Title
The Role of the Mitochondrial Apoptotic Pathway in Neuronal Development and Survival

Author
Reyes, Nichole

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The Role of the Mitochondrial Apoptotic Pathway in Neuronal Development and Survival

by

Nichole Reyes

DISSERTATION

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I would like to dedicate my thesis to my father for his unending support and love.

Your lessons of hard work and perseverance paid off Dad!

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The Role of the Mitochondrial Apoptotic Pathway in Neuronal Development and Survival

ABSTRACT

Apoptosis, a form of programmed cell death, has been shown to be critical to the pathogenesis of a number of diseases, including neurodegenerative diseases. I investigated the role of BAX/BAK-dependent apoptosis in neurons in the context of neurodegeneration and maturation.

BAX/BAK deficiency in the central nervous system of mice provides the ideal model by which to study the role of apoptosis in neurodegeneration. In a mouse model of Amyotrophic Lateral Sclerosis—a neurodegenerative disease that specifically affects motor neurons—deficiency of BAX and BAK preserves viability and function of neurons for an extended period. Moreover, this correlated with a delay in symptom onset and extension of lifespan. Therefore, BAX/BAK dependent apoptosis is activated early in the disease process and represents a viable therapeutic target for neurodegenerative diseases.

Regenerative medicine is currently one of the therapeutic strategies being considered for treatment of neurodegenerative diseases. Using this method, the patient's own stem cells are differentiated *in vitro* into the affected neuronal subtype and re-implanted back into the patient. Because of the promise of regenerative medicine, it is critical to understand the signaling pathways mediating the differentiation process in stem cells. Caspase activity has been

reported to mediate the differentiation of embryonic stem cells (ESCs), and I discovered that this process requires BAX and BAK. BAX/BAK deficient ESCs undergoing differentiation express lower levels of neural markers and higher levels of pluripotency markers. These results indicate that ESCs lacking BAX and BAK are far less effective at differentiating down the neural lineage. Furthermore, engagement of BAX/BAK dependent apoptosis induces differentiation of ESCs. Therefore, the BAX/BAK apoptotic pathway mediates differentiation of stem cells.

These results underline the importance of the BAX/BAK apoptotic pathway in the development and survival of neurons. Deficiency of BAX and BAK prolongs survival in the context of neurodegeneration and delays the maturation of stem cells down the neural lineage. The findings of my thesis provide insight into the basic biology of neurons, and how to better treat neurologic disorders.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

CHAPTER ONE

Introduction

OVERVIEW

Apoptosis, a form of programmed cell death, can occur by two major routes: extrinsic or intrinsic. The extrinsic pathway is triggered by death receptors on the cell surface, such as the receptors for Fas ligand or TNF-α (1). On the other hand, the intrinsic pathway (e.g., mitochondrial cell death pathway) is primarily governed by the BCL-2 family, which includes both anti- and pro-apoptotic members. Two multidomain pro-apoptotic BCL-2 family members, BAX and BAK, are the “gatekeepers” of the mitochondrial cell death pathway, the major apoptotic pathway in vertebrate cells (2). Upstream damage signals (e.g.-DNA damage, growth factor withdrawal) converge on and activate the BH3-only proteins, thereby triggering the homo-oligomerization of BAX and/or BAK at the mitochondrion, cytochrome *c* release, and activation of caspases. Release of cytochrome *c* facilitates formation of the apoptosome and activation of caspase 9, which then in turn cleaves and activates the abundant effector caspases like Caspase-3 (1, 3).

The importance of the mitochondrial apoptotic pathway in disease and development has been established by a number of seminal studies. BCL2, the founding member of the BCL-2 family of proteins, was discovered to be upregulated by the hallmark translocation event that occurs in follicular B-cell lymphoma (4-6). Subsequent studies have shown that dysregulation of the

apoptotic pathway results in a number of diseases. Diseases of cell loss include acute conditions (e.g.-stroke), hematologic disorders (e.g.-anemia), or neurodegenerative diseases (e.g.-Amyotrophic Lateral Sclerosis)(7-9). Alternatively, diseases of cell gain include cancer, autoimmune diseases, and developmental defects (10-12). In regards to development, BAX/BAK deficiency is generally lethal with only 10% surviving until adulthood. Moreover, BAX/BAK deficient animals display a number of physical abnormalities, including interdigital webbing and defects in the immune and central nervous systems (13).

My doctoral thesis investigated the role of the intrinsic apoptotic pathway in a neurodegenerative disease model of Amyotrophic Lateral Sclerosis (ALS). ALS is one of the most common forms of neuromuscular diseases in the United States, with approximately 6000 new cases diagnosed each year. The disease is adult-onset and is characterized by specific death of the upper and lower motor neurons. While it is well established that caspases are activated in motor neurons in ALS (14, 15), the contribution of the apoptotic pathway to disease pathogenesis remains unclear. However, it is widely believed that caspases are activated late in the disease process after motor neurons have exhausted all functional capacity. My thesis directly tests the role of the BAX/BAK pathway in neurodegeneration via both mouse and cell culture models. In the process, my thesis also led to some interesting findings regarding the role of the mitochondrial apoptotic pathway in embryonic stem cell (ESC) differentiation.

In chapter two, I describe our results for blockade of the intrinsic apoptotic pathway in a mouse model of ALS. In chapter three, I describe my attempts at

generating a cell culture model of ALS to better understand the cell signaling pathways involved in the disease. While I was able to establish a cell culture model of ALS using ESC derived motor neurons, this actually led to an interesting story detailed in chapter four. In chapter four, I describe the contribution of the BAX/BAK pathway to neuronal differentiation of ESCs. The final chapter describes the reagents and methods used in each of the studies.

APOPTOSIS IN NEURODEGENERATIVE DISEASE

The incidence of neurodegenerative diseases is increasing along with the aging population in the United States. Estimates suggest that over 12 million people in the United States alone will suffer from neurodegenerative diseases by the year 2050 (16). Current treatment paradigms are ineffective, and the need for more effective therapeutics is pressing. A better understanding of the signaling pathways contributing to neuronal death will help identify potential drug targets. However, the molecular mechanisms which ultimately lead to neuronal death in these diseases are still not well understood.

Several lines of evidence implicate apoptosis in neurodegenerative diseases, including Huntington disease (HD), Alzheimer disease (AD), and amyotrophic lateral sclerosis (ALS) (17, 18). Caspase activity has been shown to contribute to disease pathogenesis of a number of neurodegenerative diseases. In HD, mutation of the Caspase-6 cleavage site in mutant huntingtin (Htt) blocks disease development in transgenic mice (19). Similarly, mutation of the caspase cleavage site in β-amyloid precursor protein (APP) in a mouse model of AD

prevents neurodegeneration and memory loss (20). Moreover, Caspase-3 has been shown to be triggered before the onset of symptoms and its blockade preserves memory in a mouse model of AD (21). Caspase-1 is activated in a transgenic mouse model of ALS months before the onset of disease and neuronal death (22, 23). Furthermore, treatment with the pan-caspase inhibitor z-VADfmk prolongs survival and delays disease onset by about 2 weeks in an ALS mouse model (24). Moreover, several lines of evidence implicate that the mitochondrial apoptotic pathway plays a role in neurodegeneration. Minocycline, a drug that prevents cytochrome *c* release from the mitochondria, is beneficial in mouse models of HD (25) and ALS (26). Furthermore, germline deletion of *bax* (27) or overexpression of *bcl2* (28) modestly delays disease onset and prolongs survival in a transgenic mouse model of ALS. These neurodegenerative diseases share a common pathology of protein misfolding and neuronal cell death (17). Therefore, what is learned about the neurodegenerative disease process in one disease may translate across multiple neurodegenerative disorders.

DISEASE MODELS OF ALS

ALS is inherited in approximately 10% of cases (familial, FALS). Of these familial cases, approximately 10-20% are due to mutations in copper/zinc superoxide dismutase-1 (SOD1) (29). Evidence argues against a loss of function role of SOD1 in neurodegeneration as FALS associated SOD1 variants have normal enzymatic function and a mouse knockout of SOD1 does not develop neurodegeneration (30, 31). Indeed, only the SOD1 mutants have been shown to

form aggregates in ALS post-mortem patient samples, as well as in mouse and cell culture models of ALS (15).

Several mouse models of ALS have been developed, but the model that most faithfully recapitulates the human disease expresses the human mutant transgene, $SOD1^{G93A}$, under control of its endogenous promoter (32). This mouse develops apoptosis of spinal cord motor neurons and paralysis of the hind limbs at approximately 100 days of age and dies at around day 120. Neurons from these mice possess $SOD1^{G93A}$ immunoreactive aggregates, while brain and spinal cord sections display reactive gliosis and loss of motor neurons in the anterior horn. Two methods commonly used to assess loss of motor function in ALS mouse models are body weight and rotarod performance. As the mice develop motor dysfunction, they have difficulty accessing food and lose weight. Therefore, weight loss is a reliable measure to assess disease onset (33, 34). The rotarod is a machine that measures the ability of the mice to remain upright on a rotating rod (35). A decline in latency indicates a decline in motor function. In summary, the $SOD1^{G93A}$ mouse is therefore a powerful animal model by which to study the mechanism of neurodegeneration in ALS. This mouse model was utilized in chapter two.

Cell culture models for ALS include transformed motor neuron cell lines (eg-NSC34, Neuro2A), or primary motor neurons. However, both of these models possess a number of problems. For instance, NSC34 cells are a mouse neuroblastoma/spinal cord cell line (36) and there is evidence that tumor cells are resistant to apoptosis induced by the unfolded protein response (UPR) (37, 38).

In mammals, the UPR is governed by three major ER stress sensors: pancreatic ER kinase (PERK), activating transcription factor (ATF6), and inositol-requiring enzyme (IRE1), which span the ER membrane (3, 39, 40). Under normal conditions, these sensors are held inactive by the ER resident chaperone Grp78. However, when an excess of misfolded proteins accumulates in the ER, Grp78 dissociates from these stress sensors resulting in their activation. The UPR initially promotes restoration of ER homeostasis (39, 40). However, if the ER damage is extensive or prolonged, then protein misfolding triggers apoptosis through the mitochondrial apoptotic pathway (2, 41). Dysregulation of cell death pathways in tumor cells tip the balance of the UPR towards homeostatic outputs, thereby promoting cell survival (37, 38). Since $SOD1^{G93A}$ has been shown to aggregate and activate the UPR *in vitro*, these NSC34 cells may be resistant to $SOD1^{G93A}$ toxicity. Indeed, these cells require hydrogen peroxide treatment in order to potentiate $SOD1^{G93A}$ toxicity, as has been shown previously (42). Primary motor neurons, on the other hand, offer a near pure population of motor neurons (90-95%), but are difficult to obtain and are not a reproducible culture system since the cells can only survive for a few weeks in culture (43, 44). Thus, the ideal culture system would display reproducible and robust toxicity to $SOD1^{G93A}$. Recently, a novel cell culture system was described whereby mouse embryonic stem cells were isolated from the ALS mouse and grown on primary astrocyte monolayers (45, 46). This cell culture system produced a robust response to $SOD1^{G93A}$ toxicity. Moreover, since ES cells are a self-renewing population, this model provides a reproducible culture system. Although this

approach is less well established than primary motor neuron cultures, the robust toxicity and reproducibility afforded by ES cells provides a more powerful cell culture model by which to study ALS *in vitro*.

To complement our *in vivo* study on ALS (described in chapter two), BAX/BAK deficient ESCs were generated. These ESCs could then be differentiated into motor neurons *in vitro* (47) to further characterize the BCL-2 family members involved in mediating $SOD1^{G93A}$ toxicity. However, BAX/BAK deficient ESCs were unable to differentiate into motor neurons, which led to another story characterizing the role of the mitochondrial apoptotic pathway in neuronal differentiation (described in chapter four).

APOPTOSIS IN ESC DIFFERENTIATION

ESCs have gained popularity due to their therapeutic potential in regenerative medicine (48, 49). As these processes require the differentiation of ESCs into multiple tissue types, it is critical to fully understand the signaling events involved in differentiation.

Caspases have been shown to play a role in the differentiation process of a number of cell types. In *Drosophila*, caspase activity has been shown to be necessary for sperm differentiation (50). Furthermore, caspases are involved in the maturation process of several cell types, including erythrocytes and platelets (51-54). Neuronal precursor cells undergoing differentiation display increased caspase activity (55). Furthermore, Caspase-3 was shown to be necessary for ESC and hematopoietic stem cell differentiation (56, 57).

In addition to caspases, other apoptotic signaling members have been implicated in the differentiation of stem cells. Overexpression of BCL2 in ESCs delays differentiation and confers resistance to retinoic acid (RA) induced death (58). Activity of the DNA damage activated transcription factor, p53, is necessary for ESC differentiation and downregulates expression of the pluripotency factor, Nanog (59). The BH3-only protein, PUMA, directly mediates radiation-induced death in gastrointestinal stem cells and its blockade enhances regeneration of the stem cell compartment (60).

Although caspase activation during differentiation is widely believed to occur independently of apoptosis, we show evidence for interplay between these two processes in chapter four.

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CHAPTER TWO

Blocking the mitochondrial apoptotic pathway preserves motor neuron viability and function in a mouse model of amyotrophic lateral sclerosis (ALS)

SUMMARY

Apoptosis of motor neurons is a well-documented feature in amyotrophic lateral sclerosis (ALS) and related motor neuron diseases (MNDs). However, the role of apoptosis in the pathogenesis of these diseases remains unresolved. One possibility is that the affected motor neurons only succumb to apoptosis once they have exhausted functional capacity. If true, blocking apoptosis should confer no therapeutic benefit. To directly investigate this idea, we tested whether tissue-specific deletion in the mouse CNS of BCL2-associated X protein (BAX) and BCL2-homologous antagonist/killer (BAK), two proapoptotic BCL-2 family proteins that together represent an essential gateway to the mitochondrial apoptotic pathway, would protect against motor neuron degeneration. We found that neuronal deletion of *Bax* and *Bak* in a mouse model of familial ALS not only halted neuronal loss, but prevented axonal degeneration, symptom onset, weight loss, and paralysis and extended survival. These results show that motor neurons damaged in ALS activate the mitochondrial apoptotic pathway early in the disease process and that apoptotic signaling directly contributes to neuromuscular degeneration and neuronal dysfunction. Hence, inhibiting apoptosis upstream of mitochondrial permeabilization represents a possible

therapeutic strategy for preserving functional motor neurons in ALS and other MNDs.

BACKGROUND

Neuronal tissues are susceptible to a number of insults that contribute to motor neuron dysfunction and cell death, including misfolded proteins, reactive oxygen and nitrogen species, calcium entry, excitotoxicity, trophic-factor withdrawal, death receptor activation, and mitochondrial-complex inhibition(39, 40). There is abundant evidence that injured motor neurons undergo apoptosis in a variety of MNDs. For example, mouse models, cell culture systems, and/or post-mortem tissues from affected patients of Spinal Muscular Atrophy (SMA), Kennedy's Disease, and ALS show caspase activation in degenerating neurons(41-43). Caspase-3, one of the major cysteine-aspartate proteases responsible for degrading cellular components during apoptosis, is activated in both motor neurons and astrocytes contemporaneous with the first stages of motor neuron degeneration in the best-studied mouse models of ALS(4, 44). Moreover, inhibiting caspases through various approaches modestly improves outcome in several models of neurodegeneration(45-47). These findings suggest that apoptosis may actively contribute to the ongoing disease process.

In opposition to this view, recent temporal studies of neurodegenerative models have strongly argued that apoptosis is a relatively late event, preceded by earlier functional abnormalities (e.g., activation of cellular stress pathways, electrophysiological deficits) and microanatomical deficits (e.g., synapse loss,

neurite retraction)(48-50). These studies have led to the widely held view that degenerating neurons activate apoptosis only after end-stage irreversible damage and functional exhaustion have already ensued. Therefore, the contribution of apoptosis to the pathology and/or clinical manifestations of neurodegeneration remains unresolved. Given the morbidity and mortality associated of these diseases and the current lack of effective therapies, it is essential to determine whether disruption of the apoptotic program represents a valid therapeutic strategy to treat MNDs such as ALS.

RESULTS

To study the effects of disabling the mitochondrial (intrinsic) apoptotic pathway on the onset and progression of neurodegeneration in a mouse model of familial ALS, we generated mice deficient for BCL2-associated X protein (*Bax*) and BCL2-homologous antagonist/killer (*Bak*) in the CNS. In response to diverse types of cell injury, the proapoptotic BCL-2 proteins BAX and/or BAK homo-oligomerize at the outer mitochondrial membrane, which leads to efflux of proapoptotic mitochondrial matrix proteins (i.e., cytochrome c, SMAC/DIABLO) and activation of downstream effector caspases (i.e., caspase-3) (14–17). Cells doubly deficient in *Bax* and *Bak* are strikingly resistant to apoptosis in response to a wide range of intrinsic death stimuli (e.g., DNA damage, protein misfolding, reactive oxygen species). Since germline-deficient $Bax^{-/-}Bak^{-/-}$ mice generally die in utero by embryonic day 18, we used mice with a previously described floxed (f) conditional allele of *Bax* and germline deletion of *Bak* (18). These

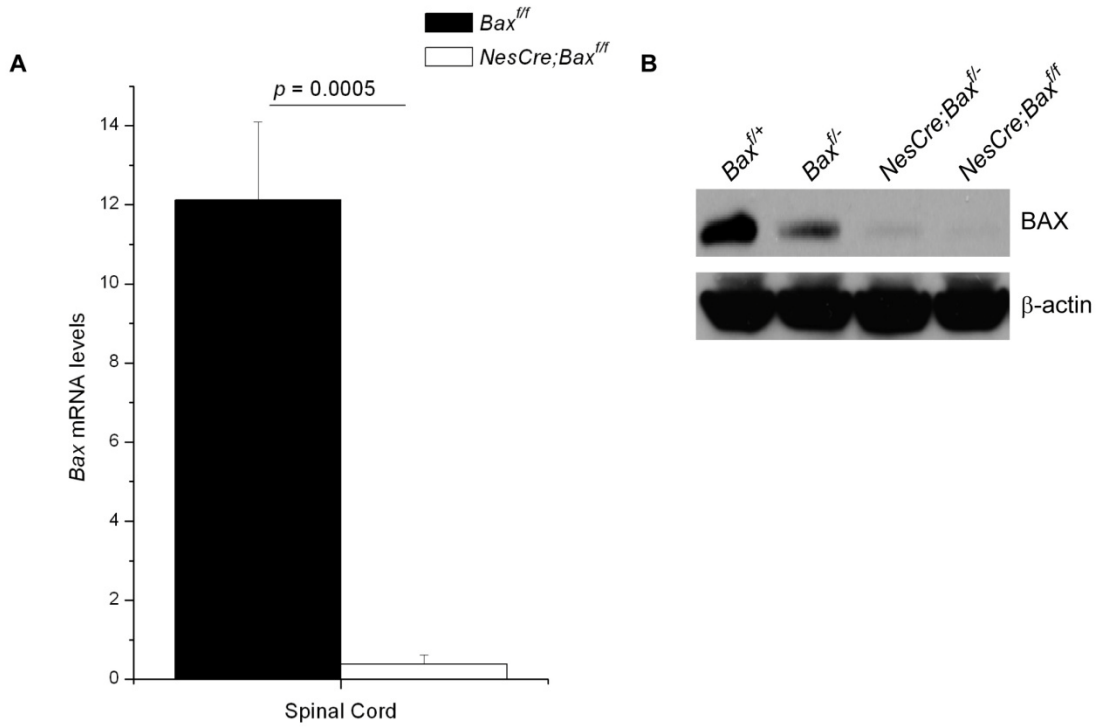

FIGURE 1
Efficient Cre mediated deletion of *Bax*$^{f/f}$ from the central nervous system.

(A) Relative Bax mRNA levels from the spinal cord of Nestin Cre (NesCre) positive versus NesCre negative Baxf/f mice determined by quantitative RT-PCR; n=3, unpaired two-tailed Student t-test. (B) Whole spinal cord extracts from 6-week old mice of indicated genotypes were immunoblotted with antibodies agains BAX and β-actin. RT-PCR, reverse transcription polymerase chain reaction; f, flox.

Bax$^{f/f}$*Bak*$^{-/-}$ mice were then bred to express *Cre* recombinase under the rat nestin promoter (Nes*Cre*) to specifically delete *Bax* in the CNS (19). We confirmed *Cre*-mediated excision of *Bax* in the spinal cord by quantitative reverse-transcription PCR (RT-PCR) and immunoblotting (Figure 1). These results indicate that *Bax*$^{f/f}$ is efficiently deleted from the CNS. The conditionally deficient *Bax* and *Bak* mice (DKO^{CNS} mice) were born according to normal Mendelian ratios and showed no gross developmental defects into adulthood (data not shown). Moreover, motor neuron numbers in DKO^{CNS} mice were essentially identical to those of mice expressing Nes*Cre* alone and similar to those published in previous studies (ref. 20, Figure 4, A and B). Hence, this is an ideal genetic model to study motor neuron degeneration in the absence of BAX/BAK-dependent apoptosis.

We bred the DKO^{CNS} mice to a model of familial ALS that expresses a toxic gain-of-function mutation in copper/zinc superoxide dismutase-1 (SOD1). Mice that express the human mutant *SOD1*^{G93A}-transgene under the control of its endogenous promoter begin developing apoptosis of spinal cord motor neurons and paralysis of the hind limbs at approximately 100 days of age and become terminally paralyzed over the next approximately 30 days (21). We followed ALS onset and survival in *SOD1*^{G93A} hemizygous mice on a BAX/BAK-positive versus DKO^{CNS} background. *Bax* and/or *Bak* heterozygosity failed to affect symptom onset or survival (Figure 2 and ref. 22); therefore, we pooled results from mice expressing at least one allele of both *Bax* and *Bak* into a single littermate cohort (Cre^{CNS} mice). To minimize differences due to environment and gender, we

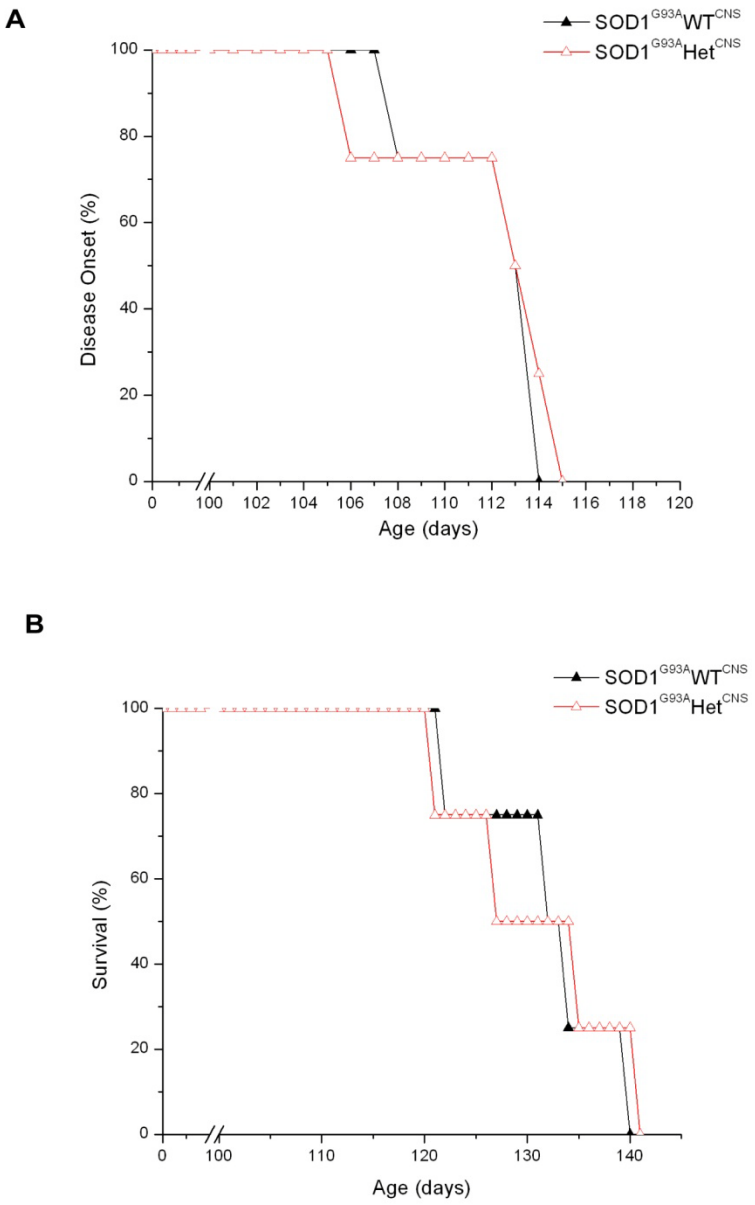

FIGURE 2
No difference in disease onset or survival between *SOD1*^{G93A} wildtype (WT) and *SOD1*^{G93A}Bax/Bak heterozygous (Het) mice.

(**A**) Disease onset in *SOD1*^{G93A}WT^{CNS} (112.5 ± 3.3) and *SOD1*^{G93A}Het^{CNS}(112 ± 4.1); *p*=0.86 (not statistically significant), *n*=4, unpaired two-tailed Student *t*-test. (**B**) Survival in *SOD1*^{G93A}WT^{CNS} (132 ± 7.5) and *SOD1*^{G93A}Het^{CNS} (131 ± 8.8); *p*=0.8 (not statistically significant), *n*=4, logrank test. WT were considered mice that contained both wildtype alleles for bax and bak, whereas heterozygous mice contained only one functional allele of bax and bak. All mice express NesCre. WT, wildtype; Het, heterozygous.

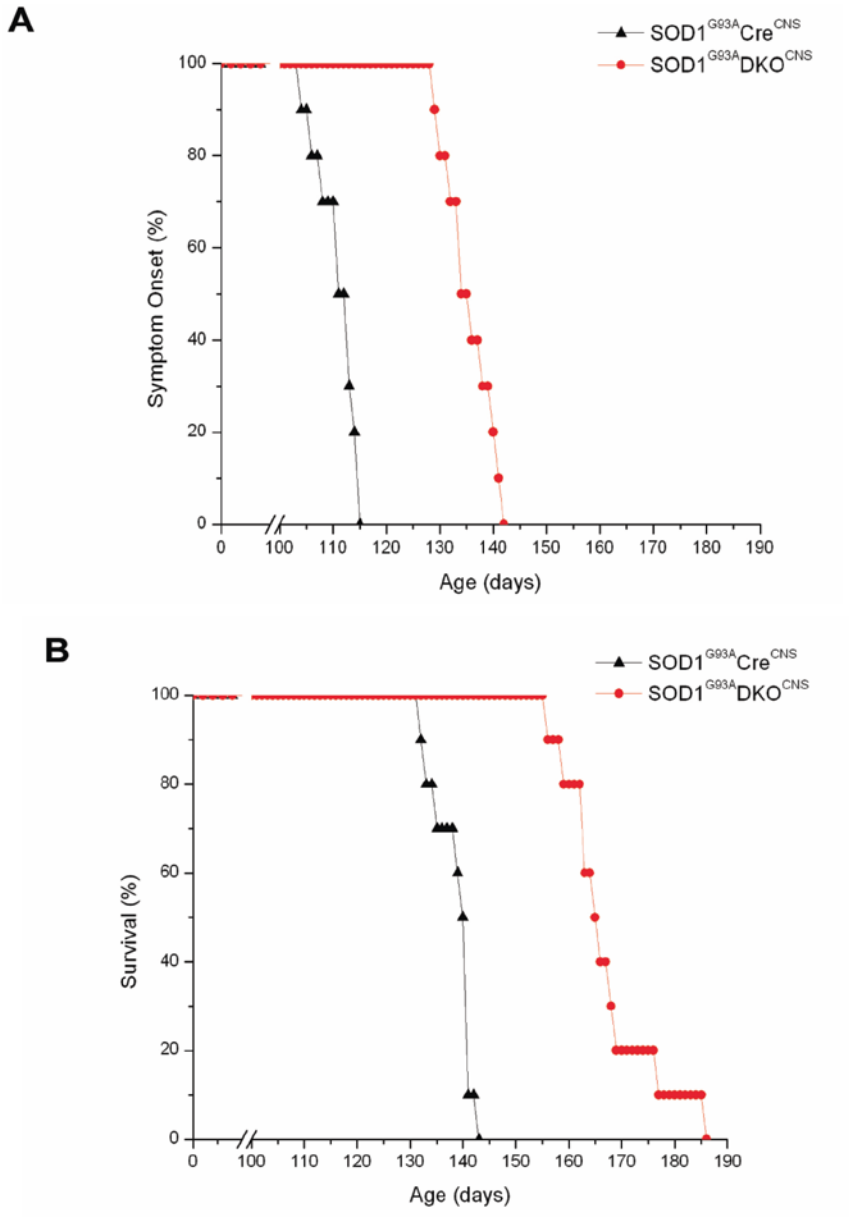

FIGURE 3
Deletion of BAX/BAK-dependent apoptosis delays symptom onset and prolongs survival in a mouse model of ALS.

(**A**) Symptom onset of *SOD1*^{G93A}Cre^{CNS} (111.3 ± 4.3 days) and *SOD1*^{G93A}DKO^{CNS} mice (135.6 ± 4.6 days); $p<0.0001$, $n=10$, unpaired two-tailed Student *t*-test. (**B**) Survival of *SOD1*^{G93A}Cre^{CNS} (138.6 ± 3.8 days) and *SOD1*^{G93A}DKO^{CNS} (167.2 ± 8.7 days); $p<0.0001$, $n=10$, logrank test.

compared identically housed, congenic cohorts with an equal number of males and females.Weight loss is an accepted measurement of symptom onset (motor dysfunction) and progression in this model of neurodegeneration (20, 23). Symptom onset was significantly delayed by approximately 3.5 weeks in the *SOD1*^{G93A}DKO^{CNS} mice as compared with that of the *SOD1*^{G93A}Cre^{CNS} mice (Figure 3A). Moreover, the *SOD1*^{G93A}DKO^{CNS} mice lived almost 1 month longer than *SOD1*^{G93A}Cre^{CNS} mice (167.2 and 138.6 days, respectively; $P < 0.0001$), representing an approximately 21% extension in life span (Figure 3B). Notably, at the age when the *SOD1*^{G93A}Cre^{CNS} mice were terminally paralyzed, the majority of the *SOD1*^{G93A}DKO^{CNS} mice showed no weight loss or paralysis. The significant delay in symptom onset and extended survival of the DKO^{CNS} ALS mice strongly suggest that the mitochondrial apoptotic pathway directly contributes to pathogenesis in this model of neurodegeneration.

Delayed paralysis and extended life span were associated with conspicuous preservation of motor neurons in the *SOD1*^{G93A}DKO^{CNS} mice that continued even through end-stage paralysis (Figure 4, A and B). As such, it took the *SOD1*^{G93A}DKO^{CNS} mice 150 days to approach the same degree of motor neuron loss seen in the *SOD1*^{G93A}Cre^{CNS} littermates at 90 days of age. Motor neuron survival in the *SOD1*^{G93A}DKO^{CNS} mice strongly correlated with decreased apoptosis, as determined by caspase-3 activation (Figure 5, A and B). This is striking in comparison with the *SOD1*^{G93A}Cre^{CNS} cohort, which showed caspase-3 activation as early as 90 days of age. These results indicate that activation of the mitochondrial apoptotic pathway is a critical route through which *SOD1*^{G93A}

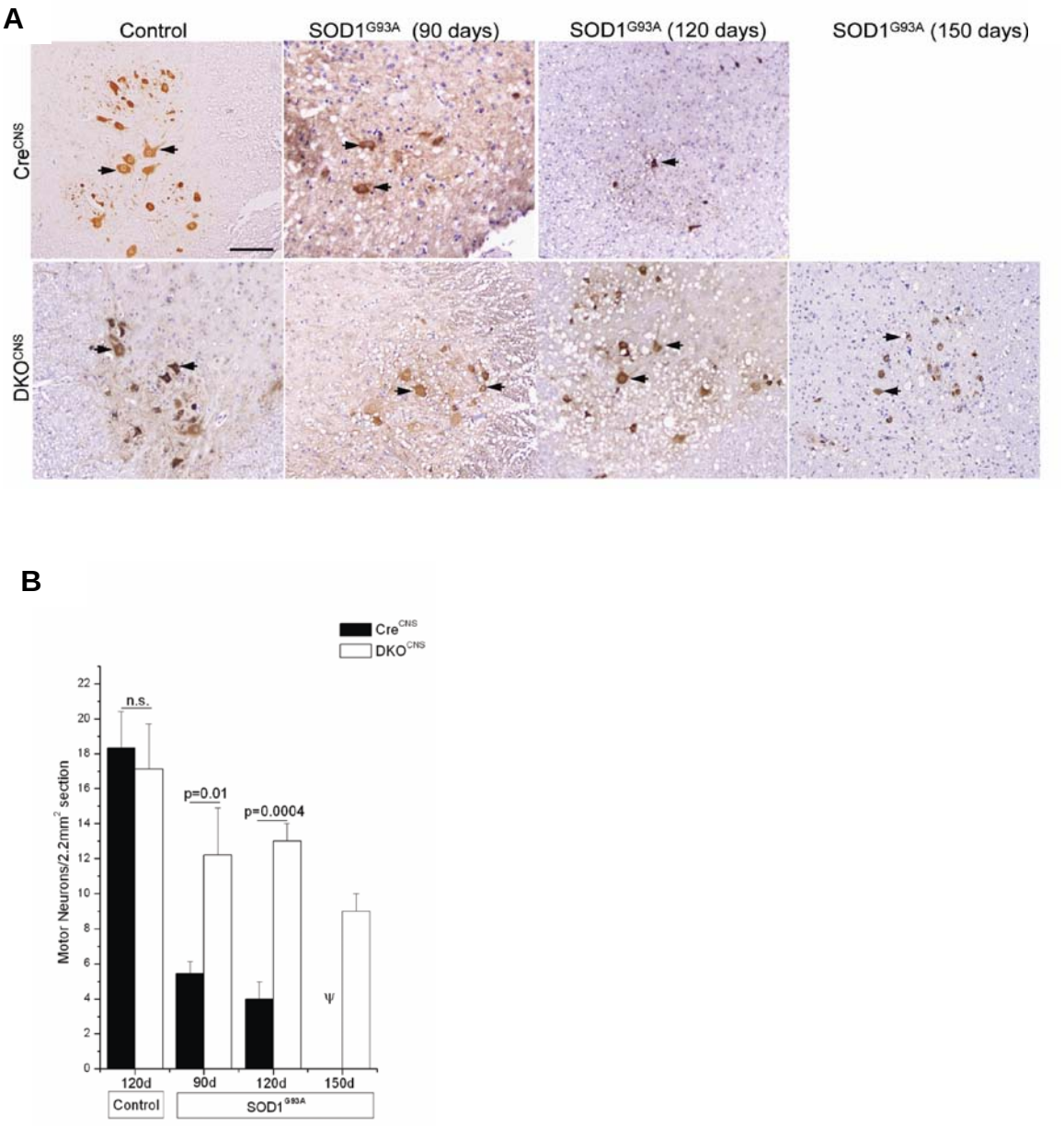

FIGURE 4
Deletion of BAX/BAK-dependent apoptosis preserves motor neurons.

Control mice were harvested at 120 days (d) of age; symptomatic at 90d or 90d/120d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively; and endstage at 120d or 150d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively. (**A**) Representative choline acetyltransferase (ChAT) staining (brown) of the anterior horn region of spinal cords from the indicated genotypes. Arrowheads indicate motor neurons. Scale bars: 200 μm. (**B**) Quantitation of anterior horn motor neurons from control and SOD1G93A mice using ChAT staining; *n*=3, unpaired two-tailed Student *t*-test.

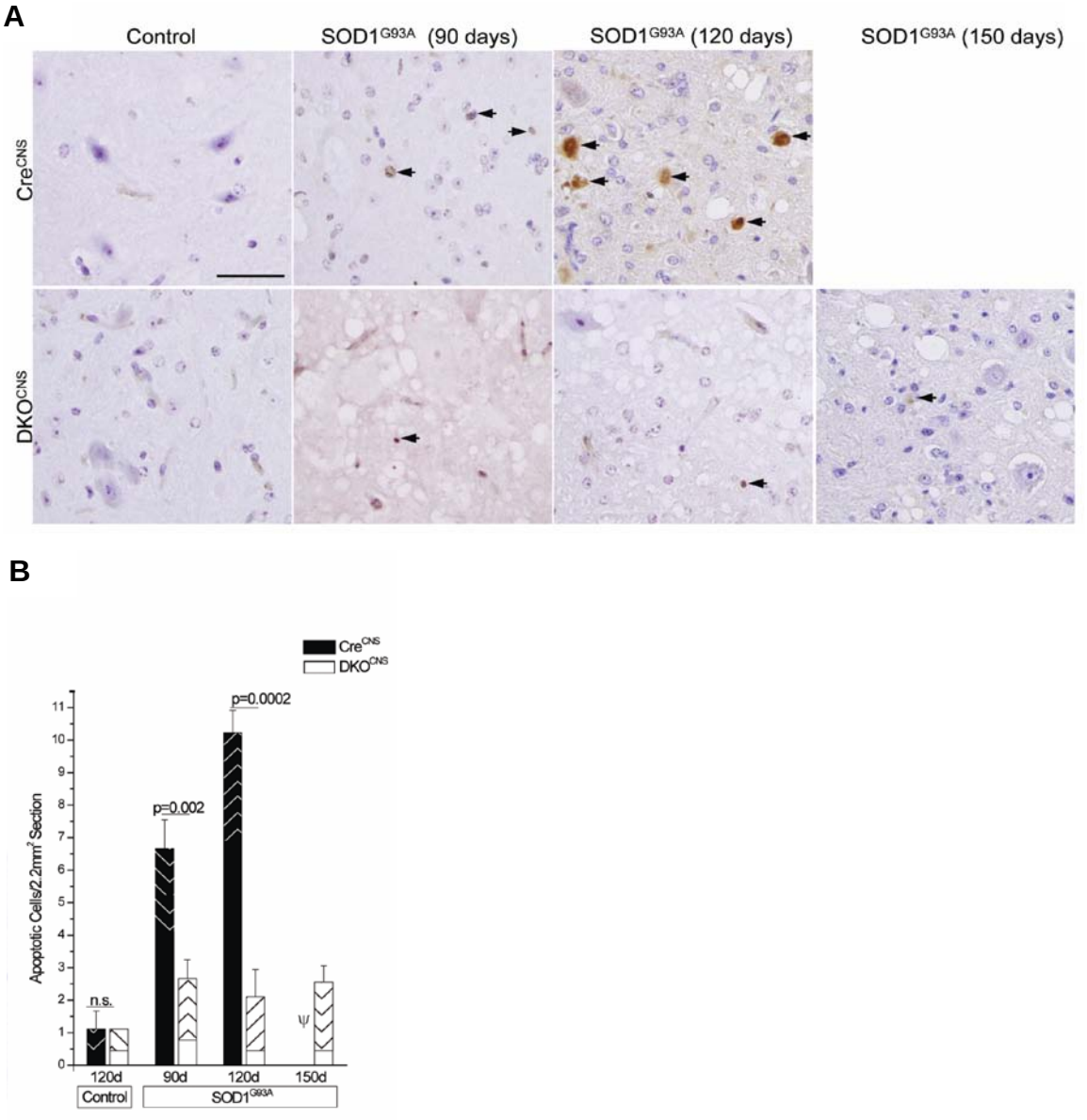

FIGURE 5
BAX/BAK-dependent apoptosis is triggered early in response to *SOD1*^{G93A} .

Mice were harvested as in Figure 3. (**A**) Representative spinal cord anterior horn sections stained with antibody to caspase-3 (brown). Arrowheads indicate activated caspase-3 staining. Scale bars: 100 µm. (**B**) Numbers of apoptotic cells (positive for activated caspase-3) from control and SOD1G93A mice. Solid colors represent motor neurons, while hatched pattern represents all other cell types; *n*=3, unpaired two-tailed Student *t*-test.

triggers neuronal cell death early in the disease process. In the absence of the mitochondrial apoptotic pathway, there is some eventual motor neuron loss in the *SOD1*^{G93A}DKO^{CNS} mice, which is apparently independent of caspase-3 activation. These findings are consistent with the delayed cell death that eventually occurs in fibroblasts from *Bax*$^{-/-}$*Bak*$^{-/-}$ mice when exposed to a range of intrinsic apoptotic stimuli (25).

To examine the morphological features of the diseased neurons, we performed EM on spinal cord sections from the ALS mice. While motor neurons from the *SOD1*^{G93A}Cre^{CNS} mice showed morphological features of apoptosis as early as 90 days of age, many *SOD1*^{G93A}DKO^{CNS} motor neurons lacked such features, even at end-stage disease (Figure 6A). With extended survival, the motor neurons from the *SOD1*^{G93A}DKO^{CNS} mice showed increased intracellular aggregates, lysosomes, and autophagosomes (Figure 6, B–D). Moreover, spinal cord axons from the *SOD1*^{G93A}DKO^{CNS} mice were dystrophic and contained prominent lysosomes at late stages of disease (Figure 6C), a hallmark of neuronal associated autophagy (26). To determine whether the increase in lysosomes and autophagosomes was due to the induction of autophagy, we stained spinal cord sections with LC3 (a marker of mature autophagosomes) (27) and p62 (a protein specifically degraded by autophagy) (28, 29). Interestingly, end-stage *SOD1*^{G93A}DKO^{CNS} motor neurons showed accumulation of LC3 and diminished p62 staining, consistent with active autophagy (Figure 7). This finding is consistent with the occurrence of autophagy in *Bax*$^{-/-}$*Bak*$^{-/-}$ fibroblasts when challenged with various stresses (30). During disease progression, the

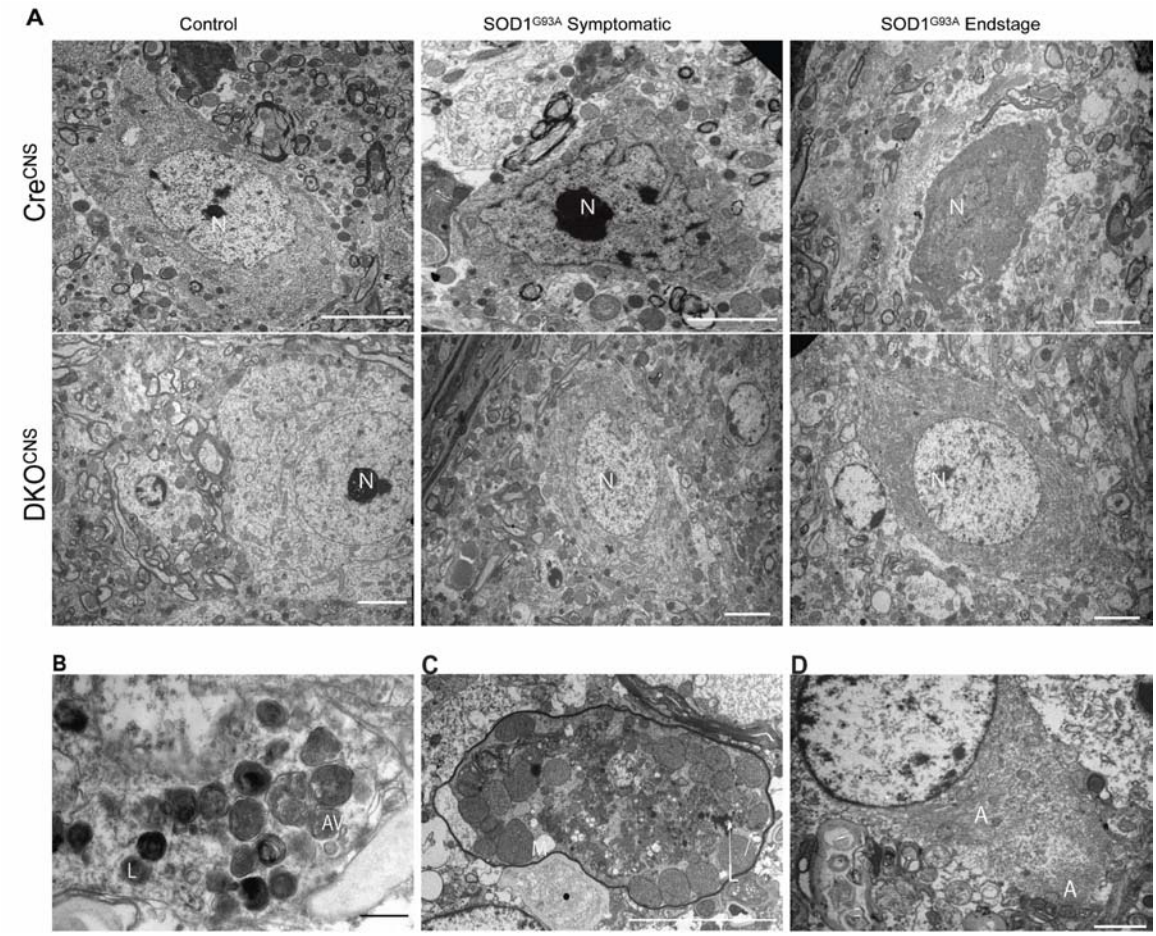

FIGURE 6
SOD1^{G93A}DKO^{CNS} neurons lack morphological features of apoptosis, but show evidence of autophagy.

Transmission electron microscope images (**A-D**) of motor neurons (MNs) from control and SOD1^{G93A} mice.For (**A, E)**, control mice were harvested at 120d; symptomatic mice at 90d and 120d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively; and endstage mice at 120d and 150d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively. For (**B-D**), the *SOD1*^{G93A}DKO^{CNS} animal was harvested at endstage (156d). (**A**) MNs from *SOD1*^{G93A}Cre^{CNS} animals appear apoptotic, while MNs from *SOD1*^{G93A}DKO^{CNS} animals appear healthy. Scale bars: 5 µm. (**B**) *SOD1*^{G93A}DKO^{CNS} MNs display morphological features of autophagy. Scale bars: 0.5 µm. (**C**) *SOD1*^{G93A}DKO^{CNS} motor axons are dystrophic and contain lysosomes. Scale bars: 5 µm. (**D**) Increased intracellular aggregates in *SOD1*^{G93A}DKO^{CNS} MNs. Scale bars: 2 µm; N, nucleus; M, mitochondria; L, lysosome; A, aggregates; AV, autophagic vesicle.

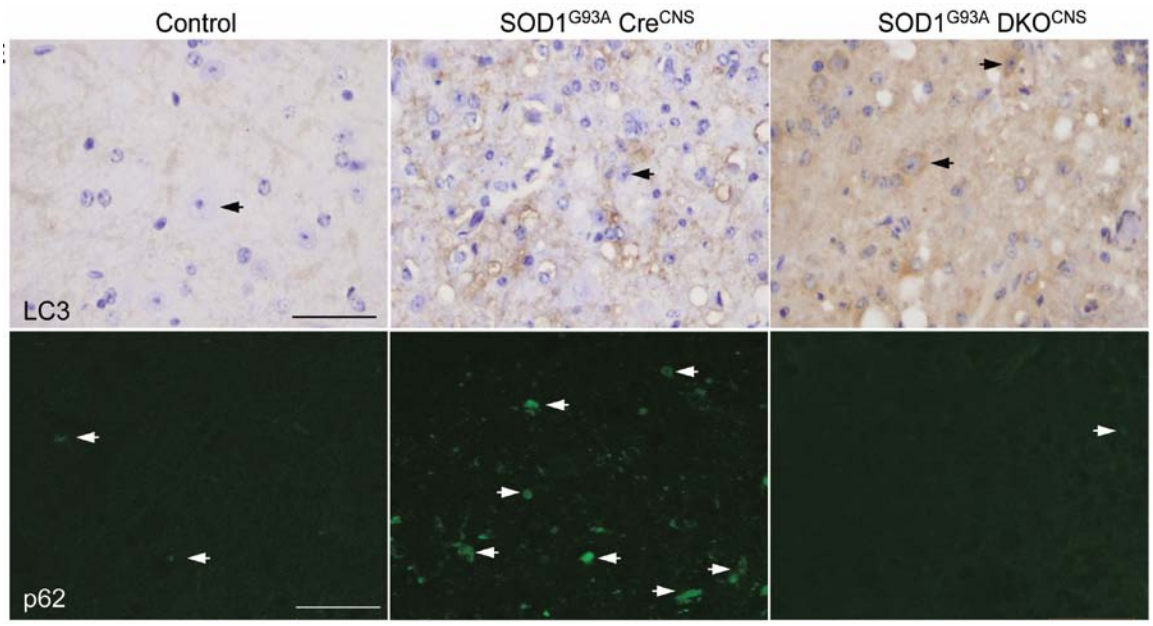

FIGURE 7
SOD1^{G93A}DKO^{CNS} motor neurons are actively undergoing autophagy.

Representative LC3 (brown) and p62 staining (green) of the anterior horn region of spinal cords from control and *SOD1*^{G93A} mice. Arrows indicate motor neurons (top panels) and positive p62 staining (bottom panels). Scale bars are 100 μm for top panels and 50 μm for bottom panels.

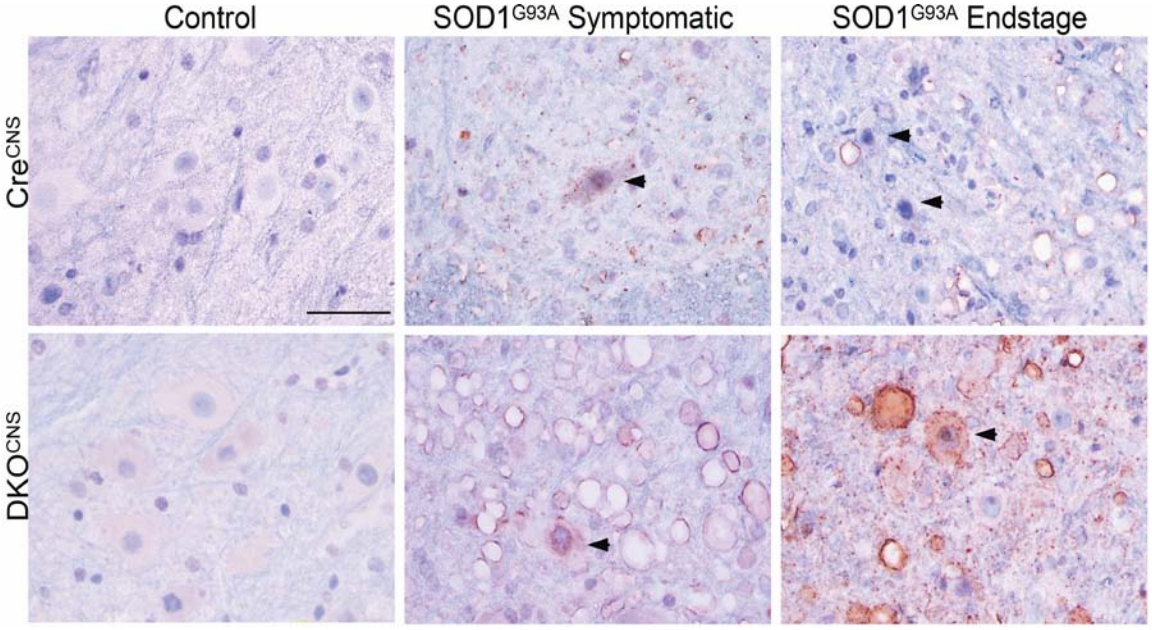

FIGURE 8
SOD1 aggregates in both Cre^{CNS} and DKO^{CNS} neurons.

Representative spinal cord anterior horn sections stained with antibody to SOD1 from the indicated genotypes. Arrowheads indicate examples of SOD1 aggregates within motor neurons. Control was taken at 120 (d) days of age; symptomatic was taken at 90 d and 120 d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively; and endstage was taken at 120 d and 150 d for *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS}, respectively. Scale bar: 100 µm.

SOD1^{G93A}DKO^{CNS} motor neurons continued to accumulate SOD1-containing aggregates (Figure 8). Thus, blocking the mitochondrial apoptotic pathway preserves motor neuron viability, despite amassing toxic protein species (31, 32).

To assess motor neuron function, we quantified the number of ventral root myelinated axons and innervated medial gastrocnemii synapses from the ALS mice. The *SOD1*^{G93A}DKO^{CNS} mice retained significantly more myelinated axons and innervated synapses compared with those of the *SOD1*^{G93A}Cre^{CNS} littermates (Figure 9 and 10), indicating functional preservation of spinal cord motor neurons. Furthermore, the neuromuscular junctions of *SOD1*^{G93A}Cre^{CNS} mice were significantly more denervated and degenerated in comparison with those of age-matched *SOD1*^{G93A}DKO^{CNS} mice (Figure 10). Finally, the *SOD1*^{G93A}DKO^{CNS} mice maintained motor function, as measured by rotarod performance, significantly longer than *SOD1*^{G93A}Cre^{CNS} littermates (Figure 11). In accordance with a previous study on *Bax–/–* mice and rotarod performance (33), our DKO^{CNS} animals exhibit impaired performance on the rotarod at higher speeds, which likely explains the discrepancy found in protection against symptom onset data, between using weight loss (3.5 weeks) versus rotarod performance (1.5 weeks) as a measure of motor function. However, using either measurement, onset is significantly delayed in *SOD1*^{G93A}DKO^{CNS} mice in comparison with *SOD1*^{G93A}Cre^{CNS} littermates. Therefore, *SOD1*^{G93A}DKO^{CNS} motor neurons not only demonstrate increased viability, but also retain functional capacity for an extended period of time after *SOD1*^{G93A}Cre^{CNS} motor neurons succumb.

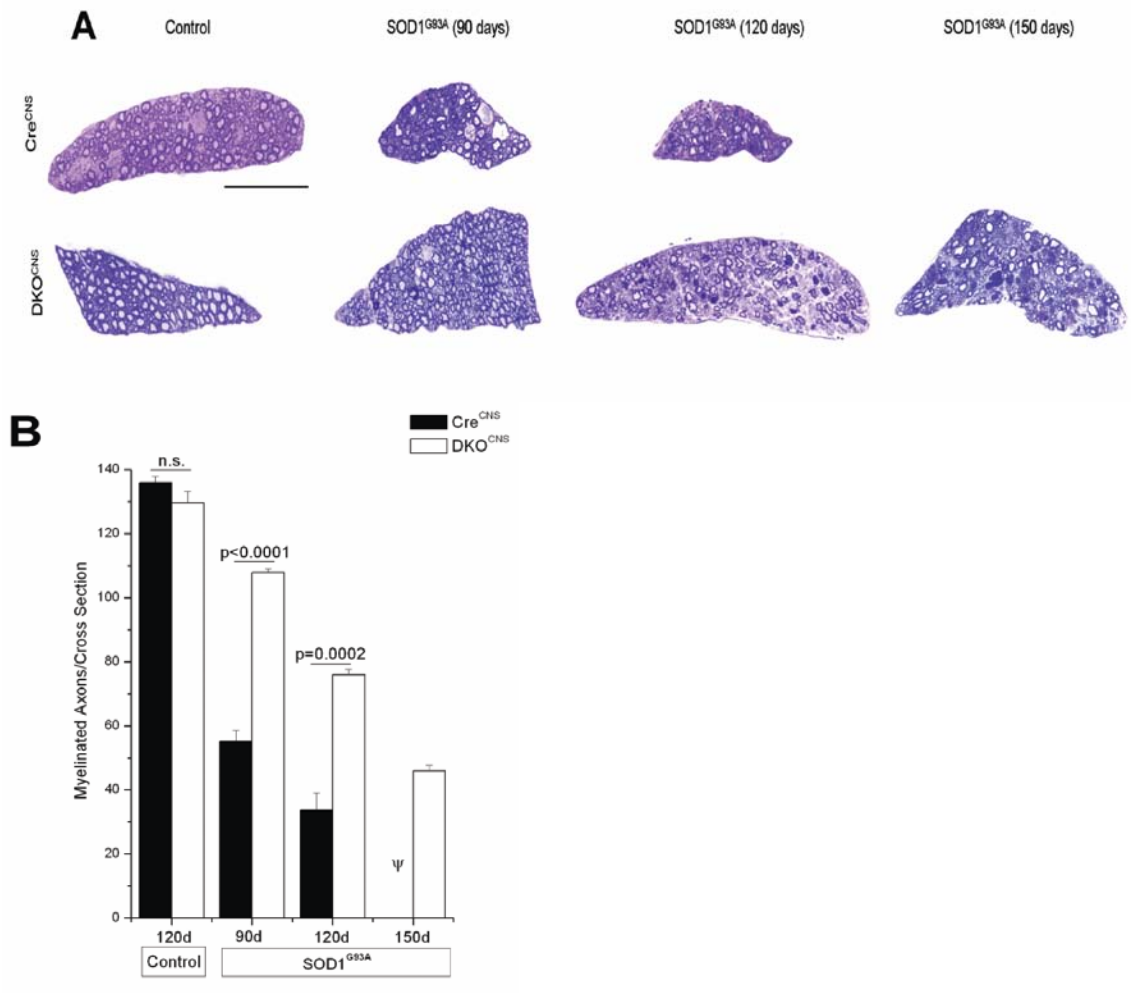

FIGURE 9
Deletion of BAX/BAK preserves motor axon myelination.

Control, symptomatic, and endstage are as defined in legend for Figure 4. (**A**) Representative ventral root sections from control and *SOD1*^{G93A} mice stained with toluidine blue. Scale bars: 200 μm. (**B**) Quantitation of myelinated ventral root axons from control and *SOD1*^{G93A} mice; *n*=3, unpaired two-tailed Student *t*-test. ψ, all mice deceased at indicated timepoint; n.s., not statistically significant.

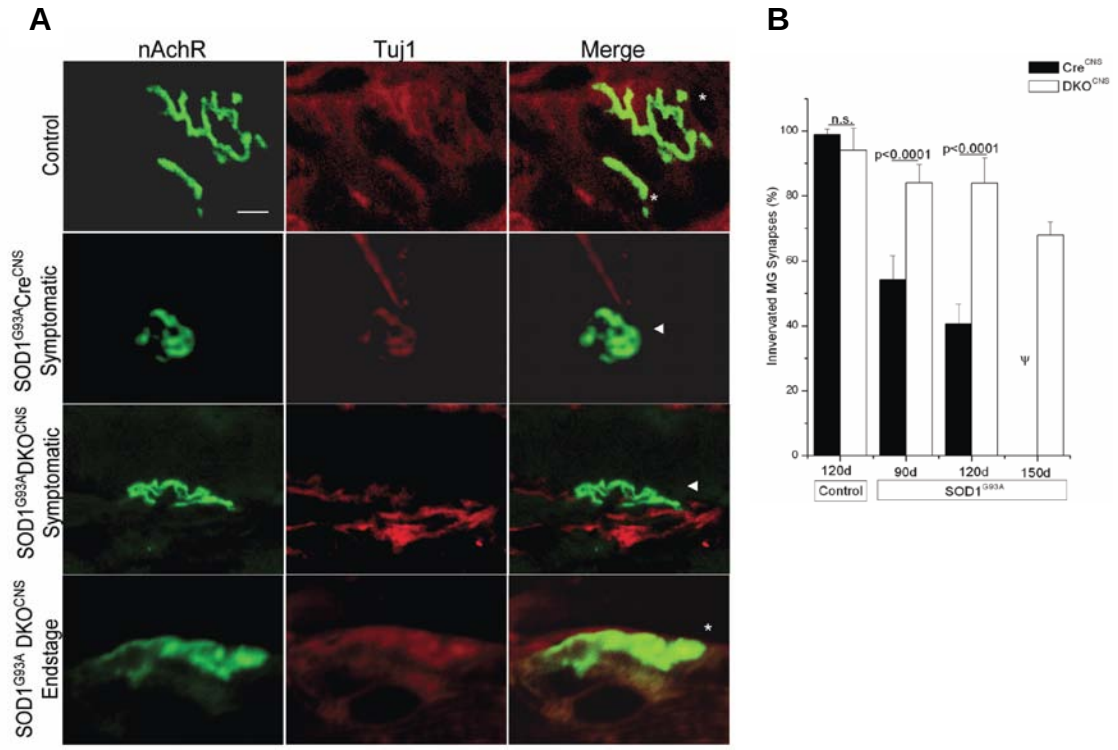

FIGURE 10
Deletion of BAX/BAK preserves neuromuscular junction innervation.

Control, symptomatic, and endstage are as defined in legend for Figure 4. (**A**) Representative neuromuscular junction (NMJ) images from control and *SOD1*^{G93A} mice stained with α-bungarotoxin-FITC (green) and antibody to Tuj1 (red). Asterisks and arrowheads indicate fully innervated and partially innervated NMJs, respectively. Notice the rounded, degenerated appearance of the *SOD1*^{G93A}Cre^{CNS} symptomatic NMJ. Scale bars: 8 μm. (**B**) Percentage of innervated synapses in control and *SOD1*^{G93A} mice as quantified from NMJ staining; *n*=2, unpaired two-tailed Student *t*-test. ψ, all mice deceased at indicated timepoint; n.s., not statistically significant.

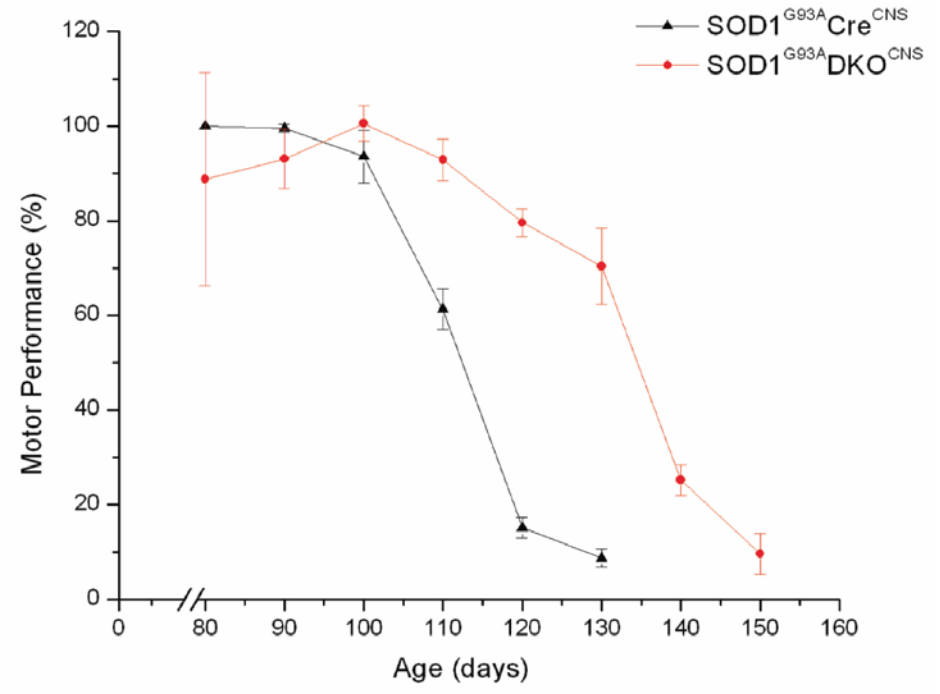

FIGURE 11
Deletion of BAX/BAK preserves neuronal function.

Percentage of maximum rotarod performance in *SOD1*^{G93A}Cre^{CNS} and *SOD1*^{G93A}DKO^{CNS} mice; n=3 for each group, p<0.05 via ANOVA; ANOVA, analysis of variance.

DISCUSSION

In this study, we show that genetic deletion of the mitochondrial apoptotic pathway significantly preserves neuronal viability, motor function, and life span in a mouse model of familial ALS. Prior attempts have been made to partially address the role of apoptosis in the pathogenesis of neurodegeneration in mouse models of ALS. Neuron-specific overexpression of antiapoptotic BCL-2 or administration of the broad-spectrum caspase inhibitor z-VADfmk extended life span by approximately 2 weeks in *SOD1*^{G93A} mice (7, 34). Bcl-2 is less effective at blocking the mitochondrial apoptotic pathway than deletion of BAX/BAK and, when overexpressed, is known to regulate tangential cell death signals unrelated to the BAX/BAK pathway, such as calcium-induced death, autophagy, and cell cycle entry (35–38). Moreover, z-VADfmk is a potent inhibitor of several key downstream effector caspases that are activated only after BAX/BAK-dependent mitochondrial permeabilization has occurred, an event that is incompatible with long-term cell viability (39, 40). Finally, a role for BAX in this process was suggested when *SOD1*^{G93A} mice bred to animals germline-deleted for *Bax* outlived *SOD1*^{G93A} wild-type controls by about 2 weeks (22), in agreement with our results for *SOD1*^{G93A} mice deficient for *Bax* in the CNS (*SOD1*^{G93A}*Bax*$^{-/-;CNS}$ mice) (data not shown).

In contrast with our findings in the *SOD1*^{G93A}DKO^{CNS} mice, Gould et al. reported no protection against neuromuscular denervation in the *SOD1*^{G93A}*Bax*$^{-/-}$ mice (22) and therefore reasoned that targeting the intrinsic apoptotic pathway would provide little functional benefit against MNDs. However, BAX and BAK are

often partly redundant in triggering apoptosis and must both be deleted for long-term protection from apoptotic stimuli in many cell types (14, 15). By conditionally deleting *Bax* and *Bak* specifically in the neurons of the ALS mouse, we show that the mitochondrial apoptotic pathway is a major contributor to the pathogenesis of this disease. Compared with the *SOD1*^{G93A}*Bcl-2* and *SOD1*^{G93A}*Bax*$^{-/-}$ mice (7, 34), the *SOD1*^{G93A}DKO^{CNS} mice show an approximately 40% greater extension in survival. Moreover, the *SOD1*^{G93A}DKO^{CNS} mice demonstrate a 1.33-fold and 3-fold increase in the preservation of innervated synapses at end-stage compared with the *SOD1*^{G93A}*Bcl-2* and *SOD1*^{G93A}*Bax*$^{-/-}$ animals, respectively (22, 34). BAX/BAK deletion not only halts neuronal loss, but prevents axonal degeneration, symptom onset, weight loss, and paralysis and extends survival by approximately 21%. Hence, complete blockade of the mitochondrial apoptotic pathway through deletion of both BAX and BAK in ALS preserves neuronal function for an extended period. These findings suggest that the same mitochondrial apoptotic machinery first causes neuromuscular degeneration and neuronal dysfunction before ultimately triggering cell death.

Interestingly, blocking apoptosis also induced autophagy, a process implicated in the clearance of intracellular protein aggregates in several neurodegenerative diseases (41, 42). Mounting evidence supports the notion that autophagy plays a largely protective role in neurodegeneration (43–46). Indeed, a recent study reports that p62 interacts with mutant SOD1, suggesting a potential role for autophagy in the degradation of misfolded SOD1 species (47).

However, further studies will need to be done to define the exact role of autophagy induction in ALS.

In summary, our findings show that the mitochondrial apoptotic pathway plays a direct role in the pathogenesis of familial ALS and suggest that inherited differences in the threshold for triggering apoptosis may be one determinant in susceptibility to disorders of motor neuron loss. As such, therapeutic interventions to inhibit apoptosis upstream of mitochondrial permeabilization represent a promising strategy to treat ALS and related MNDs (48).

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CHAPTER THREE
Building an in vitro model for ALS

SUMMARY

To compliment the *in vivo* study described in chapter two, I sought to build a cell culture model to better define the molecular mechanisms mediating SOD1 ^{G93A} toxicity. Our goal was to study signaling pathways in the absence of BAX and BAK. This would enable us to identify the mechanisms mediating SOD1 ^{G93A} toxicity in the absence of cell death. The ideal *in vitro* model would provide a reproducible cell culture system and a robust response to SOD1 ^{G93A} toxicity. Potential cell culture models included mouse embryonic fibroblasts (MEFs), primary neurons and a transformed neuronal cell line. However, each of these models possessed a number of problems. ESC derived motor neurons emerged as the most promising model system. Using lentivirus expressing SOD1 ^{G93A} with a cyan fluorescent protein (CFP) tag, we are able to infect ESC derived motor neurons (which in turn can be tracked by green fluorescent protein (GFP)). Moreover, SOD1 ^{G93A} caused specific killing of motor neurons.

Since SOD1 ^{G93A} was toxic to ESC derived motor neurons, I then generated DKO ESCs (in collaboration with Robert Blelloch's laboratory). However, we encountered an interesting problem with DKO ESCs. The absence of the mitochondrial apoptotic pathway resulted in an unexpected differentiation delay, which led to an interesting story described in chapter four. The *in vitro* cell culture model of ALS was then followed up by my colleague, Eric Wang.

BACKGROUND

Neurodegenerative diseases are difficult to study because of the lack of good model systems. Most *in vitro* models employ primary neuronal cultures or transformed cell lines (1, 2). However, these cell lines lack the complexity of the CNS, which is composed of multiple neuronal (e.g.-motor neurons, dopaminergic neurons) and non-neuronal cells (e.g.-microglia, astrocytes)(3, 4). Moreover, current models suffer from a lack of reproducibility (e.g.-ex vivo cultures) or absence of toxicity (e.g.-transformed cell lines) (2). Therefore, the ideal *in vitro* system should mimic the multifaceted landscape of the CNS, provide a constant supply of cells, and exhibit robust toxicity to misfolded disease proteins.

Embryonic stem cells (ESCs) represent a promising cell culture model to study neurodegenerative disorders (5, 6). Due to their pluripotency, ESCs retain the ability to differentiate into multiple tissue types. ESCs have successfully been differentiated into pancreatic β-cells, cardiomyocytes, and neurons, to name a few (7-9). Furthermore, these differentiation protocols yield a heterogeneous population of cells, which more closely recapitulates the cellular environment *in vivo*. Therefore, ESCs offer the opportunity to identify the molecular mechanisms involved in a number of diseases.

Indeed, ESCs are currently being utilized as a tool to study multiple neurodegenerative diseases (10, 11). In particular, human ESCs harboring disease mutations have been generated for Huntington Disease, fragile-X syndrome, and Duchenne muscular dystrophy, among others (12-14). However,

as these lines are derived from aborted embryos, their use has been somewhat controversial. Human induced pluripotent stem cells (iPSCs) have become increasingly popular as they are easy to obtain and can provide patient-specific information. However, evidence suggests that iPSCs are genetically distinct from ESCs (15, 16). Moreover, iPSCs have been successfully differentiated into only a few neuronal cell types (17).

Due to their genetic tractability and the availability of complementary *in vivo* models, mouse ESCs offer an attractive cell culture system to interrogate neurological disorders. Mouse models for AD, HD, and ALS are well characterized and provide a physiological setting to study any candidate signaling pathways identified in mouse ESCs (18). Furthermore, the ability to generate a “designer” ESC line via breeding of transgenic lines provides an added advantage to the end user.

Indeed, a transgenic mouse has been generated that expresses an *eGFP* cDNA under control of the mouse *Hb9* promoter (19). Hb9 is a homeodomain transcription factor that is specifically expressed in somatic motor neurons of the spinal cord starting from around embryonic day 10.5. Thus, ESC derived motor neurons from the *Hb9:eGFP* mouse can be monitored via GFP expression. Upon treatment with retinoic acid (RA) and Sonic hedgehog (Shh), ESCs differentiate into a mixed population of motor neurons, astrocytes, and interneurons (8). The ability to preferentially mark the affected cell type in a system that captures non-cell autonomous signaling provides a powerful cell culture model by which to study motor neuron diseases, such as ALS.

RESULTS

Transformed MEFs were initially tested as a cell culture model for their ease of transfection, amenability to biochemical studies, and availability of DKO MEFs. While SOD1 ^{G93A} is specifically toxic to motor neurons, the protein is ubiquitously expressed. Therefore, while MEFs are not the affected cell type in ALS, it is believed that expression of a toxic protein alone would be sufficient to kill any cell type. As shown in Figure 1A, MEFs are able to express SOD1^{WT} and SOD1 ^{G93A} protein at high levels. However, utilizing specific readouts of the unfolded protein response (UPR), SOD1 ^{G93A} was unable to activate the UPR in MEFs (Figure 1B and C). Moreover, SOD1 ^{G93A} did not kill MEFs (Figure 2). Therefore, MEFs were no longer pursued as a cell culture model for ALS.

We next considered primary motor neurons as a potential *in vitro* model. Utilizing previously published protocols, I attempted to isolate primary motor neurons via purification with an anti-p75 neurotrophin receptor antibody (20, 21). There were a number of difficulties encountered with the protocol. First, isolation of the spinal cords of embryonic day 13 (E13) mice requires a large number of embryos to yield a sufficient number of motor neurons. Moreover, as these motor neurons are primary cell lines, they can only survive a few passages in culture and therefore do not provide a reproducible cell culture system. Secondly, we were unable to identify an anti-p75 antibody that detected native p75 protein (data not shown). Consequently, we never obtained a pure population of motor neurons. Therefore, we next assessed whether a transformed motor neuron cell line would yield a valid cell culture system.

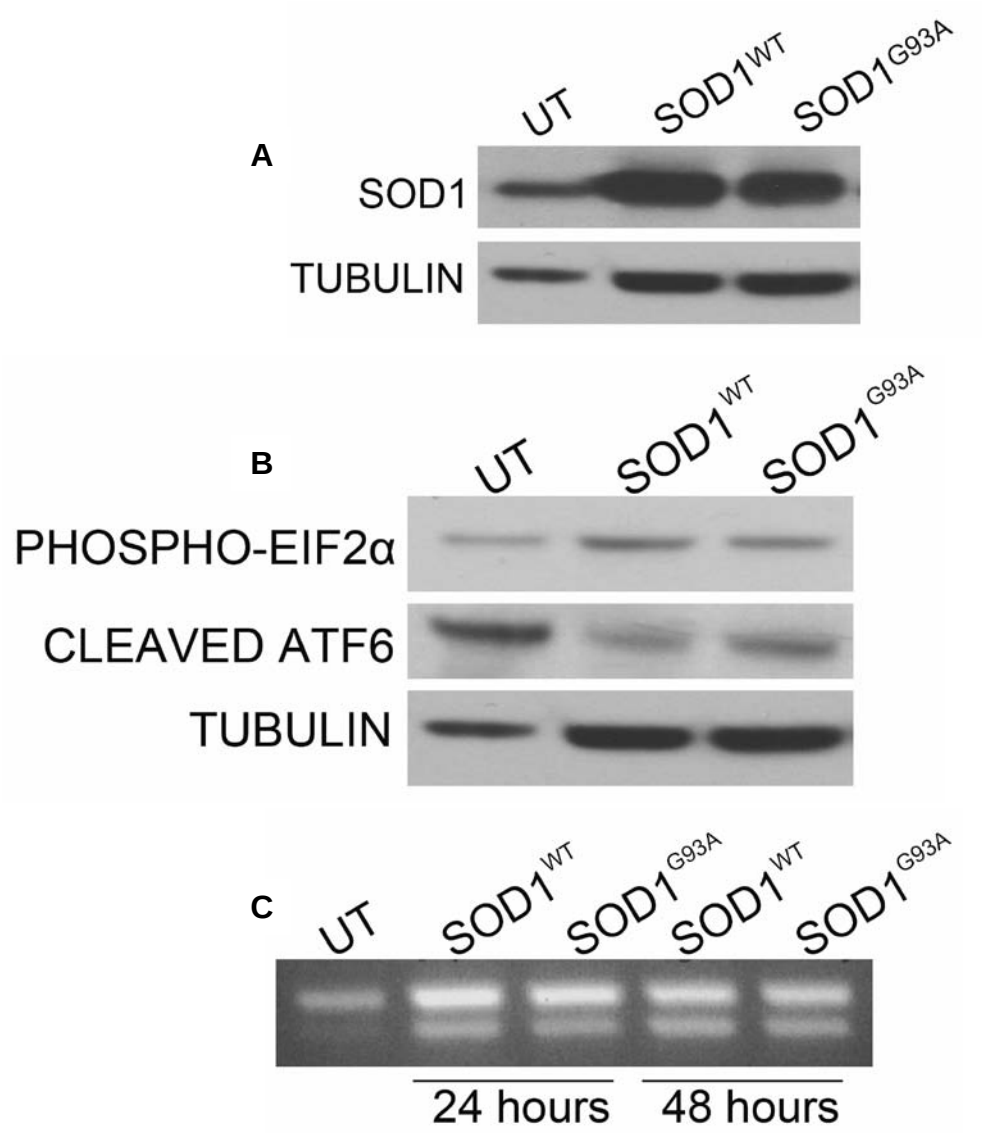

FIGURE 1
SOD1^{G93A} is unable to activate the unfolded protein response in MEFs.

(**A**) Plasmids encoding SOD1WT or SOD1G93A were transfected into MEFs and assessed for protein expression 24 hours later. (**B**) Markers of the unfolded protein response in SOD1 transfected MEFs (24 hours post-transfection). (**C**) cDNA was generated from transfected MEFs and analyzed for XBP-1 splicing via PCR. UT, untransfected; EIF2α, eukaryotic initiation factor-2α; ATF6, activation transcriptionfactor-6; XBP-1, X-box binding protein-1; PCR, polymerase chain reaction.

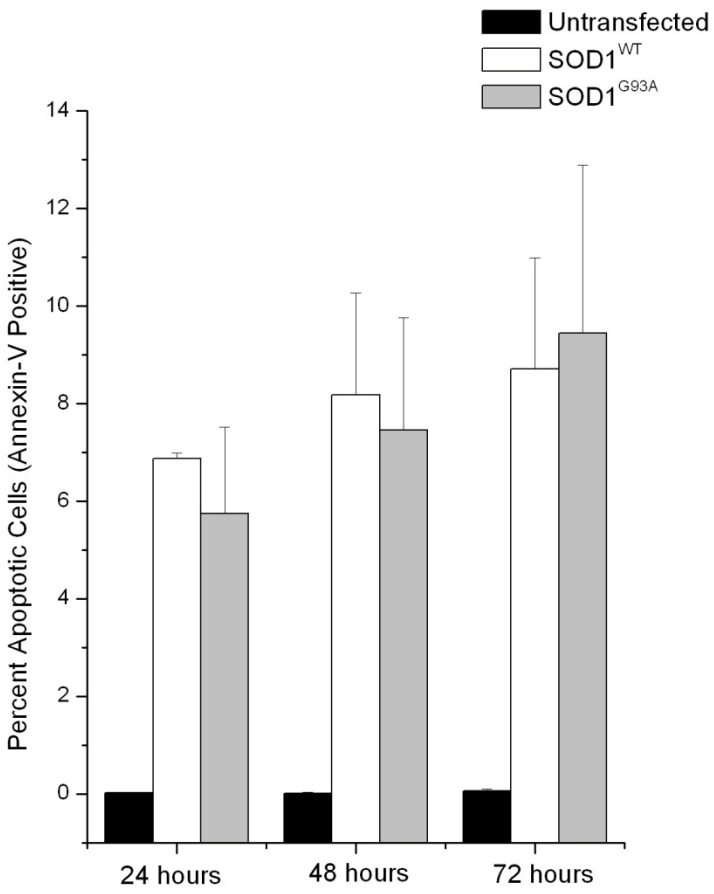

FIGURE 2
SOD1^{G93A} is not toxic to MEFs.

Plasmids encoding SOD1WT or SOD1G93A were transfected into MEFs and stained for Annexin-V 24, 48, and 72 hours later. Samples were analyzed via flow cytometry.

NSC34 cells have been used in the literature to study ALS (22-26). NSC34 cells are a mouse neuroblastoma/spinal cord cell line, and therefore have the advantage of reproducibility and tractability for biochemical studies (27). Transfection of SOD1 ^{G93A} in these cells conferred moderate toxicity compared to WT-SOD1 as assessed by propidium iodide staining (Figure 3A). Evidence suggests that tumor cells are resistant to apoptosis induced by the unfolded protein response (UPR) (28, 29). Since SOD1 ^{G93A} has been shown to aggregate and activate the UPR *in vitro* (30, 31), these NSC34 cells may be resistant to SOD1 ^{G93A} toxicity. Indeed, in comparison to MEFs, NSC34 cells are resistant to death mediated by a number of protein misfolding agents (Figure 3B). Because of these reasons, we next determined the feasibility of ESC derived motor neurons as a cell culture system.

ESCs can be differentiated into motor neurons *in vitro* by treatment with retinoic acid (RA) and Sonic Hedgehog (Shh) (8). Using *Hb9:eGFP* ESCs, approximately 50% of the cells expressed GFP as assessed by flow cytometry (Figure 4A). Additionally, the GFP expressing cells exhibit neuronal morphology, and stained positive for the motor neuron markers Islet-1 and choline acetyltransferase (ChAT) via immunocytochemistry (Figure 4B). Lastly, expression of SOD1 ^{G93A} in *Hb9:eGFP* ESC derived motor neurons is toxic (5, 6).

Since ESC derived motor neurons produced a robust response to SOD1 ^{G93A} toxicity and provide a reproducible culture system, we then sought to generate DKO ESCs. In collaboration with Robert Blelloch's laboratory, *Bax$^{f/f}$*

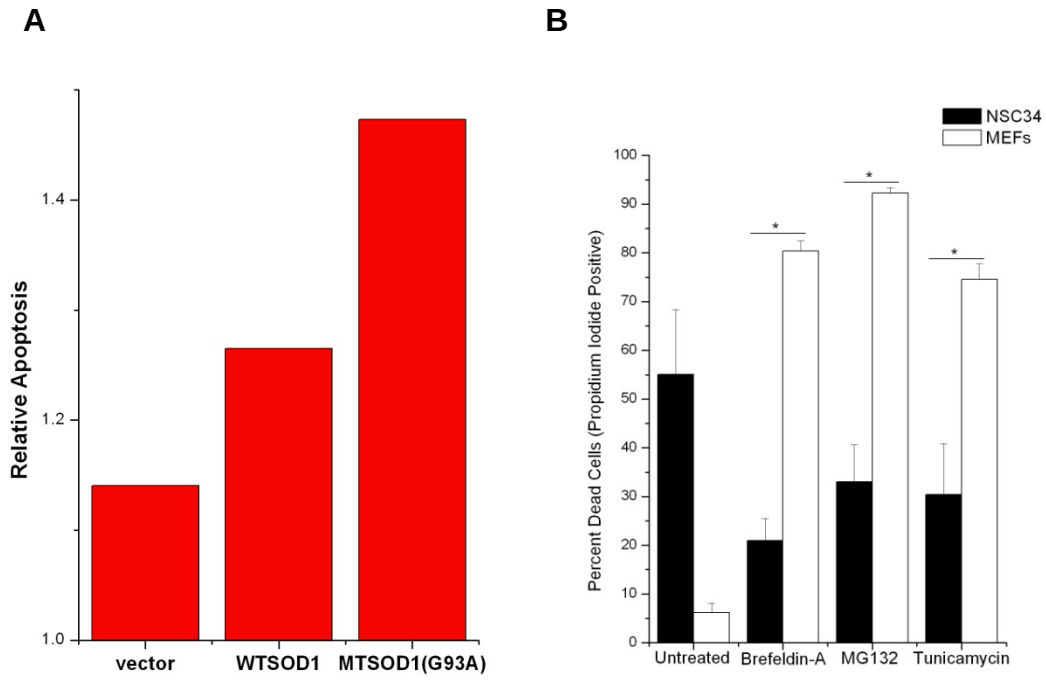

FIGURE 3
NSC34 cells are resistant to death induced by the unfolded protein response.

(**A**) NSC34 cells were transfected and analyzed for apoptosis via propidium iodide staining 24 hours later. Data was taken relative to untreated controls. SOD1G93A confers moderate toxicity. WT, wildtype; MT, mutant. (**B**) NSC34 cells were treated with drugs that induce protein misfolding and assessed for propidium iodide staining 24 hours later. MEFs were included as a positive control. $p<0.01$, $n=3$, unpaired two-tailed Student *t*-test.

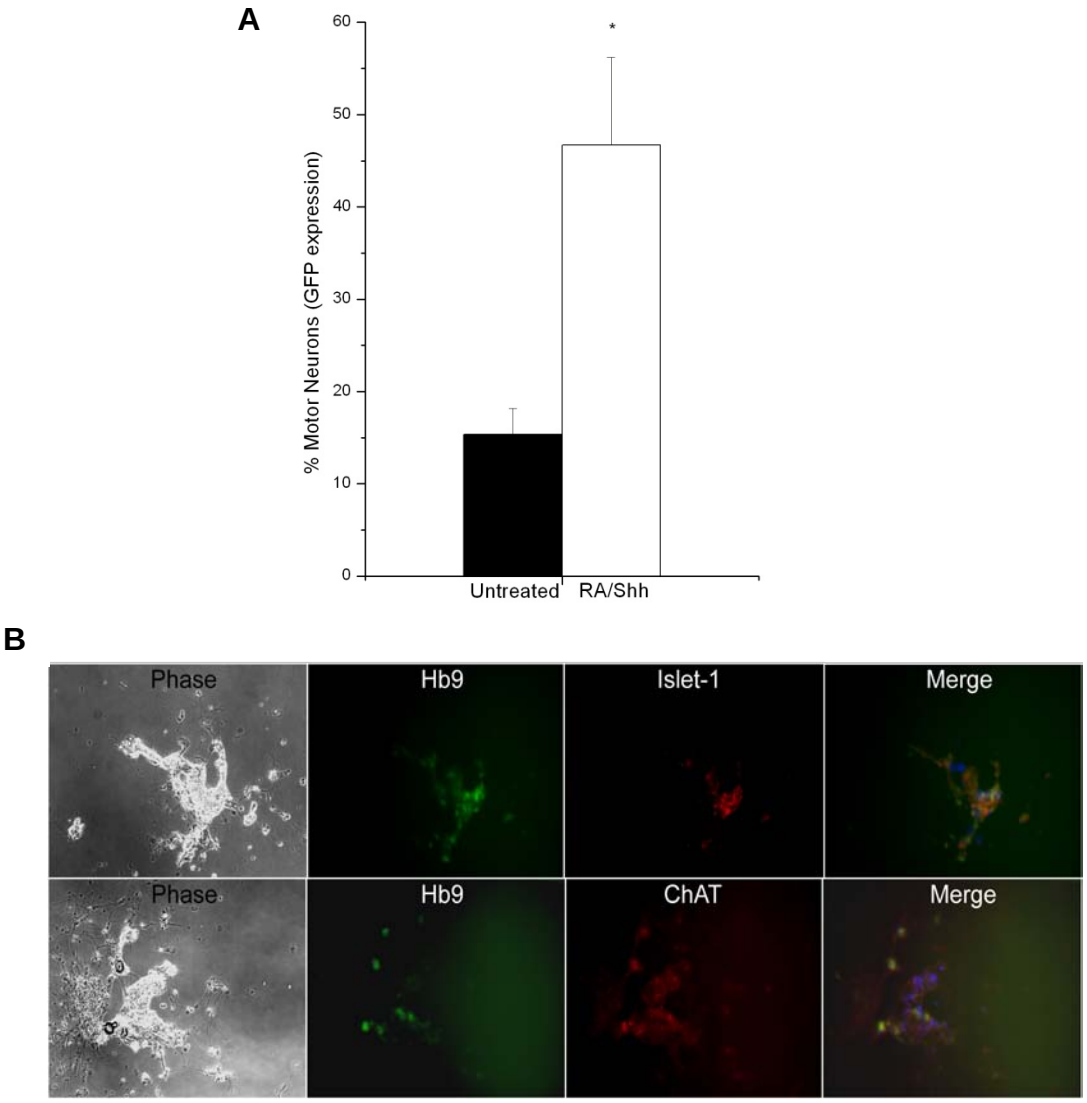

FIGURE 4
Hb9:eGFP ESCs can be differentiated into motor neurons.

(**A**) ESCs were formed into EBs and treated with retinoic acid and sonic hedgehog for approximately one week. Cells were then dissociated and assessed for GFP expression via flow cytometry. $p<0.01$, $n=3$, unpaired two-tailed Student *t*-test. (**B**) ESC derived motor neurons were stained with antibodies for the indicated lineage specific markers. EB, embryoid body; GFP, green fluorescent protein; RA, retinoic acid; Shh, Sonic hedgehog; Hb9, homeobox gene-9, ChAT, choline acetyltransferase.

Bak$^{-/-}$ ESCs were generated from the mice described in chapter two. Initial attempts at isolating these ESCs were unsuccessful due to the mixed strain background of the *Bax*$^{f/f}$*Bak*$^{-/-}$ mice. The line was then backcrossed to B6 mice for six generations, enabling the successful isolation of three different *Bax*$^{f/f}$*Bak*$^{-/-}$ ESC lines (Figure 5A) which were transiently transfected with Cre recombinase to obtain BAX/BAK deficient (Doubleknockout, DKO) ESCs. The resulting five clones were screened for BAX mRNA and protein expression (Figure 5B). From these, the three clones with the lowest BAX expression were genotyped for Cre recombinase (Figure 5C). One DKO ESC line (DKO line 3) possessed stable integration of Cre recombinase, and was not used for analysis. The other two lines were carried on for further analysis.

Deletion of BAX and BAK had no observable effects on the size, shape, and growth rate of undifferentiated ESCs (data not shown). To determine whether DKO ESCs are able to properly differentiate, ESCs were treated with retinoic acid (RA) and compared to a C57/B6 control cell line (Bax$^{+/+}$Bak$^{+/+}$), the parental cell line (Bax$^{f/f}$Bak$^{-/-}$), and Caspase-3$^{-/-}$ (*Casp-3*$^{-/-}$*)* ESCs. Since RA treatment biases ESC differentiation down the neural lineage (32), we compared transcript levels of two early neural markers, Nkx2.5 and NeuroD1, in the various ESC genotypes. Under these conditions, transcript levels of these neural markers are significantly lower in the DKO ESCs at all time points compared to the other cell types (Figure 6). Moreover, the mRNA levels for the neuronal markers in the DKO cells never reach that of the control cells, suggesting that

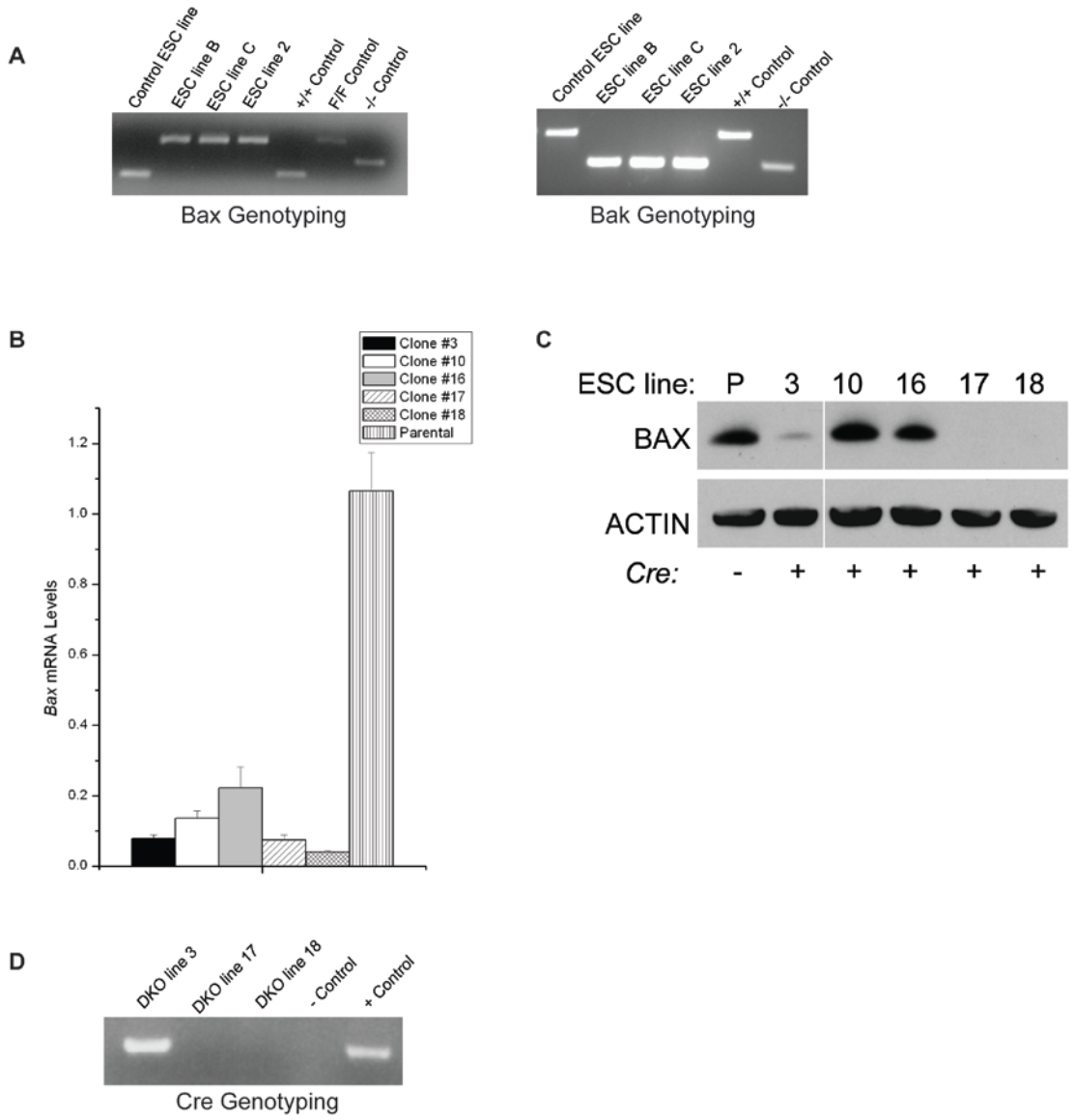

FIGURE 5
Generation of BAX/BAK deficient embryonic stem cells.

(**A**) Bax and Bak genotyping results from three independent Baxf/f Bak-/- ESCs lines. Wildtype ESCs are shown as a control. (**B**) Bax mRNA levels in five independently transfected lines via quantitative reverse -transcriptase PCR. Clones were derived from transfection of Cre recombinase in the parental line, “ESC line B”, followed by selection in Puromycin (**C**) Bax protein levels after Cre mediated excision. (**D**) Cre genotyping results in three DKO lines. ESC, embryonic stem cell; Control, $Bax^{+/+}Bak^{+/+}$; parental, $Bax^{f/f}Bak^{-/-}$; DKO, $Bax^{-/-}Bak^{-/}$.

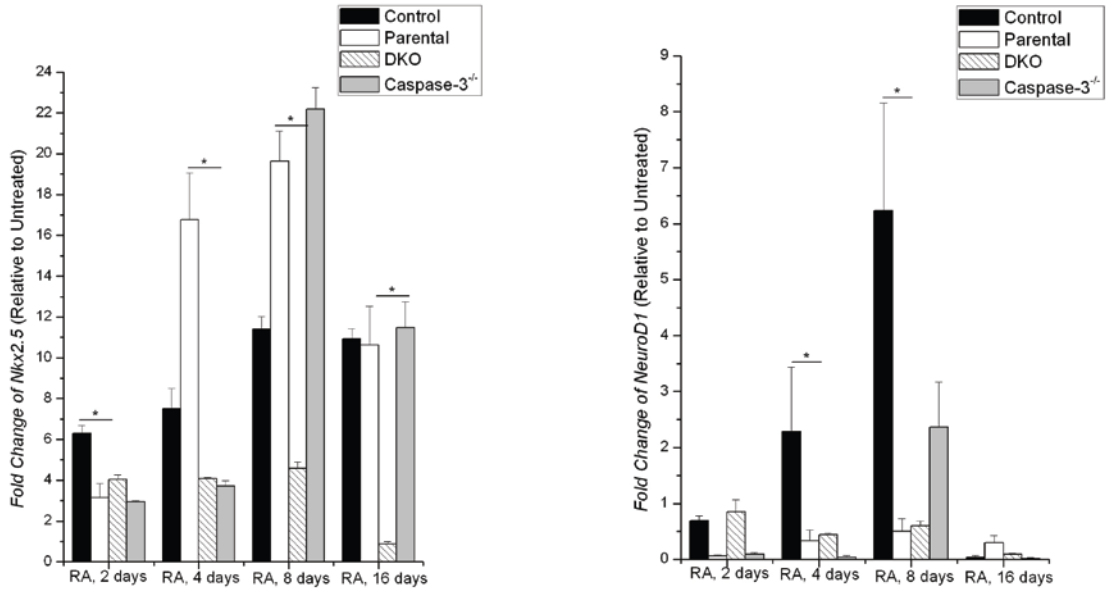

FIGURE 6
BAX/BAK deficient ESCs are ineffective at differentiating down the neural lineage.

mRNA levels of neural lineage markers from retinoic acid treated ESCs. *n*=3, *p*<0.01, unpaired two-tailed Student *t*-test; RA, retinoic acid.

ESCs lacking the intrinsic apoptotic pathway are unable to fully differentiate into neuronal precursors. Interestingly, parental (*Bax*$^{flox/flox}$ *Bak*$^{-/-}$) ESCs exhibit lower neuronal transcript levels compared to control cells, suggesting a gene dosage effect.

At this point, I realized that the differentiation delay exhibited by ESCs lacking the mitochondrial apoptotic pathway was a novel finding that begged further characterization. The findings of this project are detailed in Chapter Four. The cell culture model I established for ALS in the lab is going to be utilized by my colleague, Eric Wang, for his thesis project.

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CHAPTER FOUR

Engagement of the intrinsic apoptotic pathway mediates the differentiation of embryonic stem cells (ESCs)

SUMMARY

The pro-death BCL-2 proteins BAX and BAK are the gatekeepers to the mitochondrial apoptotic pathway. Germline deficiency in these two proteins is generally lethal, indicating a crucial role for apoptosis during development. It was recently reported that caspase-3, the major executioner caspase triggered by the mitochondrial apoptotic pathway, is important for the differentiation of embryonic stem cells (ESCs). However, it remains unclear whether the activation of caspase-3 during ESC differentiation occurs through engagement of the mitochondrial apoptotic pathway or via a distinct mechanism. Here we demonstrate that ESCs deficient in *Bax* and *Bak* are unable to activate caspase-3 and fail to properly differentiate. Moreover, we show that during ESC differentiation, a subpopulation of cells undergoes apoptosis and that cell death is critical for differentiation. Indeed, activation of the intrinsic apoptotic pathway in ESCs is sufficient to induce differentiation. These results show that BAX and BAK are required for proper differentiation of ESCs, and indicate that apoptosis plays a surprising role during this process.

BACKGROUND

The intrinsic apoptotic pathway is a highly conserved cell suicide program

shared by all higher eukaryotic cells that can be triggered by a wide variety of forms of irreversible internal injury. Healthy cells normally prevent activation of the intrinsic apoptotic pathway by sequestering several critical pro-death components within mitochondria. The multi-domain pro-apoptotic Bcl-2 proteins, BAX and BAK, serve as “gatekeepers” over outer mitochondrial membrane permeability (1). In response to upstream damage signals (eg. DNA damage, endoplasmic reticulum stress, or developmental cues), pro-apoptotic BH3-only proteins become activated through transcriptional and/or post-translational modifications, translocate to the mitochondria, and trigger homo-oligomerization of BAX and/or BAK to induce outer mitochondrial membrane permeability and release of cytochrome *c* and other pro-death molecules (SMAC/DIABLO). Release of cytochrome *c* facilitates formation of the apoptosome and activation of caspase 9, which then in turn cleaves and activates abundant effector caspases like Caspase-3 to proteolytically dismantle the cell.

BAX and BAK play a critical role in triggering programmed cell death during embryonic development, as mice deficient in these two proteins are generally lethal. The rare $Bax^{-/-}Bak^{-/-}$ mice that do survive until adulthood possess physical abnormalities, including interdigital webbing, imperforate vagina in females, splenomegaly, and lymphadenopathy (2). Interestingly, animals singly deficient in either BAX or BAK have limited morphological defects, indicating these proteins are functionally redundant (2, 3). Furthermore, mice genetically deficient for other key players in the intrinsic apoptotic pathway, such as Apaf-1 and Caspase-9, possess phenotypic defects but to a lesser degree than

BAX/BAK knockout animals (4, 5). These results indicate that BAX and BAK are the principal apoptotic proteins involved in mammalian development.

Recently, it was reported that caspase activation was required for the differentiation of embryonic stem cells (ESCs) and that activation of Caspase-3 alone was sufficient to induce ESC differentiation (6). In conjunction with other findings for cellular differentiation, Fujita et al. attributed caspase activity during ESC differentiation to a non-apoptotic process. However, we observe high levels of BAX/BAK dependent apoptosis during neuronal-targeted differentiation. Here we demonstrate that caspase activation during ESC differentiation requires flux through BAX and BAK, thereby indicating a novel role for the intrinsic apoptotic pathway in ESC differentiation.

RESULTS

As described in chapter two, we noted that ESCs lacking the BAX/BAK pathway are ineffective at differentiating down the neural lineage. We next asked whether DKO ESCs are able to give rise to all three germ layers. To do this, we injected DKO ESCs, *Casp-3*$^{-/-}$ ESCs, control, and parental cell lines into immunocompromised mice and analyzed tumor formation at the injection site. Interestingly, teratomas from DKO or *Casp-3*$^{-/-}$ ESCs formed approximately three weeks faster compared to control and parental cell lines (data not shown). Moreover, hematoxylin and eosin (H&E) stains of the tumors revealed that control, parental, and to some extent *Casp-3*$^{-/-}$ lines were able to differentiate into all germ layers *in vivo* (Figure 1). However, DKO teratomas consist largely of

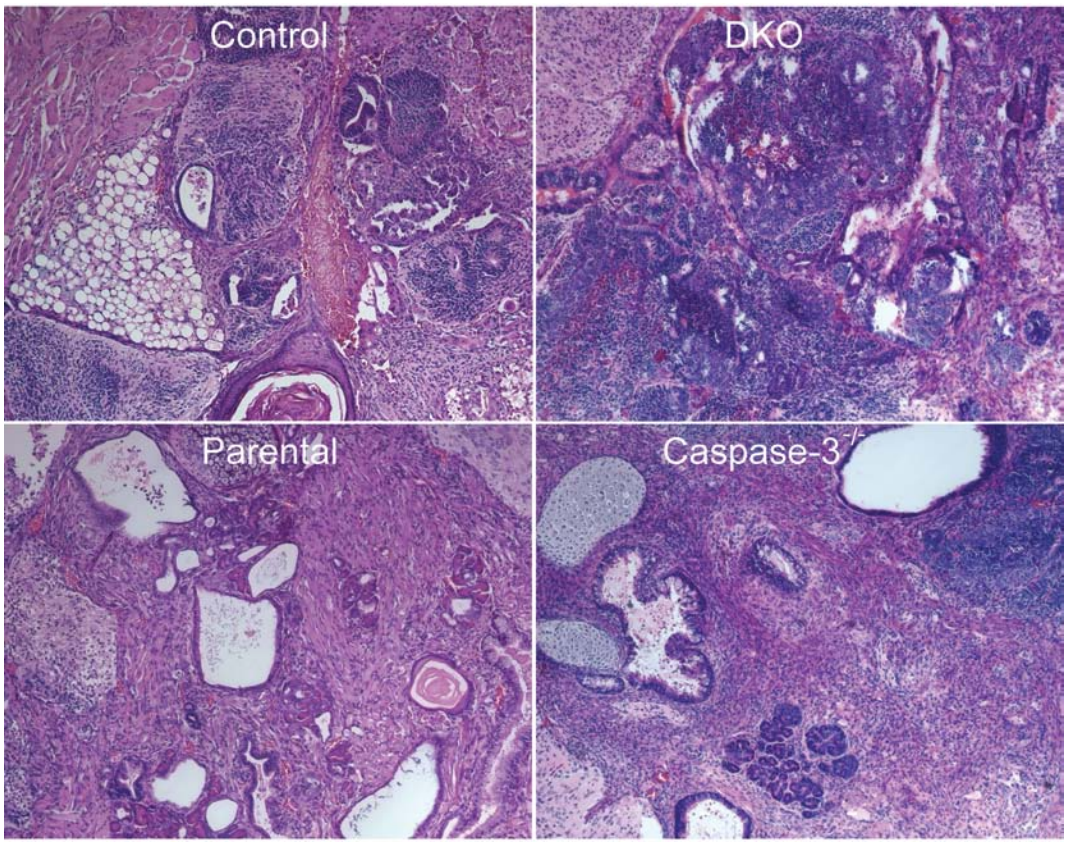

FIGURE 1
Differentiation is far less effective in the absence of BAX and BAK.

H&E stains of teratomas from immunocompromised mice. Control, *Bax*$^{+/+}$*Bak*$^{+/+}$; parental, *Bax*$^{f/f}$*Bak*$^{-/-}$; DKO, *Bax*$^{-/-}$*Bak*$^{-/-}$; H & E, hematoxylin and eosin.

sheets of undifferentiated cells, indicative of a more immature state (Figure 1, upper right panel). Interestingly, while *Casp-3*$^{-/-}$ teratomas are able to form all three germ layers, the differentiation is less complete than that of teratomas derived from control or parental lines. These results suggest that while differentiation is impaired in the absence of Caspase-3, the impediment is more significant in the absence of BAX and BAK. These results mirror the greater degree of protection against apoptosis seen in *Bax*$^{-/-}$*Bak*$^{-/-}$ cells compared to *Casp-3*$^{-/-}$ cells.

To determine whether this differentiation delay correlates with retained expression of pluripotency markers, we performed colony formation assays with RA treated ESCs. Even after treatment with RA for sixteen days, DKO ESCs retained expression of the pluripotency marker alkaline phosphatase (AP) (7, 8) (Figures 2A and B). Notably, ESCs deficient in Caspase-3 fully differentiate by day eight as they no longer express detectable levels of AP (Fig 2B). These results argue that Caspase-3 is normally activated by the BAX/BAK-dependent mitochondrial apoptotic pathway during differentiation. To further assess the differentiation defect in DKO ESCs, we measured mRNA levels of the pluripotency factors, *Nanog* and *Oct4* (9). In comparison to the control or parental cells, DKO ESCs express 6-fold and 3-fold higher levels of *Nanog* and *Oct4,* respectively (Figure 2C). However, by day 4, transcript levels for these pluripotency factors have dropped significantly (Figures 2C and D) and by day 8 are undetectable (Figure 2C). Therefore, the differentiation delay exhibited by DKO ESCs is associated with retained expression of pluripotency factors for an

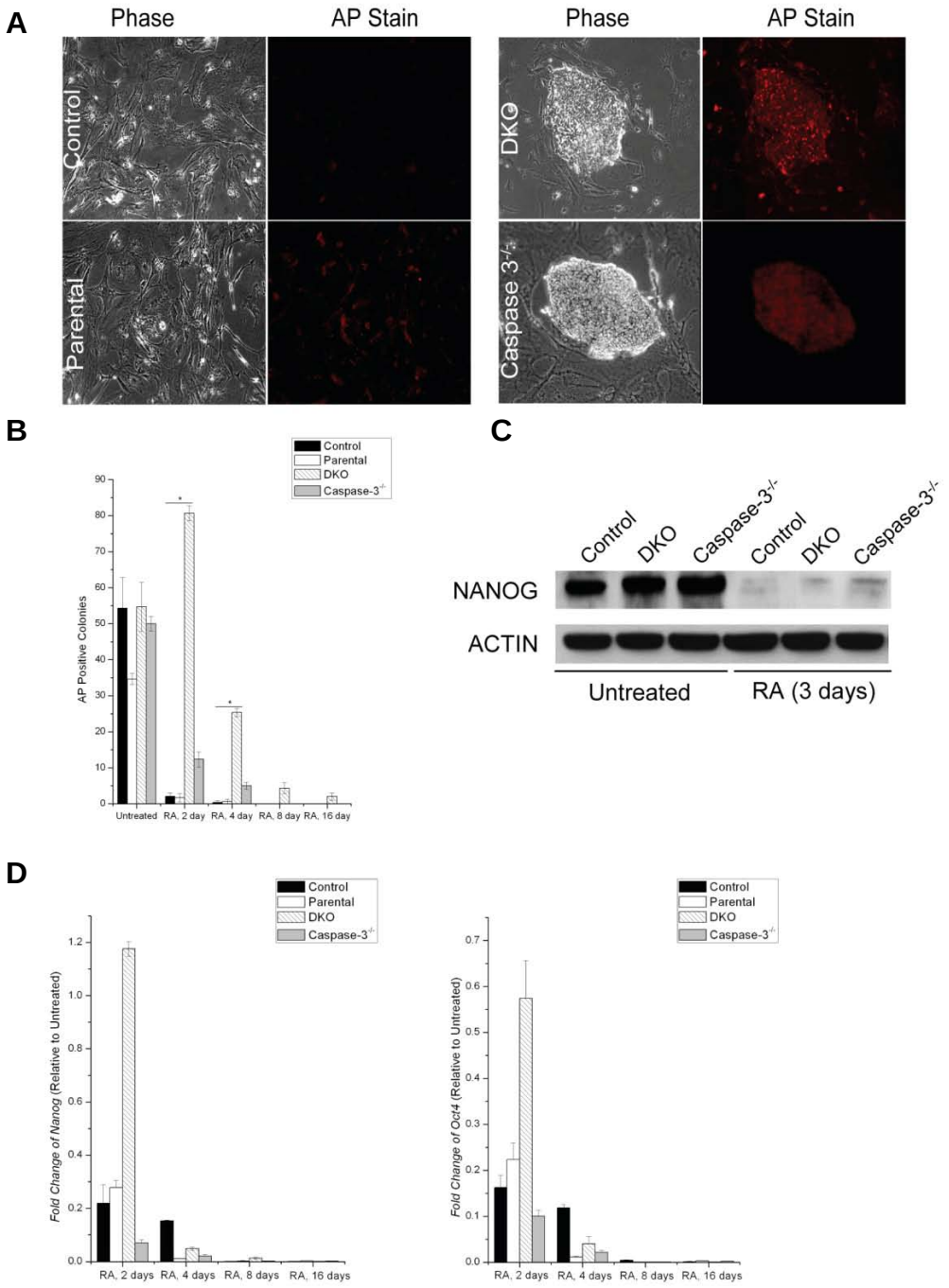

FIGURE 2

Expression of pluripotency markers is retained for an extended period in the absence of BAX/BAK.

(**A**) AP expression in ESC lines after RA treatment. (**B**) Number of AP positive colonies. (**C**) Nanog protein levels from ESCs. (**D**) Nanog and Oct4 mRNA levels from ESCs as analyzed via Q-RT-PCR. AP, alkaline phosphatase; RA, retinoic acid.

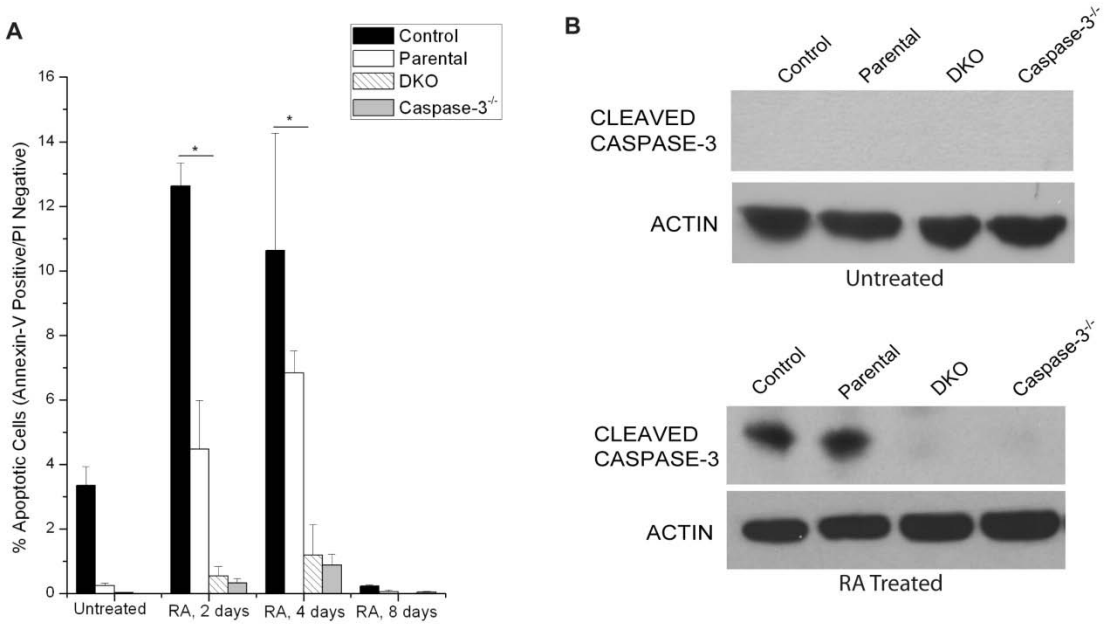

FIGURE 3
Caspase activity during differentiation requires Bax and Bak.

(**A**) ESCs deficient in BAX/BAK or Caspase-3 are resistant to RA mediated apoptosis (**B**) ESCs were treated with RA for three days and immunoblotted for activated Caspase-3. Caspase-3 cleavage is absent in BAX/BAK deficient ESCs. PI, propidium iodide.

extended period. These results indicate that differentiation is far less efficient in the absence of BAX and BAK.

Previously, it was shown that caspases are activated during differentiation (6). To determine whether caspase activity correlates with apoptosis, we stained RA treated cells with Annexin-V and propidium iodide (PI). Apoptotic cells were scored as Annexin-V positive and PI negative. In untreated ESCs, the percentage of apoptotic cells is extremely low (~3%). However, during differentiation of ESCs, apoptotic cells make up 10-12% of the cell population at 2 and 4 days post RA treatment (10) (Figure 3A). By 8 days post RA treatment, apoptosis is essentially undetectable. In contrast, the *Casp-3*$^{-/-}$ and DKO ESCs show greatly diminished levels of apoptosis under both untreated and RA treated conditions (Figure 3A). Furthermore, Caspase-3 activity during differentiation is completely absent in DKO ESCs, confirming that its activation requires the mitochondrial apoptotic pathway (Figure 3B). Interestingly, parental ESCs also exhibit decreased levels of apoptosis compared to control ESCs, suggesting a potential gene dosage effect.

We next asked whether forced activation of Caspase-3 in DKO ESCs induces differentiation. In the presence of leukemia inhibitory factor (LIF), cells were treated with chelerythrine chloride, a drug that induces apoptosis by a BAX/BAK independent mitochondrial mechanism (11). Since LIF retains cells in a pluripotent state (12), if Caspase-3 activation were sufficient to induce differentiation, chelerythrine chloride should differentiate ESCs regardless of

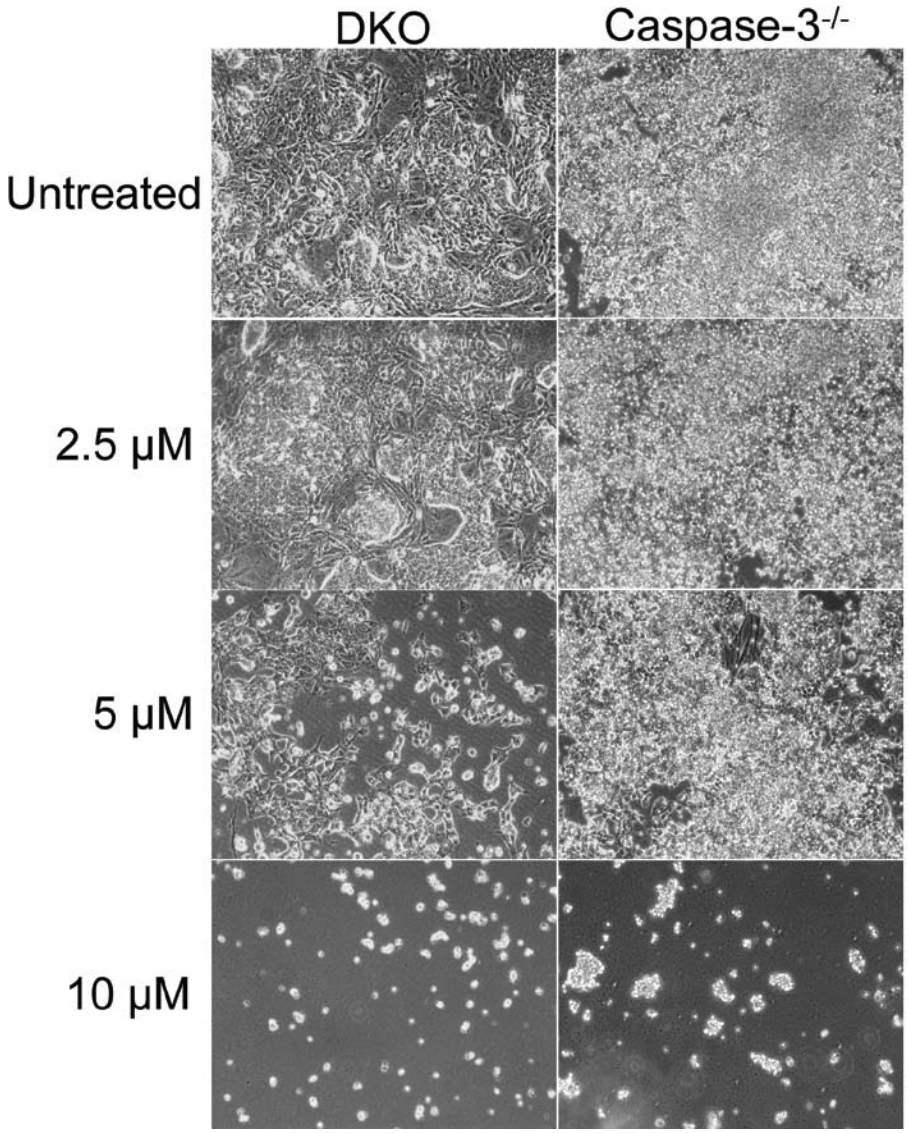

FIGURE 4
Forced caspase activation induces differentiation of BAX/BAK deficient ESCs.

Morphology of ESCs after treatment with chelerythrine chloride for two days. Caspase-3 deficient ESCs are unable to differentiate.

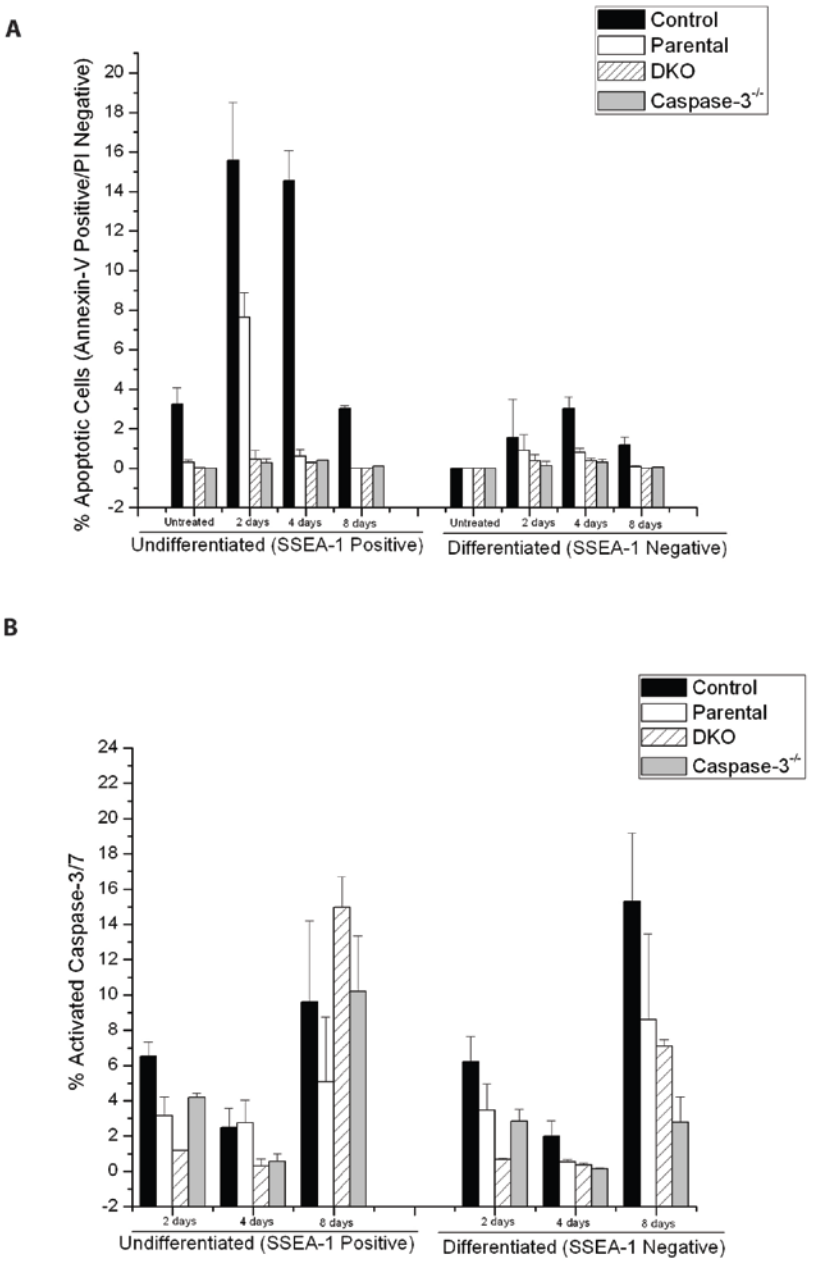

FIGURE 5
ESCs Activate Caspases and Undergo Apoptosis in a BAX/BAK Dependent Manner.

(**A**) ESCs undergoing apoptosis under RA treatment are the undifferentiated population (**B**) Activated Caspase-3 and 7 is present in both the undifferentiated and differentiated populations of RA treated ESCs. SSEA-1, stage specific embryonic antigen-1

media conditions. Control cells, parental cells, and DKO ESCs showed morphological signs of differentiation upon chelerythrine chloride treatment (Figure 4). Moreover, this process was dependent on Caspase-3, as *Casp-3*$^{-/-}$ ESCs remained undifferentiated (Figures 4). Thus, forced caspase activation induces differentiation of ESCs lacking BAX and BAK in a Caspase-3 dependent manner.

To identify the cohort of ESCs undergoing apoptosis upon RA treatment, we co-stained RA treated ESCs with Annexin-V, PI, and an antibody to SSEA-1, a pluripotency marker on the surface of ESCs (13). Interestingly, the majority of apoptosis occurs in the undifferentiated population (SSEA-1 positive), which is absent in DKO and *Casp-3*$^{-/-}$ ESCs (Figure 5A). Consistent with our previous apoptosis results (Figure 3A), undifferentiated parental ESCs exhibit a lower level of apoptosis compared to controls (Figure 5A). Interestingly, Caspase-3 was activated equally in the undifferentiated (SSEA-1 positive) and differentiated (SSEA-1 negative) populations (Figure 5B). These results suggest that tonic signaling through BAX and BAK induces a low level of caspase activity which is necessary for proper ESC differentiation. However, if caspase activity surpasses a certain threshold, then cells succumb to apoptosis.

DISCUSSION

In this study, we show that flux through the intrinsic apoptotic pathway is required for proper ESC differentiation. Previous studies have shown that other proteins in the apoptosis pathway play a role in ESC differentiation. For instance,

ESCs transgenically expressing Bcl2 (Bcl2-ESCs) remained pluripotent in serum and feeder-free conditions (10). A more recent study implicated that Caspase-3 activation was necessary and sufficient for ESC differentiation and occurred independently of apoptosis (6). Lastly, it has been shown that p53 downregulates *Nanog* expression during ESC differentiation (14).

In contrast to previous reports, we show here that the caspase activation that occurs during differentiation is due to engagement of the mitochondrial apoptotic pathway. During differentiation, a low level of apoptosis is activated and this signaling primarily occurs via the BAX/BAK pathway. The Fujita et al. study did not perform a rigorous analysis of apoptosis, but instead relied on DAPI staining to identify apoptotic figures. In accordance with Yamane et al., we see apoptosis during differentiation conditions by using Annexin-V staining, a more definitive method of identifying apoptotic cells.

The fact that apoptosis plays a pivotal role during ESC differentiation is surprising, but can be linked to the importance of the BAX/BAK pathway in embryonic development. Mice genetically deficient for BAX and BAK are mostly lethal, with only 10% of the mice surviving for a few weeks after birth. Moreover, the BAX/BAK deficient mice that do survive show numerous physical abnormalities in several tissue types, including the spleen, lymph nodes, and brain, to name a few (2). These results indicate that the intrinsic apoptotic pathway is necessary for proper tissue development. Furthermore, caspase activation has been shown to be involved in the maturation process of several cell types, including platelets, macrophages, and lens epithelial cells (15-19).

Interestingly, caspase activation during platelet maturation is BAX/BAK dependent (20). Although, it is widely believed that caspase activation in these settings occurs independently of the apoptotic pathway, it would be interesting to see whether differentiation of macrophages and lens epithelial cells are dependent on BAX and BAK.

In summary, our findings show that the mitochondrial apoptotic pathway plays a direct role in ESC differentiation, and suggest that a tonic level of apoptotic signaling is necessary for the proper maturation of stem cells, and perhaps other cell types. Our results strongly argue that cell death and differentiation are intertwined, and implicate the importance of the BCL2 family of proteins in other processes besides cell death.

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CHAPTER FIVE

Materials and Methods

Western blots and antibodies

Proteins from spinal cords were extracted in RIPA buffer (20 mM Tris-MOPS [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40) containing protease inhibitor cocktail (Roche). The extract was sonicated and then centrifuged at 15,682 g for 10 minutes at 4°C. Protein concentration was measured using BCA assay (Pierce). Thirty or sixty µg of each sample was loaded on a 10% Bis-Tris gel (Invitrogen), transferred to PVDF membranes, and immunoblotted with antibodies against BAX (1:1,000; Cell Signaling Technology), cleaved Caspase-3 (1:250, Cell Signaling Technology), nanog (1:1000, Abcam), actin (1:1,000; Chemicon), SOD1 (1:1000, Calbiochem), (ATF6 (1:500, Imgenex) or phosphoEIF2α (1:1000, Cell Signaling Technology). Horseradish peroxidase–conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (anti-mouse and anti-rabbit antibodies) or Millipore (anti-sheep). Membranes were developed with Western Lightning Chemiluminescence Reagent (PerkinElmer).

Quantitative RT-PCR and primers

Total RNA was extracted from spinal cords using the RNeasy Mini Kit (Qiagen) and from ESCs using Trizol (Invitrogen, cat. no. 15596-018). cDNA was generated using the SuperScript II Reverse Transcriptase Kit (Invitrogen). Q-

PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Bax transcript levels were normalized to hypoxanthine phosphoribosyltransferase (HPRT) or levels, while Oct4 and Nanog transcript levels were normalized to 60S ribosomal protein-7 (RPL7). Samples were run in triplicate. Primer sequences used are as follows:

Gene	***Forward Primer***	***Reverse Primer***
Bax	GCTGACATGTTTGCTGATGG	GATCAGCTCGGGCACTTTAG
Oct4	AAAGCCCTGCAGAAGGAGCTAGAA	AACACCTTTCCAAAGAGAACGCCC
Nanog	GCTCAGCACCAGTGGAGTATCC	TCCAGATGCGTTCACCAGATAG
RPL7	GATTGTGGAGCCATACATTGCA	TGCCGTAGCCTCGCTTGT
NKX2.5	CAGTGGAGCTGGACAAAGCC	TAGCGACGGTTCTGGAACCA
NeuroD1	AAGCCATGAATGCAGAGGAGGACT	AGCTGCAGGCAGCCGGCGACC

XBP-1 Splicing Assay

cDNA was generated as above and diluted 1:20. PCR was performed for XBP-1 using the following set of primers: mXXIFI: ttacgggagaaaactcacggc and mXXIB1: gcattctggacaagttggaccc. The resulting product was run on a 2.5% agarose gel made in 1X TAE buffer.

Animal models

All animal experiments were performed in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. The Bax conditionally deficient mice and Bak-null mice were a gift from O. Takeuchi (Immunology Frontier Research Center, Osaka University, Osaka, Japan) and the laboratory of the late Stanley J. Korsmeyer

(Dana-Farber Cancer Center, Boston, Massachusetts, USA). The Baxflox mice were derived from injection of RW4 ES cells (strain 129/SvJ) into C57BL/6J recipients, and the resulting chimera were bred to C57BL/6J for germline transmission (1). Baxflox mice were backcrossed to C57BL/6J background for 6 generations prior to these experiments. Bak–/– mice were backcrossed to C57BL/6J background for 6 generations before breeding to Baxflox mice. NesCre (B6.Cg[SJL]-Tg[Nes-cre]1Kln/J) and SOD1G93A mice (B6SJL-Tg[SOD1-G93A]1Gur/J) were purchased from The Jackson Laboratory and are on C57BL/6J background. Mice compared in this study were all littermates and housed together to minimize environmental factors. Mice were genotyped using PCR protocols from The Jackson Laboratory.

Survival, symptom onset, and rotarod studies

Mice were considered terminally paralyzed if they were unable to right themselves after 15 seconds of being placed on their backs. To assess symptom onset, mice were weighed daily, starting at 100 days of age. Weight data were then plotted to create a “weight curve.” The peak of the weight curve was taken as the day of symptom onset. For the rotarod studies, mice were tested twice a week for 3 trials, each starting at 60 days of age, until they were unable to remain on the rotarod for at least 10 seconds. The best trial per day was recorded and used for analysis. Data were graphed as the percentage of the longest rotarod performance by each mouse.

Immunohistochemistry and antibodies

Spinal cords were fixed in 10% formalin for 24 hours and paraffin embedded. Six-μm sections were stained with the following antibodies: SOD1 (1:1,000; Calbiochem), activated caspase-3 (1:50; Cell Signaling Technology), choline acetyltransferase (1:25; Chemicon), LC3 (1:500; gift from J. Debnath, University of California, San Francisco), and p62 (1:250; Progen Biotechnik GmbH). Staining for activated caspase-3 was performed using the PicTure-MAX Polymer Detection Kit (Zymed Laboratories). TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore). Activated caspase-3 and TUNEL stains were counterstained with cresyl violet. To quantify innervated neuromuscular junctions, medial gastrocnemii were fixed in 10% formalin for 24 hours, followed by 30% sucrose at 4°C for 24 hours. The samples were then embedded in OCT compound (Tissue-Tek) and flash frozen. Five-micron sections were fixed in acetone and stained with the following reagents: FITC-conjugated α-bungarotoxin (1:200; Sigma-Aldrich), anti-TUJ1 antibody (1:1,000; Covance), and Alexa Fluor 568–conjugated anti-mouse secondary antibody (1:1,000; Molecular Probes).

Motor neuron numbers were determined by staining lateral 6-μm sections of the lumbar spinal cord with cresyl violet. To determine the number of myelinated axons, sections of the lumbar ventral root were embedded in 1% agarose and fixed in Karnovsky's fixative (5% glutaraldehyde and 4% paraformaldehyde in 0.08 M sodium phosphate buffer) for at least 24 hours. The

sections were then postfixed in osmium tetroxide and embedded in Epon. Sections were cut at 1 μm and stained with toluidine blue.

Electron microscopy

Spinal cords were fixed in Karnovsky's fixative (5% glutaraldehyde and 4% paraformaldehyde in 0.08 M sodium phosphate buffer) for at least 24 hours and then treated with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The sections were then postfixed in osmium tetroxide and embedded in Epon. Sections were cut at 80 nm, stained with lead citrate and uranyl acetate, and examined under a Philips CM10 electron microscope.

Statistics

Kaplan-Meier survival curves were generated using Origin software and were analyzed using the log-rank test. Quantitative RT-PCR data, symptom onset, caspase-3 and TUNEL quantifications, innervated synapses, and motor neuron and myelinated axon numbers were compared using 2-tailed unpaired Student's t test. Rotarod data were compared using ANOVA. In figure legends, data are presented as mean ± SEM. P values of less than 0.05 were considered significant.

Expression of SOD1 in Cells

For MEFs and NSC34 cells, 0.5ug of pcDNA3-SOD1^{WT} or pcDNA3-SOD1^{G93A} was transfected into cells using Fugene (Roche, cat. no. 11814443001).

Treatment with Protein Misfolding Agents

Cells were incubated overnight with 1uM MG132 (Calbiochem, cat. no. 474790), 2.5ug/mL Brefeldin-A (Sigma, cat. no. B6542), or 5ug/ml Tunicamycin (Sigma, cat. no. T7765). Cells were analyzed via western blotting, XBP-1 splicing assay, or flow cytometry.

ESC Cultures

Mouse ESC lines were maintained in ESC Media: DMEM containing high glucose, 15% fetal bovine serum (JR Scientific, cat. no. 43603), 1% L-glutamine (UCSF Cell Culture Facility, cat. no. CCFGB002), 1% non-essential amino acids (UCSF Cell Culture Facility, cat. no. CCFGA001), 1% penicillin-streptomycin (UCSF Cell Culture Facility, cat. no. CCFGK003), 57.2 uM B-mercaptoethanol (Sigma-Aldrich, cat. no. M7522), and LIF (gift from the Blelloch and Goga labs). Cells were maintained on irradiated mouse embryonic fibroblasts (MEFs). For differentiation experiments, ESCs were first preplated away from MEFs in MEF Knockout Media: ESC Media containing 15% Knockout serum (Invitrogen, cat. no.10828-028) and no LIF. ESCs were then plated onto gelatinized (Gelatin from porcine skin, Sigma cat. no. G2500) coated dishes and treated with 1uM retinoic acid (RA) (Sigma, cat. no. R2625-50MG) in ESC media with LIF for two to eight days before conducting experiments.

Embryoid Body Formation

ESCs were preplated from MEFs as above and plated on 6-cm gelatinized dishes for three to four days until colonies formed. Embryoid bodies (EBs) were generated by incubation with 0.05% trypsin/EDTA and gently rocking until colonies were removed. For differentiation into motor neurons, colonies were transferred to 10-cm petri dishes in DM1 media and incubated with 2uM RA and 1uM Sonic hedgehog (R&D systems, cat. no. 461-SH) for 1-2 weeks until GFP expression was visible under the fluorescence microscope.

ESC Derived Motor Neuron Cultures

EBs were collected and spun down at 1500 rpm, 5 minutes and washed once with 1X PBS. EBs were then incubated with 20 units of papain (Sigma, cat. no. P4762) and 1000 units DNAseI (Roche, cat. no. 04 716 728 001) in Hank's balanced salt solution (UCSF Cell Culture Facility, cat. no. CCFAJ003) for 15-30 minutes at 37 degrees until colonies were dissociated. Cells were then centrifuged 5 min, 1100 rpm and washed once with 1X PBS. Cells were counted and plated at 50,000 cells/well in an 8-chamber slide or 100,000 cells/well in a 24-well plate pre-coated with 1.5ug/mL poly-L-ornithine (Sigma, cat. no. P3655) and 3ug/mL laminin (Sigma, L2020). Cells were maintained in motor neuron media: Neurobasal media (Invitrogen, cat. no. 21103-049), 2% horse serum (UCSF Cell Culture Facility, cat. no. CCFAW001), 2% B27 supplement (Invitrogen, cat. no. 17504-044), 25 uM B-mercaptoethanol, 500 uM glutamine, and 10ng/ml each of recombinant rat CNTF (R&D Systems, cat. no. 557-NT), recombinant rat GDNF (R&D Systems, cat. no. 512-GF), recombinant human

BDNF (R&D systems, cat. no. 248-BD), and recombinant human NT-3 (R&D systems, cat. no. 267-N3).

Immunofluorescence Assay

Cells were plated in 8-well chamber slides (Lab-Tek, cat. no. 154534) the day before the assay. Media was aspirated and cells fixed with 400ul 4% paraformaldehyde for 15 minutes at room temperature. Cells were then permeabilized with cold 0.5% Triton X-100 in 1XPBS for 10 minutes on ice. Slides were washed with 1XPBS-Glycine three times, 10 minutes each then incubated in 200ul blocking buffer (1X IF buffer plus 10% goat serum) for 30 minutes at room temperature. Cells were then incubated in 1:100 of goat anti-choline acetyltransferase antibody (Chemicon, cat. no. AB144P-200UL) or mouse anti-islet1 antibody (Developmental Studies Hybridoma Bank, cat. no. 40.2D6) diluted in blocking buffer overnight at 4 ºC. The next day, slides were washed with 1X IF buffer three times, 20 minutes each and then incubated with the appropriate antibody [goat anti-mouse-Alexa568 (Invitrogen, cat. no. A11004) at 1:200 or secondary anti-goat-Rhodamine at 1:100 (gift from M. Krummel)] diluted in blocking buffer for 30 minutes at room temperature. Slides were kept in the dark from this point forward. Cells were rinsed with 1X IF buffer two times, 10 minutes each and incubated with DAPI (Invitrogen, cat. no. D1306) diluted 1:10,000 in water for 15 minutes. Cells were washed quickly with water and maintained in 1X PBS for visualization under scope.

Generating Lentivirus Expressing SOD1

$SOD1^{WT}$ and $SOD1^{G93A}$ were amplified from pcDNA3-$SOD1^{WT}$ and pcDNA3-$SOD1^{G93A}$ with KpnI sites to allow insertion into pECFP-N1. $SOD1^{WT}$-CFP and $SOD1^{G93A}$-CFP were amplified from pECFP-N1- $SOD1^{WT}$ and pECFP-N1-$SOD1^{G93A}$ with NheI and EcoRI sites to allow insertion into pLentilox (gift from M. McManus). The resulting pLentilox- $SOD1^{WT}$-CFP and pLentilox- $SOD1^{G93A}$-CFP were transfected into 293T cells at 3ug each with 1ug each of pMDL, pRSV, and pVSV-G (gift from J. Debnath) with Fugene. Virus was harvested three to seven days post-transfection and spun down at 1100 rpm, 5 minutes to get rid of cell debris. Virus was aliquoted and stored at -80.

Generation of $Bax^{-/-}Bak^{-/-}$ (DKO) ESCs

ESCs were obtained from timed matings of $Bax^{f/f}$ $Bak^{-/-}$ animals. The $Bax^{f/f}$ mice were derived from injection of RW4 ES cells (strain 129/SvJ) into C57BL/6J recipients and the resulting chimera were bred to C57BL/6J for germline transmission(17). $Bax^{f/f}$ mice were backcrossed to C57BL/6J background for 6 generations prior to these experiments. $Bak^{-/-}$ mice were backcrossed to C57BL/6J background for 6 generations before breeding to $Bax^{f/f}$ mice. Blastocysts were harvested at embryonic day 3.5, plated on irradiated MEFs, trypsinized and allowed to grow until confluency. Three independent lines were obtained and transfected with pCre-pac (gift from the Blelloch lab). Cells were replated onto irradiated DR-4 MEFs (2) and selected with puromycin at 2ug/ml for 1 week until visible colonies formed. Six independent;$Bax^{-/-}Bak^{-/-}$ lines were

screened for BAX mRNA and protein levels, and genotyped for Cre-recombinase: Cre-TD 5': ctgcattaccggtcgatgcaa; Cre-TD 3': tgctaaccagcgttttcgttctgcc (PCR product size is approximately 550bp). One clone was carried on for further analysis.

Flow Cytometry and antibodies

For ESC studies, cells were harvested, washed in 1XPBS twice, and resuspended at 100,000 cells per tube. Samples were incubated with antibody to SSEA-1 (Developmental Studies Hybridoma Bank, MC-480 concentrate) at 1:50 in SSEA-1 staining media (HBSS-Ca and Mg free w/o phenol red (UCSF CCF CCFAJ005) plus 2% FBS) for 30 minutes on ice, followed by incubation with goat secondary anti-mouse AlexaDy633 (Thermo Scientific, cat. no. 35513) at 1:100 for 30 minutes on ice.. Cells were washed in 1XPBS and incubated in Annexin-V FITC (Biovision, cat. no. 1001-200) at 1:1000 and propidium iodide (PI) (Biovision, cat. no. 1056-1) at 250ug/ml in Annexin-V staining buffer (90mL HEPES (pH=7.4), 73.62g NaCl, 2.5g CaCl2. Dilute to 1X with water) for 5 min. at room temperature.

MEFs and NSC34 cells were incubated in Annexin-V or PI as described above.

Flow cytometry was run on a FACS Calibur and at least 10,000 events were collected. Data was analyzed using FloJo software or BD FACSDiva. Experiments were done in triplicate.

Alkaline Phosphatase Staining

Untreated or RA treated embyronic stem cells were plated onto irradiated MEFs in a 6-well plate at 2000 cells/well in triplicate and allowed to grow for a week or until visible colonies formed. Cells were washed with 1xPBS and fixed in 4% paraformaldehyde for 10 minutes. Cells were then washed with 1xPBS twice and stained for alkaline phosphatase using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories, cat. no. SK-5100).

For ABT-737 (Santa Cruz Biotechnology, cat. no. sc-207242) treatment, cells were treated with ABT-737 at 2uM in ESC media plus or minus LIF for two to eight days. Cells were plated back onto irradiated MEFs as above to stain colonies for alkaline phosphatase.

Teratoma formation

Parental ($Bax^{f/f}$ $Bak^{-/-}$) control ($Bax^{+/+}Bak^{+/+}$), DKO ($Bax^{-/-}Bak^{-/-}$) ESCs, and Caspase-$3^{-/-}$ ESCs were injected intradermally into nude mice at 1 million cells/mL on each side. Tumors that eventually formed three to seven weeks later were isolated and stained with haematoxylin and eosin.

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