P53 Regulates Rapid Apoptosis in Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSCs) are sensitive to DNA damage and undergo rapid apoptosis compared to their differentiated progeny cells. Here, we explore the underlying mechanisms for the increased apoptotic sensitivity of hPSCs that helps to determine pluripotent stem cell fate. Apoptosis was induced by exposure to actinomycin D, etoposide, or tunicamycin, with each agent triggering a distinct apoptotic pathway. We show that hPSCs are more sensitive to all three types of apoptosis induction than are lineage-non-specific, retinoic-acid-differentiated hPSCs. Also, Bax activation and pro-apoptotic mitochondrial intermembrane space protein release, which are required to initiate the mitochondria-mediated apoptosis pathway, are more rapid in hPSCs than in retinoic-acid-differentiated hPSCs. Surprisingly, Bak and not Bax is essential for actinomycin-D-induced apoptosis in human embryonic stem cells. Finally, P53 is degraded rapidly in an ubiquitin-proteasome-dependent pathway in hPSCs at steady state but quickly accumulates and induces apoptosis when Mdm2 function is impaired. Rapid degradation of P53 ensures the survival of healthy hPSCs but avails these cells for immediate apoptosis upon cellular damage by P53 stabilization. Altogether, we provide an underlying, interconnected molecular mechanism that primes hPSCs for quick clearance by apoptosis to eliminate hPSCs with unrepaired genome alterations and preserves organismal genomic integrity during the early critical stages of human embryonic development.

Introduction

Human pluripotent stem cells (hPSCs) have both the ability to self-renew and the ability to differentiate into all cell types in the human body [1,2]. Genomic integrity of hPSCs in utero and in tissue culture is essential for cell lineage fidelity during further development and differentiation, and therefore, a rapid response to cellular damage is required. Human embryonic stem cells (hESCs) have been shown to undergo rapid apoptosis in preference to repair in response to DNA damage when compared to differentiated cells [3–8]. The mechanisms, however, that potentiate the rapid response of DNA-damaged hESCs toward apoptosis instead of repair are still not fully understood. The tumor suppressor protein P53 is a known regulator of apoptosis in response to DNA damage in hESCs [3–5,7,8]. In somatic cells, following

DNA damage, P53 contributes to apoptosis induction and execution through transcriptional activation of pro-apoptotic genes, through sequestration of anti-apoptotic proteins, or by interacting with and positively influencing pro-apoptotic proteins, Bax and Bak, in the cytosol [9–15]. Knockdown of *P53* in hESCs eliminates the apoptotic response to DNA damage. hESCs expressing P53 lacking a nuclear localization signal can activate apoptosis in response to DNA damage, indicating that cytosol-localized P53, in addition to nuclear P53, contributes to apoptosis in hESCs [5]. Inhibition of cyclin-dependent kinase CDK1 can selectively induce the DNA damage response and P53-dependent apoptosis in hESCs, in contrast to only causing transient cell cycle arrest during DNA repair in differentiated cells [16]. Expression of P53 target genes is rapidly induced in response to DNA damage in embryonic stem cells, but this rapid response is also seen in differentiated cells [5]. Whereas P53 plays a large and potentially distinct role in the DNA damage responses of hESCs and somatic cells, no actual differences in the apoptosis-inducing behavior of P53 or its regulation have yet been identified between hPSCs and differentiated cells. Instead, what has been reported is that the mitochondria in hPSCs are primed for apoptosis due to a difference in the balance between pro-apoptotic and anti-apoptotic proteins, leading to a higher sensitivity and lower apoptotic threshold for hESCs compared to differentiated cells [5,17].

Activation of apoptosis by cell intrinsic stimuli, such as DNA damage, occurs through mitochondrial outer membrane permeabilization (MOMP), which requires the activation of pro-apoptotic BCL-2 family member protein Bax or Bak [11,18,19]. Some hESC lines show constitutively activated pro-apoptotic Bax localized to the Golgi apparatus during S phase, where it is unable to activate apoptosis until DNA damage induces its translocation to the mitochondria to induce MOMP [20]. Knockdown of *Bax* in hESCs decreases apoptosis in response to DNA damage. Additionally, P53 is required for the translocation of Bax from the Golgi apparatus to the mitochondria with DNA damage in hESCs [20]. In other cell types and hESC lines, Bax is localized to the cytosol in an inactive state. Once activated by BH3-only proteins, Bax undergoes a conformational change and insertion into the mitochondrial outer membrane [18,21]. Nonetheless, since activated Bax is not detectable in the Golgi apparatus of most hESC lines, this potential sensitizing mechanism cannot be exclusively responsible for the rapid activation of apoptosis in response to DNA damage in these hESC lines [20].

Here, we further investigate apoptotic mechanisms in hPSCs and discover that differential regulation of P53 stability sensitizes hPSCs to apoptosis. Initially, we evaluated the similarities and differences in the apoptotic machinery between hPSCs and differentiated cells to elucidate the pathways underlying the rapid activation of apoptosis in hPSCs. We discovered that hPSCs activate apoptosis rapidly not only in response to DNA damage but also in response to transcriptional inhibition and the induction of endoplasmic reticulum (ER) stress. In addition, we identified important roles for the mitochondrial fission protein Drp1 and pro-apoptotic BCL-2 family member protein Bak in hESC apoptotic hypersensitivity. Finally, we report that P53 is rapidly degraded at steady state in hPSCs, but inhibition of ubiquitin-proteasome-dependent degradation by Mdm2 causes prompt stabilization of P53 and the induction of apoptosis in hESCs.

Results

hESCs are hypersensitive to diverse mitochondria-mediated apoptotic stimuli

In addition to rapid apoptosis in response to DNA damage, hPSCs also undergo mitochondria-dependent apoptosis upon dissociation into single cells, which can be suppressed by Rho-dependent protein kinase inhibitors [22,23]. Therefore, we considered whether hPSCs are more generally hypersensitive to mitochondria-mediated apoptosis by evaluating whether hPSCs are more sensitive to multiple intrinsic apoptotic stimuli than their differentiated counterparts. We used retinoic acid (RA)-induced differentiation of hPSCs as a differentiated comparative cell derivative of hPSCs [24–26]. RA is an important morphogen during development and is commonly used for neuronal differentiation [27–29]. RA treatment of monolayer culture of hESCs causes preferential differentiation into ectodermal and mesodermal lineages [30–32]. Undifferentiated hPSCs and RA-differentiated cells were evaluated for apoptosis by incubation with various apoptosis-stimulating agents. Exposure of H9 hESCs to RA reduced the expression of Nanog and Oct4, key transcription factors for maintaining hPSC self-renewal, to undetectable levels by days 3 and 5, respectively (Fig. 1a). Actinomycin D, a potent inducer of apoptosis through transcriptional repression, was used to induce apoptosis in H9 and H1 hESCs and in a human induced pluripotent stem cell (hiPSC) line, HIPS2, on days 0, 1, 3, and 5 of RA-induced differentiation (Fig. 1b and Fig. S1b and c). The fragmentation of cells into apoptotic bodies is carried out by a family of intracellular cysteine-dependent, aspartate-directed proteases (caspases). Upon activation of mitochondria-mediated apoptosis and MOMP, procaspase-3 is cleaved and in turn cleaved caspase-3 executes apoptosis through digestion of other intracellular proteins including the DNA repair protein poly(ADP-ribose) polymerase (PARP) [33–35]. Therefore, apoptosis was evaluated
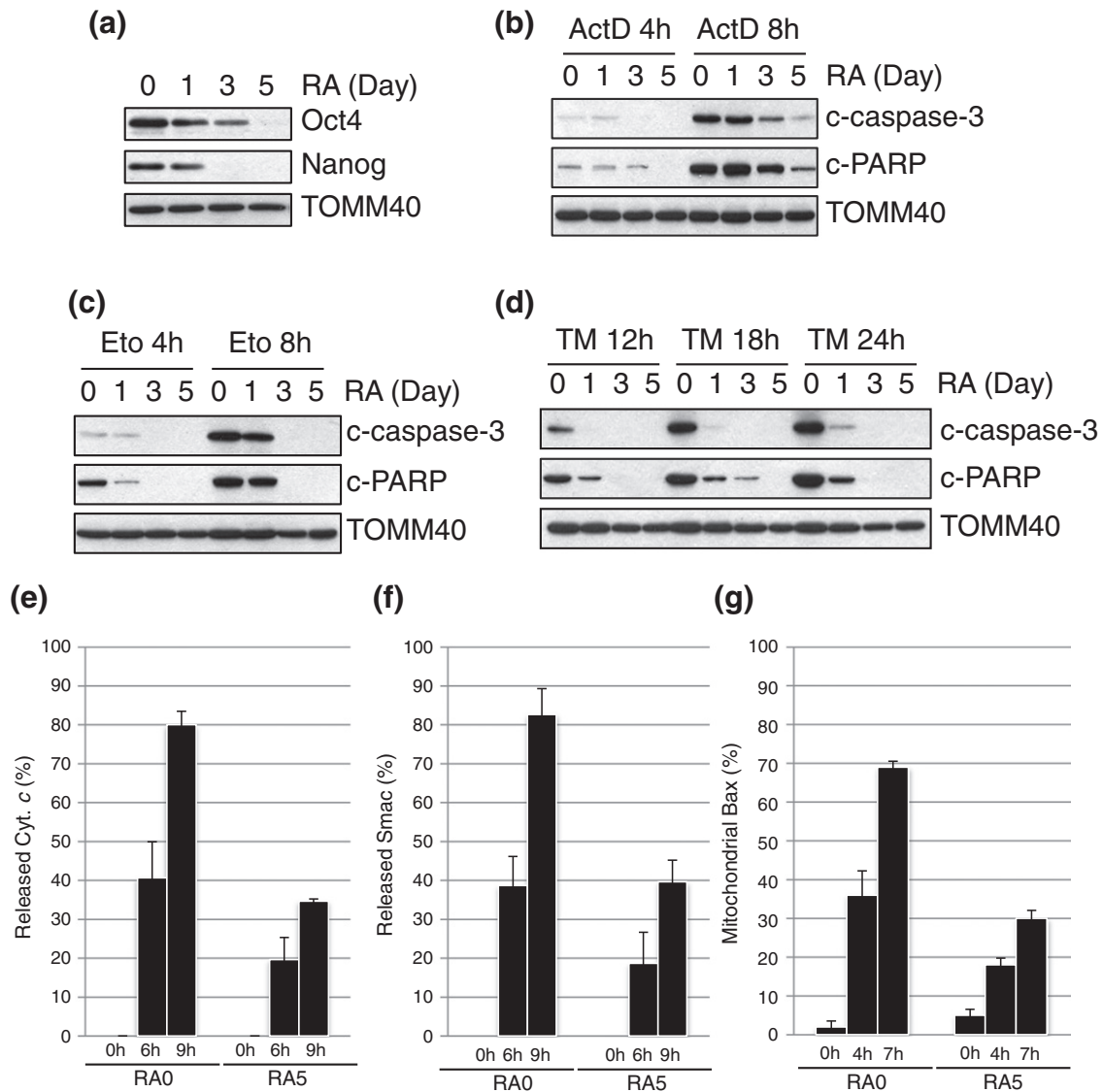
Fig. 1. Rapid apoptosis induction in hESCs. (a) Immunoblot of Oct4 and Nanog in H9 hESCs differentiated with 1 μM RA for 0, 1, 3, and 5 days. TOMM40 was included as a loading control. (b–d) H9 hESCs differentiated with 1 μM RA for 0, 1, 3, and 5 days were treated with multiple intrinsic apoptosis inducers: 1 μM actinomycin D (b), 1 μM etoposide (c), and 1 μg/ml tunicamycin (d) for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies for cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). TOMM40 was included as a loading control. (e and f) H1 hESCs were treated with actinomycin D in the presence of pan-caspase inhibitor Q-VD-OPH. Cells were fixed and IMS protein release of cytochrome *c* (e) and Smac (f) were detected by immunofluorescence confocal microscopy. Data were obtained from 100 cells in each of three independent experiments. Error bars represent standard deviation (SD). (g) Mitochondria-targeted Bax was detected by immunofluorescence confocal microscopy using anti-Bax and anti-Mortalin (mitochondrial chaperone) antibodies. Cells were treated as in (e) and (f) and Bax and TOMM20 co-localization were counted in each of three independent experiments. Error bars represent SD.

by Western blot analysis of cleaved caspase-3 and cleaved PARP. Undifferentiated hPSCs showed a robust time-dependent cleavage of caspase-3 and PARP after actinomycin D exposure (Fig. 1b and Fig. S1b and c). Although uncleaved caspase-3 showed only slightly decrease during differentiation, hPSCs that were differentiated for 5 days, however,

had only low levels of caspase-3 and PARP cleavage after actinomycin D treatment (Fig. 1b and Fig. S1a–c). Next, DNA damage was induced in undifferentiated and differentiated hPSCs by treatment with etoposide, which causes DNA double-strand breaks through the inhibition of DNA topoisomerase II [36]. Undifferentiated H9 and HIPS2 cells again show robust,

time-dependent cleavage of caspase-3 and PARP, but hPSCs differentiated for 5 days have no detectable cleavage of caspase-3 or PARP (Fig. 1c and Fig. S1d).

Apoptosis induced by ER stress is also mediated though the mitochondria [37–40]. To determine whether hPSCs are sensitive to ER stress-induced apoptosis, we treated undifferentiated and differentiated hESCs with tunicamycin, which induces ER stress through the inhibition of N-linked glycosylation [41]. Whereas high levels of cleaved caspase-3 and cleaved PARP were detected in undifferentiated hESCs, there was an absence of these cleaved apoptotic signature proteins in cells differentiated for 5 days (Fig. 1d).

Because cleavage of caspase-3 and PARP is a late event in apoptosis, we next evaluated the release of pro-apoptotic intermembrane space (IMS) proteins cytochrome *c* and Smac from the mitochondrial IMS upon MOMP [42–44]. Cytochrome *c* and Smac release was detected by imaging co-localization with the mitochondrial marker TOMM20 using confocal immunofluorescence microscopy following timed actinomycin D exposure. Cytochrome *c* and Smac were released into the cytosol from a greater percentage of undifferentiated hESCs than from differentiated cells (Fig. 1e and f and Fig. S2a and b). Bax recruitment to the mitochondria can lead to MOMP [11,18], and it precedes the release of IMS proteins from the mitochondria into the cytosol and caspase activation. Therefore, Bax localization to the mitochondria was also measured by immunofluorescence microscopy. Timed actinomycin D exposure induced the mitochondrial localization of Bax in a greater percentage of undifferentiated hESCs than in differentiated cells (Fig. 1g and Fig. S3a and b).

Mitochondrial elongation reduces the sensitivity of hPSCs to apoptosis

Mitochondria are fused, interlacing network structures that extend peripherally in the cytosol of most differentiated cell types, in contrast to the fragmented, punctate, perinuclear appearance of mitochondria in
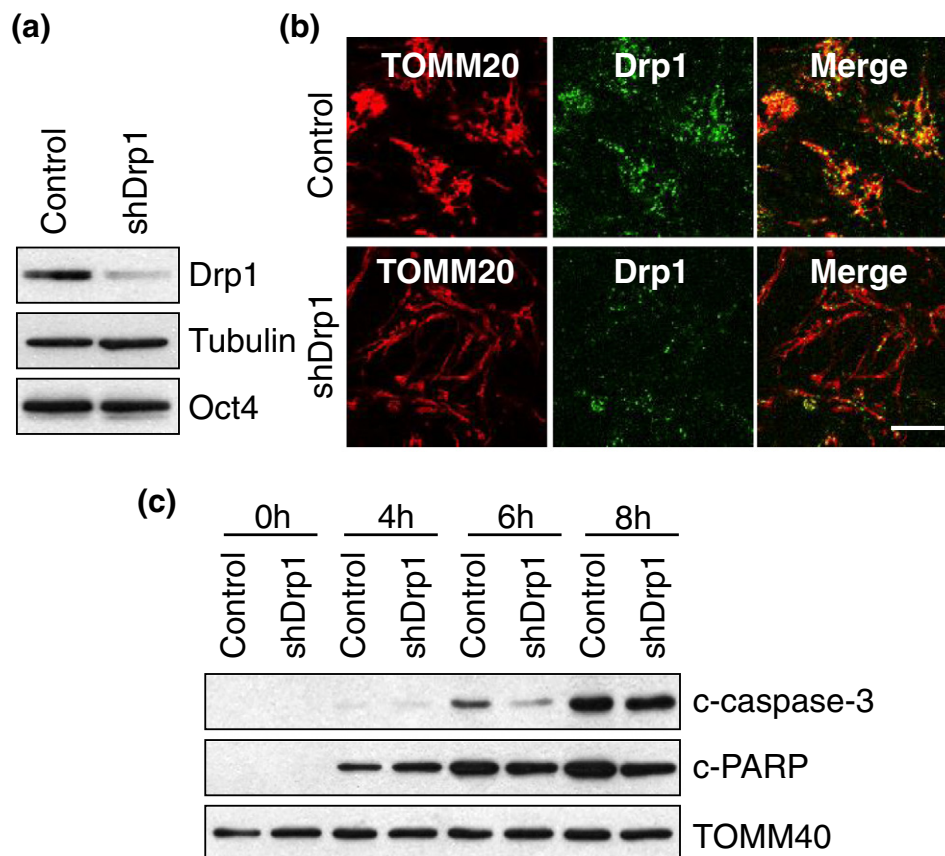

Fig. 2. Knockdown of Drp1 induces mitochondria elongation and suppressed apoptosis sensitivity in hESCs. (a) Knockdown efficiency of Drp1 in H9 hESCs was confirmed by immunoblot. (b) Mitochondrial morphology in H9 hESCs transduced with *Drp1* or control shRNAs was analyzed by immunofluorescence confocal microscopy using anti-TOMM20 antibody. (c) H9 hESCs transduced with *Drp1* or scramble shRNAs were exposed to actinomycin D for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies for cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). TOMM40 was included as a loading control.

hPSCs [24,45]. Mitochondrial fragmentation occurs during apoptosis [46–48], and therefore, cells with a more fragmented network structure may be more sensitive to apoptotic stimuli. The dynamin-related GTPase Drp1 mediates fission of the mitochondrial network and localizes to the mitochondrial outer membrane at sites of fission [46,49,50]. In somatic cells, Drp1 depletion retards the usual rate of cytochrome *c* release and caspase activation during apoptosis [46]. To determine whether the fragmented mitochondrial morphology seen in hESCs influences their hypersensitivity to intrinsic apoptotic stimuli, we inhibited mitochondrial fission by shRNA knockdown of *Drp1* (Fig. 2a). Immunofluorescent confocal imaging of TOMM20, a mitochondrial outer membrane translocon protein, in *Drp1* knockdown hESCs revealed mitochondria that were more elongated when compared to scramble control hESCs (Fig. 2b). Therefore, Drp1 depletion induces mitochondrial network fusion in hESCs. To determine whether an elongated mitochondrial morphology decreases the apoptotic hypersensitivity of hESCs, we induced apoptosis by actinomycin D treatment in *Drp1* knockdown and scramble control hESCs. Timed actinomycin D exposure resulted in less cleavage of caspase-3 and PARP in Drp1 knockdown hESCs when compared to scramble control (Fig. 2c). Thus, the fragmented mitochondrial structure in hESCs helps to promote their sensitivity to apoptosis induction.

Bak has a crucial role in hPSC hypersensitivity to apoptosis

Activation of BCL-2 family member proteins Bak and Bax causes their oligomerization and initiates MOMP, which releases IMS proteins into the cytosol and activates the caspase cascade [19,51,52]. Bak and Bax have overlapping roles during development. Bak-deficient mice develop normally, and Bax-deficient mice have limited abnormalities. Double knockouts for both Bax and Bak, however, die perinatally with multiple developmental defects related to defective apoptosis induction, such as persistent interdigital webs and excessive cells in the central nervous and hematopoietic systems [53]. In addition, Bax has been shown to be important for the apoptotic response to etoposide in hESCs, but the roles of Bax and Bak have not been analyzed in response to other apoptotic stimuli [20]. Therefore, because Bax or Bak is essential for mitochondria-mediated apoptosis and their contribution to apoptosis in hESCs is controversial, we next investigated whether either protein had a more dominant role in mediating hESC hypersensitivity to apoptosis. Bak, Bax, and BCL-XL protein levels are not markedly changed during RA-induced differentiation (Fig. 3a). To further assess the role of Bax and Bak in hESC sensitivity to apoptosis, we separately knocked down the expression of *Bax* and *Bak* with shRNA

in H1 and H9 hESCs (Fig. 3b and Fig. S4a). The expression of pluripotency biomarker proteins SSEA4, Lin28, Nanog, and Oct4 was not affected by knockdown of either *Bax* or *Bak* (Fig. 3c). Activation of apoptosis by exposure to actinomycin D was almost completely inhibited in *Bak* knockdown cells (Fig. 3d and Fig. S4b and c). Therefore, Bak is an important component contributing to the hypersensitivity of hESCs to mitochondria-mediated apoptosis. In contrast, knockdown of *Bax* only slightly decreased the induction of apoptosis in hESCs (Fig. 3d and e and Fig. S4b and c). With etoposide treatment, however, Bax played a more crucial role as previously reported (Fig. S4d) [20]. While Bax has an important role in hESC response to etoposide, Bak is important for the response to actinomycin D. This suggests that both Bax and Bak play important roles in hPSC apoptosis.

P53 turnover is rapid in hPSCs

Tumor suppressor P53 is a central regulator of growth arrest, senescence, and apoptosis in response to a broad array of cellular damage [10,13,54–56]. Regulation of P53 is mainly accomplished through ubiquitin-proteasome-dependent degradation [57]. Surprisingly, while P53 has been carefully studied in hPSCs, differences in P53 protein stability have not been examined as the potential cause of hPSC hypersensitivity. Mdm2 is the primary E3 ubiquitin ligase that negatively regulates P53 by degradation or by masking the transactivation domain of P53 [58–60]. Mdm2, in turn, is degraded following DNA damage that stabilizes P53 [61,62].

In H1 hESCs, Mdm2 protein levels decrease after RA-induced differentiation, whereas P53 protein levels do not change (Fig. 4a). To determine whether the decrease in Mdm2 was regulated transcriptionally, we performed quantitative real-time PCR to measure Mdm2 mRNA levels. No change in Mdm2 expression was detected between hESCs and RA-differentiated cells (Fig. S5a). P53 expression was 2-fold higher in H9 hESCs than in RA-differentiated H9s, but no change was seen in H1 cells (Fig. S5b). To determine whether the p53 protein that we detected was wild-type p53, we compared the molecular weight of p53 in H1 hESC cell lysate with recombinant p53 and observed equal molecular weights (Fig. S5c).

To determine whether p53 protein stability is differentially regulated in hESCs and their differentiated counterparts, we treated cells with an Mdm2 inhibitor, nutlin-3a [63]. Timed exposure of hESCs to nutlin-3a rapidly stabilizes high levels of P53 (Fig. 4b). Treatment of differentiated cells with nutlin-3a, however, stabilizes only a small amount of P53 (Fig. 4b). Furthermore, treatment of hPSCs with nutlin-3a activates apoptosis, but apoptosis is
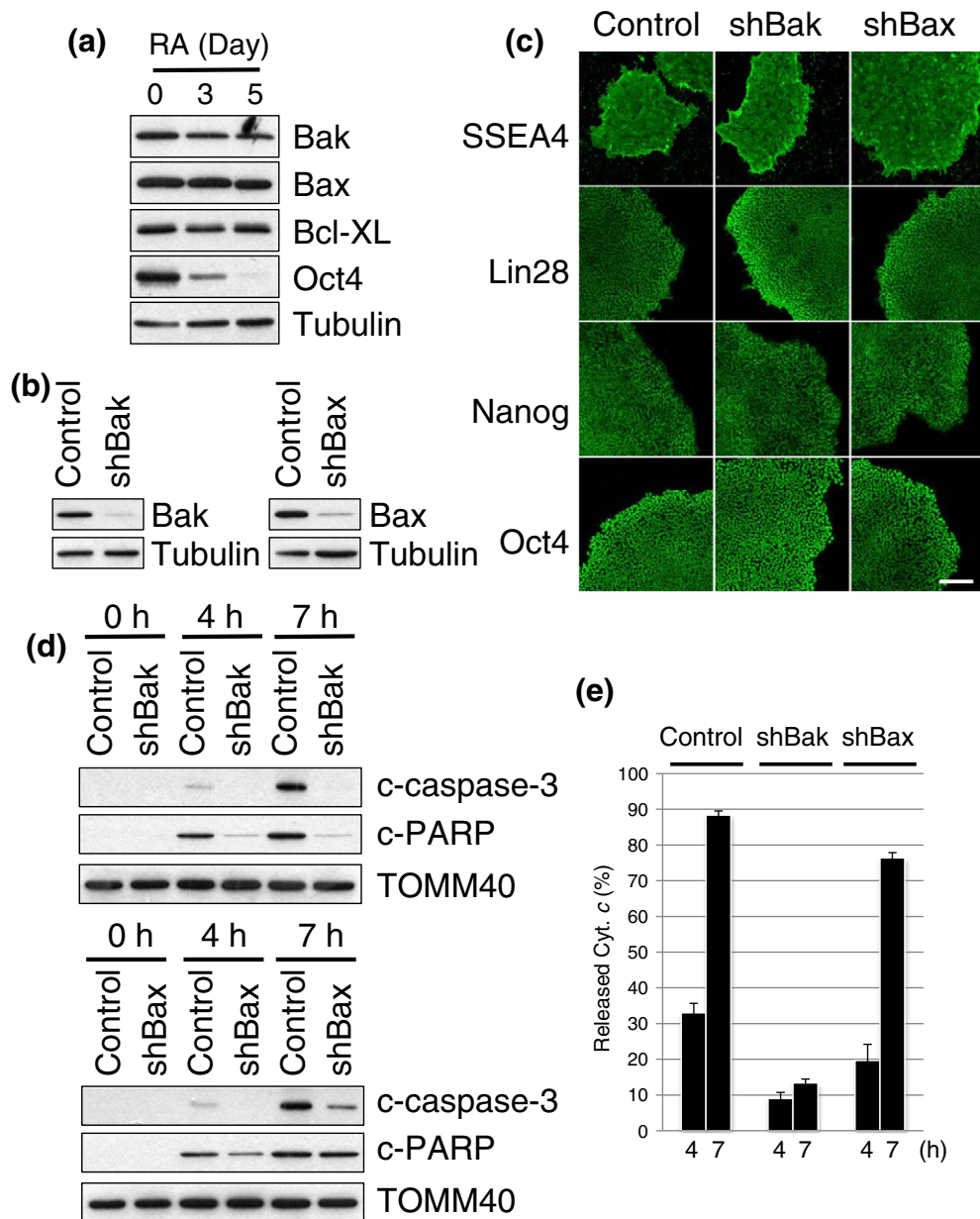
Fig. 3. Bak has a dominant role in actinomycin-D-induced apoptosis of hESCs. (a) Immunoblots of BCL-2 family proteins in H1 hESCs differentiated with 1 µM RA for 0, 3, and 5 days. (b) Immunoblots showing Bak and Bax protein levels in scramble, *Bak*, and *Bax* knockdown H1 hESCs. Tubulin was included as a loading control. (c) Immunofluorescent detection of pluripotency biomarker proteins from scramble, *Bak*, and *Bax* knockdown H1 hESCs. Scale bar is 200 µm. (d) Scramble, *Bak*, and *Bax* knockdown H1 hESCs were exposed to actinomycin D for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies for cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). TOMM40 was included as a loading control. (e) H1 hESCs were exposed to actinomycin D in the presence of the pan-caspase inhibitor Q-VD-OPH for the indicated times. Cells were fixed and analyzed by immunofluorescence confocal microscopy using antibodies against cytochrome *c*. Data were obtained from three independent experiments. Error bars represent SD.

not detectable after nutlin-3a treatment of RA differentiated cells (Fig. 4b). Therefore, p53 is rapidly degraded in hPSCs through ubiquitination by Mdm2. To further probe the difference in P53 stability, we treated H1, H9, and HIPS2 hPSCs with the protein translation inhibitor cycloheximide (CHX). After 2 h of CHX exposure, P53 levels were diminished (Fig. 4c and d and Fig. S5d and f). In contrast, P53 slowly decreases after CHX exposure in RA-differentiated hPSCs (Fig. 4e and f and Fig. S5e and g). Additional treatment with the
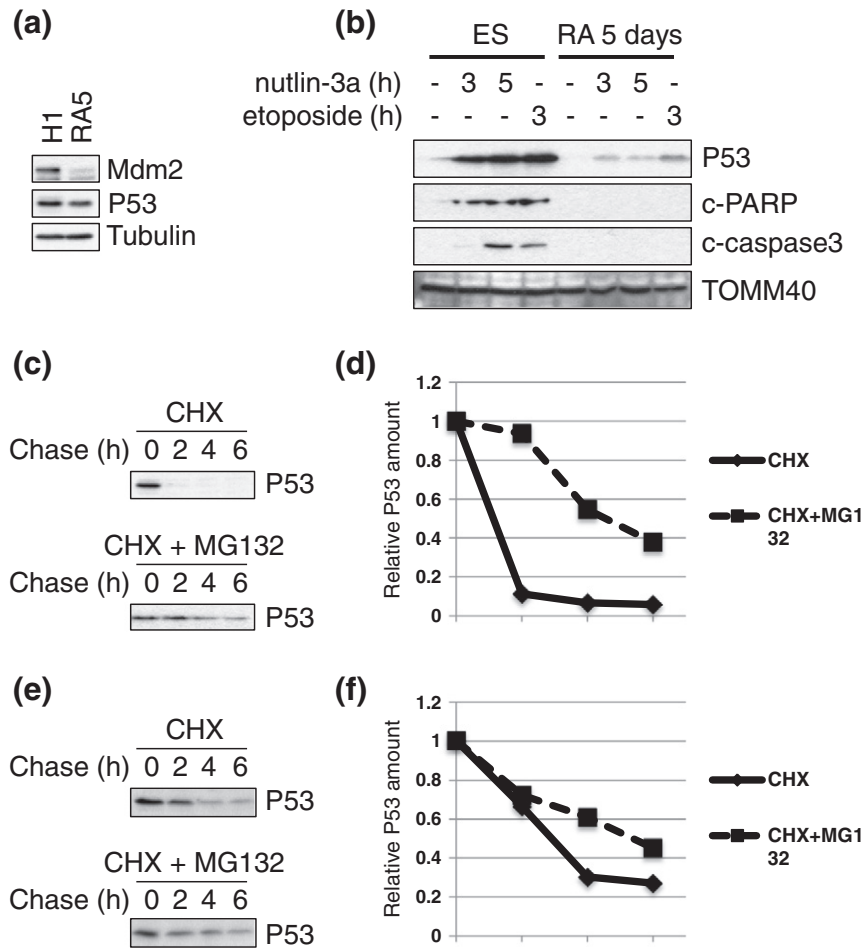
Fig. 4. P53 turnover is rapid in hESCs. (a) Immunoblot of Mdm2 and P53 in H1 hESCs and day 5 RA-induced H1 differentiated cells. (b) Immunoblot of P53, cleaved PARP (c-PARP), and cleaved caspase-3 (c-caspase-3) in H1 hESCs and RA-differentiated H1 cells after 1 μM etoposide or 4 μM nutlin-3a for the indicated times. (c and e) P53 protein stability analyzed by immunoblot in undifferentiated H1 hESCs (c) and H1 cells differentiated with RA for 5 days (e) were examined by CHX exposure for the indicated times in the presence of DMSO or MG132. (d and f) Band intensities corresponding to P53 protein levels in undifferentiated H1 hESCs (d) and H1 cells differentiated with RA for 5 days (f) were quantified by ImageJ and shown as relative amounts. Protein levels at 0 h were set as 1.0.

proteasome inhibitor MG132 slowed the decrease in levels of P53 in both hPSCs and RA-differentiated hPSCs, indicating that P53 degradation is dependent on ubiquitin proteasome (Fig. 4c and f and Fig. S5d–g). This indicates that P53 undergoes Mdm2-initiated, ubiquitin-proteasome-dependent degradation at a rate higher in hPSCs than in hPSCs induced to undergo differentiation.

Discussion

hESCs and hiPSCs have immense potential in regenerative medicine because of their ability to self-renew and to differentiate into every cell type in the human body. Therefore, maintenance of genomic integrity is essential to avoid defects that may cause aberrant development or cancer. Apoptosis is one available mechanism for eliminating damaged cells following DNA or other forms of cellular damage [64]. Recent studies have shown that hESCs undergo rapid apoptosis; however, the mechanisms underlying this enhanced apoptosis sensitivity still remain unclear [3–5,20].

Here, we show that hPSCs undergo rapid mito-chondria-dependent apoptosis in response to three independent apoptotic stimuli. Furthermore, we investigated each step of the cell death cascade and found hPSCs to be hypersensitive all stages. We further showed the importance of mitochondria in rapid apoptosis by inducing elongated mitochondria by Drp1 depletion. The inhibition of rapid apoptosis in hESCs by Drp1 depletion emphasizes the involvement of mitochondria in this unique event. We further examined the contribution of Bax and Bak, two main pro-apoptotic members of the BCL-2 protein family that directly mediate the mitochondrial

apoptosis pathway [39]. Although a recent study showed that etoposide-induced hESC apoptosis is Bax dependent and is Bak independent, our data showed a crucial role for Bak in actinomycin-D-induced apoptosis, suggesting that both Bax and Bak have an important role in hESC apoptosis that is regulated by many apoptotic factors in a complex manner.

Mdm2, a negative regulator of P53, is inactivated following DNA damage, leading to P53 stabilization, which can initiate apoptosis [61,62]. We discovered that a rapid accumulation of P53 caused by Mdm2 inhibitor nutlin-3a is sufficient to induce apoptosis in hESCs, but only has a modest effect in differentiated cells. However, the P53 protein level in steady-state cells is similar in both hESCs and RA-differentiated cells. Therefore, hESCs rapidly turn over p53 in an ubiquitin-proteasome-dependent manner that correlates with high expression of Mdm2. A previous study reported that P53 exhibits no difference in apoptosis-inducing behavior in both differentiated and undifferentiated hESCs and, therefore, the rapid apoptosis in hESCs is due to their highly mitochondrial primed state [5]. However, because P53 interacts with mitochondrial proteins [7], P53 could be a key factor that primes the mitochondria for rapid induction of apoptosis in hESCs. Given that suppression of P53 increases the efficiency of hiPSC generation up to 10- to 20-fold [65], inactivation of P53 by Mdm2 in healthy hPSCs may be crucial for their survival and maintenance. Our study identifies a unique regulation of P53 in hESCs that prepares them to induce rapid apoptosis to maintain genome integrity while assuring survival at steady state.

Materials and Methods

Cell culture

H1, H9 [1], and HIPS2 [66] hPSCs were grown on matrigel and fed daily with mTeSR-1 medium (Stemcell Technologies), maintained at 37 °C and 5% CO_2. The medium was changed daily and passaged with Dispase (Stemcell Technologies) before confluent. Differentiation of hPSCs was induced by 1 μM RA treatment, with a fresh medium change every other day.

Antibodies and reagents

Antibodies were used against Oct4 (Santa Cruz Biotechnology), Nanog (Millipore), SSEA4 (Millipore), Lin28 (Cell Signaling), Tubulin (Sigma), cytochrome *c* (BD Biosciences), Smac/Diablo (BD Biosciences), Bax-NT (Millipore), Bak-NT (Millipore), Drp1 (BD Biosciences), Mdm2 (Santa Cruz Biotechnology), TOMM20 (Santa Cruz Biotechnology), BCL-XL (Cell Signaling), c-caspase-3 (Cell Signaling), full-length caspase-3 (Cell Signaling), c-PARP (Cell Signaling), and P53 (Cell Signaling) proteins. A polyclonal antibody against recombinant TOMM40 (Pacific Immunology) was described previously [67]. CHX, MG132, actinomycin D, and etoposide were obtained from Sigma; Q-VD-OPH was obtained from SM Biochemicals; recombinant human p53 protein was obtained from BD Pharmingen; and nutlin-3a was obtained from Cayman Chemical.

Western blotting

Cultured cells were harvested into SDS buffer [40 mM Tris–HCl (pH 6.8), 3% glycerol, and 1% SDS]. Protein concentrations were quantified using the BCA assay (Pierce). Immunodetection was performed with ECL reagent (GE Healthcare). For protein turnover analyses, cells were incubated with 50 μg/ml CHX with or without 10 μM MG132 for the times indicated in each figure legend.

Lentivirus-mediated shRNA knockdown in hESCs

To generate hESCs that are knocked down for *Drp1*, *Bak*, and *Bax*, we obtained shRNAs that specifically target *Drp1* (TRCN0000001097), *Bak* (TRCN0000033466), and *Bax* (TRCN0000033472) from Sigma. On day 1, hESC colonies on matrigel were transduced with 25 μl of 1×10^7 TU/ml of viral particles and 7 μg/ml polybrene for 8 h. On days 2 and 3, 75 μl of 1×10^7 TU/ml of viral particles was also added. Selection with 1 μg/ml puromycin was started on days 5–7 post-infection. Cell culture medium supplemented with puromycin was replaced daily.

Immunofluorescence confocal microscopy

Cells were grown on matrigel-coated chambered cover glass (Thermo Scientific). For cytochrome *c*/Smac release assays, as well as for Bax targeting analysis, cells were exposed to apoptosis stimuli in the presence of 25 μM Q-VD-OPH, a broad caspase inhibitor. Cells were fixed for 15 min at room temperature with pre-warmed 4% PFA and then permeabilized for 5 min with 1% Triton X-100 in PBS followed by incubation with primary antibodies. After incubation with AlexaFluor-conjugated secondary antibodies (Invitrogen), images were acquired with either LSM 5 PASCAL or LSM 780 confocal microscopes (Carl Zeiss).

Quantitative real-time PCR

Cultured cells were harvested with TRIzol reagent (Life Technologies). RNA was extracted according to manufacturer's instructions. RNA was converted into cDNA with iScript (Bio-Rad). Quantitative real-time PCR was performed on a LightCycler480 (Roche) using SYBR green (Roche). Primers used to detect *P53* were agg cct tgg aac tca agg at and ccc ttt ttg gac ttc agg tg. Primers used to detect *Mdm2* were gac tcc aag cgc gaa aac and ggt ggt tac agc acc atc agt. Primers used to detect *Eifb2* were ttg agg cga tta atg agc tg and tgg agt gaa tgt gct cca ga.

Conflict of Interest: The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.07.019.

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