Regulation of Apoptosis and Differentiation by p53 in Human Embryonic Stem Cells

Received for publication, November 9, 2006, and in revised form, December 18, 2006 Published, JBC Papers in Press, December 19, 2006, DOI 10.1074/jbc.M610464200

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The essentially infinite expansion potential and pluripotency of human embryonic stem cells (hESCs) makes them attractive for cell-based therapeutics. In contrast to mouse embryonic stem cells (mESCs), hESCs normally undergo high rates of spontaneous apoptosis and differentiation, making them difficult to maintain in culture. Here we demonstrate that p53 protein accumulates in apoptotic hESCs induced by agents that damage DNA. However, despite the accumulation of p53, it nevertheless fails to activate the transcription of its target genes. This inability of p53 to activate its target genes has not been observed in other cell types, including mESCs. We further demonstrate that p53 induces apoptosis of hESCs through a mitochondrial pathway. Reducing p53 expression in hESCs in turn reduces both DNA damage-induced apoptosis as well as spontaneous apoptosis. Reducing p53 expression also reduces spontaneous differentiation and slows the differentiation rate of hESCs. Our studies reveal the important roles of p53 as a critical mediator of human embryonic stem cells survival and differentiation.

Human embryonic stem cells (hESCs) are capable of essentially unlimited self-renewal and retain the developmental potential to differentiate into almost any cell type. These characteristics of hESCs make them attractive for tissue and cell-based therapies (1, 2). Previously, basic fibroblast growth factor and activin A were identified as self-renewal factors (3–6). However, for reasons that are not clear, hESCs often display high rates of spontaneous apoptosis and differentiation in culture, thus making the process of expanding these cells highly inefficient (3, 7–10). For example, Dravid et al. (8) reported that, under routine culture conditions, >30% of hESCs undergo spontaneous apoptosis. Furthermore, Ezashi et al. (12) showed that nearly 40% of hESCs undergo spontaneous differentiation after 12 days of culture in normoxic conditions. Finally, Maitra et al. (13) reported that multiple passages of hESCs can cause genomic alterations, which may limit the therapeutic application of hESCs. In contrast to hESCs, mouse embryonic stem cells (mESCs) undergo lower rates of spontaneous apoptosis and differentiation (14). Moreover, they maintain their pluripotency and genomic stability longer than hESCs (15). The reason for these different species-specific phenotypes in embryonic stem cells is currently unknown.

The p53 tumor suppressor gene is a strong candidate for playing a role in the observed phenotypes of hESCs, because it regulates various cellular processes, including apoptosis, differentiation, and genomic integrity (16). In many cell types p53 plays a crucial role in controlling apoptosis and cell cycle arrest when these cells are exposed to stress-inducing conditions (17). In response to stress, p53 accumulates and transactivates downstream target genes such as mdm2 (responsible for the feedback degradation circuitry of p53), p21 (responsible for cell cycle control), bax, noxa, and puma (responsible for DNA damage-induced apoptosis) (18).

Besides the transcription-dependent induction of apoptosis, p53 also induces apoptosis through a mitochondrial pathway. In this pathway, p53 binds to the outer mitochondrial membrane, induces its permeabilization, and forms complexes with the protective bcl XL and bcl 2 proteins. The binding of p53 to these factors triggers cytochrome c release and caspase activation (19–22). In addition to activating apoptotic pathways, p53 has also been shown to regulate cell differentiation and play a role in DNA repair (23, 24).

In this study, we examined the role of p53 during the expansion of hESCs. We found that p53 contributed to spontaneous and DNA damage-induced apoptosis of hESCs through a transcription-independent mitochondrial pathway. Reducing p53 expression can reduce spontaneous apoptosis of hESCs and promote their survival. Reducing p53 expression also reduced spontaneous differentiation of hESCs. This critical role of p53 in preventing differentiation of hESCs was consistent with a recent report indicating p53 can also induce differentiation in...
mESCs (9). Our studies reveal the important roles of p53 in regulating the survival and differentiation of hESCs.

EXPERIMENTAL PROCEDURES

Cell Culture and Karyotyping—Experiments were performed with H1 and H9 human embryonic stem cell lines, which were obtained from WiCell Research Institute (Madison, WI). Cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) in hESCs culture medium consisting of 80% Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen), 20% knock-out serum replacement (Invitrogen), 1 mM l-glutamine, 1% non-modified Eagle’s medium/F-12 (Invitrogen), 20% knock-out serum replacement (Invitrogen), 1 mM l-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (all from Invitrogen). Undifferentiated hESCs from passages 42–68 for H1 and passages 45–62 for H9 were used in this study. Cells were passaged with dispase (Invitrogen) every 5–7 days. A normal karyotype was confirmed in both cell lines (data not shown). For karyotyping, hESCs grown in log phase were harvested and karyotyped by using Giemsa stain (Genzyme). Twenty cells were scored in each case. For feeder-free cultures, hESCs used in assay were cultured on growth factor-reduced Matrigel (BD Biosciences) at 1:30 in the medium conditioned from MEFs, as previously described (1, 2). For human embryoid body formation, hESCs passaged with 1 mg/ml collagenase IV (Invitrogen) were cultured in an uncoated, 3.5-cm Petri dish in the presence of Iscove’s modified Dulbecco’s medium supplemented with 15% fetal bovine serum, 1 mM l-glutamine, 1% non-essential amino acids, 1% insulin transferrin selenium, 0.1 mM β-mercaptoethanol (all from Invitrogen). After 6 days of suspension culture, the embryoid bodies were formed, and the cells were transferred to a plate coated with 5 ng/μl fibronectin (Sigma). The cells attached to the plate and were cultured for 6 days, then used for further analysis. For activin A-induced direct differentiation toward definitive endoderm lineage, hESCs were passaged to Matrigel, and the medium was changed to RPMI 1640 (HyClone) plus insulin transferrin selenium and activin A (PeproTech) the next day. After 4 days of culture, cells were used for further analysis. Brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4 were purchased from PeproTech. Experiments were performed in both H1 and H9 except for claiming.

Annexin V Analysis and TUNEL Staining—To assess the extent of apoptosis after irradiation, cells were harvested and resuspended in binding buffer (10 mM Heps, pH 7.4/140 mM NaCl, 2.5 mM CaCl2) and were stained with 5 μl of annexin V-fluorescein isothiocyanate (BD Pharmingen) and 2.5 μg/ml propidium iodide (PI). The cell suspension was incubated for 15 min at room temperature and analyzed by flow cytometry (MoFlo High-Performance Cell Sorter; DakoCytomation). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining was carried out using the DeathEnd Fluorometric TUNEL System kit (Promega, Madison, WI) according to the manufacturer’s instructions.

Immunofluorescence and PI Staining—Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then blocked in 3% horse serum in PBS at room temperature for 1 h. Cells were incubated with primary anti-bodies at 4°C overnight. For all the immunostaining, cells stained without primary antibodies were used as a negative control. After five washes with PBS, fluorescein isothiocyanate or TRITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the mixture was incubated at 37°C for 1 h. Nuclei were detected by DAPI (Sigma) staining. PI staining was performed at the concentration of 50 μg/ml in PBS. Images were captured using an Olympus IX-71 microscope or a Leica confocal TCS-SP2 microscope.

p53 Transactivity Luciferase Reporter Assay—hESCs were transfected with a p53 transactivity luciferase reporter plasmid PG-13 kindly provided by Dr. Bert Vogelstein, together with a trace amount of a pRL-tk plasmid (Promega) to express Renilla luciferase as an internal control. The cells were plated on Matrigel in 24-well plates and split 24–48 h before transfection. Co-transfection of reporter plasmid was carried out with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Per well, 0.4 μg of PG13 and 0.4 μg of pRL-tk formulated into liposomes, were applied. The final volume was 600 μl per well. Cells were incubated for 24 h after transfection and appeared healthy thereafter when variously treated and analyzed. Cell lysates were extracted, and both the firefly and Renilla luciferase activities were tested by using the Dual-luciferase Reporter Assay System (Promega) on a Luminometer (Beckman Coulter). Results are shown as the firefly luciferase activity normalized to Renilla luciferase activity.

Detection of Caspase 9 Activity and Mitochondrial Fractionation—The activity of caspase 9 was determined by using a fluorometric assay kit (R & D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. Briefly, 5 × 10⁶ cells were collected and lysed in 50 ml of lysis buffer and incubated with fluororhromic caspase substrate Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin. After incubation at 37.5°C for 1 h, the fluorescence was measured by using a spectrophluorophotometer (Model RF540, Shimadzu, Tokyo) with excitation at 400 nm and emission at 550 nm. Mitochondria were prepared as described previously (25).

Lentiviral Transduction—To reduce p53 expression, siRNAs that specifically targeted p53 were constructed in a Lentilox3.7 (pLL3.7) vector. P53-specific siRNA expression was driven by the U6 promoter. The oligonucleotides encoding the p53-specific siRNA were 5’-TGACTCCAGTGTGTTATCTAC- TTCAAGAGAGTAGATTACCACTGGAGTCTTTTTTC-3’ and 5’-TCGAGAAAAAGACCTCCAGTGTGTTATCTACTCCTTGAAGTAGATTACCACTGGAGTCACA-3’. This siRNA sequence is specific for p53, and no off-target effects have been reported (26). These oligonucleotides were annealed and ligated into the lentiviral vector EF-V-CMV.GFP (kindly provided by Linzhao Cheng) by insertion into EcoRV restriction enzyme sites under the control of an elongation factor (EF) promoter. Lentiviral stocks were prepared as previously described (26). Viral supernatants were combined with hESC medium at the ratio of 1:1. The hESCs used for this study were of the WiCell H1 line. The cells were plated on Matrigel in 6-well plates and split 24–48 h before transduction. A total of 1 × 10⁶ H1 hESCs was transduced on MEFs by
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A flow cytometric analysis of apoptotic cells using Annexin-V surface staining and PI. Shown are dot plots of the fluorescein isothiocyanate-conjugated Annexin-V versus PI staining for hESCs (a) and hESCs 12 h post 20 J/m² UV irradiation (b). B, immunofluorescent detection of p53 in hESCs before and 6 h after, 20 J/m² UV. Nucleus is stained by DAPI (bar, 20 μm). C, real-time RT-PCR analysis of p53 target genes in undifferentiated/differentiated H1-null and H1-p53si cells before and 6 h after 20 J/m² UV irradiation. D, p53 transcription activity by PG13-luciferase reporter assay in undifferentiated/differentiated hESCs before and 6 h after irradiation with different doses of UV. Shown are normalized values by TK-renilla luciferase. Data represent the average of triplicates.

activity luciferase reporter assay. For other experiment procedures please see the supplemental section.

RESULTS

p53 Accumulation, but Not Its Transcription Activity, Can Be Induced by DNA Damage Agents in hESCs —To induce hESCs to undergo apoptosis, we irradiated the H1 human embryonic stem cell line with UV. We found that, after exposure of hESCs to UV irradiation for 5 h, the cells began to die; after 12 h, 67% of the irradiated cell underwent apoptosis or necrosis as indicated by Annexin-V staining (Fig. 1A); and after 40 h all of the irradiated cells were dead (data not shown). Similar results were found using the H9 human embryonic stem cell line (data not shown).

To determine whether p53 was activated in the UV-induced apoptosis of hESCs, we assayed for changes in p53 protein levels. hESCs exclusively accumulated p53 after UV irradiation compared with the non-irradiated cells as indicated by immunofluorescence staining (Fig. 1B). The overall p53 protein levels also increased dramatically after UV irradiation as indicated by Western blot (data not shown).

We then performed real-time RT-PCR analysis to examine whether the expression levels of various p53 target genes were up-regulated after UV irradiation in hESCs. p53 accumulation usually correlates with an elevation of its transcription activity after UV irradiation in many cell types, including mESCs (18). Surprisingly, we found that the expression levels of various p53 target genes, including mdm2, p21, bax, and puma, were down-regulated after 20 J/m² UV irradiation in H1 cells (Fig. 1C). This finding was confirmed by at least five independent experiments. Similar results were found in H9 cells (data not shown). To exclude the possibility that UV at the dosage of 20 J/m² was too low to induce the expression levels of p53 target genes, we also used a higher dosage of UV at 100 J/m² and found similar results (data not shown). Moreover, we also lowered the UV dosage to exclude the possibility that UV at a high dosage caused too many lesions in the DNA and thus inhibited the transcription activity of p53 (28). We found that, even when 5 J/m² UV (the lowest dosage at which UV can

single round infections for 10 h. Positive cells were purified by selecting clones under a fluorescence microscope.

oct-4 Promoter-luc Reporter Assay—The phOCT4-EGFP plasmid containing 3.4 kb of human oct-4 promoter was kindly provided by Dr. Wei Cui (27). The oct-4 promoter fragment between Xhol and BglII was cloned into vector pGL3-Basic (Promega) by insertion into Xhol and BamHI restriction enzyme sites upstream of luciferase. hESCs were transfected with the oct-4 promoter-luc plasmid together with a trace amount of a pRL-tk plasmid (Promega) to express renilla luciferase as an internal control. The transfection and luciferase activity detection procedures were the same as the p53 transfection levels of various p53 target genes, including mdm2, p21, bax, and puma, were down-regulated after 20 J/m² UV irradiation in H1 cells (Fig. 1C). This finding was confirmed by at least five independent experiments. Similar results were found in H9 cells (data not shown). To exclude the possibility that UV at the dosage of 20 J/m² was too low to induce the expression levels of p53 target genes, we also used a higher dosage of UV at 100 J/m² and found similar results (data not shown). Moreover, we also lowered the UV dosage to exclude the possibility that UV at a high dosage caused too many lesions in the DNA and thus inhibited the transcription activity of p53 (28). We found that, even when 5 J/m² UV (the lowest dosage at which UV can
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induce apoptosis in hESCs was used, the expression of p53 target genes was still not activated.

It has been reported that p53 functions differently in undifferentiated mESCs compared with the differentiated mESCs (29). Therefore, we allowed hESCs to differentiate in Dulbecco’s modified Eagle’s medium/F-12 plus 20% fetal bovine serum for 12 days before assaying for the expression levels of p53 target genes after exposure to UV irradiation. In contrast to the results observed in hESCs, RT-PCR analysis showed a notable increase in the expression levels of p21, mdm2, bax, and noxa in differentiated hESCs (Fig. 1C). These results indicate that the failure of p53 to activate the transcription of its target genes is only observed in undifferentiated hESCs but not in differentiated cells.

To further assess p53 transcription activity in hESCs, we assayed for p53 transcription activity using a luciferase reporter plasmid PG-13 (30). This plasmid contained 13 copies of the p53-binding consensus sequences upstream of a luciferase reporter gene and was transfected into UV-irradiated hESCs. As a control a second vector, MG-15, containing a mutated version of the p53-binding consensus sequences was used. In agreement with our previous findings, there was no up-regulation of p53 transcription activity after UV irradiation at the dosage of 5 J/m² or 20 J/m² in hESCs. However, when hESCs were differentiated, p53 transcription activity gradually increased along with the increasing doses of UV irradiation (Fig. 1D).

To confirm our initial observations we used Western blotting to analyze changes in p53 target gene expression after UV irradiation at the protein level. We found that p53 began to accumulate as early as 2 h after irradiation. No obvious changes in the expression of the p53 target genes mdm2 and bax were found (Fig. 1E). However, p21 protein levels increased by ∼2-fold after UV irradiation. Interestingly, we did not observe an increase in p21 mRNA levels (Fig. 1C). We believe the increase in p21 expression occurs post-translationally, as previously demonstrated in mESCs (31). These results were further confirmed by immunofluorescence staining (supplemental Fig. S1).

We next tested whether other DNA damage agents induced similar effects on hESCs. Similar to UV irradiation, γ-radiation also induced p53 protein expression, but not the transcription activity of p53 (supplemental Fig. S2). As a result, neither UV irradiation, nor other forms of DNA damage, can activate p53 transcription activity in hESCs.

Post-translation modifications of p53 play an important role in the regulation of p53 stability and activity (32). As a result of our observation that the ability of p53 to transactivate target genes after DNA damage was different in hESCs compared with differentiated cells, we assessed p53 post-translation modifications in both cell types to determine if this was the cause of the different phenotypes. With the induction of p53 expression by UV and γ-radiation, p53 was phosphorylated at Ser-15. This modification is known to be responsible for the accumulation and activation of p53 after DNA damage in many cell types (33). Notably, we found several different p53 modifications in hESCs as compared with differentiated cells. Acetylation at Lys-382 was only found in hESCs, and phosphorylation at Ser-9 was found mostly in the differentiated cells (Fig. 1F). A slight phosphorylation at Ser-9 was found in hESCs, correlating with the failure of p53 to activate the transcription of its target genes after DNA damage. No obvious changes were detected at other sites, including Ser-6, Thr-18, Ser-20, Ser-33, Ser-37, Ser-46, Thr-81, Ser-315, and Ser-392 (data not shown).

The above data demonstrate p53 cannot activate the transcription of its target genes after DNA damage in hESCs despite an accumulation of the p53 protein product. To test whether p53 transcription activity contributed to UV-induced apoptosis in hESCs, we treated mESCs and hESCs with pifithrin-α, a p53 transcription inhibitor (34). Pifithrin-α rescued UV-induced apoptosis in mESCs by ∼30%, but no effect was observed in hESCs (Fig. 1G). However, pifithrin-α did inhibit p53 transcription activity in hESCs as assessed by p53 transactivity luciferase reporter assay (Fig. 1H). Even when high concentrations of pifithrin-α were used, no rescue was observed in hESCs (data not shown). We also found that pifithrin-α had no influence on the UV-induced apoptosis rate of H1 cells (Fig. 1I). Similar results were found in H9 cells (data not shown). These data further support p53 transcription activity as not being involved in the UV-induced apoptosis in hESCs. This finding is very different from previous observations of mESCs (14, 29).

\textit{p53 Induces Apoptosis through the Mitochondrial Pathway in hESCs—}It has been reported that p53 can induce apoptosis through a mitochondrial pathway (19–22). Because p53 cannot activate the transcription of its target genes, we tested whether hESCs apoptosis was induced by p53 associated with the mitochondria. We stained hESCs for p53 and hsp75, a mitochondria marker. We found that cytoplasmic p53 clearly co-stained with hsp75 in hESCs after UV irradiation (Fig. 2A). Furthermore, we isolated mitochondria from hESCs and assayed for p53 association by Western blot. Blotting of Cox IV, a mitochondrial inner-membrane protein, and histone H3, a nuclear protein, verified the enriched mitochondrial fraction was free of nuclear contamination. Our results show that p53 accumulates in the mitochondrial fraction after UV irradiation (Fig. 2B). Moreover, incubation of cells with pifithrin-α, a p53 transcription inhibitor, had no effect on the overall accumulation of p53 and little effect on its translocation into mitochondria (Fig. 2B).

To test whether p53 accumulation in the mitochondria led to the cleavage of caspase 9, a penultimate biochemical effector for apoptosis, we analyzed cell lysates for the enzymatic activity of cleaved caspase 9 using a preferential-fluorogenic substrate. Caspase 9 activity increased nearly 3-fold in hESCs after UV irradiation (Fig. 2C). This result suggests that p53 accumulates in mitochondria and activates caspase 9 to induce apoptosis in hESCs after UV irradiation.

To further investigate the role of p53 accumulation in the mitochondria, we knocked down p53 in hESCs and examined whether reducing p53 expression inhibited caspase 9 activation and the induction of hESCs apoptosis upon exposure to UV irradiation. We first established a stable p53 knockdown H1 human embryonic stem cell line. The sequence of siRNA-specific for p53 was chosen as previously described (26), and it was delivered into H1 cells by lentiviral transduction (35). RT-PCR showed that p53 mRNA decreased in the p53 knockdown H1 cells (H1-p53si) by
more than 15-fold compared with the control cells (H1-null) (data not shown). Immunofluorescence staining (Fig. 2D) and Western blot analysis (Fig. 2E) revealed that p53 protein levels were reduced by >10-fold. We then found that p53 knockdown in H1 cells inhibited caspase 9 elevation by ~50% (Fig. 2C). Moreover, p53 knockdown rescued 40% of H1 cells from apoptosis induced by UV (Fig. 2F). Similar results were obtained when we knocked down p53 in another human embryonic stem cell line, H9, using synthesized siRNA duplexes (supplemental Fig. S3).
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We then utilized pifithrin-μ, a recently reported small molecule that specifically inhibits p53 binding to the mitochondria (36), to further test the role of the mitochondria associated p53 in hESCs. We found that pifithrin-μ significantly increased the survival rate of H1 cells after UV irradiation (Fig. 2G). Pifithrin-μ also enhanced the survival rate of H9 cells after UV irradiation (data not shown). In contrast, the p53 transcription inhibitor pifithrin-α had no effects on UV-induced apoptosis in hESCs. Moreover, when p53 was knocked down in H1 cells, treatment of the cells with pifithrin-μ did not enhance the survival rate. Taken together, these results suggest that p53 contributes to UV-induced apoptosis in hESCs through the mitochondrial pathway and the effects of p53 can be attenuated by reducing p53 expression using siRNA.

*p53 Knockdown Reduces Spontaneous Apoptosis of hESCs—Because our previous findings indicated that p53 played an important role in the DNA damage-induced apoptosis in hESCs, we wondered whether it also contributed to the spontaneous apoptosis of hESCs. We co-stained TUNEL-positive cells, which specifically recognized DNA fragments of apoptotic cells, for p53 in H1 cells. We found that TUNEL-positive cells strongly expressed p53 (Fig. 3A). We also found some H1 cells with strong p53 expression but negative TUNEL staining. These cells may represent hESCs in an early stage of apoptosis undetectable by TUNEL. Similar results were observed in H9 cells (data not shown). This observation raises the possibility that p53 accumulation induces the apoptosis of hESCs.

To further investigate the role of p53 in spontaneous apoptosis of hESCs in culture, we examined whether p53 knockdown reduced the spontaneous apoptosis of hESCs. In situ PI staining, an indicator for cell viability, showed that the H1-p53si cells displayed fewer PI-positive cells as compared with the control H1-null cells in routine culture conditions (Fig. 3B). The expression of the p53-specific siRNA reduced the spontaneous apoptosis rate by >50% compared with the control cells (Fig. 3C).

We then tested whether reducing p53 expression could have influence on the single cell survival rate of hESCs. This survival rate was reported to be quite low because of the high rate of spontaneous apoptosis of the cells (10). The results of the low density survival assay showed that 4.5% of single H1-p53si cells survived and formed AP-positive clones, compared with 1.3% of the H1-null cells (Fig. 3, D and E). As a result, the single cell survival rate of hESCs is evidently elevated by reducing p53 expression. It has been reported recently that neurotrophins can elevate the single cell survival rate and prevent apoptosis of hESCs (10). We also tested the effect of neurotrophins on the p53 knockdown hESCs. Brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4 treatment of hESCs reduced the spontane-
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Reducing p53 expression in low concentrations of activin A-treated hESCs reduced the percentage of endoderm cells by 3-fold (Fig. 4D).

Because reducing p53 expression slows the differentiation rate of hESCs (including spontaneous, random, and directed differentiation), we hypothesized that the differentiation potential of H1-p53si cells into all three germ layers might be impaired. We found that the H1-p53si cells readily formed embryoid bodies in suspension culture. Day 7 embryoid bodies were plated for further differentiation, and immunofluorescence staining was performed to monitor differentiation using markers for all three germ layers. Neurofilament heavy chain-positive ectoderm cells, muscle actin-positive mesoderm cells, and SOX 17, or alpha fetal protein-positive endoderm cells were detected among the differentiated cells (Fig. 4E). These results indicate that p53 knockdown hESCs maintain pluripotency and possess multilineage differentiation potential.

FIGURE 4. p53 knockdown slows down differentiation rate of hESCs. A, spontaneous differentiation of H1-null and H1-p53si colonies grown on Matrigel for 10 days after passage. Phase contrast images and oct-4 staining are shown (bar, 200 μm). Cells stained without primary antibodies were used as a negative control (data not shown). B, morphometric analysis of differentiated areas within colonies of H1-null and H1-p53si cells after passage for 10 days. Data for each condition were obtained from the analysis of 15 colonies randomly sampled from three independent culture wells. Shown are the total colony area and the overt differentiated area. C, percentage of undifferentiated clones at each passage after basic fibroblast growth factor was withdrawn from the culture medium of H1-null and H1-p53si cells. *** p < 0.001. D, percentage of SOX 17-positive endoderm cells 3 days after 5 and 100 ng/ml activin A induction in H1-null and H1-p53si cells. E, immunofluorescence staining of ectoderm (neurofilament), mesoderm (muscle actin), and endoderm (SOX17, α-fetal protein) markers in differentiated H1-p53si cells. Nucleus is stained by DAPI (bar, 10 μm). Cells stained without primary antibodies were used as a negative control (data not shown). F, real-time PCR analysis of oct-4 and nanog in H1-null and H1-p53si cells before and 6 h after 20 J/m² UV. G, oct-4 promoter-driven luciferase activities in H1-null and H1-p53si cells before and 6 h after 20 J/m² UV. H, chromatin immunoprecipitation analysis of DNA binding of p53 to p53-dependent promoters (p21 and noxa) and to the oct-4, nanog promoter in hESCs before and 6 h after 20 J/m² UV irradiation. Chromatin immunoprecipitation assay was performed using an antibody against p53 followed by PCR with primers amplifying the promoter regions of p21, mdm2, oct-4, and nanog, PCR products were separated by agarose gel electrophoresis. I, Western blot of oct-4, nanog, and p53 in hESCs, H1-null, and H1-p53si cells 6 h after 20 J/m² UV.

It has been reported that p53 deletion might cause instability of the genome in certain cell types (14). We analyzed the karyotype of H1-null and H1-p53si cells (25 passages) by standard G-banding techniques. Both maintained a normal karyotype (H1-p53si in Fig. 3H, H1-null not shown). As a result, p53 knockdown does not cause observable changes in karyotype of hESCs.

p53 Knockdown Reduces Spontaneous Differentiation of hESCs—When we cultured the p53 knockdown H1 cells, we found that they were harder to differentiate compared with the control cells. As shown in Fig. 4A, spontaneous differentiated areas, which contained larger, flatter, and Oct4-negative cells, appeared in normal H1 cells and those expressing no siRNA after 10 days of culture on Matrigel. However, in the H1 cells expressing a p53-specific siRNA, the differentiated areas were much smaller in size and less numerous. Morphometric analysis revealed that the percentage of differentiated areas was reduced by 4-fold in the H1-p53si cells (Fig. 4B). Our results indicate that reducing p53 levels in hESCs reduces spontaneous differentiation of these cells.

Because our data show that reducing p53 expression using siRNA in hESCs leads to lower rates of spontaneous apoptosis and higher rates of cell survival, we hypothesized that reducing p53 expression would facilitate the expansion of routinely cultured hESCs. We demonstrated that the H1-p53si cells possessed a higher proliferation rate compared with H1-null cells, and when cell numbers were assessed each day after passage in both cell lines by a 3-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 3G).

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The transcription factors oct-4 and nanog play essential roles in controlling pluripotency and self-renewal of hESCs (40). To test whether the expression of p53 affected the steady-state levels of oct-4 and nanog in UV-irradiated hESCs, we followed oct-4 and nanog expression using RT-PCR. The expression of oct-4 and nanog decreased by ~3-fold after UV irradiation in H1-null cells. In contrast, oct-4 down-regulation was totally inhibited, and nanog down-regulation was partially inhibited in the H1-p53si cells (Fig. 4F). Similar results were found when an oct-4 promoter-driven luciferase vector was used as a reporter transfected into H1-null and H1-p53si cells (Fig. 4G). These data suggest that the down-regulation of oct-4 and nanog after UV irradiation, particularly oct-4, is dependent on the expression of p53.

To test whether p53 directly regulates the transcription of oct-4 and nanog, we performed a chromatin immunoprecipitation assay in H1 cells. We found that, in hESCs exposed to UV irradiation, p53 binds to the promoter region of oct4 and nanog, as well as to two well-known p53 target genes, p21 and noxa (Fig. 4H). Similar results were found in H9 cells (data not shown).

At the protein level, oct-4 and nanog were decreased in UV-irradiated H1-null cells but not in H1-p53si cells as assessed by Western blot (Fig. 4I).

p53 Accumulation Can Be Inhibited by mdm2 Overexpression—

Based on our original findings that reducing p53 expression reduces both spontaneous apoptosis and spontaneous differentiation of hESCs, we present a tentative model to explain how p53 functions in these cells. As shown in Fig. 5A, p53 protein accumulates in response to DNA damage agents in culture conditions. However, p53 cannot activate the transcription of its target genes, including mdm2. mdm2 is able to negatively regulate p53 by mediating its degradation through the ubiquitin pathway (33, 41, 42). As a result, the lack of mdm2 induction abolishes the feedback degradation circuitry required to maintain the proper amount of p53 in response to stress-inducing conditions in culture. The accumulated p53 can effectively induce apoptosis through the mitochondrial pathway (Fig. 2) or induce differentiation by suppressing the transcription of oct-4 and nanog (Fig. 4).
the H1-mdm2 cells (Fig. 5B). mdm2 protein levels were also higher in the H1-mdm2 cells (Fig. 5C). Moreover, fewer H1-mdm2 cells showed potent p53 expression, which represents the spontaneous apoptosis hESCs, compared with the control H1-GFP cells (Fig. 5C). These results indicate that the overexpression of mdm2 can inhibit p53 accumulation in hESCs.

Because overexpression of mdm2 reduced p53 levels, we reasoned that mdm2 overexpression might reduce the spontaneous apoptosis of hESCs. In situ P1 staining showed that the H1-mdm2 cells displayed fewer PI-positive cells compared with the control H1-GFP cells (Fig. 5D), and the spontaneous apoptosis rate was reduced from 9.6% in H1-GFP cells to 3.5% in H1-mdm2 cells (Fig. 5E). As a result, mdm2 overexpression modestly reduced the rate of spontaneous apoptosis in routinely cultured hESCs. We also tested whether mdm2 overexpression affected the single cell survival rate of hESCs. The results of the low density survival assay showed that the single cell survival rate of H1-mdm2 cells was as nearly 2-fold as the control cells (Fig. 5F).

Finally, we compared the differentiation rate of H1-null and H1-p53si cells and found no differences (data not shown). It might be because p53 expression was not reduced enough to affect the transcription of oct-4 and nanog.

**DISCUSSION**

In this report we demonstrate that, in hESCs, p53 cannot activate the transcription of its target genes after DNA damage, thus abolishing the feedback degradation circuitry mediated by mdm2. As a result, culturing hESCs under stress-inducing conditions leads to p53 over-accumulation. The accumulated p53 induces spontaneous apoptosis of hESCs through the mitochondrial pathway. p53 accumulation can also suppress the expression of oct-4 and nanog genes as well as inducing the spontaneous differentiation of hESCs. p53 knockdown reduces both spontaneous apoptosis and differentiation and promotes the survival and expansion of hESCs. These studies demonstrate a novel p53 regulatory mechanism in promoting the spontaneous apoptosis and differentiation of hESCs.

The p53 transcription-dependent induction of apoptosis and the p53 mitochondrial pathway are two independent pathways to induce apoptosis, both of which can be activated in most cell types (16). However, in hESCs, p53 cannot activate the transcription of its target genes (Fig. 1), and the ability of p53 to induce apoptosis is only sustained through the mitochondrial pathway (Fig. 2). In contrast, in mESCs p53 transactivates target pro-apoptotic genes and induces apoptosis after DNA damage (14, 29). To verify that different pathways are activated in mESCs and hESCs, we used the p53 transactivation inhibitor pifithrin-α and found that it inhibits apoptosis in mESCs but not in hESCs (Fig. 1G). It is worthwhile to point out that, in certain cell types, pifithrin-α can also inhibit p53 from binding to mitochondria, because the sites of p53 used to transactivate genes and to bind to mitochondria are quite close to each other (25). However, in hESCs, the results show that pifithrin-α only inhibits p53 transcription activity and has little effect on the binding of p53 to mitochondria (Fig. 2B). As a result, it does not inhibit DNA damage-induced apoptosis in hESCs (Fig. 1G). On the other hand, inhibition of p53 binding to the mitochondria, as a result of pifithrin-μ treatment, can significantly inhibit DNA damage-induced apoptosis in hESCs (Fig. 2G).

We demonstrate that p53 knockdown promotes the survival of hESCs (Fig. 3F). It has been recently reported by Pyle et al. (10) that neurotrophins are also able to mediate the survival of hESCs. We found that neurotrophins can promote the survival of normal hESCs but had no effect in the p53 knockdown hESCs (Fig. 3F). This finding suggests that neurotrophins might function through the p53 pathway. Our model predicts that p53 over-accumulation and induction of apoptosis is caused by stress-inducing culture conditions. Therefore, it should be possible to suppress p53-dependent spontaneous apoptosis of hESCs by improving the culture conditions. This hypothesis is supported by our recent finding that the spontaneous apoptosis rate of hESCs was reduced from 26% in conditioned medium to 10% in an N2B27 supplemented chemical-defined medium (43). It has been reported that oxygen at a normal atmospheric concentration is hazardous to hESCs (12). N2B27 contains many antioxidants that can help reduce the free radicals produced by oxygen and thus reduce the stress on hESCs induced by oxygen. Altogether, these findings suggest that the expansion of hESCs can be enhanced by p53 knockdown, down-regulation of p53 pathway by overexpressing mdm2, using certain growth factors, or by removing any stress-inducing conditions during the routine culture of hESCs.

We find that p53 directly regulates the expression of oct-4 and nanog and is responsible for the spontaneous differentiation of hESCs (Fig. 4). The transcription factors oct-4 and nanog have essential roles in maintaining the pluripotency and self-renewal of both hESCs and mESCs (44–46). Disruption of oct-4 or nanog results in the differentiation of both human and mouse embryonic stem cells (47–49). Currently, little is known about how these self-renewal genes are regulated. It has been reported that in mESCs p53 induces differentiation through suppression of nanog expression (9), and in this study we find that p53 can also regulate these genes directly in hESCs. When p53 accumulates in response to the stress-inducing conditions of hESCs, it suppresses the expression of the self-renewal genes oct-4 and nanog (Fig. 4F), and thus induces spontaneous differentiation of hESCs, as illustrated by our working model in Fig. 5A. On the other hand, our results demonstrate that, in the p53 knockdown hESCs, oct-4 and nanog cannot be down-regulated efficiently (Fig. 4F). Consequently, the differentiation rate (spontaneous, random, and directed differentiation) of p53 knockdown hESCs is slowed (Fig. 4).

One of the most important findings from this study is that p53 cannot activate the transcription of its target genes in hESCs after DNA damage. We demonstrated this using a range of doses of UV and γ-radiation and assayed at both the p53 mRNA and the protein levels (Fig. 1, C and E; supplemental Figs. S1 and S2). These results were further confirmed by a p53 transactivity luciferase reporter assay (Fig. 1D) and were supported by the results using the p53 transcription inhibitor pifithrin-α (Fig. 1, G–I). The fact that p53 cannot activate the transcription of its target genes in hESCs after DNA damage is quite different from many other cell types, including mESCs (18).
Regulation of Apoptosis and Differentiation by p53 in hESCs

Why is p53 unable to activate its target genes in stress-induced hESCs? Previous reports have shown that certain p53 mutations result in the failure of p53 to activate the transcription of its target genes (50–52). However, we do not believe that the inability of p53 to activate its target genes in stress-induced hESCs results from a mutation in p53. When hESCs differentiate, the ability of p53 to activate the transcription of its target genes is restored (Fig. 1, C and D). Therefore, the inability of p53 to activate its target genes after DNA damage in hESCs is most likely not caused by a p53 mutation.

Secondly, the failure of p53 to activate the transcription of its target genes is most likely related to the special post-translation modification patterns of p53 in hESCs. It has been previously demonstrated that post-translation modification of p53 is crucial to p53 stability and activity (32). For example, Lin et al. reported that in humanized p53 knock-in (p53hki) mESCs, Ser-315 phosphorylation is a common event during cell differentiation and is involved in further modifications and activities of p53 induced by DNA damage agents (9). We compared the post-translation modification patterns of p53 in undifferentiated and differentiated hESCs (Fig. 1F), in which the ability of p53 to transactivate target genes after DNA damage is different. We found that p53 modifications differ at Lys-382 and Ser-9. Lys-382, a residue previously shown to be important for p53 activity during the stress response, is only acetylated in undifferentiated hESCs (53). Ser-9 is phosphorylated in the differentiated hESCs but not in undifferentiated hESCs. Ser-9 of p53 is phosphorylated by casein kinase in response to DNA damage agents, and phosphorylation of this residue regulates the activity of p53 (54). In the undifferentiated hESCs, Ser-9 phosphorylation is barely observable. This is likely related to the failure of p53 to activate the transcription of its target genes in hESCs. A point mutation study of this site of p53 in hESCs should address this question. Although currently the genetic modification of hESCs still faces many technical problems (55), eventually these problems will be overcome and direct evidence will be obtainable.

Third, the failure of p53 to activate the transcription of its target genes might also be related to the cofactors required for this process. We found that, although p53 cannot transactivate its target genes in hESCs, its ability to transcriptionally repress oct-4 and nanog is not impaired (Fig. 4F). Notably, p53 still occupies the promoter of p21 and noxa after DNA damage in undifferentiated hESCs (Fig. 4H). Therefore, the failure of p53 to transactivate its target genes is not caused by the failure of p53 to bind to the promoter of specific genes. This observation raises the possibility that the failure of p53 to activate the transcription of its target genes may lie in various cofactors required for p53 to transactivate its target genes. In response to DNA damage agents, p53 must be able to recruit co-activators or co-repressors to form an active complex required to transcriptionally activate or repress target gene expression. For example, the histone acetyltransferase p300/CBP (CAMP response element binding protein-binding protein) has been reported to be a co-activator of p53 to transactivate target genes (56–59). On the other hand, for transcriptional repression p53 uses other sets of cofactors, e.g., the histone deacetylase mSin3a (60). Therefore, p53 can suppress oct-4 and nanog transcription despite its failure to activate the transcription of its target genes. It has been reported that the assembly of the active transcription complex of p53 could be regulated by its post-translation modification (61). It is possible that the post-translation modification pattern of p53 in hESCs prevents the recruitment of p53 cofactors. Failure to recruit the appropriate cofactors prevents the formation of an active transcription complex and transactivation of p53 target genes.

To date, such a failure of p53 to activate the transcription of its target genes after DNA damage has only been reported in certain nucleotide excision repair-deficient fibroblast cell lines (28, 62). Nucleotide excision repair can overcome the effect of DNA damage agents on inhibiting mRNA synthesis. As a result, the activity of nucleotide excision repair is evidently critical for DNA repair (63). The fact that the failure of p53 to activate the transcription of its target genes in hESCs is similar to nucleotide excision repair-deficient fibroblasts, together with the recent report that hESCs suffer genomic instability after multiple passages (13), indicates a deficiency in the hESC DNA repair system. This situation is different from mESCs, in which the frequency of spontaneous mutation is quite low because of the tightly maintained DNA repair activity (11). It will be of interest to further examine the hESC DNA repair system to determine whether it contributes to the failure of p53 to activate the transcription of its target genes and the genomic instability of hESCs.

Acknowledgments—We thank Dr. Bert Vogelstein for kindly providing the PG-13 and MG-15 vectors, Dr. Ygal Haupt for providing cDNA of mdm2, Dr. Linzhao Cheng for providing the lentiviral vector EF.V-CMV.GFP, Dr. Wei Cui for providing the promoter of oct-4, and Dr. Matthew Stremlau, Dr. Tung-Tien Sun, Dr. Hui Zhang, Dr. Guangwen Wang, and Dr. Hong Zhang for critical reading of the manuscript. We also thank Yan Shi, Aihua Zheng, Fei Yuan, Wei Wei, Xiaolei Yin, Chengyuan Wang, Jun Yong, Zan Tong, Lichen Ren, Wei Jiang, Jiefang You, Yaxin Lv, Yan Shen, Xiaoran Xiong, and other colleagues in our laboratory for technical assistance and advice during experiments, Liyung Du for providing the technical support of flow cytometric analysis, and Yizhe Zhang for providing the technical support of real-time PCR analysis.

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