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Research article

Immortalization of mouse myogenic cells can occur without loss of p16^{INK4a}, p19^{ARF}, or p53 and is accelerated by inactivation of Bax

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Abstract

Background: Upon serial passaging of mouse skeletal muscle cells, a small number of cells will spontaneously develop the ability to proliferate indefinitely while retaining the ability to differentiate into multinucleate myotubes. Possible gene changes that could underlie myogenic cell immortalization and their possible effects on myogenesis had not been examined.

Results: We found that immortalization occurred earlier and more frequently when the myogenic cells lacked the pro-apoptotic protein Bax. Furthermore, myogenesis was altered by Bax inactivation as Bax-null cells produced muscle colonies with more nuclei than wild-type cells, though a lower percentage of the Bax-null nuclei were incorporated into multinucleate myotubes. In vivo, both the fast and slow myofibers in Bax-null muscles had smaller cross-sectional areas than those in wild-type muscles. After immortalization, both Bax-null and Bax-positive myogenic cells expressed desmin, retained the capacity to form multinucleate myotubes, expressed p19^{ARF} protein, and retained p53 functions. Expression of p16^{INK4a}, however, was found in only about half of the immortalized myogenic cell lines.

Conclusions: Mouse myogenic cells can undergo spontaneous immortalization via a mechanism that can include, but does not require, loss of p16^{INK4a}, and also does not require inactivation of p19^{ARF} or p53. Furthermore, loss of Bax, which appears to be a downstream effector of p53, accelerates immortalization of myogenic cells and alters myogenesis.

Background

Under cell culture conditions in which almost all primary mouse cells cease proliferation after 10 – 30 population doublings in serial subcultures, a small number of cells escape this proliferation block, spontaneously immortalize, and continue to proliferate indefinitely. Many types of mouse cells undergo this spontaneous immortalization, including fibroblasts obtained from mouse embryos and

myogenic cells obtained from skeletal muscles [1-3]. In the case of myogenic cells, immortalization does not impair the ability of the cells to respond to differentiation signals by ceasing to proliferate and fusing to form multinucleate myotubes [1,3]. Such immortalized myogenic cell lines have been very valuable in studies of myogenesis, but the molecular alterations underlying myogenic cell immortalization have not been examined. In this study, we examine the mechanisms of myogenic cell immortalization and differentiation with a focus on apoptosis regulators. We focused on apoptosis regulators because we have found that myogenic cells express several members of the Bcl-2 family of apoptosis regulators and that Bcl-2 is required for normal fast myofiber development [4,5]. Furthermore, muscle cell apoptosis is found in diseased and injured muscle [6,7]. Finally, inactivation of apoptosis pathways, including inactivation of members of the p53 and pRb pathways, is one possible route to cell immortalization [8].

The molecular and cellular mechanisms which allow some types of mouse cells to circumvent proliferation limits in vitro have begun to be identified. For example, improved culture conditions allow some types of rodent cells to circumvent this usual replication limit and continue to proliferate indefinitely [9-11]. Inadequate culture conditions may produce stress-related changes in rodent cells that rapidly lead to cessation of growth, a mechanism distinct from the cessation of growth due to telomere shortening seen with cultured human cells [8,12,13].

Even under culture conditions that do not support long-term growth of most cells, however, immortalization of mouse cells can occur upon inactivation of one or more cell cycle regulators including p19^{ARF}, p53, or the Cyclin D regulator p16^{INK4a}. For example, mouse embryo fibroblasts and pre-B cells escape proliferation limits and grow indefinitely upon inactivation of either p19^{ARF} or p53 [2,14-16]. Some cell types, such as mouse bone marrow macrophages, immortalize upon inactivation of p16^{INK4a} rather than p19^{ARF} or p53 [15]. Inactivation of p16^{INK4a} also accelerates immortalization of mouse embryo fibroblasts [17]. On the other hand, mouse cells that are deficient in DNA repair due to mutation of ATM, Brca2, Ku80, XPG, or Ligase IV cease replicating even sooner than wild-type cells in culture [8]. These results have raised the possibility that spontaneous immortalization of rodent cells under inadequate culture conditions may require inactivation of either the p16^{INK4a}-regulated Cyclin D/Rb pathway or the p19^{ARF}/p53 pathway that responds to DNA damage by inducing apoptosis [8].

Though immortalized myogenic cells will proliferate indefinitely under high serum conditions or at low density, these cells retain the ability of non-immortalized

myoblasts to respond to low serum or high density by ceasing proliferation and fusing to form multinucleate myotubes in which the myonuclei are post-mitotic. Thus, myogenic cell immortalization must occur via molecular alterations that promote continued proliferation without impairing differentiation. With respect to the p53 and pRb pathways, myogenesis is known to proceed normally in p53-null mice in vivo and with a moderately lowered fusion index in p53-null myoblasts in vitro [18,19], whereas myogenesis is highly impaired in pRb-null mice in vivo and by pRb-null myoblasts in vitro [20,21]. Though loss of p53 pathway function thus appears compatible with immortalization of myogenic cells, at least one mouse myogenic cell line, C$_2$C$_{12}$, has previously been shown to have normal p53 function [22].

Several lines of evidence suggest that the response of rodent cells to culture conditions may be under control of apoptosis regulators that are downstream of p19^{ARF}/p53, particularly the pro-apoptotic protein Bax. First, cells that lose the capacity to proliferate in culture often undergo apoptosis, a process that is regulated in many cases by members of the Bcl-2 family, including Bax which promotes apoptosis [8,23]. Second, Bax appears to act downstream of p53 in the apoptotic pathway. For example, p53 and Bax undergo nuclear translocation upon chemotherapy-induced apoptosis in human melanoma cells [24]; and inactivation of p53 prevents Bax translocation upon apoptosis induction in embryonic neurons [25]. Studies of tumorigenesis in double knock-out mice also suggest that Bax acts downstream of p53 [26], and inactivation of Bax alters the tumor type and number, though not age of tumor appearance, in p19^{ARF}-null mice [27].

In this study, we found that, unlike mouse embryo fibroblasts, mouse myogenic cells undergo spontaneous immortalization via a mechanism that does not require inactivation of p19^{ARF} or loss of p53 functions. In some, but not all cases, immortalization of muscle lines was accompanied by loss of p16^{INK4a}. Furthermore, we identified a new mechanism, inactivation of Bax, that accelerates the immortalization of mouse myogenic cells. We also found that Bax functions in myogenesis, because Bax-null myogenic cells produced relatively more mononucleate cells and fewer multinucleate myotubes than wild-type myogenic cells in culture, and the myofibers in Bax-null muscles in vivo were smaller than those in wild-type muscles. Thus, we find that mouse postnatal myogenic cells can spontaneously immortalize by a different mechanism than mouse embryo fibroblasts; that inactivation of Bax accelerates immortalization of myogenic cells, and that Bax functions in regulation of myogenesis.

Results

During derivation of spontaneously immortalized mouse myogenic cell lines, we found that myogenic cells from homozygous Bax-null mice immortalized more rapidly in culture than myogenic cells from wild-type or heterozygous Bax (+/-) mice, though not as rapidly as myogenic cells from p53-null mice (Fig. 1). In multiple independent experiments, skeletal muscles were obtained from homozygous-null Bax (-/-), heterozygous Bax (+/-), and wild-type Bax (+/+) littermates at 4 – 6 weeks after birth. The muscles were individually dissociated to produce single cell suspensions, and primary cultures of the Bax (-/-), Bax (+/-), and Bax (+/+) muscle cells were established and serially passaged as described in Materials and Methods. For comparison, a serial culture was also prepared using muscle cells from similarly aged p53 (-/-) mice, because previous work showed that mouse embryo fibroblasts are immortalized upon inactivation of p53 [14].

In Figure 1, we show results from three separate experiments in which we started four independent cultures of Bax-null myogenic cells. Each of these cultures produced immortalized Bax-null cells at no later than 30 days after the beginning of the experiment (Fig. 1, Expts. 1 – 3). In contrast, only one of the three wild-type, and neither of the two heterozygote, cultures produced immortalized cells; and the one line of immortalized wild-type cells that did arise was not produced until ~40 days after the beginning of the experiment (Fig. 1, Expt. 3). The Bax-null, heterozygous, and wild-type myogenic cells all showed an early period of rapid growth lasting about ten days (3 – 4 passages), which was followed by a plateau period of slower or no growth in the wild-type and heterozygous cultures. In Bax-null cultures, the early period of fast growth was followed by either a similar, though shorter, plateau period of slower or no growth (Fig. 1; Expts. 2 and 3) or by little change in growth rate (Fig. 1; Expt. 1). We found immortalized cells by day 50 of culture in 7 of 7 Bax-null cultures, but in only 3 of 9 Bax-positive cultures which included 1 of 4 Bax (+/-) cultures and 2 of 5 Bax (+/+) cultures (P = 0.011 by Fisher's exact test comparing Bax-null to Bax-positive results). As noted before [4], loss of Bax is not required for immortalization, because spontaneously immortalized wild-type and Bax (+/-) heterozygous myogenic cells, as well as NIH3T3 cells, retained Bax protein expression (Fig. 5 and not shown).

Because p53-null mouse embryo fibroblasts immortalize very efficiently [14], we also examined proliferation of p53-null myogenic cells. The p53-null myogenic cells, similar to the p53-null mouse embryonic fibroblasts [14], showed rapid growth without the period of slower growth seen in the Bax-null and wild-type cells (Fig. 1, Expt. 3). The proliferation rate of the immortalized myogenic cells appeared to be independent of genotype, because the immortalized Bax-null, p53-null, and wild-type cells all had a proliferation rate at 60 days of culture of approximately one doubling per day.

Immortalized Bax-null, p53-null, and wild-type muscle cells taken from rapidly growing, late passage cultures grew in clonal cultures at high efficiency, with >50% of single cells forming colonies after cloning. Starting with muscle-derived cells from long-term (>20 passages) cultures, we used two rounds of cloning to obtain multiple independent cell lines of different genotypes (Bax-null, p53-null, wild-type). These lines of immortalized cells were further characterized and compared to cells in primary, non-clonal cultures at early passage (<4 passages).

Immortalized and cloned Bax-null cells retained many molecular and functional properties of satellite cell myoblasts. In particular, when proliferating at low density, the immortalized Bax-null cells expressed desmin and had the typical bipolar morphology of myoblasts (not shown). In the clonal lines, all of the cells expressed desmin, whereas some cells in the primary cultures, which were not cloned and thus contained a mixture of myogenic and non-myogenic cells, did not express desmin. Upon reaching confluence, Bax-null cells of the clonal lines fused and formed myosin-expressing, multinucleate myotubes (not shown). The clonal lines derived from p53-null and wild-type cells similarly showed desmin expression and the ability to form multinucleate myotubes. Multinucleate myotubes formed by Bax-null, wild-type, and p53-null cells appeared to have identical morphologies.

The immortalized and cloned Bax-null myogenic cells failed to grow in soft agar, suggesting that they were not tumorigenic, and they did not express SA-β-Gal, suggesting that they were not senescent and/or "stressed" [8]. In addition, the immortalized and cloned Bax-null cells did not express either Sca-1 or Bcl-2, two proteins that appear to mark muscle-derived cells that are at an early stage of myogenesis or have stem cell-like properties [4,5,28-32]. In contrast, a small proportion (~1–5%) of the desmin-positive cells in uncloned primary cultures did express the Sca-1 and Bcl-2 proteins (not shown), just as in previous work [4,5].

Though both non-immortalized (early passage) and immortalized Bax-null cells were capable of forming multinucleate myotubes in culture, the percentage of nuclei in multinucleate cells (the fusion index) was lower in Bax-null than in wild-type clonal cultures (Table 1 and not shown). A similar reduction in fusion index was seen in differentiated cultures of p53-null myoblasts (not shown), a result also seen in an earlier study [19]. When grown at high density and allowed to form multinucleate cells upon reaching confluence in growth medium, we
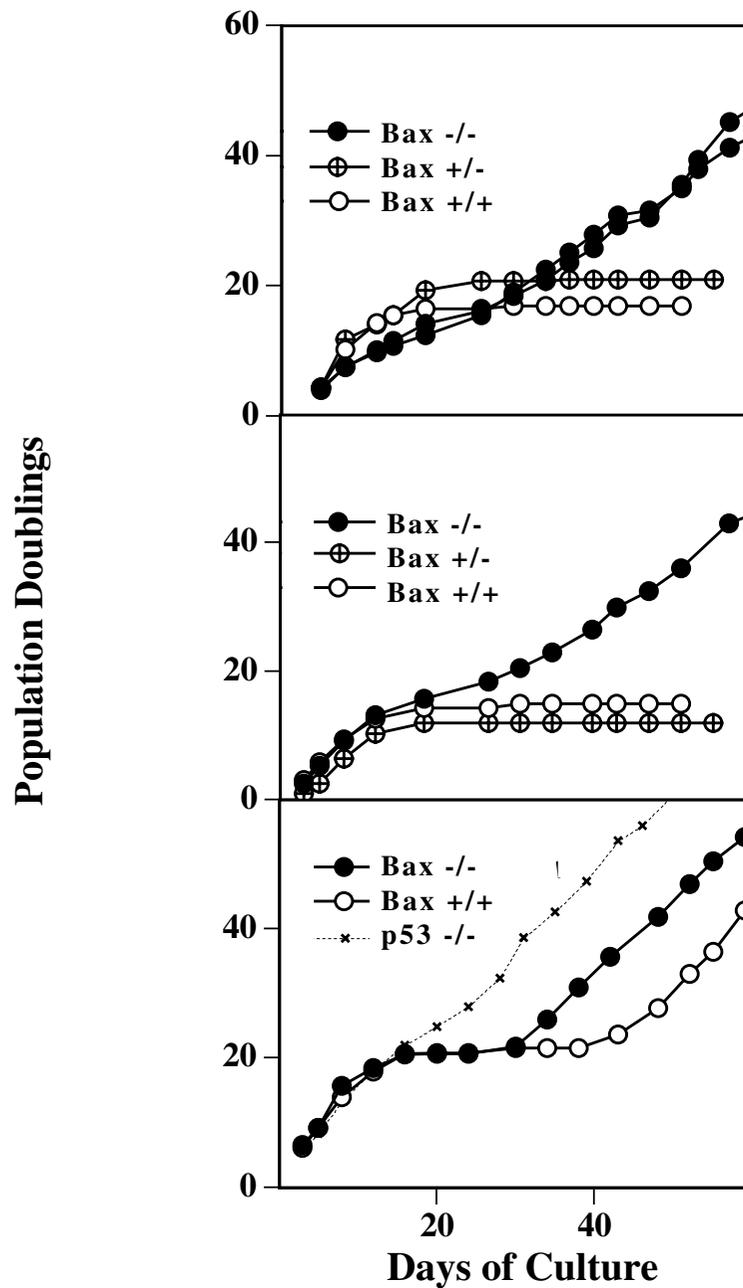
Figure 1
Cells derived from Bax-null muscles immortalized more rapidly than cells from Bax-positive muscles. Primary cultures of Bax (-/-); Bax (+/-); Bax (+/+); and p53 (-/-) myogenic cells were established, serially passaged, and enumerated at each passage as described in Materials and Methods. Results of three independent experiments are shown. As described in the text, myogenic cells from Bax-null muscles (closed circles) immortalized more rapidly and more frequently than myogenic cells from either Bax (+/-) heterozygous muscles (crossed circles) or Bax (+/+) wild-type muscles (open circles). However, myogenic cells from p53 (-/-) muscles (stars with dotted line in Expt. 3) immortalized faster than Bax-null cells without showing the temporary plateau in growth rate shown by Bax-null cells. Each symbol denotes a time at which cells were collected and passaged onto new culture dishes as described in Materials and Methods.

Table 1: Effect of *Bax* genotype on properties of muscle colonies formed by the progeny of individual myogenic cells *in vitro*.

Property	Bax-positive ave ± SE (n)	Bax-null ave ± SE (n)	Significance*
Number of nuclei per colony	428 ± 17 (341)	486 ± 22 (303)	P < 0.05
Number of mononucleate cells per colony	131 ± 7.0 (341)	191 ± 8.6 (306)	P < 0.0001
Percentage of nuclei in multinucleate cells	65.1 ± 0.9 (341)	55.5 ± 1.1 (306)	P < 0.0001
Number of nuclei per multinucleate cell	3.9 ± 0.1 (341)	3.9 ± 0.1 (305)	P > 0.5

*Unpaired, two-tailed, t-test.

saw the same lower fusion index for Bax-null than for Bax-positive cells as when the cells were grown at clonal density (not shown).

This difference in fusion index appeared to be due to a higher number of mononucleate cells, rather than a lower number of nuclei in myotubes, in the Bax-null cultures. For example, muscle colonies formed by the progeny of individual Bax-null cells contained both significantly more total nuclei and significantly more mononucleate cells than those formed by Bax-positive cells (Table 1). In contrast, neither the average number of nuclei per multinucleate myotube (Table 1) nor the average number of myotubes per colony (not shown) differed between Bax-positive and Bax-null colonies.

To determine if Bax functions to regulate apoptosis in myogenic cells, as it does in other cell types, we compared the response of Bax-null and Bax-positive myogenic cells to three treatments that can induce apoptosis: exposure to adriamycin (doxorubicin), staurosporine, or serum-free medium [11,32]. Bax-null myogenic cells were significantly more resistant than Bax-positive cells to cell death induced both by adriamycin or staurosporine (Fig. 2A) and by serum-free medium (Fig. 2B). The active, cleaved form of caspase-3, which was undetectable by immunoblot in untreated cultures, appeared more slowly in staurosporine-treated Bax-null cultures than in similarly treated wild-type cultures (not shown). Additional assays based on appearance of pyknotic nuclei, propidium iodide permeability, and annexin V externalization also showed that Bax-null cells were more resistant to induced cell death.

To test the possibility that Bax inactivation might affect muscle development *in vivo*, we examined muscle fiber size, density, and fiber type in soleus muscles obtained from Bax-null and wild-type littermates at seven weeks of age. Muscles were examined from three pairs of age- and gender-matched, Bax-null and Bax-positive littermates. Each pair gave similar results and the aggregated results are presented in Table 2. The average cross-sectional areas of both fast and slow myofibers were significantly smaller in Bax-null than in wild-type soleus muscles. The fast and slow fibers in the diaphragm were also smaller in Bax-null

than in wild-type mice. Furthermore, the density (number per mm²) of both fast and slow myofibers was significantly higher in Bax-null than in Bax-positive soleus muscles. In contrast, the percentages of fast and slow myofibers were the same in Bax-null and wild-type soleus muscles.

To examine possible mechanisms of muscle cell immortalization, we analyzed expression and function of three proteins – p53, p19^{ARF}, and p16^{INK4a} – that when inactivated lead to immortalization of mouse cells in non-myogenic lineages. To eliminate the possibility that spontaneous immortalization of myogenic cells was due to loss of p53 function, we used three types of experiments, all of which suggested that normal p53 function is retained after immortalization of both wild-type and Bax-null myogenic cells. First, we used karyotyping to show that immortalized Bax-null myogenic cells did not show the chromosome instability that is seen when p53 function is lost. Previous work had shown that immortalized p53-null mouse embryo fibroblasts have about twice as many chromosomes as p53-positive cells [14]. Multiply passaged (>20 passages), immortalized wild-type and Bax-null myogenic cells had the normal number of chromosomes, whereas similarly passaged and immortalized p53-null myogenic cells had about twice as many chromosomes as the Bax-null and wild-type myogenic cells (Fig. 3). Second, in cultures at the time that multinucleate myotube formation was beginning, we found that a promoter fragment that requires p53 for activation was expressed in immortalized Bax-null and wild-type, but not in p53-null, myogenic cells (Fig. 4A,4B,4C,4D,4E,4F). In contrast, a control promoter that did not require p53 function for activation was equally well expressed in Bax-null, p53-null, and wild-type cells (Fig. 4A,4B,4C,4D,4E,4F). Previous work with the C$_2$C$_{12}$ line of myogenic cells, which are Bax-positive [4], had shown that p53 activity and activation of this promoter fragment are both upregulated as fusion of myoblasts to form multinucleate myotubes begins in culture [33]. Finally, we found that in both Bax-null and Bax-positive immortalized myogenic cells p53 protein levels were markedly upregulated upon actinomycin treatment, which is the normal response of p53 to this DNA-damaging drug (Fig.
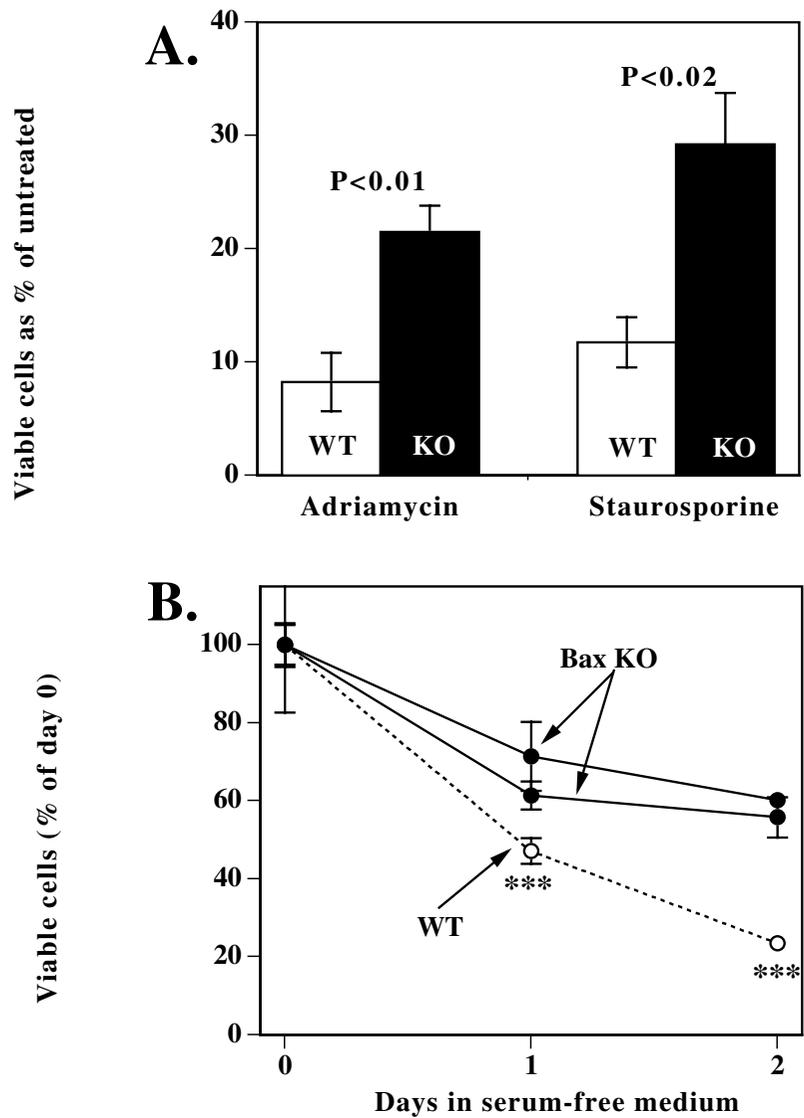
Figure 2
Bax-null myogenic cells were more resistant than wild-type myogenic cells to cell death induced by adriamycin, staurosporine, or serum-free medium. **A.** Myogenic cells from Bax-null (KO, filled bars) and wild-type (WT, open bars) were grown in culture until near confluence and then exposed to either 1.7 μM adriamycin, 50 nM staurosporine, or left untreated as a control. After 24 h of treatment, the number of viable cells in each culture was determined and expressed as a percentage of the number of viable cells in the untreated cultures. After either adriamycin or staurosporine treatment, signficantly more viable cells remained in the Bax-null than in the wild-type cultures. Error bars equal SE with n = 4. P values of significance from unpaired, two-tailed, t-test. This experiment was repeated an additional two times with similar results. **B.** Myogenic cells from two independent, immortalized Bax-null lines (Bax KO, filled circles) and one immortalized wild-type line (WT, open circles) were grown in culture until near confluence and then switched to serum-free medium. After 1 day and 2 days of treatment, the number of viable cells in each culture was determined and expressed as a percentage of the number of viable cells on day 0 when the medium switch was made. After both one and two days in serum-free medium, significantly more viable cells remained in the each of the Bax-null cultures than in the wild-type culture (P < 0.001 by Welch alternate T-test as indicated by ***). In constrast, the two Bax KO experiments did not differ significantly from each other. Error bars equal SE with n = 12 to 24.

Table 2: Effect of *Bax* genotype on properties of myofibers *in vivo*.

Property	Muscle	Bax-positive ave ± SE (n)	Bax-null ave ± SE (n)	Significance*
Cross-sectional area (μm^2) of fast‡ myofibers	Soleus	1045 ± 17.0 (451)	785 ± 11.9 (500)	P < 0.0001
	Diaphragm	873 ± 7.7 (1364)	717 ± 6.9 (1570)	P < 0.0001
Cross-sectional area (μm^2) of slow‡ myofibers	Soleus	1399 ± 33.7 (193)	1096 ± 16.6 (270)	P < 0.0001
	Diaphragm	640 ± 15.0 (136)	547 ± 12.8 (150)	P < 0.0001
Percentage of fast myofibers	Soleus	70.2 ± 1.8 (3)	70.8 ± 0.1 (3)	P > 0.7
	Diaphragm	90.7 ± 0.7 (5)	91.2 ± 0.8 (5)	P > 0.7
Cross-sectional area (mm^2) of whole muscle	Soleus	1.54 ± 0.12 (4)	1.18 ± 0.06 (5)	P = 0.024
Total number of myofibers	Soleus	1081 ± 36 (4)	1102 ± 17 (5)	P > 0.5

‡Fast and slow myofibers were identified by ATPase staining as described in Methods. *Comparison of Bax-positive to Bax-null with unpaired, two-tailed, t-test.

4G). Levels of the p53-inducible protein p21^{Cip1} were also raised as expected by actinomycin treatment (not shown).

Thus, immortalized wild-type and Bax-null myogenic cells had normal p53 function as assayed by maintenance of normal karyotype, promoter activation, and induction by actinomycin.

In addition to normal p53 function, immortalized myogenic cells continued to express p19^{ARF} protein (Fig. 5). For p19^{ARF} assays, we examined expression in primary cultures (not immortalized) of myogenic cells obtained from postnatal skeletal muscle, and in cultures of spontaneously immortalized mouse embryonic fibroblasts (NIH3T3), wild-type postnatal myogenic cells, and Bax-null postnatal myogenic cells. Immunoblotting showed that, as expected [2], NIH3T3 cells did not express detectable amounts of p19^{ARF}; however, p19^{ARF} was present in all Bax-null and wild-type myogenic cells, including both early passage primary cells and late passage immortalized cells (Fig. 5). The amount of p19^{ARF} protein appeared to be increased in both Bax-null and wild-type immortalized myogenic cells, a result also seen in late passage, wild-type mouse embryo fibroblasts [34].

In contrast to p53 and p19^{ARF}, which were found in all tested myogenic lines, p16^{INK4a} was deleted in two of the four Bax-null myogenic lines that were examined (Fig. 5). As found previously in mouse embryo fibroblasts [2], we found that p16^{INK4a} protein was undetectable in rapidly growing cultures of early passage (not immortalized) mouse myogenic cells (Fig. 5), but became highly expressed when the cultures reached the slow growing "crisis" stage after 4–5 passages (not shown). After immortalization, we found that p16^{INK4a} was expressed by two independently-derived Bax-null myogenic lines and one Bax-positive line but was not expressed in two additional Bax-null lines (Fig. 5 and not shown). Thus, immortalization was accompanied by lack of p16^{INK4a} expression in some, but not all, myogenic cell lines.

Discussion

We found that mouse myogenic cells can undergo spontaneous immortalization via a mechanism that can include, but does not require, loss of p16^{INK4a} protein expression, and also does not require loss of p19^{ARF} protein or p53 function. Furthermore, we identified a new mechanism, inactivation of Bax, that accelerates the immortalization of mouse myogenic cells. We also found that Bax-null myogenic cells produced relatively more mononucleate cells than wild-type myogenic cells in culture and that the myofibers in Bax-null muscles in vivo were smaller than those in wild-type muscles. We discuss these findings below.

Mouse embryo fibroblasts and some immune cells are highly dependent on either mutations of p53 or deletions of p19^{ARF} to escape replicative limits in culture [2,14-16]. In contrast, we did not find such alterations in immortalized myogenic cells, whether Bax-positive or Bax-null, suggesting that immortalization of postnatal myogenic cells and embryonic fibroblasts can occur by different mechanisms. As shown previously, spontaneously immortalized myogenic cell lines that are derived from wild-type mouse cells typically retain p53 function [19,22,33,35] and Bax protein expression [4]. Here we showed that immortalized Bax-null myogenic cells also retained p53 functions, as well as p19^{ARF} protein. Cell cycle checkpoints required for ceasing mitosis and differentiation were also normal in immortalized myogenic cells, because both Bax-positive and Bax-null cells were able to cease dividing and form multinucleate myotubes that contained post-mitotic myonuclei.

Inactivation of p16^{INK4a}, which occurred in some myogenic cell lines, also leads to immortalization of macrophages [15] and accelerated immortalization of mouse embryo fibroblasts [17]. Inactivation of p16^{INK4a} is also associated with establishment of immortalized cell lines from mouse liver and lung epithelial tissues [36,37]. Though deletion of their overlapping reading frames often
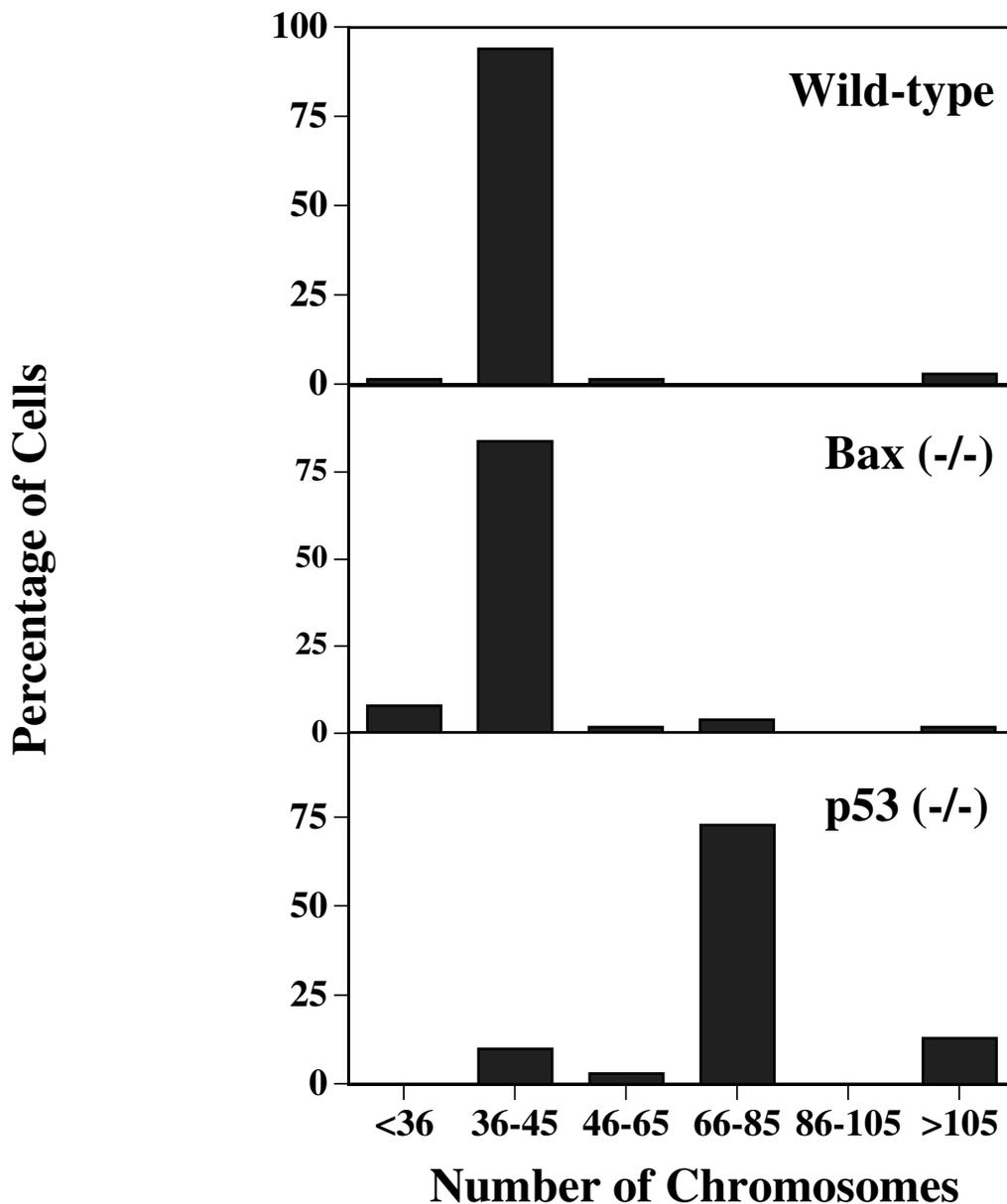
Figure 3
Immortalized Bax-null myogenic cells had normal chromosome numbers. Chromosome numbers of individual cells were determined in (i) primary cultures of two independent, early passage number <4, wild-type myogenic cells (n = 66); (ii) late passage cultures of two independent, immortalized, Bax-null myogenic lines (n = 49); and (iii) a late passage culture of one line of immortalized p53-null myogenic cells (n = 32). (In this and subsequent figures, late passage, immortalized lines were examined at passage numbers 17 – 26 corresponding to >60 days of total culture duration; and early passage, non-immortalized lines were examined at passage numbers < 4 corresponding to less than 12 days of total culture duration and prior to the slow growth period.) Pooled results are shown for the two wild-type and the two Bax-null lines (BaxKO lines #1 and #2, see also Figs. 4 and 5), because, in each case, the two lines had statistically identical chromosome numbers. Both wild-type and Bax-null myogenic cells had normal karyotypes with approximately 40 chromosomes, whereas p53-null myogenic lines had approximately twice the normal number of chromosomes.
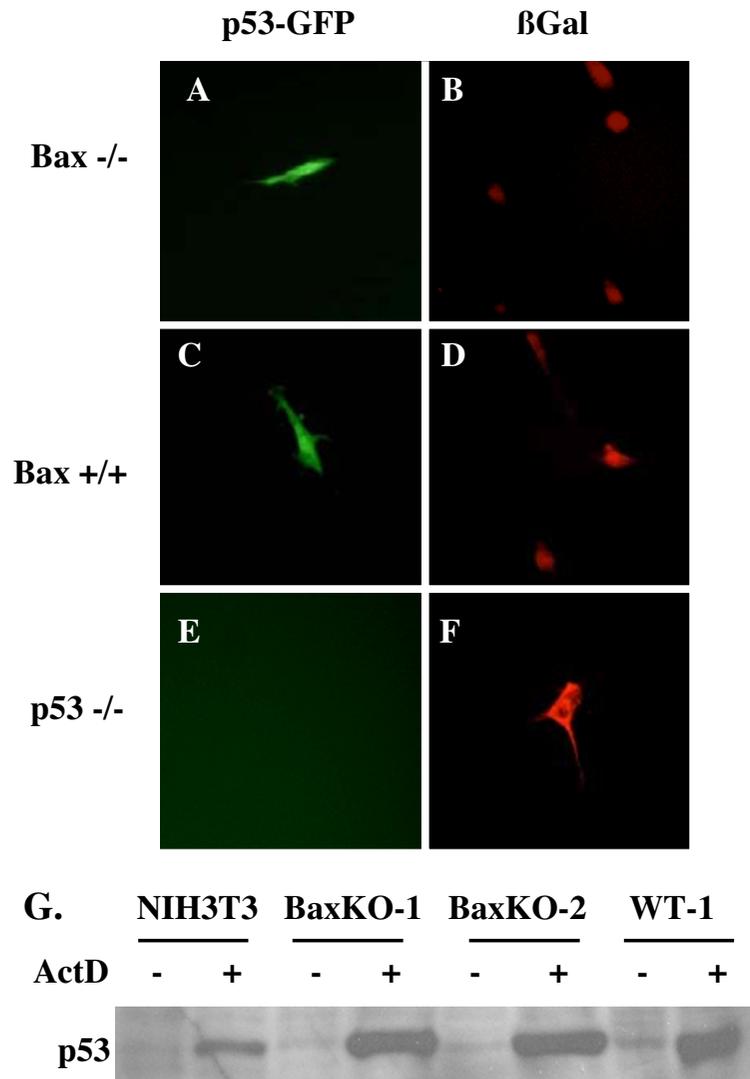
Figure 4
Activation of a p53-dependent promoter and induction by Actinomycin D were normal in immortalized Bax-null myogenic cells. As indicated in panels A-F, wild-type, immortalized Bax-null, and immortalized p53-null myogenic cells were transfected with either PG13-GFP, a plasmid from which GFP expression requires normal p53, or with the control pCMV-β-Gal, a plasmid from which β-Galactosidase is expressed independently of p53. GFP was detected by endogenous green fluorescence, and β-Galactosidase was detected by immunocytochemistry with a specific antibody (red fluorescence). GFP was expressed in wild-type and Bax-null cells, but not in p53-null cells, whereas β-Galactosidase was expressed in all three cell types. Transfection efficiency, measured as percentage of β-Galactosidase-expressing cells, was independent of genotype and ranged from 5.0 – 15.7% in different experiments. Bar = 20 μm. For panel G, cultures of NIH3T3 cells, two immortalized Bax-null lines (BaxKO-1 and BaxKO-2), and one immortalized wild-type line (WT-1) were examined by immunoblotting for p53 expression. Expression of p53 was barely detectable in untreated cultures (ActD-), but was abundant in parallel Actinomycin D-treated cultures. Treated cells were exposed to 60 ng/ml of Actinomycin D for 8 hours and cells were collected for immunoblot analysis 16 hours after the Actinomycin D was removed and replaced with fresh medium.
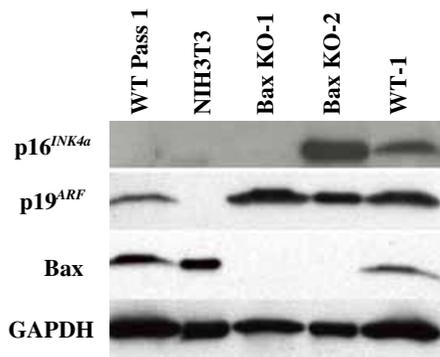
Figure 5
Immortalized myogenic cells expressed p19^{ARF} but sometimes did not express p16^{INK4a}. Immunoblotting was used to examine p16^{INK4a} and p19^{ARF} expression by non-immortalized, rapidly growing, Bax-positive myogenic cells (WT Pass 1); NIH3T3 cells; two independently-derived lines of immortalized Bax-null myogenic cells (BaxKO-1 and BaxKO-2); and one line of immortalized Bax-positive myogenic cells (WT-1). Immunoblotting for Bax was used to verify genotypes, and immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify that similar amounts of protein were analyzed in each lane. As expected [14], NIH3T3 cells (immortalized mouse embryo fibroblasts) did not express either p16^{INK4a} or p19^{ARF}. Early passage, rapidly growing wild-type cells expressed p19^{ARF} but not p16^{INK4a}, though p16^{INK4a} was expressed when cells entered "crisis" (not shown). The p19^{ARF} protein was expressed by all immortalized myogenic cell lines that were examined, whereas the p16^{INK4a} protein was expressed by some (e.g., BaxKO-2), but not all (BaxKO-1) lines. See text for details.

leads to simultaneous inactivation of p16^{INK4a} and p19^{ARF} in cells such as mouse embryo fibroblasts [2], we did not observe such double inactivation in immortalized mouse myogenic cells. Promoter-specific hypermethylation is one mechanism that can inactivate p16^{INK4a} but not p19^{ARF} expression [*e. g.*, [37]]. Our results show that myogenic cell immortalization can sometimes occur without loss of the three proteins – p16^{INK4a}, p19^{ARF}, and p53 – that are typically found to be inactivated upon immortalization of mouse cells of many non-myogenic lineages. Thus, though loss of p16^{INK4a} protein expression is likely to be one of the mechanisms underlying myogenic cell immortalization, further work is needed to identify the additional mechanisms that appear to exist in mouse myogenic cells. Though p16^{INK4a} is an indirect regulator of pRb function, it is unlikely that loss of pRb function itself can underlie myogenic cell immortalization, because loss

of pRb function causes a drastic loss of muscle differentiation capacity [20,21].

How might the absence of Bax increase the speed and frequency of myogenic cell immortalization? A likely explanation is that many potentially immortal Bax-null cells can arise and replicate instead of being destroyed by Bax-dependent cell death. Bax is a pro-apoptotic member of the Bcl-2 family, and inactivation of Bax can render cells more resistant to apoptosis, as shown previously for several cell types [38,39] and here for myogenic cells (Fig. 2). Spontaneous immortalization of myogenic cells under usual (perhaps sub-optimum) culture conditions is a rare event [1]. Primary cultures of myogenic cells, as well as other cell types [11,12,15], typically undergo a "crisis" during which most cells cease replicating, followed by emergence of the progeny of rare immortalized cells (*cf.* Fig. 1, Expt. 3).The mouse C$_2$ myogenic cell line, for example, was initially derived from such rare spontaneously immortalized cells [3]. It may be that many cultured cells which carry potential immortalizing alterations are normally subject to apoptosis. Disabling the apoptosis mechanism, such as by Bax inactivation, would increase the number of such potentially immortal cells that survive. Further work is needed to determine if other mutations that inactivate apoptosis will also increase myogenic cell immortalization in culture.

Bax inactivation appeared to affect the balance between myoblast proliferation, survival, and fusion to form multinucleate myotubes. Differentiated cultures of Bax-null myogenic cells contained relatively more mononucleate cells, but had a lower fusion rate, than parallel wild-type cultures. These differences could also arise due to increased resistance of Bax-null myogenic cells to apoptosis. As muscle cell differentiation begins in culture, some myoblasts typically undergo apoptosis rather than fusing to form myotubes or surviving in a quiescent state [40-42]. If myoblasts normally destined for apoptosis at the beginning of differentiation instead survive but do not fuse when Bax is inactivated, then Bax-null cultures would have more mononucleate cells, and relatively fewer multinucleate myotubes, than wild-type cultures. A role for inhibition of apoptosis in myoblast survival is supported by the finding that apoptosis of myoblasts is prevented by the heat shock protein, alpha B-crystallin, which also inhibits activation of caspase-3 [41].

Though a small percentage of the desmin-positive, myogenic cells in Bax-null cultures expressed Sca-1, this protein was not expressed by immortalized Bax-null myogenic cells. Expression of the cell surface protein Sca-1, at least on some muscle-derived cells, appears to identify rare cells that are at a very early stage of the myogenic pathway and may have stem cell properties

[4,28,30-32,44]. Because none of the immortalized Bax-null myogenic cells expressed Sca-1, it is likely that they were derived by immortalization of Sca-1-negative myoblast precursors.

The smaller myofibers in the Bax-null soleus might have been due to indirect, rather than direct, effects on muscle development. Unlike Bcl-2 inactivation which leads to a specific loss of fast myofibers [4], Bax inactivation affects both fast and slow myofibers equally. Bax (-/-) mice have normal viability, but have abnormalities in reproductive cells, lymphoid hyperplasia, and decreased apoptosis during development [45,46]. No difference in the growth rates of Bax-null and Bax-positive mice has been reported, and skeletal muscles had not previously been examined in Bax-null mice. In skeletal muscles, the size of myofibers is regulated by a number of factors, including motor neuron activity and exercise patterns; protein growth factors such as growth hormone, IGF1, and myostatin; steroid hormones; availability of muscle precursor cells (myoblasts); and food intake [47]. Myofiber size in Bax-null muscles would likely be affected if any of these regulatory factors is altered by Bax-deficiency. As one possibility, for example, excess motor neurons which are eliminated during neuromuscular development in Bax-positive mice survive in Bax-null mice [48], which could perhaps lead to inappropriate nerve activity and decreased myofiber size. Further studies are necessary to determine if Bax inactivation leads to alterations in factors that indirectly regulate myofiber size.

A direct, muscle cell autonomous, effect of Bax inactivation that would produce smaller myofibers is perhaps less likely. Increased resistance to apoptosis in muscle precursor cells would seem to be consistent with the production of more myogenic cells and larger myofibers. If, however, Bax-null myoblasts are less likely to fuse in vivo as in vitro, smaller myofibers could result. In this case, however, one would then expect to see higher numbers of myoblasts in Bax-null muscles, but we did not observe an increased density of nuclei outside of myofibers in Bax-null muscles (not shown). Studies of mice with muscle-specific inactivation of Bax, or with muscle-specific expression of Bax in Bax-null mice could determine whether Bax inactivation affects myofiber size directly or indirectly.

Conclusions

We found that mouse myogenic cells can undergo spontaneous immortalization via a mechanism that can include, but does not require, loss of p16^{INK4a}, and also does not require inactivation of p19^{ARF} or p53. Furthermore, we identified a new mechanism, inactivation of Bax, that accelerates the immortalization of mouse myogenic cells. We also found that Bax-null myogenic cells produced rel-

atively more mononucleate cells and fewer multinucleate myotubes than wild-type myogenic cells in culture and that the myofibers in Bax-null muscles in vivo were smaller than those in wild-type muscles. These results, as well as our earlier work with Bcl-2 [4,5], implicate Bcl-2 family-mediated processes in controlling the proliferation and differentiation of myogenic cells. In addition, this study emphasizes that mouse cells of different lineages take different routes to immortalization.

Methods
Mice and cell culture
Breeding pairs of heterozygous B6.129X1-Bax^{tm1Sjk} mice [45] were obtained from the Jackson Laboratory and mated in our laboratory. Progeny were genotyped [45], and muscle tissue or cells for primary cultures were obtained from tissues of the resulting wild-type, heterozygous, and Bax-null littermates at 4–6 weeks of age. At this age, there was no difference in body weights between Bax-null and Bax-positive mice. To obtain myogenic cells for culture, hindlimb muscles were isolated and treated with pronase to release mononucleate cells as described [4]. To obtain highly enriched myogenic cell populations, each mononucleate muscle cell preparation was fractionated by 2-step (35%, 70% Percoll) gradients, and cells were collected from the 35–70% interface as described [4,49]. Unless otherwise noted, cells were cultured on ECL Matrix (Upstate Biotechnology) in proliferation medium (PM) which consisted of DMEM with 15% horse serum, 4% chicken embryo extract, 2 mM L-glutamine, 10 mM HEPES pH 7.4, 100 U/ml penicillin, and 1 mM pyruvate. Myoblasts were induced to differentiate by transfer to differentiation medium (DM) which consisted of DMEM without chicken embryo extract and with only 2% horse serum.

Cell proliferation and clonal assays
To measure proliferation of cells obtained from adult tissues, myogenic cell cultures were initiated at ~100 cells per cm^2. Growth medium was used at ~0.1 ml per cm^2 and medium was replaced each day with fresh medium. Myogenic cells were passaged and reseeded at the initial density after 3 – 6 days of culture, at which time the cells had not reached confluence and fusion to form multinucleate myotubes had not begun. For all proliferation assays, cells were released from the culture dish by treatment with trypsin, and cell numbers were determined using a hemocytometer to enumerate viable cells that excluded Trypan Blue (GIBCO-Invitrogen). Cell growth was also assessed by counting cells per unit area in culture using an inverted microscope with results equivalent to those obtained with a hemocytometer.

To determine the properties of clonally-derived muscle colonies, cultures of myogenic cells that were initiated at

clonal density (~1–10 cells/cm²) were fixed with paraformaldehyde (see below) after 8 days of growth, immunostained for the muscle-specific intermediate filament protein desmin (typically >80% of the colonies were composed of cells that were desmin-positive), and examined to determine the number of nuclei per colony and the percentage of nuclei contained within multinucleate myotubes (fusion index). Measurements of muscle colonies were carried out by observers unaware of the genotypes of the cells. Clonal cell lines were derived by 2 consecutive rounds of subcloning carried out using limiting dilution. Statistical analysis was done using the InStat program (v. 2.0, Graphpad Software, San Diego CA).

Immunostaining, immunoblotting, and β-Galactosidase assays

Antibodies included rabbit anti-desmin (ICN/Cappel, Costa Mesa CA) used at a dilution of 1:500; rabbit anti-p16^{INK4a} (sc-1207, Santa Cruz Biotechnology, Santa Cruz CA) used at 2 μg/ml, rabbit anti-p19^{ARF} (Novus Biologicals, Littleton CO) used at 0.5 μg/mL, and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used at 2 μg/ml (Research Diagnostics, Flanders NJ). Antibody binding was visualized using a horseradish peroxidase-based detection system (Vectastain Elite kit; Vector Laboratories) with diaminobenzidine substrate. Detection of Sca-1 and Bcl-2 was performed as previously described [4], except only a single mAb (clone E13-161.7; Pharmingen Laboratories, San Diego CA) was used to detect Sca-1.

Protein extraction from cultured cells and immunoblotting was carried out as described [4].

To detect senescence- (or stress-) associated β-galactosidase activity (SA-βGal) [10,11,13], cells were washed in PBS, fixed for five minutes in 4% paraformaldehyde, washed two times with PBS and incubated overnight at 37°C in fresh staining solution consisting of 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal; stored as a 20 mg/ml stock in DMSO) per ml / 40 mM citric acid/ sodium phosphate, pH 6.0 / 5 mM potassium ferrocyanide /5 mM potassium ferricyanide /150 mM NaCl /2 mM MgCl$_2$.

p53, karyotyping, soft agar growth

To analyze p53 transcriptional activity, target cells were transfected with the PG$_{13}$-GFP reporter plasmid from which expression of Green Fluorescent Protein (GFP) is under control of a p53-dependent promoter consisting of thirteen copies of the p53 consensus binding sequence [33]. As a control for transfection efficiency, target cells were simultaneously transfected with a plasmid on which β-galactosidase is under control of a constitutively active fragment of CMV promoter (BD Biosciences, San Diego

CA). FuGENE 6-mediated transfection was carried out using supplier-provided protocols (Roche Applied Sciences, Indianapolis IN), using 2 μg of the PG$_{13}$-GFP reporter plasmid and 0.5 μg of the control plasmid per 35 mm culture dish. At 72 hours after transfection, cultures were switched into differentiation medium, and, after an additional 48 hours, cells were examined for expression of GFP by fluorescence microscopy. Finally, cells were paraformaldehyde-fixed and immunostained for β-galactosidase using a specific primary antibody (Molecular Probes, Eugene OR) and a rhodamine F(ab)-conjugated secondary antibody (ICN/Cappel, Costa Mesa CA).

Induction of p53 by Actinomycin D was determined by treating cells with 60 ng/ml of Actinomycin D for 8 hours. Cells were collected for immunoblot analysis 16 hours after the Actinomycin D had been removed and replaced with fresh medium.

Karyotypes of colcemid-treated and fixed cells were determined essentially as in previous work [14,50], except that chromosomes were not eosin-stained. Chromosomes of 20 – 30 cells of each genotype were counted in metaphase spreads.

Soft agar assays to test for anchorage-independent growth were performed as described [30]. For each assay, 2×10^4 cells were cultured per 6 cm dish in proliferation medium.

Induced cell death assays

Cultures of cloned, immortalized Bax-null and wild-type myogenic cells were grown to near confluence in growth medium, at which time fresh growth medium containing adriamycin (doxorubicin, 1.7 μM), staurosporine (50 nM), or no addition as a control was added. After 24 h, the number of viable cells in each culture was determined as above. Untreated Bax-null and Bax-positive cultures had statistically identical numbers of cells, and the number of cells in the adriamycin- and staurosporine-treated cultures was converted to percentage of the untreated control cell number.

ATPase stain

Sections (10 μm) were prepared [4,51] from frozen soleus muscles dissected from Bax-null and Bax-positive littermates at seven weeks of age. Muscles were examined from three pairs of gender- and age-matched littermates. ATPase staining of muscle sections at pH 10.2 and 4.3 was used to identify fast and slow myofibers [52]. The cross-sectional area of every fast and slow myofiber within a 0.3 mm² area of each soleus was determined by computer-assisted morphometry (NIH Image program, http://rsb.info.nih.gov/nih-image/), with average cross-sectional areas, average density of myofibers, and statistical comparisons determined with the InStat program.

Author's contributions

JN designed and carried out experiments presented in Fig. 1; JN and JM produced the data for Fig. 2; JAD did the experiments for Fig. 3; JM did the experiments for Figs. 4 and 5. Data in Table 1 were from JN and AK, and data in Table 2 were from CAK and MG. JBM conceived and coordinated the study, performed the statistical analyses, and drafted the manuscript.

Acknowledgements

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