Please cite this article in press as: Liu et al., High Mitochondrial Priming Sensitizes hESCs to DNA-Damage-Induced Apoptosis, Cell Stem Cell (2013), http://dx.doi.org/10.1016/j.stem.2013.07.018

Cell Stem Cell
Short Article

High Mitochondrial Priming Sensitizes hESCs to DNA-Damage-Induced Apoptosis

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http://dx.doi.org/10.1016/j.stem.2013.07.018

SUMMARY

Human embryonic stem cells (hESCs) are highly sensitive to DNA damage and have low survival ability relative to differentiated cells. We investigated the source of this difference by comparing damage response pathways in hESCs and differentiated cells. We found that hESCs undergo more rapid p53-dependent apoptosis after DNA damage than differentiated cells do. However, p53 localization and function are similar between hESCs and differentiated cells, suggesting that p53 alone cannot explain the difference in sensitivity. Instead, we show that mitochondrial readiness for apoptosis, known as mitochondrial priming, differs between hESCs and differentiated cells. Specifically, the balance between proapoptotic and antiapoptotic proteins is shifted closer to the apoptotic threshold in hESCs than in differentiated cells. Altering this balance in differentiated cells increases their sensitivity and results in cell death, suggesting that manipulation of mitochondrial priming could potentially alter the sensitivity of other stem cells, including cancer stem cells.

INTRODUCTION

Of the diverse array of possible cellular responses to DNA damage—including DNA repair, cell cycle arrest, senescence, and apoptosis—human embryonic stem cells (hESCs) primarily undergo apoptosis (Momcilovic et al., 2010; Wilson et al., 2010). hESCs are able to self-renew indefinitely and can differentiate into all cell lineages in the body, making it particularly important that they maintain genomic integrity. Their high sensitivity to DNA damage and ability to differentiate make them a good model system for studying the regulatory networks that control apoptosis and how they differ between hESCs and their differentiated progeny.

One important protein controlling cell fate decisions in response to DNA damage is the tumor suppressor protein p53 (Vogelstein et al., 2000; Vousden and Lane, 2007). p53 was previously shown to be induced in response to DNA damage in hESCs, primarily triggering apoptosis (Filion et al., 2009; Grandela et al., 2007; Qin et al., 2007). In somatic cells, p53 is known to contribute to cell death through two primary mechanisms. First, nuclear p53 activates the transcription of proapoptotic genes, such as *PUMA*, *BAX*, and *NOXA* (Villunger et al., 2003). Second, cytoplasmic p53 directly interacts with mitochondrial proteins, acting as a direct activator of Bak and/or Bax oligomerization, or as a sensitizer, by sequestering antiapoptotic proteins (Green and Kroemer, 2009). In hESCs, there is evidence that p53 triggers apoptosis exclusively through the mitochondrial pathway after UV irradiation (Qin et al., 2007). However, other work showed that p53 not only associates with mitochondria but also translocates to the nucleus in response to DNA breaks caused by the topoisomerase-II poison etoposide, suggesting that induction of apoptosis by p53 involves a transcription-dependent pathway (Grandela et al., 2007). Additionally, the genome-wide transcriptome of hESCs after γ irradiation revealed altered gene expression of primarily p53-dependent, proapoptotic transcripts (Sokolov et al., 2011).

The cellular responses of differentiated cells to DNA damage differ widely depending on the tissue of origin. For example, tissues vary drastically in their survival ability after irradiation: some tissues, including those comprising bone marrow, small intestine, thymus, and spleen, are acutely affected by even low doses of radiation, while tissues from kidney, heart, liver, and lung are relatively resistant (Gudkov and Komarova, 2003). While it is thought that sensitivity to DNA damage tends to be characteristic of highly proliferating cells, some rapidly dividing tumors are resistant to chemotherapy, while other slowly dividing tumors are chemosensitive, suggesting that additional factors affect the cellular damage response (Gudkov and Komarova, 2003). Distinct p53 expression and activity in cells from different tissues can be a factor contributing to differential tissue sensitivity to DNA damage (Komarova et al., 1997). In addition, recent studies have found that cells' relative mitochondrial priming affects their sensitivity to DNA damaging drugs (Certo et al.,

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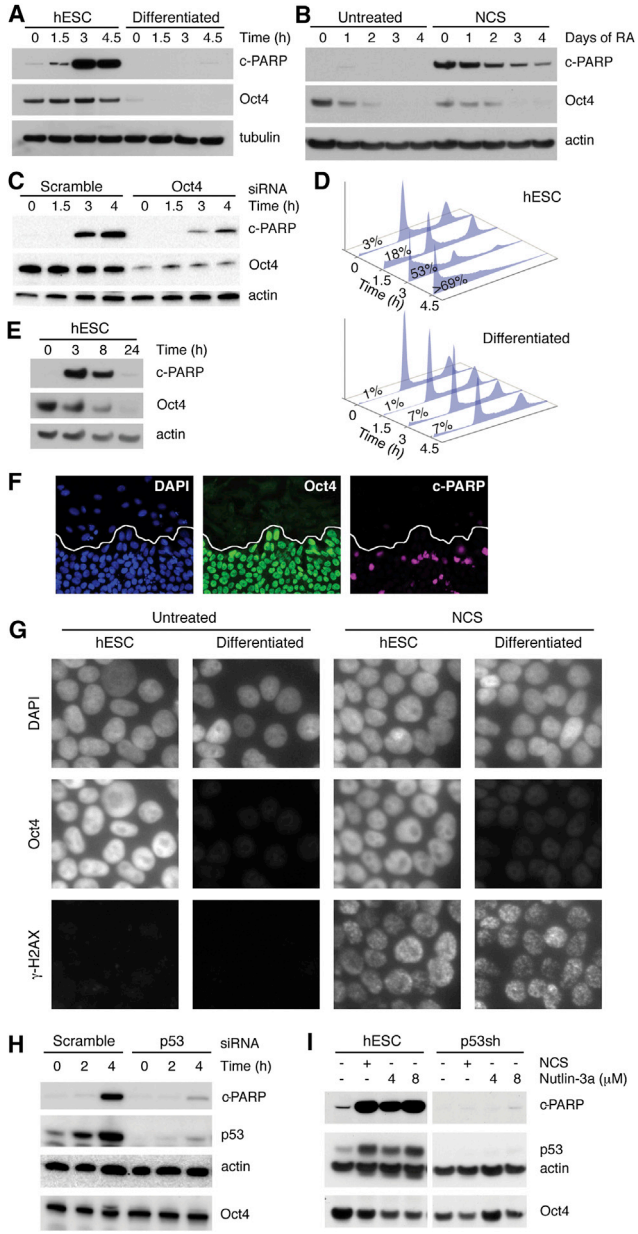

Figure 1. DNA Damage Leads to Rapid p53-Dependent Apoptosis in Undifferentiated hESCs, but Not in Differentiated Cells

(A) Immunoblot of c-PARP and Oct4 in hESCs and differentiated cells treated with 100 ng/ml of NCS.

(B) Immunoblot of c-PARP and Oct4 of cells differentiated with 1 μM RA for 0, 1, 2, 3, and 4 days, before and after NCS treatment for 3 hr.

(C) Immunoblot of c-PARP and Oct4 in hESCs transfected with scramble or Oct4 siRNA and treated with NCS.

(D) DNA content (propidium iodide incorporation) measured by flow cytometry of hESCs and differentiated cells, treated with NCS. The percentage of cells in the sub-G1 fraction is marked.

(E) Immunoblot of c-PARP and Oct4 in hESCs at later time points after NCS treatment.

(F) Immunofluorescence images of an hESC colony costained with DAPI and antibodies against Oct4 and c-PARP, fixed 2.5 hr after damage with NCS.

(G) Immunofluorescence images of hESCs and differentiated cells before and 15 min after treatment with NCS. Cells were costained with DAPI and antibodies against Oct4 and γ-H2AX.

(H) Immunoblot of c-PARP, p53, and Oct4 in hESCs transfected with scramble or p53 siRNA and treated with NCS.

(I) Immunoblot of c-PARP, p53, and Oct4 in hESCs and p53shRNA hESCs after 3 hr of NCS or 3 hr of Nutlin-3a treatment at the indicated concentrations.

See also Figure S1.

2006; Ni Chonghaile et al., 2011; Vo et al., 2012). Mitochondrial priming denotes the intrinsic potential of cells to undergo apoptosis due to the balance of proapoptotic and antiapoptotic Bcl-2 family proteins at the mitochondria and can be assessed by BH3 profiling (Ni Chonghaile et al., 2011). This assay measures mitochondrial outer membrane permeabilization (MOMP) after mitochondria are exposed to proapoptotic promiscuously interacting BH3 peptides. Higher mitochondrial priming determined by BH3 profiling has been shown to correlate with clinical response in several cancers (Ni Chonghaile et al., 2011) as well as increased chemosensitivity (Vo et al., 2012).

Here we determined the origin of the sensitivity of hESCs to DNA damage compared with their differentiated progeny. Specifically we investigated whether the p53 pathway is differentially induced in hESCs and differentiated cells or whether other intrinsic cellular properties can explain the difference in sensitivity. We found that DNA damage rapidly induced p53-dependent apoptosis in hESCs. p53 target genes were also induced; however, inhibition of p53's transcriptional activity or expression of exclusively cytoplasmic p53 did not prevent apoptosis, suggesting that p53's cytoplasmic function is the main contributor to apoptosis of hESCs. p53 was also induced in differentiated cells. Its localization and function did not differ between hESCs and differentiated cells. Instead, we found that differential mitochondrial priming, measured by BH3 profiling, determined the sensitivity of hESCs to DNA damage. hESCs showed high mitochondrial priming relative to their differentiated progeny. Increasing the low priming of differentiated cells increased their sensitivity to DNA damage and led to apoptosis.

RESULTS

DNA Damage Leads to Rapid p53-Dependent Apoptosis in hESCs, but Not in Differentiated Cells

We first characterized the response of hESCs to DNA damage and compared their behavior with that of differentiated cells from the same background. DNA damage was induced using the radiomimetic drug neocarzinostatin (NCS), which creates double-stranded DNA breaks (DSBs) within 5 min following its addition to cell culture medium (Shiloh et al., 1983). Cells were differentiated with 1 μM retinoic acid (RA) for 4 days (Andrews, 1984), and differentiation was confirmed using an antibody against Oct4, one of the key pluripotency genes in hESCs (Niwa et al., 2000). Induction of apoptosis was measured using an antibody against the 89-kDa fragment of cleaved PARP (c-PARP), which is cleaved during apoptosis by caspase-3. We found that hESCs accumulated c-PARP within 3 hr after DNA damage. In contrast, differentiated cells exhibited no c-PARP (Figure 1A). Treatment of hESCs with RA for shorter periods allowed us to investigate cells in the process of differentiation. We observed that decrease in Oct4 during RA treatment

was correlated with reduction of c-PARP after damage (Figure 1B). We further demonstrated the strong connection between pluripotency and induction of apoptosis by silencing Oct4. Oct4 knockdown cells show a drastic reduction in c-PARP levels after damage in comparison with hESCs treated with scramble siRNA (Figure 1C). Sensitivity to DNA damage was confirmed in two additional hESC lines (Figures S1A and S1B available online), suggesting that the differential sensitivity between stem and differentiated cells is not limited to one specific line. Furthermore, the higher induction of c-PARP in hESCs compared with their differentiated progeny was consistently reproduced in response to additional DNA-damage-inducing agents, including γ irradiation, UV radiation, and etoposide (Figure S1C).

Annexin V measurements confirmed that hESCs, but not differentiated cells, undergo apoptotic death after DNA damage (Figure S1D). Consistently, cell cycle analysis showed that the sub-G1 fraction of the population, which represents dead cells, increases rapidly in undifferentiated cells (Figure 1D). Over 50% of cells died within 3 hr after damage. In contrast, the sub-G1 fraction of the differentiated cells remained low following DNA damage. We noticed that a small fraction of hESCs survived the damage. To determine whether these were resistant stem cells or spontaneously differentiated cells, we monitored the levels of Oct4 and c-PARP in the surviving cells 24 hr after damage. We found that the remaining cells were no longer Oct4 positive (Figure 1E), suggesting that they had already differentiated in culture or in response to the damage. These surviving differentiated cells were also c-PARP-negative, supporting pluripotency as a determinant of sensitivity to damage. In agreement, immunofluorescence (IF) experiments revealed that in a colony comprising both undifferentiated (Oct4-positive) and spontaneously differentiated (Oct4-negative) cells, c-PARP was only detectable in the undifferentiated cells (Figure 1F). This difference in sensitivity was not due to different extents of DNA damage incurred by NCS because the levels of γ-H2AX, the canonical marker for DSBs (Löbrich et al., 2010), were comparable between undifferentiated and differentiated cells (Figure 1G). Taken together, these results show that hESCs, unlike their differentiated progeny, respond to DNA damage by rapidly undergoing apoptosis. This highly sensitive damage response is linked to pluripotency.

We next determined whether p53 plays a role in the induction of apoptosis in hESCs in response to DNA damage. p53 levels were induced in hESCs following DSBs (Figure 1H). Knocking down p53 using siRNA abrogated the c-PARP response (Figure 1H). In addition, cultures treated with siRNA against p53 did not show the colony shrinkage or the floating cells observed in damaged hESC cultures (Figure S1E). This shows that p53 is required for activation of the apoptotic pathway in hESCs in response to DNA damage. To determine whether apoptosis in hESCs can be triggered solely by elevation of p53 and independently of DNA damage, we treated cells with Nutlin-3a. Nutlin-3a is a small molecule that inhibits the binding of Mdm2, a major negative regulator of p53, to p53, thereby increasing p53 stability (Vassilev et al., 2004). Nutlin-3a led to an accumulation of p53 and c-PARP to levels comparable to those observed in response to DNA damage (Figure 1I). The ability of Nutlin-3a to trigger apoptosis in hESCs suggests that stabilization of p53 is sufficient to induce apoptosis without requiring additional damage-dependent posttranslational modifications. To further strengthen the dependency of cell death on p53, we used a short hairpin RNA (shRNA) against p53 that is stably integrated into hESCs via lentiviral infection. Cells carrying the p53shRNA lost induction of p53 and showed no c-PARP after DNA damage and Nutlin-3a treatments (Figure 1I). Similar results were obtained when we used a different hESC line, HUES9, and compared c-PARP levels after NCS treatment between wild-type and *p53*$^{-/-}$ cells (Figure S1F).

p53 Activates Transcription of Proapoptotic Genes in Both hESCs and Differentiated Cells

In somatic cells, p53 acts as a transcription factor activating many target genes, including proapoptotic genes (Riley et al., 2008). We asked whether p53 also activates transcription of proapoptotic genes in damaged hESCs, potentially contributing to their death in response to DNA damage. We used qRT-PCR to measure mRNA levels in hESCS of known p53 transcriptional targets involved in apoptosis (Müller et al., 1998; Nakano and Vousden, 2001; Oda et al., 2000a, 2000b; Riley et al., 2008; Robles et al., 2001; Thornborrow et al., 2002). Two genes (*BAX* and *TP53AIP1*) showed almost no induction (Figure 2A). Three genes (*FAS*, *APAF1*, and *NOXA*) showed between 2- to 3-fold induction 3 hr after damage. Notably, *PUMA*, a gene encoding a proapoptotic BH3-only protein that inhibits antiapoptotic proteins (Chipuk et al., 2005), showed a more than 6-fold induction in mRNA level after DNA damage. *PUMA* mRNA and protein levels were significantly reduced in cells silenced for p53, suggesting that activation of *PUMA* following DNA damage is p53 dependent (Figures 2B and 2C).

Why do differentiated cells, which originated from the stem cell population, show such a remarkable difference in their sensitivity to DNA damage? One possibility is that p53 does not activate transcription of proapoptotic genes in differentiated cells. We compared mRNA levels of three proapoptotic p53 target genes (*PUMA*, *APAF1*, and *FAS*) between undifferentiated and differentiated cells. We found that these proapoptotic genes were induced in both undifferentiated and differentiated cells (Figure 2D). In the case of *APAF1* and *FAS*, mRNA levels in differentiated cells even exceeded those in undifferentiated cells. Moreover, Puma protein levels in differentiated cells reached comparable levels to those in undifferentiated cells, though the accumulation was slightly delayed in differentiated cells (Figure 2E). Taken together, these results show that p53 activates the transcription of proapoptotic genes in both differentiated and undifferentiated cells. Therefore, p53's transcriptional activity cannot explain the high sensitivity of hESCs to DNA damage compared with differentiated cells.

Cytoplasmic p53 Contributes to Apoptosis in hESCs

It has been shown that in addition to acting as a transcription factor, p53 can also activate apoptosis through other mechanisms, for example by interacting with mitochondrial proteins in the cytoplasm (Green and Kroemer, 2009). To determine whether such transcription-independent mechanisms contribute to apoptosis in hESCs, we used the RNA polymerase-II poison α-amanitin to inhibit transcription during the DNA damage response. Surprisingly, we found that hESCs still activated

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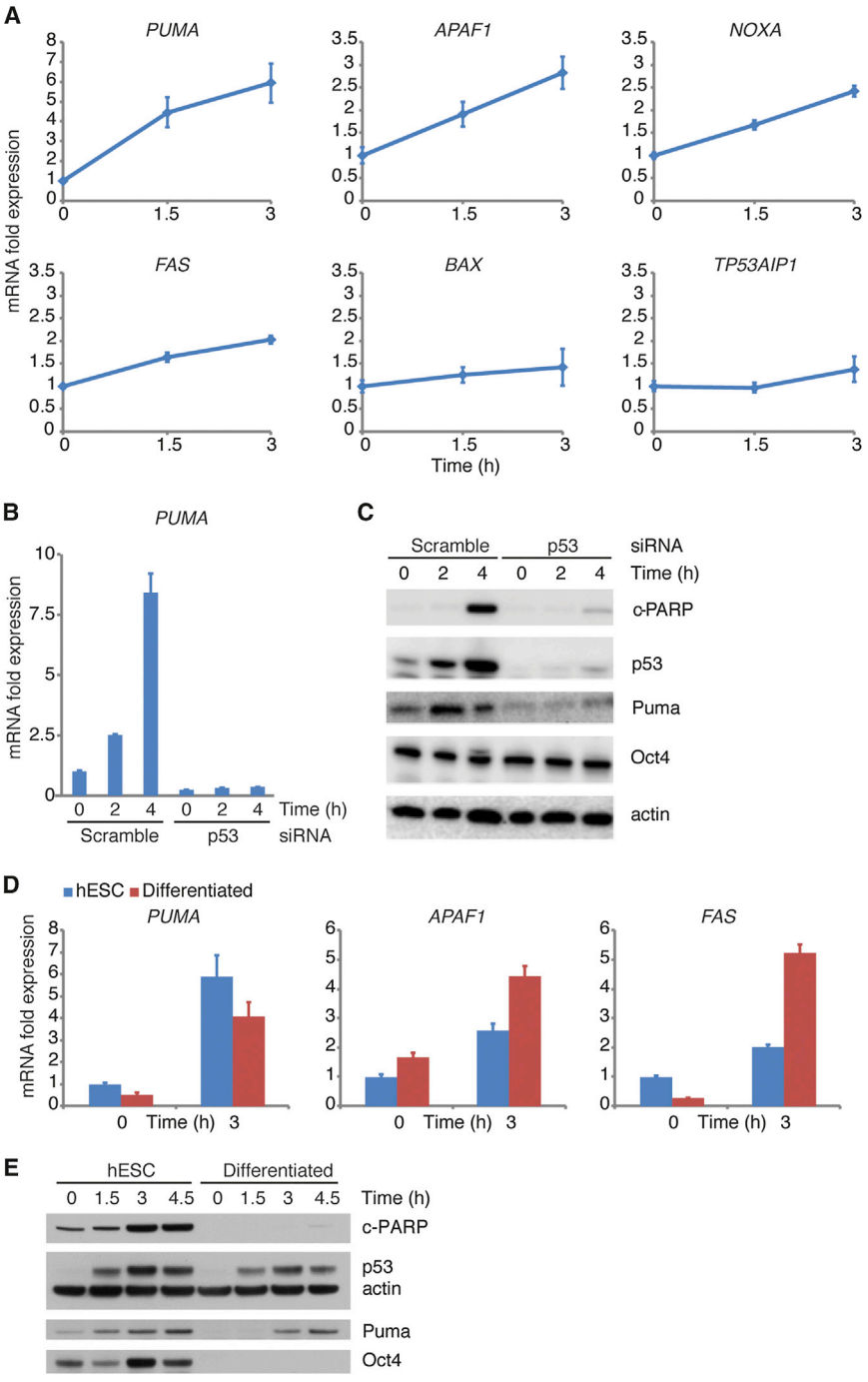

Figure 2. p53 Transcriptional Activity Is Induced after DNA Damage but Is Not Distinct between Undifferentiated and Differentiated Cells

(A) Relative mRNA expression of proapoptotic p53 target genes in hESCs treated with 100 ng/ml NCS, as measured by RT-qPCR. mRNA levels were normalized to *GAPDH*.

(B) Relative *PUMA* mRNA expression after treatment with NCS in hESCs transfected with scramble or p53 siRNA.

(C) Immunoblot of c-PARP, p53, Puma, and Oct4 after treatment with NCS in hESCs transfected with scramble or p53 siRNA.

(D) Relative mRNA expression of proapoptotic p53 target genes in hESCs and differentiated cells, undamaged and after 3 hr of NCS. For all genes in both hESCs and differentiated cells, predamage and postdamage mRNA levels were statistically significant ($p < 0.05$) as determined by Student's t test.

(E) Immunoblot of c-PARP, p53, Puma, and Oct4 in hESCs and differentiated cells, damaged with NCS.

Data are represented as mean ± SD.

apoptosis after DNA damage (Figure 3A) even when p53 target genes were not induced (Figure 3B). This apoptosis was p53 dependent, because the p53shRNA hESCs did not activate c-PARP following damage and α-amanitin treatment (Figure 3A). These results show that even though p53 target genes are upregulated in damaged hESCs, p53 transcriptional activity is not the main contributor to the rapid apoptosis seen in these cells. We therefore checked whether the cytoplasmic activity of p53 contributes to the apoptotic response in hESCs. Using IF to probe the localization of p53 in damaged hESCs, we found that p53 appeared primarily nuclear (Figure 1F and Figure 3C). However, by separating cell lysates into nuclear and cytoplasmic fractions, we found that a fraction of p53 (~20%) resided in the cytoplasm following DNA damage (Figure 3D). These low levels of cytoplasmic p53 may be below the detection threshold of IF. To determine whether cytoplasmic p53 can activate apoptosis in hESCs, we expressed a mutant form of p53 confined to the cytoplasm and tested its ability to activate apoptosis. p53 contains a bipartite nuclear localization signal (NLS), part of which includes Lys305 and Arg306. The two amino acid substitutions K305A and R306A cause p53 to be excluded from the nucleus (O'Keefe et al., 2003). We cloned the K305A and R306A mutant p53 and fused it with an mVenus fluorescent tag. Using lentiviral infection, we introduced this mutant p53 (cytop53) into hESCs expressing p53shRNA. Cytop53 contains synonymous substitutions that render it resistant to shRNA knockdown. Cytop53 was exclusively expressed in the cytoplasm (Figure 3E) and showed no transcriptional activity (Figure 3F), in contrast to a wild-type p53-mVenus (wtp53). We found that cells expressing cytop53 successfully induced c-PARP after damage (Figure 3G). These results show that cytoplasmic p53 contributes to the activation of apoptosis in hESCs. The levels of c-PARP were lower in cells expressing cytop53 in comparison to cells expressing wtp53. This may result from reduced cytoplasmic function or misfolding due to the inserted mutations.

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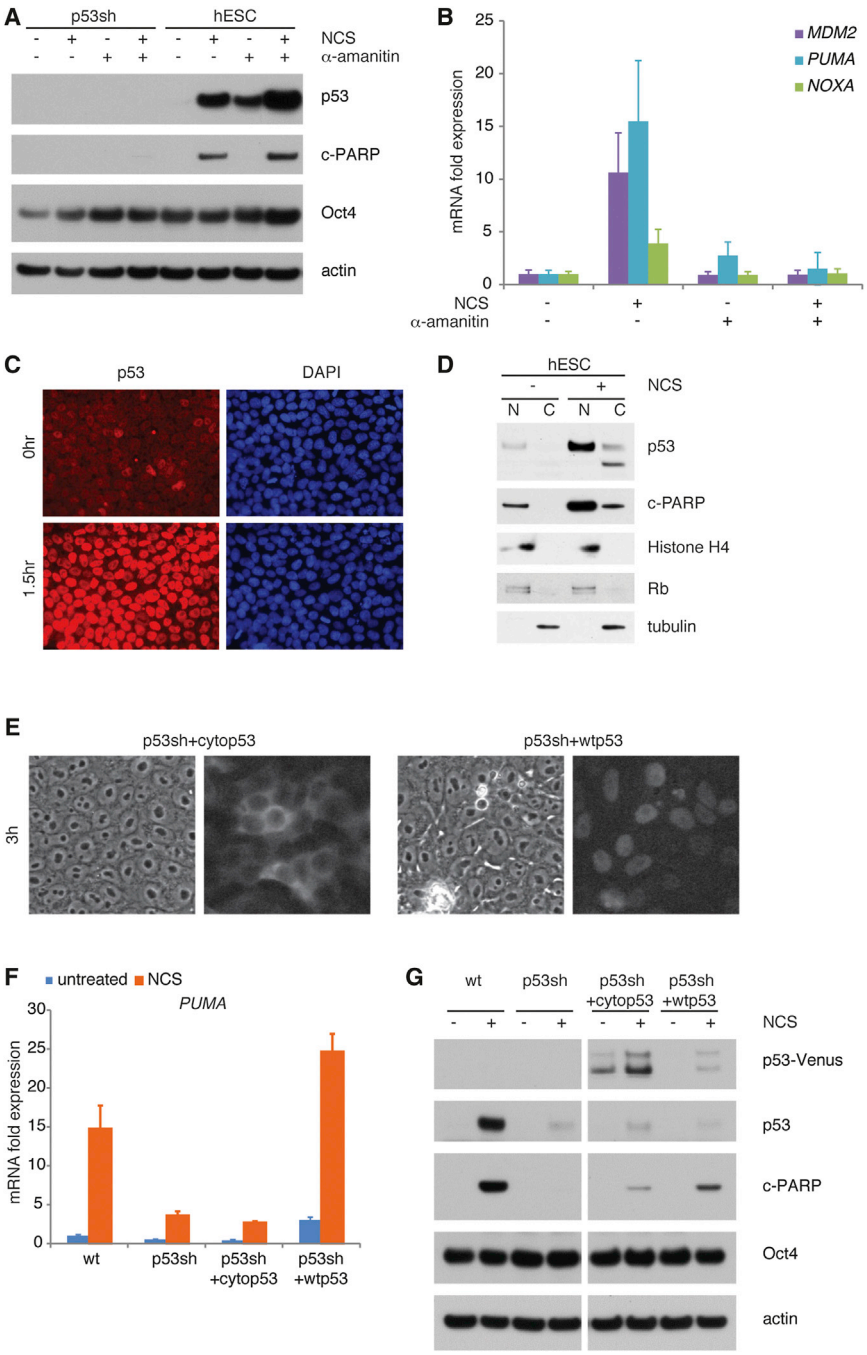

Figure 3. Though p53 Localization Appears Primarily Nuclear, Residual Cytoplasmic p53 Is Active in Contributing to Apoptosis in hESCs

(A) Immunoblot of c-PARP, p53, and Oct4 in hESCs and a p53shRNA hESC line after 3 hr of 100 ng/ml NCS and/or 4 hr of 15 μg/ml α-amanitin treatment (1 hr pretreatment when combined with NCS).

(B) Relative *MDM2*, *PUMA*, and *NOXA* mRNA expression of hESCs after 3 hr of NCS and/or 4 hr of α-amanitin treatment (1 hr pretreatment when combined with NCS).

(C) p53 and DAPI immunofluorescence images of hESCs fixed before and after 1.5 hr of treatment with NCS.

(D) Immunoblot of p53 and c-PARP in the nuclear and cytoplasmic fractions of hESCs. Cells were undamaged or damaged with NCS for 3 hr. Histone H4 and the transcription factor Rb serve as nuclear markers, and tubulin as a cytoplasmic marker. N, nuclear; C, cytoplasmic.

(E) Phase and fluorescent images of p53shRNA hESC lines with reintroduced p53-mVenus (wtp53) and p53K305A,R306A-mVenus (cytop53) after 3 hr of damage with NCS. These cells were then collected for the immunoblot in 4G.

(F) Relative *PUMA* mRNA expression in parental hESCs (wt), p53shRNA hESCs (p53sh), and p53shRNA hESC lines with reintroduced wtp53 and cytop53 before and after 3 hr of treatment with NCS.

(G) Immunoblot of p53-mVenus, p53, c-PARP, and Oct4 in parental hESCs (wt), p53shRNA hESCs (p53sh), and p53shRNA hESC lines with reintroduced wtp53 and cytop53 before and after 3 hr of damage with NCS.

Data are represented as mean ± SD.

hESCs, Unlike Differentiated Cells, Are Highly Primed toward Apoptosis Independently of p53

Because p53's transcriptional activity is similar between hESCs and differentiated cells and because cytoplasmic p53 can activate apoptosis in hESCs, we next examined two potential mechanisms that might explain the p53-dependent apoptosis observed only in undifferentiated cells. The first is that cytoplasmic p53 is active exclusively in undifferentiated cells. The second is that cytoplasmic p53 is active in both differentiated and undifferentiated cells, but other intrinsic properties of hESCs affect their sensitivity to damage. Specifically, recent studies showed that mitochondrial priming determines the survival of various cancer cells (Ni Chonghaile et al., 2011; Vo et al., 2012). We therefore first explored whether undifferentiated cells are intrinsically more "primed" for apoptosis than differentiated cells. Using BH3 profiling to measure MOMP induced by proapoptotic BH3-only peptides (Ni Chonghaile et al., 2011), we found that undifferentiated cells underwent MOMP more readily than differentiated cells in response to each peptide in the panel, at all concentrations (Figure 4A). To better connect mitochondrial priming with differentiation, we measured the priming level during the course of RA differentiation. Our results show a consistent trend: mitochondrial priming was highest in undifferentiated cells and decreased gradually with each additional day of differentiation (Figure 4B). We further showed that two other hESC lines were more primed than their differentiated progeny (Figure S2A), suggesting that the correlation between priming and pluripotency is not limited to one specific hESC line.

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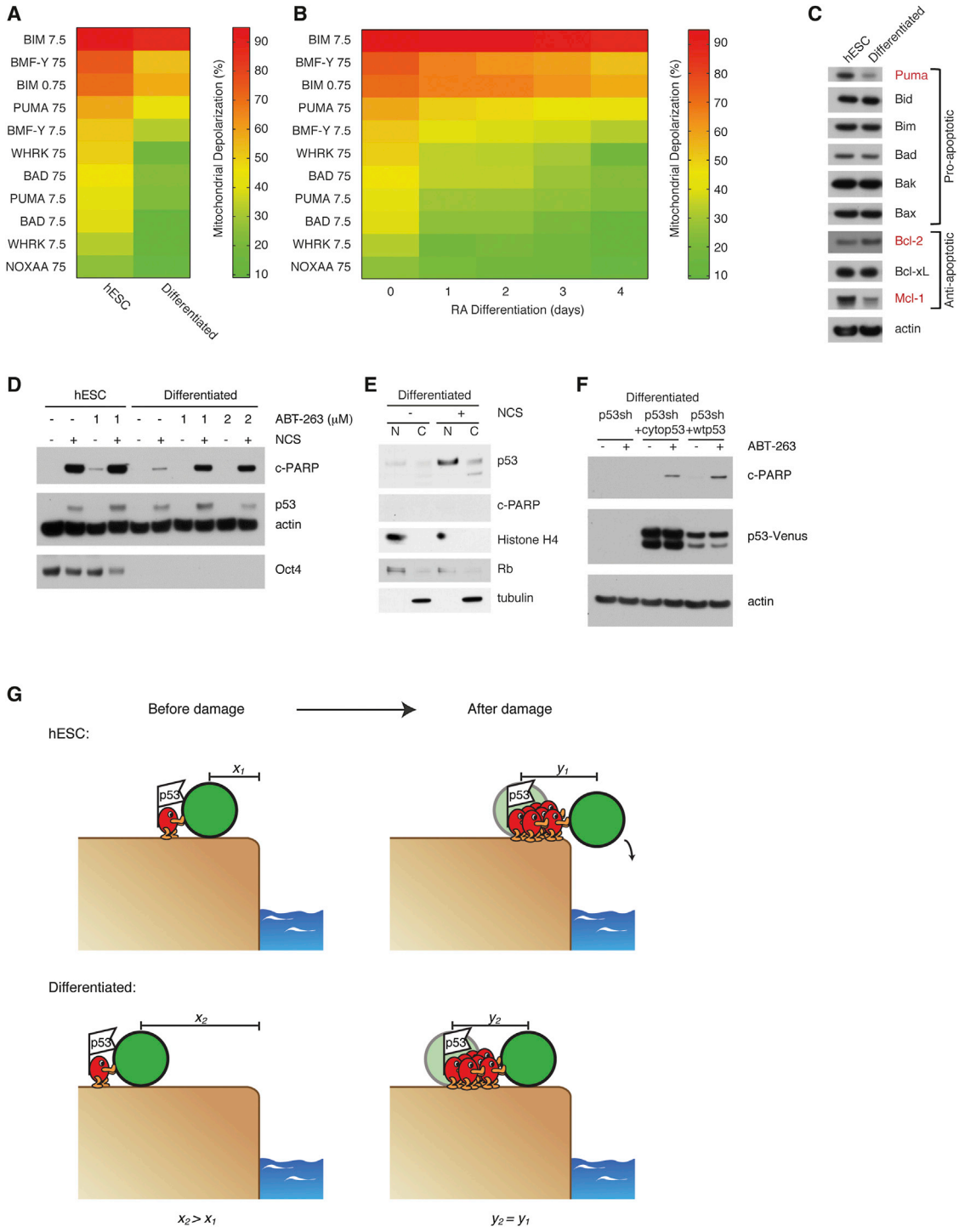

Figure 4. BH3 Profiling Reveals High Priming of hESCs toward Apoptosis

(A) Heat map of mitochondrial depolarization (percentages indicated by color bar) caused by BH3-only peptides at the indicated concentrations (μM) in hESCs and differentiated cells.

(B) Heat map of mitochondrial depolarization caused by BH3-only peptides in hESCs treated with 1 μM RA for 0, 1, 2, 3, and 4 days.

(C) Immunoblots of proapoptotic and antiapoptotic proteins in hESCs and differentiated cells. Colored in red are proteins that show differential levels between hESCs and differentiated cells.

(D) Immunoblot of c-PARP, p53, and Oct4 in hESCs and differentiated cells, untreated, treated with 100 ng/ml NCS for 3 hr, or treated with the indicated concentration of ABT-263 for 4 hr (1 hr pretreatment when combined with NCS).

(E) Immunoblot of p53 and c-PARP in the nuclear and cytoplasmic fractions of hESCs and differentiated cells. Cells were undamaged or damaged with NCS for 3 hr. Histone H4 and the transcription factor Rb serve as nuclear markers and tubulin serves as a cytoplasmic marker. N, nuclear; C, cytoplasmic.

(legend continued on next page)

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What might lead to high mitochondrial priming in hESCs? We first tested whether p53 itself contributes to high priming in undifferentiated cells. Silencing of p53 did not reduce priming; in fact, we found a slight increase in priming compared with hESCs expressing scramble shRNA (Figure S2B). Hence, hESCs can remain highly primed toward apoptosis independently of p53. Next, we sought to probe the status of the apoptotic machinery by measuring levels of proapoptotic and antiapoptotic proteins in hESCs and their differentiated progeny (Figure 4C). While most proteins showed comparable levels between undifferentiated and differentiated cells, we observed two differences that are consistent with the correlation between priming and pluripotency. Specifically, hESCs had lower levels of the antiapoptotic protein Bcl-2 and higher levels of the proapoptotic protein Puma (Figure 4C). Counterintuitively, undifferentiated cells showed higher levels of the antiapoptotic protein Mcl-1. Taken together, these results suggest that Mcl-1 is not a major determinant of priming in hESCs; instead, the basal balance between other antiapoptotic and proapoptotic proteins, such as Bcl-2 and Puma, contributes to the differential priming of undifferentiated and differentiated cells.

We next asked whether perturbing the balance between proapoptotic and antiapoptotic proteins can enable differentiated cells to activate apoptosis. We used ABT-263, a BH3-mimetic drug that binds and inhibits antiapoptotic proteins including Bcl-2 (Tse et al., 2008), and we measured c-PARP after DNA damage. We found that differentiated cells treated with ABT-263 successfully activated apoptosis after damage (Figure 4D). This result suggests that the intrinsic balance of proapoptotic and antiapoptotic proteins is closer to the apoptotic threshold in undifferentiated cells than it is in differentiated cells. Perturbation of this balance in differentiated cells changes their priming and leads to apoptosis. Notably, accumulation of c-PARP after damage in hESCs treated with ABT-263 did not exceed that in cells not treated with ABT-263 (lanes 2 and 4 of Figure 4D), suggesting that hESCs are already highly primed.

The observation that differentiated cells can trigger apoptosis when treated with ABT-263 suggests that p53 is fully functional in these cells. We have shown that apoptosis in undifferentiated cells results from p53 cytoplasmic activity. To determine whether cytoplasmic p53 is active only in hESCs and not in differentiated cells, we tested the localization of p53 in differentiated cells and the ability of cytoplasmic p53 to activate apoptosis in differentiated cells treated with ABT-263. First, we found that a small fraction of p53 was also localized to the cytoplasm in differentiated cells (Figure 4E). Next, we differentiated hESCs silenced for p53 and infected them with cytop53 and wtp53. Differentiated cells expressing the exogenous wtp53 showed behavior similar to that of their parental cells; they did not die after damage unless treated with ABT-263 (Figure 4F). Interestingly, differentiated cells expressing cytop53 also induced apoptosis after DNA damage under ABT-263 treatment, showing that cytoplasmic p53 is functional in differentiated cells, but lower mitochondrial priming in these cells prevents the activation of apoptosis in response to DNA damage.

In summary, we have shown that p53 exhibits similar apoptosis-inducing behavior in both undifferentiated and differentiated cells. However, differentiated cells are far from the apoptotic threshold, and p53 is unable to overcome this barrier. Only when the balance of proapoptotic to antiapoptotic proteins is altered in differentiated cells can p53 push cells toward apoptosis. In contrast, p53 can successfully push hESCs past the apoptotic threshold due to their highly primed state (Figure 4G).

DISCUSSION

A cell's decision among different cell fates is especially critical in response to challenging inputs such as DNA damage. Different cell types meet this challenge in a variety of ways. In cell types where maintenance of genomic integrity is crucial, such as hESCs, one might expect that signaling pathways are poised to cope with DNA damage aggressively by selecting terminal cell fates such as apoptosis. Here we show that hESCs use multiple pathways to ensure apoptosis is induced after damage, including activation of p53 transcription and cytoplasmic functions and high mitochondrial priming. We have shown that in hESCs p53's transcriptional activity is not required to induce apoptosis; rather, cytoplasmic p53 is sufficient for induction of apoptosis without transcription of downstream p53 target genes. Yet proapoptotic p53 target genes such as *PUMA*, *NOXA*, and *APAF1* are upregulated after damage in a p53-dependent manner, suggesting that hESCs may use the p53 transcriptional pathway as a second line of defense to ensure cell death in case cytoplasmic p53 fails to do so.

We have shown here that cytoplasmic p53 contributes to induction of apoptosis in hESCs. However, the mechanisms by which cytoplasmic p53 exerts its function remain open. A recent study showed that hESCs maintain active Bax that rapidly localizes from the Golgi to the mitochondria after damage and that this translocation is p53 dependent (Dumitru et al., 2012). The role of p53 in generating the translocation of active Bax, however, remains unclear. Interestingly, this mechanism was employed to various extents by different hESC lines; the cell line primarily used in our study, H1, did not show active Bax under basal conditions (Dumitru et al., 2012). Yet we show

(F) Immunoblot of c-PARP, p53, and Oct4 in cells differentiated from p53shRNA hESCs (p53sh) and, where indicated, infected with wtp53 and cytop53 constructs. Prior to infection, cells were differentiated with 1 μM RA and passaged, maintaining RA in the media. All cells were treated with NCS for 3 hr and, where indicated, pretreated with 1 μM ABT-263 for 1 hr before NCS addition.

(G) The proximity of hESCs, unlike differentiated cells, to the apoptotic threshold makes them sensitive to increased p53 after damage. Mitochondrial priming is depicted as proximity to the cliff's edge (the apoptotic threshold). A cell, shown as a green ball, is at distance x_1 from the edge if it is an hESC, and at distance x_2 from the edge if it is differentiated, where $x_2 > x_1$. Prior to damage, basal low levels of p53, represented as a red cartoon, are insufficient to push either an hESC or a differentiated cell over the cliff. Damage causes p53 to accumulate in both hESCs and differentiated cells to similar levels, with similar functions. The distance that p53 pushes the cell toward the cliff is represented by the displacement y_1 for the hESC and y_2 for the differentiated cell, where $y_1 = y_2$. For an hESC, $y_1 > x_1$, sufficing to push the cell over the cliff, crossing the apoptotic threshold and leading to cell death. In contrast, a differentiated cell is too far away from the edge ($y_2 < x_2$), so p53 function is insufficient to push the cell beyond the apoptotic threshold, allowing the cell to survive after damage.

See also Figure S2.

here that H1 cells also undergo rapid apoptosis after DNA damage, suggesting cytoplasmic p53 can trigger the same end result via other mechanisms.

While we have shown that the levels of the antiapoptotic protein Bcl-2 and the proapoptotic protein Puma are consistent with the high priming in hESCs in comparison with differentiated cells, the complete network that determines mitochondrial priming is likely to involve additional players. Even for apoptosis-regulating proteins that show similar levels in undifferentiated and differentiated cells (Figure 4C), specific modifications, localization, and protein interactions might affect their function and therefore generate differential priming. Furthermore, the relationship between priming and pluripotency might depend on other networks. For example, pluripotency is known to be linked to rapid cell cycle progression (Filipczyk et al., 2007), which may directly or indirectly affect the apoptotic machinery. Complete understanding of the mechanisms controlling priming will require global analysis of protein function and interaction in multiple pathways.

EXPERIMENTAL PROCEDURES

Standard procedures were followed for cell culture, flow cytometry, qRT-PCR, immunoblotting, IF, and siRNA knockdown, as described in the Supplemental Experimental Procedures.

Cell Line Construction

The p53shRNA construct with a blasticidin resistance cassette was kindly provided by the Agami lab (Brummelkamp et al., 2002). The cytop53 and wtp53 constructs are resistant to this p53shRNA due to silent point mutations described in the Supplemental Experimental Procedures. The lentiviral vectors for cytop53 and wtp53 were created using standard molecular biology techniques to include an upstream ubiquitin promoter, p53, and an mVenus tag. Point mutations for the K305A and R306A amino acid substitutions in the NLS region of p53 for the cytop53 construct were introduced using site-directed mutagenesis (Quikchange kit, Agilent). Further details regarding primers, viral production, and infection are included in the Supplemental Experimental Procedures.

BH3 Profiling

hESCs were dissociated with Accutase, counted, and suspended in DTEB buffer (135 mM trehalose, 10 mM HEPES, 50 mM KCl, 20 μM EDTA, 20 μM EGTA, 5 mM potassium succinate, final pH 7.5) at 2.67×10^6 cells/ml. The cells were then added to an equal volume of 4× staining mastermix (4 μM JC-1, 40 μg/ml oligomycin, 20 mM 2-mercaptoethanol, 100 μg/ml digitonin in DTEB buffer) and allowed to stain for 10 min at RT. 15 μl of stained cells were added to wells containing 15 μl of peptides at 2× final concentration in DTEB to yield the final profiling plate with 20,000 cells/well. Fluorescence at 590 nm was monitored using 545 nm excitation on a Tecan Safire 2 at a controlled temperature of 30°C with automated readings every 5 min. The area under each peptide response curve was calculated using Graphpad Prism, and these areas were normalized to the internal FCCP and DMSO controls as Depolarization (%) = 1 – ([sample-FCCP]/[DMSO-FCCP]).

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.07.018.

ACKNOWLEDGMENTS

We thank R. Agami for the p53shRNA construct; G. Daley for the CHB8 cell line; D. Melton for the HUES9 cell line; Y. Xu for the HUES9 *p53*$^{-/-}$ cell line; A. Baccei, B. Gorman, and K. Karhohs for assistance with IF imaging and analysis; J. Moore and J. Stewart-Ornstein for assistance with flow cytometry and analysis; J. Reyes for assistance with illustration; and E. Batchelor and all members of our laboratory for comments and discussions. J.C.L. was supported by the Molecular Biophysics Training Grant (NIH/NIGMS T32008313) and the National Science Foundation Graduate Research Fellowship. A.G.R. was supported by the Harvard College Program for Research in Science and Engineering and the FAS Center for Systems Biology. P.H.L. was supported by the Charles H. Hood Foundation and NIH/NICHD HD061981.

Received: February 13, 2013
Revised: June 12, 2013
Accepted: July 24, 2013
Published: August 15, 2013

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