-regulated, p53 phosphorylation and transcriptional activation were reduced, while neurogenesis and gliogenesis were significantly delayed. The effect of siRNA-mediated silencing of p53 on FOXO3A/Id1 was similar to that of z-VAD.fmk only at 3 days of differentiation. Interestingly, caspase inhibition further increased the effect of p53 knockdown during neurogenesis. In conclusion, apoptosis-associated factors such as caspases and p53 temporally modulate FOXO3A/Id1 signaling and differentiation of mouse NSCs. J. Cell. Biochem. 107: 748–758, 2009.

KEY WORDS: APOPTOSIS; CASPASES; DIFFERENTIATION; NEURAL STEM CELLS; PROLIFERATION; p53

Neural stem cells (NSCs) are defined by their capacity for self-renewal and ability to generate major cell types of the mammalian central nervous system [Doe, 2008]. These features make them a therapeutic tool to treat a broad spectrum of human neurological disorders, including Parkinson's, Alzheimer's, and Huntington's diseases as well as acute stroke [Schwarz et al., 2006]. Despite the therapeutic potential of NSCs, major hurdles must be overcome to render these cells a reliable and efficient system to produce specific cell types for neuro-replacement therapies [Hsu et al., 2007]. Tight regulation of the differentiation process remains a critical issue. In fact, the mechanisms controlling differentiation and lineage specification of NSCs are still poorly understood, and many molecules and their specific functions are yet unknown.

Apoptosis is often thought to result from a failure of cells to correctly exit the cell cycle and differentiate. In this respect, it has been postulated that apoptotic pathways may also regulate other basic cell functions that do not involve cell death per se [Garrido and Kroemer, 2004]. It was recently suggested that several conserved elements of apoptosis are also integral components of terminal differentiation. Therefore, it is not surprising that during differentiation, specific cellular changes occur in a similar manner to those observed during apoptosis [Chasis and Schrier, 1989]. Terminal erythroid differentiation, for example, is characterized by major morphological changes that include chromatin condensation and complete removal of the nucleus [Garrido and Kroemer, 2004]. In addition, it has been demonstrated that the fate of

Abbreviations used: Id1, inhibitor of differentiation 1; NSC, neural stem cells.

Márcia M. Aranha and Susana Solá contributed equally to this work.

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erythroid precursors is determined downstream of caspase activation by the pattern of cleaved targets [Ribeil et al., 2005].

Caspases have recently been implicated in the differentiation process for a variety of model systems [Schwerk and Schulze-Osthoff, 2003]. Specifically, caspase-8 was shown to be required for T cell activation [Misra et al., 2007], while bone morphogenetic protein-induced differentiation of osteoblastic cells has been associated with transient and potent activation of caspase-8, -2, and -3 [Mogi and Togari, 2003]. In addition, it has been demonstrated that NSC differentiation depends on endogenous caspase-3 activity that may influence the kinase activity associated with changes in phenotype [Fernando et al., 2005]. The time of engagement and activity level of these pathways is thought to ultimately determine the choice between cell death or maturation [Fernando and Megeney, 2007]. Nevertheless, the precise role of caspase-3 in NSC differentiation is still largely unknown.

Curiously, widespread neurogenesis and gliogenesis occur in the adult mammalian nervous system in response to certain stress conditions, including hypoxia [Magavi et al., 2000]. The tumor suppressor p53 is known to regulate key cellular functions involved in differentiation. p53 is activated by cellular stress, hyperproliferative signals, and developmental stimuli. One well characterized outcome of p53 activation is irreversible cell cycle exit required for either apoptosis, senescence, or differentiation [Stiewe, 2007]. In fact, p53 was shown to contribute to differentiation of mouse embryonic stem cells by suppressing expression of *Nanog*, which is known to maintain self-renewal and the undifferentiated state of stem cells [Lin et al., 2005]. In addition, reduced p53 expression in human embryonic stem cells has been shown to decrease spontaneous differentiation and slow differentiation [Qin et al., 2007]. Other studies have shown that p53 is required for differentiation of PC12 cells [Zhang et al., 2006], myoblasts [Porrello et al., 2000], and epithelial cells [Saifudeen et al., 2002].

Interestingly, p53 may contribute to differentiation by limiting self-renewal and the undifferentiated state of cells through inactivation of the Akt-mediated phosphoinositide 3-kinase (PI3K) survival pathway. In fact, p53 may inhibit the Akt proliferation pathway by modulating important downstream targets, such as the lipid phosphatase and tensin homolog deleted from chromosome 10 (PTEN) [Stambolic et al., 2001]. Moreover, differentiation of PC12 cells has been associated with enhanced expression of proapoptotic molecules and reduced activation of Akt survival signaling [Mielke and Herdegen, 2002]. Finally, Akt represses cellular differentiation by up-regulating the inhibitor of differentiation 1 (Id1), through phosphorylation of its repressor FOXO3A. Akt regulates the sub-cellular localization of FOXO3A by phosphorylation, thereby preventing its nuclear translocation and inhibition of Id1 [Birkenkamp et al., 2007].

Having provided a rationale for similarities between apoptosis and differentiation cell machineries, the next challenge will be to further define and manipulate an operative pathway common to each process. Half of all neurons produced during neurogenesis die through apoptosis before maturation of the nervous system. This could be explained, at least in part, by the high rate of cell death that occurs during the G1/S cell cycle checkpoint [Liu and Greene, 2001]. In this regard, it is crucial to understand which apoptosis mechanism(s) overlaps with neuronal differentiation pathways that reduce cell death and improve lineage specification for potential neuro-replacement therapies.

Here, we further investigate the role of caspases and p53 during mouse NSC differentiation, and elucidate the cross-talk between apoptosis-associated elements and the Akt survival pathway. Our data strongly indicate that caspases and p53 act in a synergistic manner in suppressing p-FOXO3A/Id1 signaling and, thereby inducing neurogenesis of NSCs.

MATERIALS AND METHODS

MOUSE NSC CULTURE AND DIFFERENTIATION

Mouse NSCs containing a constitutively expressed marker for green fluorescence protein (GFP) were used to investigate the process of neuronal differentiation. Cells were obtained from E14 mouse embryo central nervous system and cultured as described by Rietze and Reynolds [2006] and Reynolds and Weiss [1992]. Mouse NSCs were maintained as neurospheres in undifferentiation medium, serum-free, 1:1 mix of DMEM/F12 (Invitrogen Corp., Grand Island, NY) with 1× N-2 supplement (Invitrogen Corp.), 20 ng/ml EGF, 20 ng/ml b-FGF (R&D Systems Inc., Minneapolis, MN), and 1% penicillin–streptomycin (Invitrogen Corp.), at 37°C in humidified atmosphere of 5% CO_2. Subculture occurred at day 7 with mechanical dissociation of neurospheres. Cellular plating was performed at 5×10^4 cell/ml density on T75 flasks and half of the culture medium was changed after 3 days. The differentiation of mouse NSCs in vitro was induced by culturing dissociated cells in differentiation medium containing DMEM/F12 with 1× N-2 supplement, 100 ng/ml b-FGF, 10% FBS (Invitrogen Corp.), 500 nM all-*trans* retinoic acid (Sigma Chemical Co., St. Louis, MO), 50 μM taurine (Sigma Chemical Co.), 10 ng/ml TGF-β2 (R&D Systems Inc.) and 1% penicillin–streptomycin in tissue culture plates pre-coated with poly-D-lysine (Sigma Chemical Co.). The culture medium was changed every 3 days. Differentiated cells at 5×10^4 cells/ml were fixed at days 3, 6, 8, 10, 12, 14, and 17 and processed for immunostaining and evaluation of differentiation. Neurons and astrocytes were obtained at 3 and 8 days, respectively, in differentiation medium. For western blot analysis, cultures at 5×10^5 cells/ml were processed at days 1, 3, and 8 to assess the role of apoptosis during both neuron and astrocyte differentiation. All experiments were performed using adherent cells only to exclude detached apoptotic cells.

IMMUNOCYTOCHEMISTRY

Mouse NSCs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min during differentiation. Cells were then blocked for 1 h at room temperature in PBS containing 0.1% Triton-X-100, 1% FBS, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Subsequently, cells were incubated with either monoclonal antibodies to glial fibrillary acidic protein (GFAP) (1:1,000), neuronal nuclei (NeuN) (1:10) and oligodendrocyte marker O4 (1:100) (Chemicon Int., Temecula, CA) or the polyclonal antibody to β-III tubulin at a dilution of 1:1,000 (Covance, Princeton, NJ) and to active caspase-3 (1:50) (R&D Systems, Inc.) in blocking solution, overnight at 4°C.

After three washes with PBS, cells were incubated with the AMCA-anti-mouse (Invitrogen Corp.) or the Alexa Fluor 594-anti-rabbit conjugated secondary antibodies (Invitrogen Corp.) for 2 h at room temperature. The localization of GFAP, NeuN, O4, β-III tubulin, and caspase-3 were visualized using an Axioskop fluorescence microscope (Carl Zeiss, Jena, Germany). Total GFP-positive cells were counted on a computer screen grid from at least four random fields (400×). Results were expressed as the average percentage of GFP-positive cells, which co-labeled for β-III tubulin or GFAP.

EVALUATION OF APOPTOSIS

Hoechst labeling and the TUNEL staining of mouse NSCs were used to detect apoptotic nuclei. In brief, for morphologic evaluation of apoptosis, the medium was gently removed at the indicated times with minimal detachment of the cells. Attached cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 μg/ml in PBS for 5 min, washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample were counted and mean values expressed as the percentage of apoptotic nuclei. For TUNEL staining, apoptotic cells were quantitated using the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Cells were fixed with 4% formaldehyde and processed using an Apoptag in situ apoptosis detection kit (Chemicon Int.). The number of TUNEL-positive cells was counted on a computer screen grid from at least three random fields (400×).

TOTAL, CYTOSOLIC, AND NUCLEAR PROTEIN EXTRACTION

For total protein extracts, adherent mouse NSCs were lysed in ice-cold buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 1% Nonidet P-40, 2 mM DTT, and protease inhibitor cocktail tablets (Complete; Roche Applied Science, Mannheim, Germany)) for 30 min, and then homogenized with 20 strokes in a loose fitting Dounce. The lysate was centrifuged at 3,200*g* for 10 min at 4°C and the supernatant recovered. For nuclear and cytosolic extracts, cells were lysed with hypotonic buffer (10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM DTT, and protease inhibitors), homogenized with 20 strokes in a loose fitting Dounce, and centrifuged at 500*g* for 10 min at 4°C. The cytosolic proteins were recovered in the supernatant while the nuclear pellet was washed in buffer containing 10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 0.25 M sucrose, 0.5% Triton X-100, and protease inhibitors, then resuspended and sonicated in buffer containing 10 mM Tris–HCl, pH 7.6, 0.25 M sucrose with protease inhibitors. Finally, the suspension was centrifuged through 0.88 M sucrose at 2,000*g* for 20 min at 4°C, and nuclear proteins were recovered in the supernatant.

IMMUNOBLOTTING

Steady-state levels of p53, p-p53 (Ser15), p-Akt (Ser473), Akt1/2, p-FOXO3A (Thr32), FOXO3A, and Id1, as well as caspase-3 processing were determined by Western blot, using primary rabbit polyclonal antibodies reactive to p-p53 (Ser15) (Calbiochem, Darmstadt, Germany), p-Akt (Ser473), total Akt, Id1 and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), FOXO3A and p-FOXO3A (Thr32) (Chemicon Int.) or primary mouse monoclonal antibody to p53 (Santa Cruz Biotechnology); as well as secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). Membranes were processed for protein detection using Super Signal™ substrate (Pierce, Rockford, IL). β-Actin was used to control for lane loading while acetyl-histone H3 was used as a marker for nuclear protein extraction. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacture's specifications.

p53–DNA BINDING ELISA ASSAY

The Universal EZ-TFA p53 Transcription Factor Assay Chemiluminescent Kit (Upstate-Millipore, Billerica, MA) was used according to the manufacturer's protocol. Nuclear extracts of mouse NSC cells were applied to plates containing biotinylated oligonucleotides with a p53 consensus binding site (5′-GGACATGCCCGGGCATGTCC-3′). After 1 h incubation at room temperature, plates were washed and incubated with diluted p53 antibody (1:1,000) for an additional 1 h. Diluted anti-rabbit horseradish peroxidase-conjugated antibody (1:500) was then added to previously washed plates. The chemiluminescence substract solution was added and incubated for 5–8 min to allow luminescence development. The reaction was stopped and samples read at 1-s integration time in a microplate luminometer. A p53 specific competitor oligonucleotide having the same consensus sequence as the p53 capture probe, but not biotinylated was used as negative control.

INHIBITION OF CASPASE ACTIVITY

Mouse NSC were exposed to 50 μM of the pan-caspase inhibitor z-VAD.fmk (Calbiochem, La Jolla, CA) 23 h after platting in differentiation medium, and then collected after 1, 3, and 8 days. The culture medium supplemented with z-VAD.fmk was changed after 3 days in culture. Total proteins were extracted for immunobloting. Attached cells were fixed for Hoechst staining and immunocytochemistry analysis.

SHORT INTERFERENCE RNA AND TRANSFECTION

A pool of four short interference RNA (siRNA) nucleotides designed to knockdown p53 gene expression in mouse was purchased from Dharmacon (Waltham, MA). A control siRNA containing a scrambled sequence that does not result in degradation of any known cellular mRNA was used as control. Twenty-four hours after platting, the culture differentiation medium was changed to medium without 1% penicillin–streptomycin. Mouse NSCs were transfected using Lipofectamine™ Transfection Reagent for siRNA (Polyplus Transfections, Illkirch, France), according to the manufacturer's instructions for an additional 24 h. The final concentration of

siRNAs was 100 nM. To assess gene silencing, transcript and protein levels of p53 were determined by RT-PCR and Western blot, respectively. Our results indicated that the silencing efficiency of the siRNAs for p53 was ~90% in mouse NSC. To investigate the effect of p53 siRNA throughout the differentiation process, attached cells treated or non-treated with z-VAD.fmk were either harvested for immunoblot or used for immunocytochemistry assays after 3 and 8 days of differentiation. Attached cells were also fixed for Hoechst staining.

RNA ISOLATION AND RT-PCR

Transcript expression of p53 was determined by RT-PCR. Total RNA was extracted from mouse NSCs using the TRIZOL reagent (Invitrogen Corp.). For RT-PCR, 5 μg of total RNA was reverse-transcribed using oligo(dT) (Integrated DNA Technologies Inc., Coralville, IA) and SuperScript II reverse transcriptase (Invitrogen Corp.). Specific oligonucleotide primer pairs were incubated with cDNA template for PCR amplification using the Expand High Fidelity^{PLUS} PCR System from Roche Applied Science. The following sequences were used as primers: p53 sense 5′-GTGAAGCCCTCC-GAGTGTCAGGAGC-3′; p53 antisense 5′-GGTGGGCAGCGCTC-TCTTTGCGC-3′; β-actin sense 5′-GTGGGCCGCTCTAGGCACCAA-3′; and β-actin antisense 5′-CTCTTTGATGTCACGCACGATTTC-3′. The product of the β-actin RNA was used as control.

DENSITOMETRY AND STATISTICAL ANALYSIS

The relative intensities of protein and nucleic acid bands were analyzed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad Laboratories). Results from different groups were compared using the Student's *t*-test, or one-way ANOVA. Kruskal–Wallis or the Mann–Whitney *U*-tests were also used whenever the assumptions of the parametric test were not satisfied. Values of $P < 0.05$ were considered statistically significant. All statistical analysis was performed with GraphPad InStat software (GraphPad Software, Inc., San Diego, CA).

RESULTS

THE Akt/p-FOXO3A/Id1 PATHWAY IS DOWN-REGULATED DURING DIFFERENTIATION

To investigate the potential cross-talk between survival and differentiation pathways during mouse NSC differentiation, we first determined the time of neurogenesis and gliogenesis by immunocytochemistry of differentiated mouse NSC. Neurogenesis was detected at ~3 days, when ~5% of cells were β-III tubulin-positive, but no astrocytic GFAP staining was detected (Fig. 1). Gliogenesis, in turn, was detected at 8 days of differentiation, when ~16% of cells were GFAP positive. At 8 days, the number of β-III tubulin-positive cells was increased; however, the absence of NeuN staining indicates that neurons were not yet fully differentiated (data not shown). In these conditions, we did not find any evidence for the presence of immature oligodendrocytes, as visualized by the absence of O4 staining (Fig. 1).

Activation of the Akt survival pathway may result in FOXO3A phosphorylation [Brunet et al., 1999]. p-FOXO3A is retained in the cytosol, and does not repress Id1 [Birkenkamp et al., 2007]. Thus, we next evaluated the involvement of Akt/p-FOXO3A/Id1 signaling during differentiation of mouse NSCs. Interestingly, our results showed a marked reduction in total levels of p-Akt (Ser473) and p-FOXO3A (Thr32) both at 3 and 8 days of differentiation ($P < 0.05$; Fig. 2A). Id1 was also strongly down-regulated throughout differentiation ($P < 0.01$).

The regulation of FOXO3A was further characterized by evaluating cytosolic p-FOXO3A (Thr32) and nuclear FOXO3A (Fig. 2B). Curiously, the inactive form of FOXO3A, p-FOXO3A (Thr32), decreased by ~60% in the cytosol at 3 days, and was almost undetectable at 8 days of differentiation ($P < 0.01$). In contrast, nuclear levels of active, non-phosphorylated FOXO3A, increased by ~2- and 3-fold at 3 and 8 days, respectively. Thus, modulation of the Akt/p-FOXO3A/Id1 survival pathway during differentiation of mouse NSCs appears to be an important regulatory event. Specifically, downregulation of Id1, possibly through FOXO3A, appears to be required for the induction of mouse NSC differentiation.

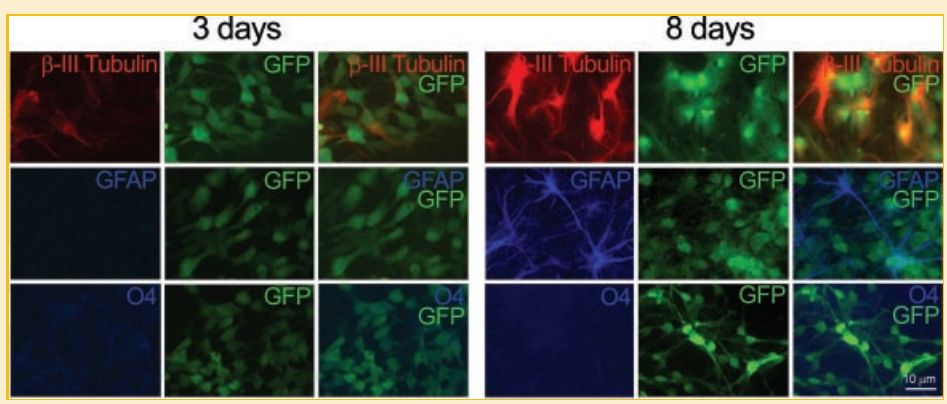


Fig. 1. Time course of neurogenesis and gliogenesis. Differentiated mouse NSCs were fixed at different time points and processed for immunostaining analysis. Neurons and astrocytes were identified with a fluorescent-labeled antibody raised against β-III tubulin and glial fibrillary acidic protein (GFAP), respectively. In these conditions, we found no evidence for the presence of oligodendrocytes (O4). Representative immunostaining images from at least three independent experiments.

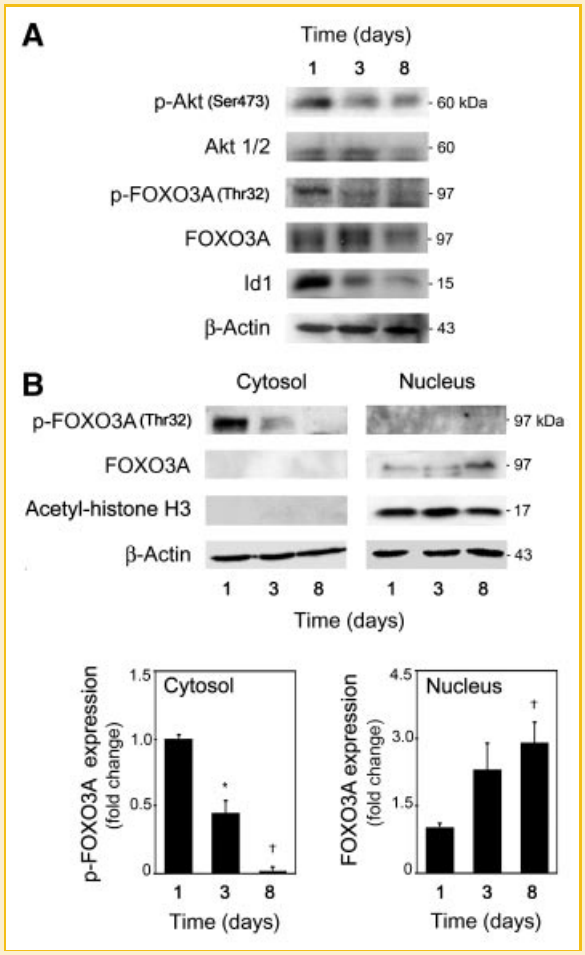


Fig. 2. The Akt/p-FOXO3A/Id1 signaling pathway is downregulated during mouse NSC differentiation. NSCs were induced to differentiate for 1, 3, and 8 days. Total, nuclear, and cytosolic proteins were extracted for immunoblot analysis. A: Representative immunoblots of p-Akt (Ser473), Akt1/2, p-FOXO3A (Thr32), FOXO3A, and Id1 protein expression. B: Representative immunoblots (top) and corresponding histograms (bottom) of cytosolic levels of p-FOXO3A and nuclear levels of FOXO3A in mouse NSCs through differentiation. The results are expressed as mean ± SEM arbitrary units of at least 6 independent experiments. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ from 1 day of differentiation. β-Actin was used as loading control. Cytosolic and nuclear fractions were also immunobloted for acetyl-histone H3.

APOPTOSIS FACTORS ARE INCREASED DURING DIFFERENTIATION

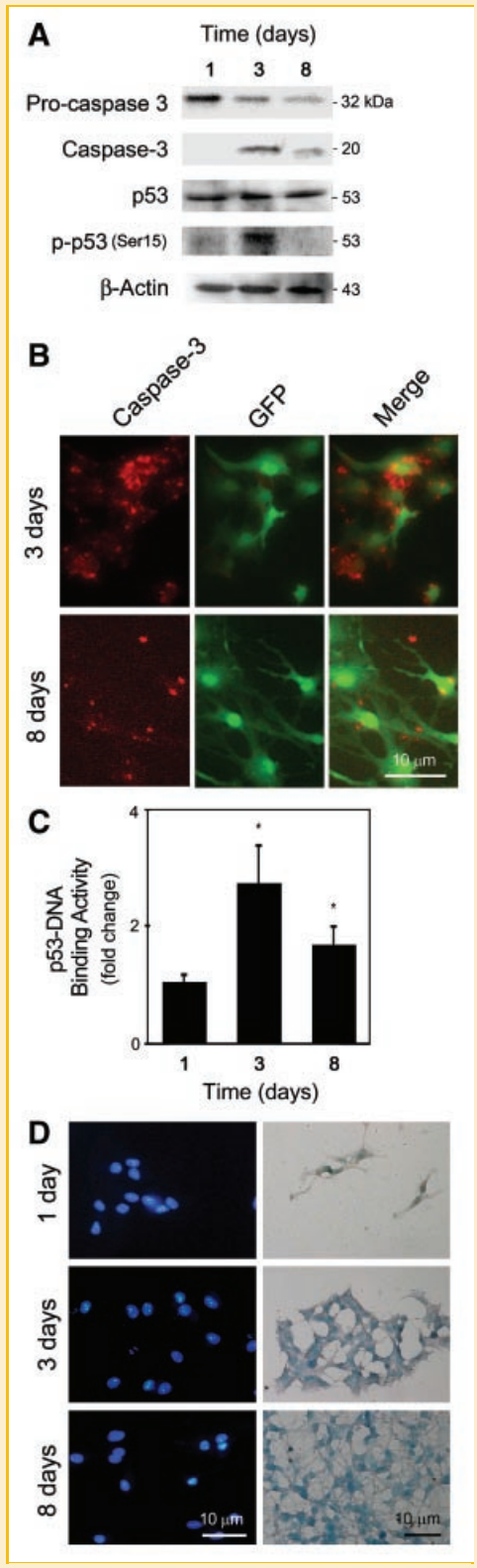
Components of the cell death machinery have been implicated in the differentiation program [Lin et al., 2005; Fernando and Megeney, 2007]. We next evaluated activation of caspase-3 and total levels of p53 during mouse NSC differentiation using only adherent viable cells. Interestingly, the results demonstrated that caspase-3 processing increased three- and two fold at 3 and 8 days of differentiation, respectively ($P < 0.05$; Fig. 3A). This was confirmed through detection of active caspase-3 by immunocytochemistry (Fig. 3B). Caspase-3 activity increased 20% from day 3 to day 8 of differentiation (data not shown). Total levels of p53 were not significantly modulated during differentiation (Fig. 3A). Nevertheless, phosphorylation of p53 at serine 15, which regulates p53 stability and activity [Xu, 2003] and is typically required for transactivation was up-regulated in early stages of differentiation. In fact, p-p53 (Ser15) increased by >2-fold at 3 days, returning to control levels at 8 days of differentiation. In addition, p53 DNA binding activity was increased by ~3- and 2-fold at 3 and 8 days of differentiation ($P < 0.05$; Fig. 3C), respectively, further indicating that regulation of p53 transcriptional activity is an important event for differentiation of mouse NSCs. Finally, both nuclear morphology analysis and TUNEL assays showed only ~2% of apoptotic cells during differentiation (Fig. 3D). This indicates that changes in pro-apoptotic molecules were not associated with increased cell death.

CASPASES INHIBIT p-FOXO3A/Id1 SIGNALING DURING DIFFERENTIATION

We next characterized possible interactions between apoptosis executioners and regulation of the Akt/p-FOXO3A/Id1 pathway during differentiation of mouse NSCs. For this purpose, we suppressed caspase activity with a pan-caspase inhibitor, z-VAD.fmk, and re-evaluated changes in Akt/p-FOXO3A/Id1 expression, p53 phosphorylation (Ser 15), and p53-DNA binding activity. As expected, cells treated with z-VAD.fmk showed reduced caspase-3 activation at both 3 and 8 days of differentiation (Fig. 4A). Notably, caspase suppression markedly decreased p-p53 (Ser15) expression (Fig. 4A) and p53 transcriptional activation (Fig. 4B) ($P < 0.05$). More importantly, in the absence of caspase activity, the p-FOXO3A/Id1 pathway was not down-regulated during differentiation. Indeed, p-FOXO3A (Thr32) increased ~2-fold at 3 ($P < 0.01$) and 8 ($P < 0.05$) days. Id1, in turn, increased 40% at 3 days ($P < 0.05$) and remained unchanged at 8 days of differentiation. p-Akt (Ser473) was maintained throughout differentiation (data not shown). Finally, the absence of significant nuclear fragmentation and TUNEL-positive cells after caspase inhibition (Fig. 4C) suggests that z-VAD.fmk-induced changes were not associated with increased cell death. Thus, our results indicate that caspases are required, at least in part, to down-regulate p-FOXO3A and Id1, facilitating differentiation of mouse NSCs.

CASPASE INHIBITION DELAYS DIFFERENTIATION

To further clarify the role of caspases in regulating the differentiation process, we tested the influence of caspase inhibition on the ability of NSCs to differentiate by immunocytochemical analysis (Fig. 5). Caspase suppression by z-VAD.fmk significantly inhibited differentiation ($P < 0.05$). In fact, β-III tubulin-positive cells were reduced by ~40% at 3 days, and GFAP-positive cells by ~60% at 8 days. Interestingly, the effect of caspase inhibition was not detectable at 8 and 17 days of neurogenesis and gliogenesis, respectively. This suggests that caspase suppression delays differentiation of mouse NSCs, although cells may have additional mechanisms of differentiation. Nevertheless, caspases appear to be integral components of both neurogenesis and gliogenesis of mouse NSCs.



p53 REPRESSES p-FOXO3A/Id1 SIGNALING IN EARLY STAGES OF DIFFERENTIATION

We next investigated whether caspases require p53 to regulate differentiation of NSCs. First, we determined the effect of siRNA-mediated silencing of p53 on p-FOXO3A and Id1. p53 mRNA and protein expression decreased by ~90% and 70%, respectively, in mouse NSCs after transfection with siRNAs for p53 ($P < 0.01$; Fig. 6A). More importantly, siRNA-mediated silencing of p53 reversed the down-regulation of p-FOXO3A (Thr32) and Id1 at 3 days of differentiation, but not at 8 days (Fig. 6B). In fact, at 3 days of differentiation, p-FOXO3A (Thr32) and Id1 increased by ~50 ($P < 0.01$) and 30% ($P < 0.05$) after p53 suppression, respectively. Inhibition of p53 resulted in increased caspase-3 cleavage (data not shown), suggesting that caspases may act upstream of p53 to decrease p-FOXO3A and Id1 during early stages of differentiation. In contrast, the absence of p53 does not influence p-FOXO3A/Id1 signaling at 8 days of differentiation. We then treated p53 siRNA-transfected cells with z-VAD.fmk and evaluated changes at 3 days of differentiation. Notably, the suppression of both caspases and p53 induced a ~4-fold increase in p-FOXO3A compared with 2-fold in cells only transfected with p53 siRNA ($P < 0.05$). Id1, in turn, was ~4-fold greater after z-VAD.fmk incubation in p53 siRNA-transfected cells compared with control siRNA-transfected cells ($P < 0.05$). Thus, at 3 days of differentiation, caspase inhibition further enhanced p53 silencing effects, indicating that caspases do not act simply in a p53-dependent manner. Rather, caspases might work with p53 in regulating p-FOXO3A and Id1.

Finally, morphology and TUNEL assays revealed that p53 siRNA-mediated effects were not associated with increased cell death throughout differentiation of mouse NSCs (Fig. 6C, top and bottom). Therefore, our results demonstrated that p53 might be required for early differentiation, and that p53 effects in repressing p-FOXO3A and Id1 is further enhanced by caspase activation.

CASPASES AND p53 ACT IN A SYNERGISTIC MANNER TO MODULATE NEUROGENESIS

To confirm the role of p53 in regulating the differentiation process, we tested the influence of p53 knockdown on the ability of NSCs to differentiate into neurons and astrocytes through immunocytochemical analysis of cells transfected with either control or p53 siRNA. Our results showed that p53 suppression significantly

Fig. 3. p53 activity and caspase-3 cleavage are modulated during mouse NSC differentiation. NSCs were induced to differentiate for 1, 3, and 8 days. Cells were fixed and processed for morphological evaluation of apoptosis. Total proteins were extracted for immunoblot analysis, while nuclear extracts were used for p53-DNA binding assays. A: Representative immunoblots of caspase-3 processing, and total p53 and phosphorylated p53 (Ser15) expression. β-Actin was used as loading control. B: Detection of active caspase-3. Representative immunostaining images from at least three independent experiments. C: p53-DNA binding activity. Nuclear protein extracts were prepared as described in Material and Methods Section. The level of p53 present in nuclear lysates that can bind to its DNA consensus recognition sequence was determined by the Universal EZ-TFA p53 assay and expressed as fold change relative to day 1 of differentiation. $^{*}P < 0.05$ from day 1. D: Evaluation of apoptosis by Hoechst staining and TUNEL assay. Representative images from at least three independent experiments. GFP, green fluorescent protein.

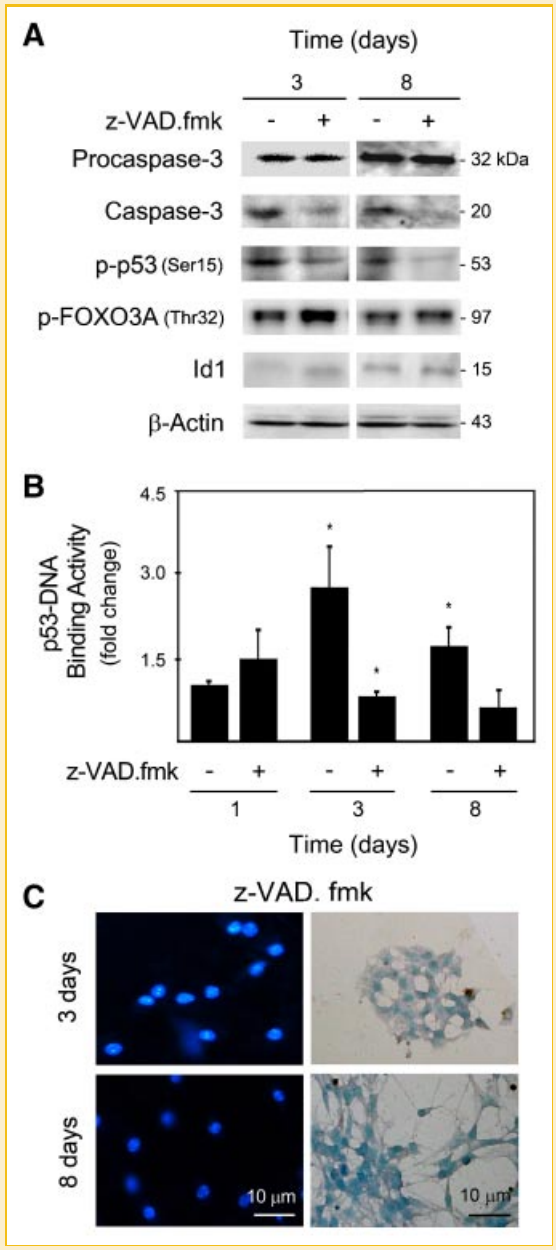


Fig. 4. Pan-caspase inhibition modulates p53 activity and interferes with the Akt/p-FOXO3A/Id1 pathway during mouse NSC differentiation. NSCs were incubated with a pan-caspase inhibitor, z-VAD.fmk, and collected after 3 and 8 days in differentiation medium. Cells were fixed and stained for morphological evaluation of apoptosis. Total proteins were extracted for immunoblot analysis, while nuclear extracts were used for p53-DNA binding assays. A: Caspase-3 processing, and p-p53 (Ser15), p-FOXO3A (Thr32) and Id1 total levels in control and z-VAD.fmk-treated cells. β-Actin was used as loading control. B: Representative immunoblots are shown from at least three independent experiments. The level of p53 present in nuclear lysates that can bind to its DNA consensus recognition sequence was determined by the Universal EZ-TFA p53 assay and expressed as fold-change relative to controls. C: Evaluation of apoptosis by Hoechst staining and TUNEL assay in z-VAD.fmk-treated cells at 3 and 8 days of differentiation. Representative images are shown from at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

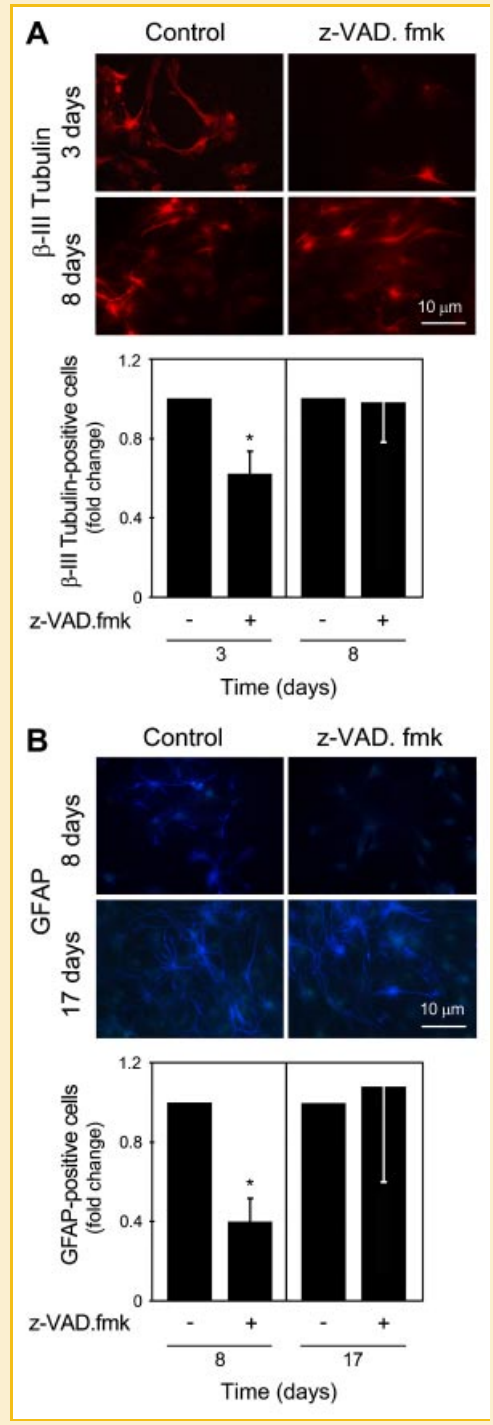


Fig. 5. Inhibition of caspase activation delays neuronal and glial differentiation of mouse NSCs. NSCs were incubated with a pan-caspase inhibitor, z-VAD.fmk, and collected after 3, 8, and 17 days in differentiation medium. Differentiated cells were fixed at indicated times and processed for immunostaining assays. Neurons and astrocytes were identified with a fluorescent-labeled antibody raised against β-III tubulin and glial fibrillary acidic protein (GFAP), respectively. A: Representative immunocytochemistry images from at least three independent experiments. B: Corresponding histograms of β-III tubulin and GFAP positive cells throughout differentiation. The results are expressed as mean ± SEM arbitrary units of at least three independent experiments. $^{*}P < 0.05$ from controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

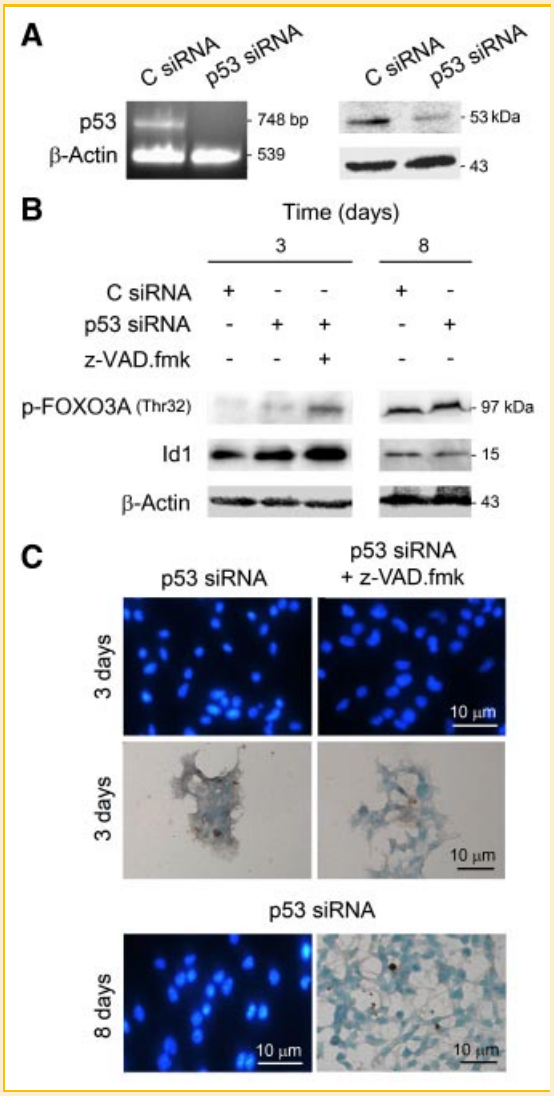


Fig. 6. Silencing of p53 decreases p-FOXO3A and Id1 expression levels during mouse NSC differentiation, without potentiating z-VAD.fmk effects. NSCs were incubated with either control or p53 siRNA and collected after 3 and 8 days in differentiation medium. Cells were fixed and stained to assess apoptosis. Total proteins were extracted for immunoblot analysis. A: Representative RT-PCR (left) and Western blot (right) of p53 and β-actin in cells transfected with either control or p53 siRNA. B: Representative immunoblots of p-FOXO3A (Thr32) and Id1 in cells transfected with either control siRNA, p53 siRNA, or p53 siRNA plus z-VAD.fmk. β-Actin was used as loading control. C: Evaluation of apoptosis by Hoechst staining and TUNEL assay in p53 siRNA transfected cells at 3 days (top) and 8 days (bottom) of differentiation in the presence or absence of z-VAD.fmk. Representative images from at least three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

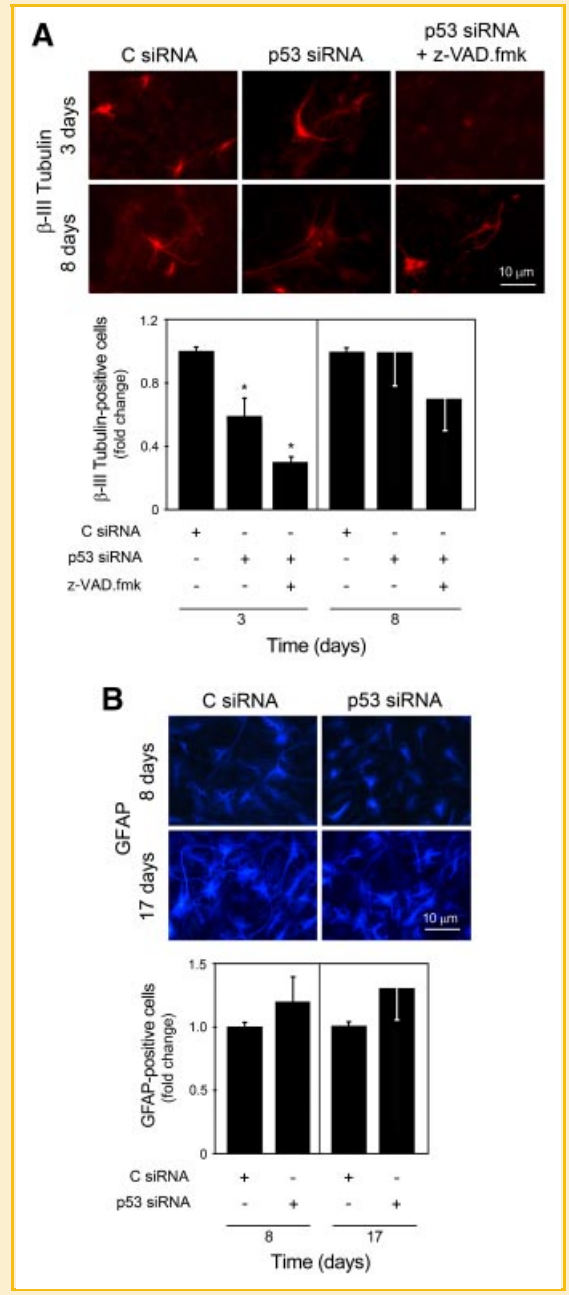


Fig. 7. Downregulation of p53 delays neuronal and glial differentiation of mouse NSCs. NSCs transfected with control siRNA, p53 siRNA, or p53 siRNA plus z-VAD.fmk, and collected after 3, 8, and 17 days in differentiation medium. Cells were fixed at indicated times and processed for immunostaining assays. Neurons and astrocytes were identified with a fluorescent-labeled antibody raised against β-III tubulin and glial fibrillary acidic protein (GFAP), respectively. A: Representative immunocytochemistry from at least three independent experiments. B: Corresponding histograms of β-III tubulin and GFAP positive cells throughout the differentiation period. The results are expressed as mean ± SEM arbitrary units of at least three independent experiments. $^{*}P < 0.05$ from controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reduced the number of positive cells for β-III tubulin compared with control siRNA-transfected cells (Fig. 7A). In addition, inhibition of neurogenesis by p53 siRNA increased after caspase suppression at 3 days, again implying that caspases and p53 might act in a synergistic manner. In contrast, siRNA-mediated silencing of p53 did not change the number of positive cells for GFAP (Fig. 7B), suggesting that p53 is not crucial for astrocyte maturation.

Curiously, the silencing effect of p53, in the presence or absence of caspase inhibition, was not present at 8 days of neurogenesis. This indicates that downregulation of p53 only delays neurogenesis in mouse NSCs. Nevertheless, caspases and p53 are crucial elements of differentiation in mouse NSCs, acting in a synergistic manner for regulating neurogenesis. In contrast, the mechanism that regulates gliogenesis in mouse NSCs appears to involve caspases, which in turn bypass p53 to promote glial cell differentiation.

DISCUSSION

Neural stem cell differentiation and commitment to either neuronal or glial lineage have important implications in developmental, pathological, and regenerative processes. It is, therefore, crucial to thoroughly understand the molecular mechanisms involved in NSC processing. It has been previously reported that cell death and differentiation processes may use similar molecular pathways [Fernando and Megeney, 2007]. Our results provide an extended mechanism of action for the apoptosis executioners throughout the differentiation process. Specifically, they show that caspases are integral components of differentiation of mouse NSCs that together with p53 can induce neurogenesis or gliogenesis. Moreover, these specific apoptotic factors interfere with elements of the Akt/p-FOXO3A/Id1 intracellular signaling pathway to promote differentiation of NSCs.

It has been demonstrated that Akt/p-FOXO3A/Id1 signaling is an important regulator of cell differentiation. In fact, the phosphorylation of the forkhead transcription factor FOXO3A was shown to play a pivotal role in maintaining the hematopoietic stem cell pool [Miyamoto et al., 2007]; and the down-regulation of active FOXO3A accelerates cellular senescence in human dermal fibroblasts [Kyoung Kim et al., 2005]. In addition, it was recently demonstrated that active FOXO3A is a negative target of Notch signaling [Mandinova et al., 2008], thus supporting its role of maintaining cells in an undifferentiated state. Our results indicate that this downstream target of Akt is indeed altered during differentiation of mouse NSCs. In fact, Akt phosphorylation was decreased throughout differentiation and this was associated with reduced levels of cytosolic p-FOXO3A. Akt expression was also attenuated in the early stages of differentiation in human endothelial progenitor cells and gradually upregulated during cell maturation [Mogi et al., 2008]. Subsequently, we showed that a reduction in Akt-induced FOXO3A phosphorylation lead to increased activity of FOXO3A, and subsequently decreased Id1 in the nucleus. The link between FOXO3A and Id1 in triggering differentiation has already been established in other cell types [Birkenkamp et al., 2007]. The decrease in Id1 expression in mouse NSCs may, in turn, allow basic helix loop helix (bHLH) transcription factors to induce expression of differentiation-associated genes [Norton, 2000].

Importantly, the present study reveals that apoptosis executioners interfere with FOXO3A/Id1 signaling to promote both neurogenesis and gliogenesis in mouse NSCs. Several studies have already elucidated the importance of programmed cell death in establishing cell lineage for model systems of neural differentiation. However, a precise role for apoptosis-associated elements in the generation of both neurons and astrocytes is still largely unknown. Curiously, it was recently reported that members of the Bcl-2 family such as Bcl-X_L and Bax play an instructive role in determining the fate of embryonic cortical precursor cells [Chang et al., 2007]. Bcl-X_L and Bax mediate differentiation of neurons and astrocytes, respectively, independently from their roles in cell survival and apoptosis.

Herein, we investigated whether caspases and p53 were upregulated during mouse NSC differentiation. Our data indicated that cleavage of caspase-3 was significantly increased at both 3 and 8 days of differentiation. In fact, the non-proteolytic functions of caspases in the regulation of cell survival, differentiation, and inflammation had already suggested that caspases may become activated without inducing an apoptotic cascade [Lamkanfi et al., 2007]. For example, in erythroblasts, keratinocytes, and lens epithelial cells undergoing differentiation, enucleation has been regarded as a caspase-mediated incomplete apoptotic process. In addition, others have previously shown that caspase activation specifically contributes to the differentiation of monocytes into macrophages, in the absence of cell death [Sordet et al., 2002]. Differentiation-associated caspase activation involved the release of cytochome *c* from the mitochondria and led to cleavage of the protein acinus, while poly (ADP-ribose) polymerase remained uncleaved. In our study, the role of caspases during differentiation of mouse NSCs was further characterized. We evaluated the potential cross-talk of caspases with the proliferation pathway Akt/p-FOXO3A/Id1 in mouse NSCs. Our results revealed that both p-FOXO3A (Thr 32) and Id1 increased after caspase inhibition, which in turn resulted in delayed neuronal and astrocyte formation at 3 and 8 days of differentiation, respectively. In addition, although total levels of p53 were unchanged, its DNA-binding activity significantly increased throughout differentiation, suggesting that p53 might be also important for both neuronal and astrocyte formation.

The role of p53 on NSC differentiation was further investigated by using siRNA-mediated p53 silencing. In fact, the results showed that p53 is pivotal for inducing neurogenesis, and its function is not associated with cell death. Although, we did not observe an increase of p-p53 (Ser15) at 8 days of differentiation, this does not exclude the involvement of p53, since this transcription factor may be activated by phosphorylation in many other residues. Nevertheless, p53 silencing did not compromise both p-FOXO3A and Id1 at 8 days of differentiation, and even astrocyte generation. The absence of p53 silencing effect at 8 days of differentiation could be explained by several potential mechanisms. First, silencing of p53 at day 1 of differentiation may not be sufficient to inhibit p53 expression throughout the course of differentiation. Indeed, mouse NSCs may re-establish normal levels of p53 mRNA at 8 days of differentiation. Nevertheless, this hypothesis was excluded when we further transfected cells with p53 siRNA at 4 days of differentiation, and both p-FOXO3A and Id1 total levels remained unchanged (data not shown). An alternate explanation is the potential substitution of p53 function by other family members at day 8 of differentiation [Stiewe, 2007]. In fact, it has been demonstrated that knockouts for p53, p63, or p73 show only slight delays on development. However, viability is compromised when double or triple knockouts for these

genes are generated, indicating that other members of this family are able to substitute the lack of one transcription factor [Stiewe, 2007].

Importantly, we have demonstrated that at 3 days of differentiation, caspases and p53 act in a synergistic manner to induce p-FOXO3A/Id1 repression. In fact, caspase suppression not only decreased p53-DNA binding activity but it also further increased siRNA p53 effects during early differentiation of mouse NSCs. Interestingly, in contrast with the apoptosis cascade, where p53 acts upstream of caspases, our data strongly suggest that caspases may act upstream of p53 during neurogenesis. Indeed, caspase inhibition significantly reduced both p53-DNA binding activity and p-p53 (Ser15) expression levels, and p53 silencing did not decrease caspase-3 cleavage (data not shown). However, caspases might act in a p53-independent manner, since the inhibition of both caspases and p53 did not promote the same level of inhibition in neurogenesis, but rather a cumulative effect. Therefore, caspases and p53 may act, at least in part, independently to induce neuronal differentiation of mouse NSC.

Our results coupled with previous studies showing that overexpression of Bcl-2, a negative target of p53, significantly reduced the number of neurons [Esdar et al., 2001], support the pivotal role of p53 activation during neurogenesis. Further, the critical involvement of p53 for the fate of neuronal cells was indeed demonstrated by others, showing that p53 is required for neurite outgrowth in cultured cells including primary neurons as well as for axonal regeneration in mice [Di Giovanni et al., 2006].

It remains to be investigated whether p53 downregulates Akt activity or whether Akt repression induces p53 activity during neurogenesis of NSC. Interestingly, it has been demonstrated that Akt downregulation may induce p53 transcriptional activation through Sirt2. In fact, 14-3-3 β/γ augment deacetylation and downregulate p53 transcriptional activity by Sirt2 in an Akt-dependent manner [Jin et al., 2008]. However, p53 may also repress the Akt pathway through PTEN [Stambolic et al., 2001].

In addition, the transcription factor FOXO3A might be phosphorylated by either serum- and glucocorticoid-inducible kinase (SGK1) [You et al., 2004] or IκB kinase (IKK) [Hu et al., 2004]. In fact, SGK1 expression was markedly increased by p53 [You et al., 2004]. In this respect, p53 silencing would lead to decreased p-FOXO3A. On the other hand, corroborating our results, the β subunit of IKK may be negatively regulated by p53 [Gu et al., 2004]. Indeed, p53 might have acted on differentiation via the IKK kinase, after levels of p-FOXO3A were markedly increased by p53 knockdown in mouse NSCs. Importantly, it has been shown that Id1 may be downregulated in a p53-dependent manner [Qian and Chen, 2008].

Finally, it will be important to investigate which cofactors and/or molecular pathways interfere with caspases and p53 to influence differentiation versus apoptosis in mouse NSCs. One explanation for this commitment derives largely from caspase targeting and activation of substrates or cofactors that are unique to each event. Most likely, levels of activation of apoptosis executioners are different between apoptosis and differentiation. In addition, our results do not exclude the possibility that sulfhydryl-dependent enzymes other than caspases might also be involved. Thus, the precise role of caspases and the identification of specific pertinent caspases during differentiation should be further elucidated.

This study provides an extended mechanism of action for apoptosis-associated elements, linking major components of cell death with the differentiation process of mouse NSCs. Caspases modulate differentiation, further enhancing p53 effects at 3 days of differentiation. Nevertheless, both caspases and p53 were shown to signal the p-FOXO3A pathway and inhibit repression of differentiation by Id1. Collectively, our results may contribute to the identification of novel targets for NSC differentiation procedures that may translate ultimately into improved neuro-replacement therapies.

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