ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage

Mirit I. Aladjem*, Benjamin T. Spike*, Luo Wei Rodewald*, Thomas J. Hope†, Martina Klemm‡, Rudolf Jaenisch‡ and Geoffrey M. Wahl*

Background: Embryonic stem (ES) cells can contribute precursors to all adult cell lineages. Consequently, damage to ES cell genomes may cause serious developmental malfunctions. In somatic cells, cell-cycle checkpoints limit DNA damage by preventing DNA replication under conditions that may produce chromosomal aberrations. The tumor suppressor p53 is involved in such checkpoint controls and is also required to avoid a high rate of embryonic malformations. We characterized the cell-cycle and DNA-damage responses of ES cells to elucidate the mechanisms that prevent accumulation or transmission of damaged genomes during development.

Results: ES cells derived from wild-type mice did not undergo cell-cycle arrest in response to DNA damage or nucleotide depletion, although they synthesized abundant quantities of p53. The p53 protein in ES cells was cytoplasmic and translocated inefficiently to the nucleus upon nucleotide depletion. Expression of high levels of active p53 from an adenovirus vector could not trigger cell-cycle arrest. Instead, ES cells that sustained DNA damage underwent p53-independent apoptosis. The antimetabolite-induced p53-dependent arrest response was restored in ES cells upon differentiation.

Conclusions: Cell-cycle regulatory pathways in early embryos differ significantly from those in differentiated somatic cells. In undifferentiated ES cells, p53 checkpoint pathways are compromised by factors that affect the nuclear localization of p53 and by the loss of downstream factors that are necessary to induce cell-cycle arrest. A p53-independent programmed cell death pathway is effectively employed to prevent cells with damaged genomes from contributing to the developing organism. The p53-mediated checkpoint controls become important when differentiation occurs.

Addresses: *Gene Expression Laboratory and †Infectious Disease Laboratory, The Salk Institute, San Diego, California 92037, USA. ‡Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA.

Correspondence: Geoffrey M. Wahl
E-mail: wahl@salk.edu

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Background

Cells are constantly subjected to environmental and metabolic conditions that may cause genetic damage. Ionizing radiation, mutagenic and clastogenic chemicals can induce mutations or large-scale genome alterations. DNA synthesis or cell division under suboptimal growth conditions may also cause chromosomal aberrations [1–4]. In response to these challenges, eukaryotes have developed cell-cycle control and repair mechanisms. Cell-cycle checkpoints are signal transduction pathways that ensure that all of the processes required to execute each phase of the cell cycle are completed before the next phase is initiated [5]. Cells depleted of nucleotides, for example, may arrest to avoid replication under suboptimal conditions [6]. Likewise, cells do not enter mitosis before completing DNA replication [7,8], or if they have unrepaired DNA damage [5,9].

The loss of cell-cycle checkpoints does not affect cell viability under nondisruptive conditions, but increases genetic instability subsequent to the challenge [2,3,5,10–12]. The tumor suppressor p53 is part of a signal transduction pathway that converts signals emanating from DNA damage, ribonucleotide depletion and other stresses into responses ranging from cell-cycle arrest to apoptosis (for reviews, see [11,13,14]). Cells that are deficient in p53 may fail to undergo cell-cycle arrest or apoptosis in response to conditions that either lead to DNA damage [3,15] or perturb the cell cycle [15–22]. These aberrant responses explain why p53-deficient mice have increased rates of tumorigenicity and genomic instability [23–25]. The resistance of p53-deficient cells to DNA-damage-induced apoptosis and their increased rate of genetic variance result in significant selective advantages to such cells during tumor progression. As expected, cells deficient in components of the p53-mediated DNA-damage-response pathway, such as the cyclin-dependent kinase inhibitor p21 or the retinoblastoma protein Rb, also show altered responses to environmental challenges [22,26–28].

Cells originating from different tissues have dramatically different responses to genotoxic challenge. For example, γ-irradiation leads to a prolonged cell-cycle arrest resembling senescence in fibroblasts [29–32], whereas the same challenge leads to apoptosis in thymocytes and intestinal epithelial cells [33–35]. It is striking that genetically identical cells situated adjacent to one another in the same tissue can also have profoundly different responses to DNA damage. For instance, prostate stromal (fibroblast) cells induce p53 and undergo cell-cycle arrest in response to γ–irradiation but adjacent epithelial cells fail to induce p53 or undergo cell-cycle arrest [36]. These data are compatible with observations during embryonic differentiation *in vivo*, demonstrating that various differentiating tissues show considerable heterogeneity in stress-induced transcription from p53-dependent promoters [37]. These differences in induction of p53 and cell-cycle arrest imply that signal processing and response within the DNA-damage-response pathway are regulated in a tissue-specific fashion.

Although several studies have shown that mouse embryo fibroblasts (MEFs) are capable of inducing the p53-dependent cell-cycle arrest in response to diverse stresses [20,22,24,26,38], the behavior of pluripotent undifferentiated embryonic cells towards genotoxic challenge has not been investigated in detail. Because these cells contribute to all adult cell lineages [39], such cells should have a highly sensitive and finely tuned p53-dependent signal transduction pathway to limit the emergence of genetic variants. This prediction is consistent with previous reports describing robust p53 expression during early murine embryogenesis [37,40] and with the modest increase in teratogenicity of benzo[a]pyrene [12] and irradiation [10] in p53-deficient mice. Nevertheless, these studies did not examine whether the conditions that activate p53 in adult tissues have similar effects on undifferentiated embryonic stem (ES) cells.

This report analyzes the consequences of challenging murine ES cells with antimetabolites or with DNA-damaging agents. We show that p53 is expressed in murine ES cells and appears to be present mainly in the cytoplasm. ES cells failed to undergo cell-cycle arrest in response to rNTP depletion, DNA damage, and p53 overexpression, however. Interestingly, ES cells were exquisitely sensitive to DNA damage through the activation of an apoptotic program that was effectively initiated in the absence of p53. These results suggest a p53-independent, DNA-damage-induced response as the front-line strategy to insure genomic stability in ES cells. Finally, we show that an active p53-mediated DNA-damage-response pathway is activated upon differentiation of ES cells.

Results

ES cells do not arrest after challenge with antimetabolites and genotoxic agents

To determine whether ES cells have intact p53-mediated cell-cycle responses to rNTP depletion and DNA damage, we analyzed the effects of the antimetabolite *n*-phosphonacetyl-L-aspartate (PALA), γ-irradiation and adriamycin on the cell cycle [6,15,29]. A baseline assessment of the cell cycle for ES cells was obtained by pulse labeling exponentially growing ES cells with bromodeoxyuridine (BrdU) and analyzing the cells using dual-parameter flow microfluorimetry (FMF). More than 65% of the ES cells were in S phase (that is, BrdU-positive cells with DNA contents ranging from 2N to 4N) after a 10 minute exposure to BrdU. BrdU exposure exceeding 40 minutes resulted in >90% of cells being labeled. By contrast, only 5–12% of isogenic MEFs were in S phase after a 30 minute BrdU pulse, and a substantial number were in G1 and G2/M (Figure 1). These data show that ES cells have very short G1 and G2/M phases and, most probably, oscillate between S phase and mitosis.

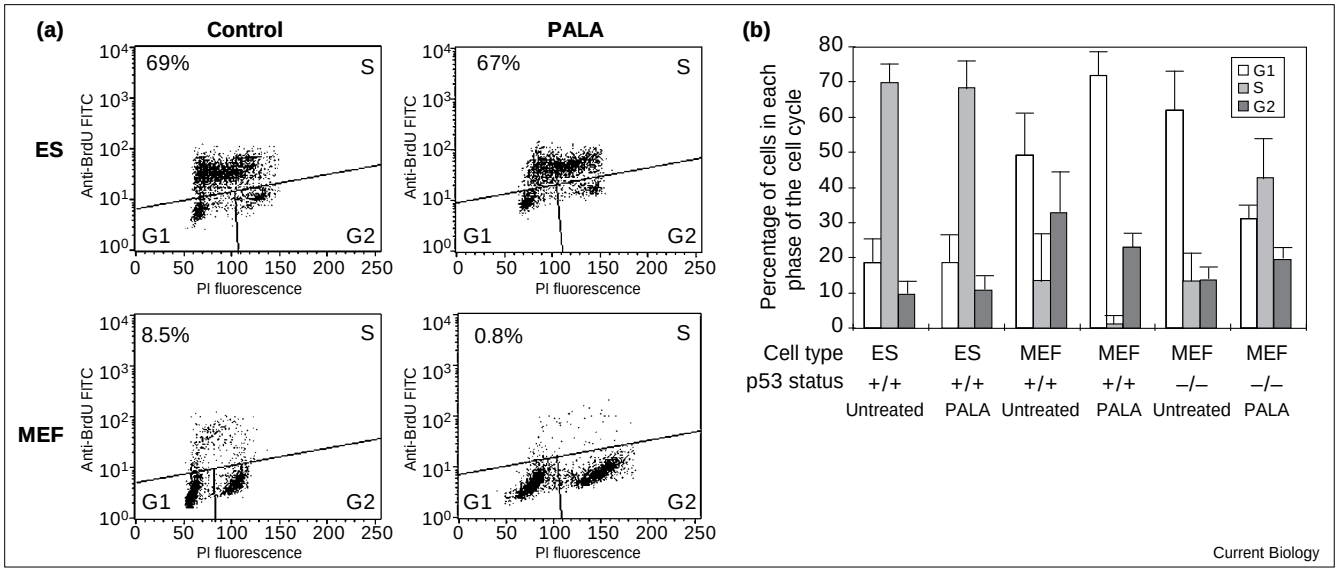
A comparison of the cell-cycle responses of MEFs and ES cells following challenge with PALA is shown in Figure 1. The S-phase fraction of MEFs was markedly reduced after treatment with 0.1 mM PALA, whereas ES cells treated with as much as 1 mM PALA continued to enter and progress through S phase (Figure 1a). The cell-cycle profile of ES cells under PALA treatment was similar to that of PALA-treated p53-deficient MEFs in that both failed to arrest during G1 and entered readily into S phase in the presence of high PALA concentrations (Figure 1b).

In somatic cells, DNA damage results in a rapid decrease in the S-phase population because a p53-dependent mechanism prevents G1 cells with DNA damage from entering S phase [15,29]. As shown in Figure 2, the S-phase fraction in ES cells harvested 16 hours after exposure to 6 Grays (Gy) ionizing radiation or treated with 1 μg/ml adriamycin was similar to the S-phase fraction in exponentially growing control cells, and no reproducible increase was seen in the G1 and/or G2 fractions. These results are similar to the radiation response of p53-deficient MEFs [23,38].

Expression of cell-cycle proteins

The lack of G1 arrest elicited by either rNTP depletion or DNA damage suggests that, despite their normal genotype, ES cells do not have a functional p53-mediated DNA-damage-response pathway. To gain an insight into the mechanisms that prevent cell-cycle arrest in ES cells, we first determined whether p53 is expressed in ES cells, and whether its abundance increases subsequent to appropriate challenges. The activation of p53 should result in transactivation of downstream target genes encoding, for example, $p21^{Cip1/Waf1/Sdi1}$ [41,42] or Mdm2

Figure 1



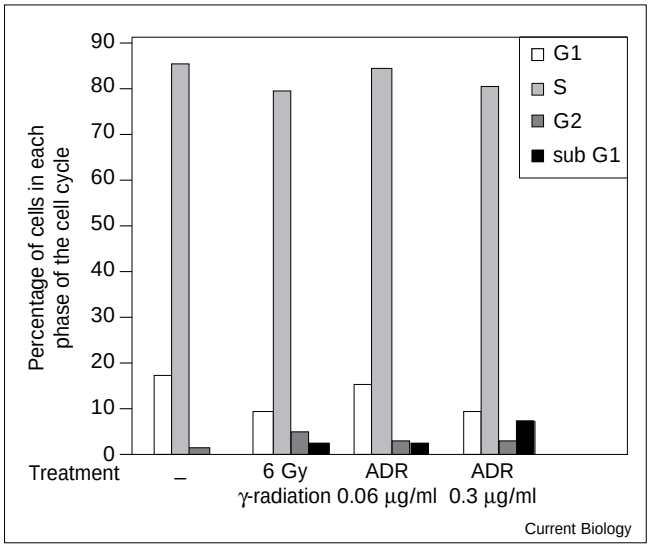
Cell-cycle response of ES cells to rNTP depletion by the antimetabolite PALA. (a) ES cells were exposed for 48 h to 1 mM PALA and MEFs to 0.1 mM PALA. The exposed cells and untreated control cells were labeled with BrdU, harvested and analyzed by FMF. The horizontal axis depicts the DNA content and the vertical axis indicates BrdU incorporation on a logarithmic scale. The percentage of cells in S phase is indicated in the upper left corner of each panel. PI, propidium iodide; FITC, fluorescein isothiocyanate. (b) Summary of cell-cycle analyses (4–10 repetitions) of ES cells, MEFs and $p53^{-/-}$ MEFs exposed to PALA.

[43,44]. To test directly for the function of the p53 pathway, we determined whether Mdm2 and p21 are present in ES cells, and whether their abundance increases subsequent to irradiation.

The p53 and Mdm2 proteins were immunoprecipitated from extracts obtained from exponentially growing, PALA-treated or γ–irradiated ES cells (Figure 3). ES cells contained a protein that is immunoprecipitated by antiserum directed against p53 (IP:p53), but not by a control antiserum that does not recognize p53 (IP:IA). A small increase in p53 levels above control levels was observed in γ-irradiated (Figure 3, lanes 2 and 4), but not in PALA-treated (Figure 3, lane 6) ES cells. These results demonstrate that the p53 protein is expressed in ES cells, but that it is not induced significantly following PALA treatment or irradiation.

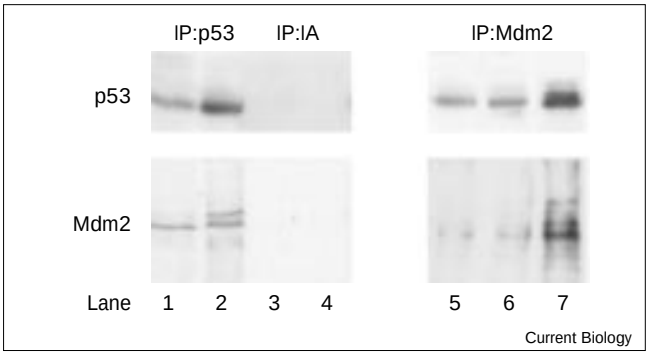
The p53 protein forms complexes with Mdm2 that target p53 for degradation [45,46]. Figure 3 shows that p53–Mdm2 complexes exist in ES cells, but that Mdm2 levels were not increased significantly by PALA treatment or γ-irradiation. The precipitating antibody used, sc-965, is a monoclonal antibody directed against a region of the Mdm2 protein (amino acids 154–167) which shares low homology with MdmX, a related protein that is not induced by p53 [47]. The detected protein migrated with an apparent molecular weight of 90 kDa, which is characteristic of Mdm2 and not of MdmX, which has an apparent molecular weight of 70 kDa. Exogenous p53, expressed by the adenovirus ACNp53, was able to

Figure 2



Cell-cycle response of ES cells to DNA-damaging agents. Cells were exposed to 6 Gy γ-irradiation and to adriamycin (ADR) at the indicated concentrations. Cells were harvested 16 h after exposure to irradiation or to the drug. Similar cell-cycle profiles were obtained after exposure to 4–10 Gy of γ-irradiation and to 1 μg/ml adriamycin in at least four independent experiments.

Figure 3



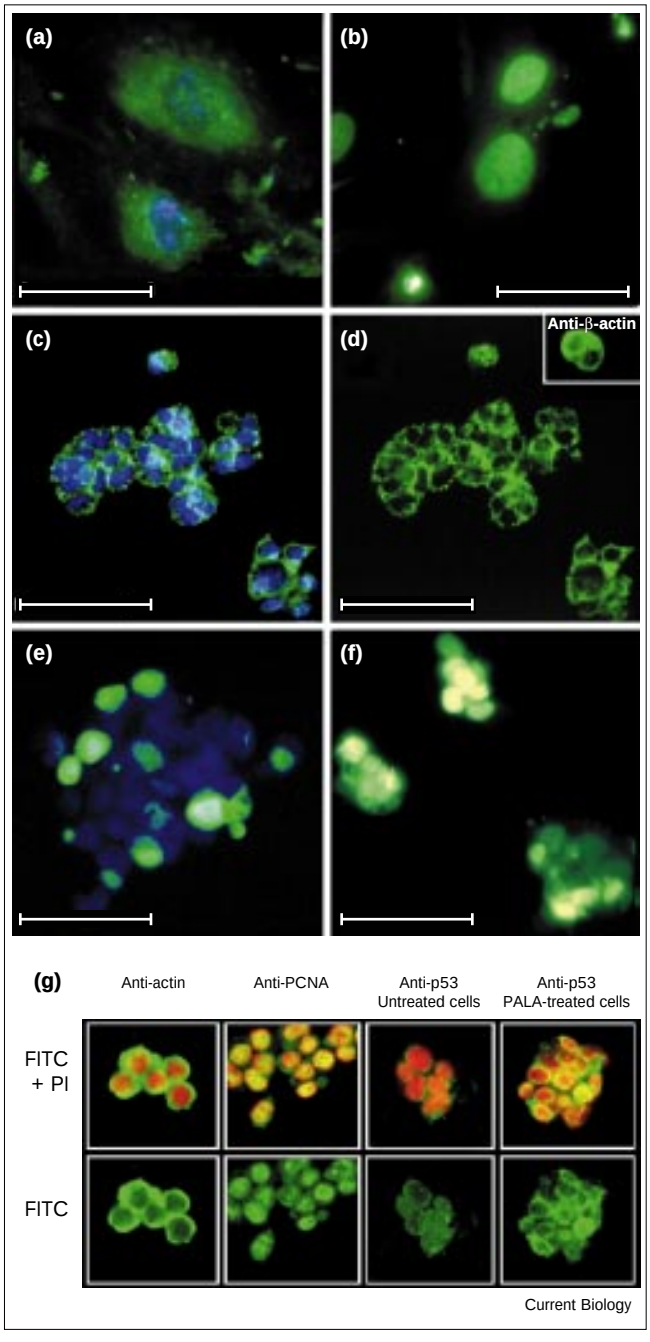
Expression of p53 and Mdm2 in ES cells. Extracts from ES cells were precipitated with antibody 421 against p53 (IP:p53) or antibody sc-965 against Mdm2 (IP:Mdm2). An irrelevant antibody (anti-BrdU) that does not react with p53 was used as a control for immunoprecipitation specificity (IP:IA). Immunocomplexes were detected with antibody sc-812 against Mdm2 or antibody Do-1 against p53. Lanes 1,3 and 5, control untreated cells; lanes 2 and 4, γ-irradiated cells (extracts prepared 18 h after irradiation); lane 6, cells exposed to PALA for 18 h; lane 7, cells infected with an adenovirus (ACNp53) expressing wild-type human p53.

increase the levels of both p53 and Mdm2 (Figure 3, lane 7). It should be noted that the overall steady-state level of Mdm2 was very low; we were unable to detect it directly in immunoblots or by immunocytochemistry (data not shown). Similarly, we were unable to detect p21 in ES cells using immunoblots, by immunofluorescence or northern hybridization analyses (data not shown), indicating that p53 was not functioning as an efficient transcriptional activator for these targets in ES cells. These data are consistent with the results of Savatier *et al.* [48], who did not observe p21 in undifferentiated ES cells.

The p53 protein is cytoplasmic in ES cells and is translocated inefficiently into the nucleus in response to rNTP depletion

PALA treatment and irradiation typically result in efficient translocation of p53 from the cytoplasm to the nucleus [6,49,50]. We used immunocytochemistry to determine whether the apparent inactivity of p53 in ES cells might result from inefficient nuclear translocation. As shown in Figure 4a,b, PALA treatment of MEFs resulted

Figure 4



Expression and cellular localization of p53 in ES cells and MEFs. Cells were grown on coverslips (5×10^5 ES cells and 1×10^5 MEFs), infected with p53 expression viruses or treated with PALA and expression was detected by immunofluorescence. **(a–f)** Antibody Do-1 was used to detect p53. Antibody staining was detected with fluorescein isothiocyanate (FITC; green) and nuclei were counterstained with Hoechst 33352 (blue). Panels (a), (c) and (e) are composites showing FITC stain and nuclear Hoechst 33352 counterstain. Panels (b), (d) and (f) were photographed with an FITC filter (to detect FITC only). (a) MEFs immunostained for p53 expression; (b) MEFs treated with PALA, immunostained for p53 expression; (c) ES cells immunostained for p53 expression; (d) Same cells as in (c), photographed with an FITC filter (the inset shows similar cytoplasmic staining of cells to the cytoplasmic marker β-actin; (e) ES cells infected with 5×10^9 particles of the p53-expressing virus AMNp53; (f) ES cells infected with 5×10^{10} particles of the p53-expressing virus AMNp53. The size bars are 50 μm. **(g)** Confocal micrographs. ES cells were grown in chamber slides, treated as indicated and processed for microscopy as above, except that the Hoechst staining was replaced by staining with propidium iodide (PI; red) in the presence of RNAse. The p53 protein was immunostained using antibody pAb421. Upper panels, combined FITC and PI signal; lower panels, FITC signal only. PCNA, proliferating cell nuclear antigen.

in a striking nuclear localization of p53 in >90% of the cells. By contrast, most of the p53 was localized in the cytoplasm of ES cells (Figure 4c,d,g) and a significant heterogeneity in p53 distribution was observed in PALA-treated cells (Figure 4g). Some PALA-treated cells had a significant increase in nuclear p53 levels, but many showed little, if any, nuclear accumulation. These results indicate that the mechanism responsible for cytoplasmic–nuclear shuttling of p53 is less efficient in ES cells than in fibroblasts.

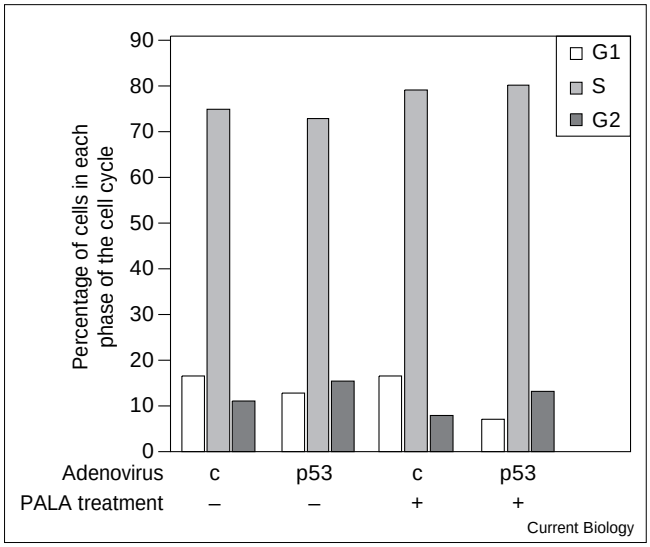
Inability to restore cell-cycle arrest upon infection with wild-type human p53

We tested whether high levels of wild-type p53 might overcome the inability of ES cells to activate cell-cycle arrest. ES cells were infected with replication-deficient adenoviruses expressing wild-type human p53, that had previously been shown to induce cell-cycle arrest in human and murine cells [6,51]. Immunoblot analyses indicated that infection with the adenovirus resulted in p53 overexpression (Figure 3, lane 7; Figure 4e,f). The p53 protein was present in the cell nuclei, but because of the fluorescent intensity of the overproduced protein and the small size of the cells, it was impossible to discern whether some p53 was also present in the cytoplasm. As shown in Figure 5, infected cells had a cell-cycle profile that was similar to that of uninfected ES cells. PALA treatment of the infected cells did not cause cell-cycle arrest despite the high levels of p53. These data suggest that ES cells have a very effective mechanism for rendering them refractory to p53 function, even when they contain substantial quantities of nuclear p53.

Induction of p53-independent apoptosis in ES cells upon DNA damage

The apparent inability of ES cells to mount a p53-dependent cell-cycle arrest in response to rNTP depletion or DNA damage raises the important issue of how such cells minimize the generation of descendants with chromosomal aberrations. One likely possibility is that ES cells with genetic damage undergo apoptosis. We observed that a considerable fraction of ES cells did not attach to a solid support following irradiation, and some cultures exhibited a sub-G1 fraction (Figure 2). Adriamycin treatment also caused a dose-dependent death response, most cells detaching from the plate 24 hours after irradiation (Figure 6a). DNA isolated from such cells displayed the ladder pattern commonly generated in the later stages of apoptosis (Figure 6b). We further substantiated that ES cells undergo apoptosis in response to DNA damage using a terminal-deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay. Figure 6c shows that many ES cells treated with adriamycin for more than eight hours were TUNEL positive and exhibited the pattern of nuclear DNA fragmentation that typifies apoptosis [52].

Figure 5

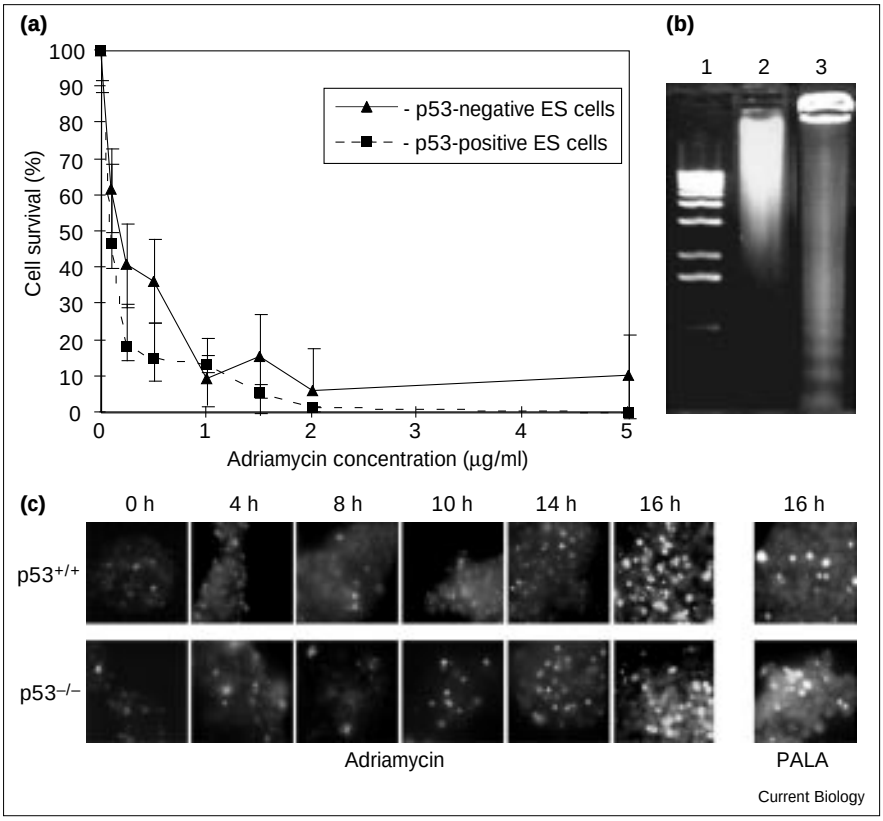


Cell-cycle profiles of adenovirus-infected ES cells in the presence and absence of PALA. Cells were infected with a control adenovirus expressing β-galactosidase (c; 10^{10} virus particles per 5×10^5 cells) or with the p53-expressing ACNp53 virus (p53; 5×10^9 particles per 5×10^5 cells) in the presence or absence of 1 mM PALA. Similar results were obtained in two other independent repetitions of the infection and treatment on two independently derived strains of ES cells.

As ES cells do not arrest in G1 phase upon challenge with antimetabolites, such cells may acquire DNA damage due to replication with an insufficient nucleotide supply [6,15,29]. Under these conditions, ES cells with normal p53 may be susceptible to apoptosis because continued DNA synthesis after nucleotide-pool depletion may lead to DNA breakage. We therefore determined whether PALA treatment induced apoptosis in ES cells. TUNEL analysis showed that 10–15% of the cells treated with PALA for 16 hours demonstrated the massive DNA breakage characteristic of apoptosis (Figure 6c). This proportion was less than that seen when ES cells were treated with adriamycin whereby >40% of the cells were TUNEL positive (Figure 6c), so we infer that PALA is less effective at inducing DNA damage than adriamycin under the stated conditions.

We determined whether apoptosis in ES cells required p53 by measuring the apoptotic response of ES cells in which the *p53* gene was inactivated by homologous recombination. The p53-positive and p53-negative cell populations were killed by adriamycin in a similar dose-dependent fashion (Figure 6a) and equivalent fractions of p53-positive and p53-deficient cells underwent apoptosis (Figure 6c). Furthermore, although the exact frequency of apoptosis was difficult to quantitate in ES cell clumps, it is clear that kinetics of the apoptotic response were similar

Figure 6



p53-independent apoptosis in adriamycin-treated ES cells. (a) Survival of p53-positive and p53-negative ES cell populations after a 16 h exposure to increasing doses of adriamycin. (b) Agarose gel electrophoresis of low molecular weight DNA from untreated ES cells (lane 2) or ES cells that were exposed to adriamycin for 16 h (lane 3). Lane 1, 1 kb ladder (BRL). (c) TUNEL assay for detection of DNA breaks in ES cells. Upper panels, $p53^{+/+}$ cells; lower panels, $p53^{-/-}$ cells. ES cells were exposed to 1 μg/ml adriamycin for the indicated times or with 0.5 mM PALA for 16 h. Fluorescent cells were visualized with an FITC filter.

in p53-positive and p53-negative cells (Figure 6c). These results indicated that p53 is not required for the damage-induced apoptotic response in ES cells.

Restoration of the PALA-induced cell-cycle-arrest response upon differentiation

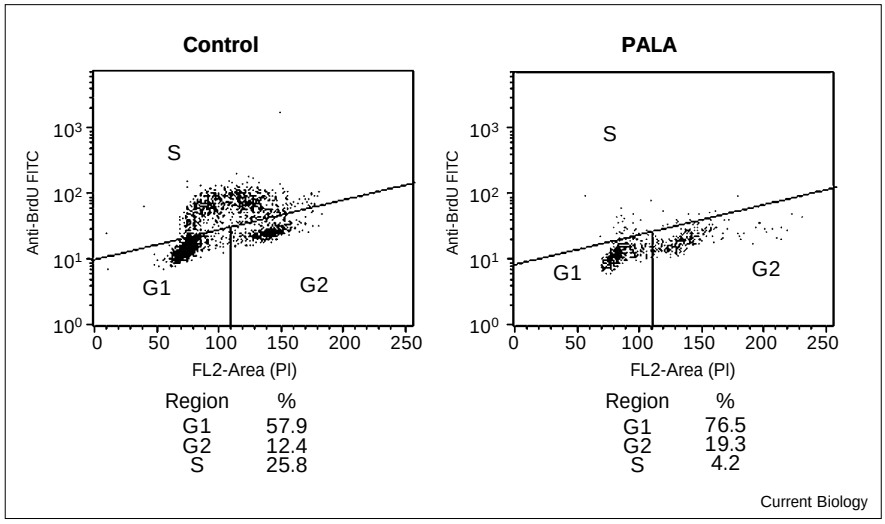
The lack of the p53-mediated cell-cycle arrest response in ES cells strongly contrasts with the PALA-induced arrest response observed in normal MEFs. Because p53 levels in ES cells are high initially and are subsequently reduced and because expression becomes limited to specific cell lineages during development [40], it is possible that the p53 response is designed to ensure that the cell-cycle arrest pathway can be activated rapidly upon differentiation of the inner cell mass. To test whether differentiation restores the arrest response, we analyzed the cell-cycle profile of ES cells grown in a medium without the cytokine leukemia inhibitory factor (LIF), supplemented with retinoic acid and treated with PALA. As shown in Figure 7, the S-phase fraction in the differentiated ES cells was reduced to about 25% and was further diminished by PALA treatment. These results show that the ability to arrest the cell cycle in response to antimetabolite challenge could be restored when ES cells differentiate.

Discussion

Chemical and physical challenges that may lead to genetic instability failed to induce cell-cycle arrest in undifferentiated murine ES cells but were able to induce apoptosis. The normal cell cycle of ES cells consists predominantly of alternating phases of DNA synthesis and mitosis, with very short G1 and G2 intervals, leading to an 8 hour doubling time (data not shown). These data are compatible with earlier inferences of 5–6 hour cell cycles of ES cells *in vivo* [53]. Very short cell cycles have been observed during early embryogenesis, such as in *Drosophila* [54] and *Xenopus* (the first 12 divisions) [55]. In these systems, replication is accelerated and occurs in the absence of transcription. Conversely, cells from the murine inner cell mass do not change the length of the S phase but appear to abbreviate G1 and, perhaps, G2 ([56]; this work). The lack of G1 and G2 correlates with the inactivation of the cell-cycle checkpoint controls that usually operate during these intervals. In early embryogenesis, guaranteeing accurate repair may be incompatible with rapid replicative cycles, and elimination of damaged cells may represent a safer alternative to cell-cycle arrest. Although this strategy exposes ES cells to the hazards of DNA replication and cell division under suboptimal conditions, the efficient apoptotic mechanism described here provides effective

Figure 7

Cell-cycle profiles of differentiated ES cells after exposure to PALA. ES cells were differentiated by removal of LIF and exposure to retinoic acid in the growth medium. Cells were then left untreated (control) or exposed to 100 μM PALA for 24 h, then allowed to take up BrdU for 30 min, harvested and analyzed for cell-cycle profiles as detailed in the legend to Figure 1.



insurance against the possibility of genetically damaged cells contributing to the developing organism.

Two components of the damage-induced cell-cycle checkpoint control pathway, p53 and Mdm2, were detected in ES cells. We could not, however, detect any p21 protein, basal levels of which are largely determined by transcriptional activation by p53 [57]. The p53 protein was found mainly in the cytoplasm of ES cells and formed a complex with Mdm2. Nevertheless, our data suggest that neither sequestration of p53 in the cytoplasm nor inactivation by Mdm2 was entirely responsible for the lack of cell-cycle arrest upon DNA damage, because PALA-treated ES cells manifested some nuclear p53, and overexpression of normal nuclear p53 by viral infection did not induce cell-cycle arrest. The lack of cell-cycle arrest therefore derives from additional dysfunctions downstream of p53 in the damage response pathway.

Our results show that conditions leading to a p53-dependent cell-cycle arrest in MEFs activate a p53-independent apoptotic mechanism in ES cells. Apoptosis can be induced by rapid growth in the presence of excess p53 [58], by induction of viral oncogenes [17,59,60], or by deletion of Rb protein in the presence of normal levels of p53 [26,61]. Growth factor modulations may also induce apoptosis in fibroblasts that would normally arrest following DNA damage [17,18]. In such fibroblasts, rapid growth may bypass the arrest pathway by not allowing cells to linger long enough before the G1 restriction point, leading to unscheduled DNA replication, DNA damage and apoptosis. We note that apoptotic indices and survival curves in p53-positive and p53-negative ES cells were experimentally indistinguishable after adriamycin treatment, indicating little if any contribution of p53 function to apoptosis in the ES cells. Apoptosis that is p53 independent was also observed in somatic cells [14,62]. One mechanism for p53-independent apoptosis may be through another protein, related to p53, that responds to different challenges in different tissues, such as the recently described p73 protein [63]. It is also formally possible that the p53-independent apoptotic pathway in ES cells is activated concomitant with a p53-dependent pathway. Nevertheless, because p53 overexpression did not induce apoptosis, we infer that a p53-dependent apoptotic pathway in the absence of DNA damage is difficult to activate in ES cells. This stringent inactivation may allow ES cells to undergo rapid cell divisions without the potential inhibitory effects produced by transactivation of p53 targets.

Another mechanism for p53-independent apoptosis is suggested by experiments in somatic cells, in which apoptosis is induced by elevated activity of the transcription factor E2F-1. This can be achieved by Rb inactivation [64], overexpression of Myc [17,18] or by transfection with cDNA encoding the E2F-1 DNA-binding domain [65,66]. Tumor cells deficient in both p53 and Rb undergo apoptosis in response to DNA damage and this apoptosis can be prevented by the expression of Rb [67]. Thus, it is tempting to speculate that a dysfunction in the Rb-mediated inhibition of E2F activity may lead to deregulation of E2F in ES cells and may render the cells exquisitely sensitive to apoptosis upon DNA damage. Indeed, most of the Rb protein is present in the proliferative form in ES cells ([56]; data not shown). Furthermore, cyclin D and its associated cyclin-dependent kinase CDK4, which modify Rb in somatic cells, are not functional in ES cells [48].

Our data suggest that the lack of arrest in ES cells reflects a cell cycle driven by fewer cyclins than in somatic cells,

and some checkpoint controls may be inactivated as a consequence. This interpretation is consistent with other observations suggesting that some early embryonic cell cycles differ from the somatic cell cycle [68,69]. Cell divisions during early *Xenopus* embryogenesis, for example, seem to be driven by a single cyclin-dependent kinase, $p34^{cdc2}$ (also known as maturation promoting factor, MPF) [69]. Extracts of *Xenopus* eggs continue to cycle in the absence of DNA replication or spindle formation [55]. There are other examples, such as fission yeast, in which the cell cycle is driven by a single functional and essential cyclin [70], while other non-essential cyclins enable further layers of control to be implemented.

The absence of components of the p53-mediated cell-cycle-arrest-response pathway in ES cells may explain how embryonic cells can divide very rapidly while expressing high levels of the normal p53 protein [40]. High levels of apparently normal p53 have also been observed in embryonic carcinoma cells [71]. The expression of p53 is down-regulated upon differentiation of embryonic carcinoma cells and during later stages of normal embryogenesis, eventually becoming restricted to only a few specific organ systems [37,40,72,73]. The fluctuations in p53 levels during early embryogenesis imply some involvement of p53 in embryonic development, yet studies in p53-deficient mice demonstrate that p53 does not play an essential role in ontogenesis [23]. On the other hand, upon exposure to genotoxic stress, mice that express p53 demonstrate a two- to four-fold lower frequency of mutant or deformed embryos than mice deficient in p53, suggesting that p53 may limit the effects of teratogens [10,12]. Our observations suggest that the p53-mediated DNA-damage response pathway can become activated upon differentiation of the inner cell mass. These results are consistent with data suggesting that, *in vivo*, differentiated embryonic tissues activate p53 upon DNA damage in a heterogeneous manner [37]. The activation of p53 response after differentiation may explain why p53-positive, Mdm2-deficient mouse embryos survive beyond the inner cell mass stage and then undergo apoptosis *in utero* [74]. Presumably, Mdm2 creates a buffer against p53 and becomes essential for survival only after the p53 pathway becomes active in differentiated cells. According to this view, Mdm2 would titrate p53 function to prevent it from inducing cell-cycle arrest or apoptosis. Our data indicate that, in early embryogenesis, cell survival does not depend on the buffer activity of the Mdm2 protein because the p53 pathway is not yet active.

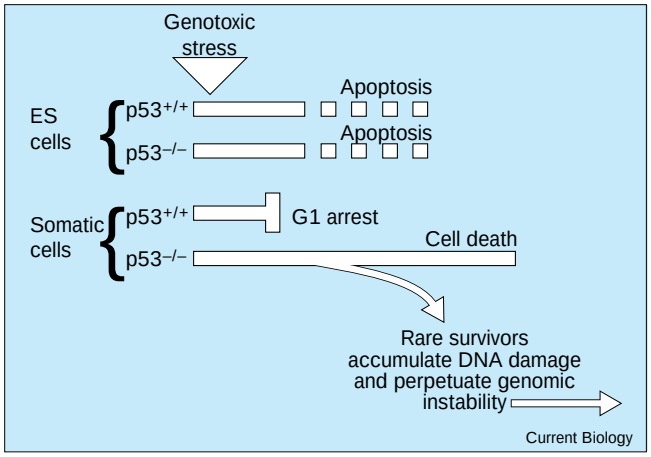
Tumors derived from pluripotent cells, such as from teratocarcinomas, are extremely susceptible to apoptosis upon DNA damage. These tumors express p53 that is normal in sequence but is not functional [75,76]. In contrast to ES cells, teratocarcinoma cells respond to DNA damage by undergoing p53-dependent apoptosis, but will also undergo limited apoptosis in the absence of p53. Similar to ES cells, the lack of damage-induced cell-cycle arrest reflects the lack of function of a downstream factor because high levels of p53-dependent transcripts were not restored upon stabilization of the p53 protein. Taken with our results, these data suggest that the susceptibility of these tumors to DNA damage and the lack of a cell-cycle-arrest pathway that is inducible by DNA damage may be an inherent characteristic of undifferentiated cells rather than a peculiar outcome of tumor progression in these malignancies.

The lack of checkpoint controls in genotypically normal ES cells and the acquisition of such checkpoints upon differentiation suggest that checkpoint controls may be regulated to various degrees upon differentiation into diverse cell types. Such variation may explain the apparent redundancy of cell-cycle proteins and pathways and may also indicate a specialized tissue-specific role for some of the cell-cycle or damage-sensor proteins. Tissue specialization of DNA damage and other stress-response arrays may explain why some cells, such as prostate epithelial cells, fail to arrest or to induce p53 in response to challenges that normally activate the p53-dependent cell-cycle-arrest pathway in genetically identical, adjacent prostate stromal cells [36]. Heterogeneity in DNA-damage responses may explain the varied etiologies of malignancies in different tissues and may also prove relevant to understanding the molecular basis for the non-random distribution of cancers in different cell types.

Conclusions

Wild-type ES cells do not undergo cell-cycle arrest under conditions, including ribonucleotide depletion and DNA breakage, that would normally activate the p53-mediated cell-cycle-arrest pathway in somatic cells (Figure 8). ES cells express abundant quantities of p53, but the p53-mediated response is inactive because of cytoplasmic sequestration of p53, low efficiency of p53 translocation to the nucleus, and an apparent lack of factors downstream of p53 in the signal transduction pathway. Nevertheless, ES cells are still sensitive to conditions that induce DNA damage, at least in part, because they activate a p53-independent apoptotic response. These results suggest that the relatively low level of developmental abnormalities in p53-deficient animals is due to the efficient elimination of damaged cells from the population during early embryogenesis. The p53-mediated cell-cycle-arrest response is restored upon differentiation of ES cells. These data provide one explanation as to why the embryonic lethality of Mdm2-deficient mice is delayed until after the inner cell mass stage. These observations further suggest that embryonic carcinomas are unable to induce cell-cycle arrest despite having wild-type p53 because they preserve the inactive p53-mediated cell-cycle-arrest pathway characteristic of their stem-cell precursors.

Figure 8



Cell-response summary. Cells respond to genotoxic stress in different ways according to cell type. ES cells activate an apoptotic pathway in response to genotoxic stresses regardless of their p53 status, whereas differentiated cells require a p53-regulated cell-cycle arrest mechanism to prevent accumulation of errors and consequent neoplasia.

Materials and methods

Cells culture, treatment and virus infection

Strain 129 ES cells were grown as described [39]. For retinoic acid treatment, cells were plated on non-gelatinized plates containing medium without LIF, in the presence of 1 μM retinoic acid. Cells were allowed to grow to confluence in this medium, then trypsinized and seeded at a lower density on new non-gelatinized plates. Cells were allowed to settle on the plates before treatment with 500 μM PALA. The $p53^{-/-}$ ES cells were isolated from embryos derived by a cross between $p53^{-/+} \times p53^{-/-}$ mice (strains 129×C57BL6). Blastocysts were explanted and cultured on feeder cells essentially as described [77]. ES cell lines were sexed by hybridization to a Y-chromosome-specific probe and genotyped by Southern analysis. Cells were supplemented with 1:100 dilution of conditioned medium from Chinese Hamster Ovary cells producing LIF [39]. MEFs were obtained from normal and p53-deficient embryos as described [78]. Adenoviruses expressing p53 were gifts from Canji, Inc. PALA was obtained from the National Cancer Institute and adriamycin from Sigma. Dose-response curves were obtained by exposure of cells in triplicate to increasing concentrations of the respective drug for the required period. Cells were then trypsinized, fixed in 70% ethanol and counted using a hemocytometer. About 50% of the cells were dying 10 h after treatment with 1 μg/ml adriamycin, and this concentration was used for further treatments. Cells were irradiated in liquid medium using a ^{60}Co source (gamabeam 150°C), at a rate of 2.9 Gy/min at a distance of 40 cm.

Cell-cycle analysis

Cells were incubated in 30 μM BrdU (Fluka) in the growth medium at 37°C, collected by trypsinization, washed with a non-enzymatic PBS-based cell dissociation medium (Specialty Media Inc.), and fixed in 70% ethanol. ES cells and MEFs were incubated in BrdU for 10 min and 30 min, respectively, prior to harvesting for cell-cycle analysis, unless noted otherwise. Each assay involved 5×10^5 cells. The BrdU content was determined by reaction with a fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (PharMingen) according to the manufacturer's protocol, using propidium iodide–RNase (100 μg/ml) counterstaining to determine DNA content. Samples were analyzed on a Becton-Dickinson FACScan using the CellQuest software.

Protein expression

Cells were lysed in NP40 lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, pH 8 [79]. Lysates were precleared with protein G-conjugated agarose beads (Santa Cruz), then reacted with agarose beads covalently linked to antibody 421 (Santa Cruz), antibody sc-812 (Santa Cruz) or to a control antibody (anti-BrdU, PharMingen). Lysates from 10^7 cells were used for each immunoprecipitation reaction. Immune complexes were collected by centrifugation, washed three times in the NP40 lysis buffer and eluted by boiling in gel-loading buffer [79]. Proteins were separated on 9% discontinuous polyacrylamide gels, transferred to BA-83 membrane (Schleicher and Schuel), blocked in a solution of 5% dry milk and overlaid with antibody according to the manufacturer's instructions. Antibodies Do-1 (Santa Cruz) or pAb421 (Oncogene Science) were used to detect p53 in the immunocomplexes and antibody sc-926 (Santa Cruz) was used to detect Mdm2. The apparent molecular weight of the proteins was calculated relative to molecular weight standards (BRL) run on the same gel. Proteins were visualized by enhanced chemiluminesence (DuPont NEN) using an HRP-conjugated goat anti-mouse antibody (Amersham). No residual p53 protein was detected in the supernatants following immunoprecipitation (data not shown), suggesting that the lack of increase was not due to an insufficient amount of antibody.

Immunofluorescence

Cells were grown for at least 16 h on gelatinized 18 mm coverslips (Sigma), then treated with antimetabolites or DNA damaging agents. Cells were fixed on coverslips with acetone:methanol (1:1) for 2 min, permeabilized in PBS containing 0.05% Tween-20 and blocked in 10% horse serum. The coverslips were then overlaid with antibody 421 or Do-1 for 1 h, or with a negative control antibody (anti-BrdU). Excess antibody was washed four times with the permeabilizing solution. Proteins that reacted with the antibodies were visualized using biotinylated anti-mouse IgG followed by overlay of avidin–fluorescein. Nuclei were counterstained with 13 μg/ml Hoechst 33352. Fluorescence was detected using FITC-specific filters (to detect FITC only) or triple-band-pass filters to detect FITC and Hoechst staining. All filters were from Chroma and were used on a Zeiss epifluorescence microscope. Photographs were taken using Ektachrome 400 ISO color slide film and scanned to Adobe Photoshop images using a Nikon scanner. For confocal analysis, cells were processed in the same way except that the nuclei were stained for 30 min with propidium iodide in the presence of 40 μg/ml RNAseA. Images were scanned in a BioRad Laser Sharp confocal microscope and processed in Adobe Photoshop.

TUNEL assay

This was performed using an *in situ* Cell Death Detection kit (Boehringer Mannheim) according to the manufacturer's instructions except that cells were permeabilized for 2 min in acetone:methanol (1:1). Treatment with DNAse (1 μg/ml) for 10 min was used as a positive control for DNA breaks. Fluorescence imaging and photography were performed as described above.

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