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ORIGINAL ARTICLE

hTERT: a novel endogenous inhibitor of the mitochondrial cell death pathway

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hTERT is the catalytic subunit of the telomerase and is hence required for telomerase maintenance activity and cancer cell immortalization. Here, we show that acute hTERT depletion has no adverse effects on the viability or proliferation of cervical and colon carcinoma cell lines, as evaluated within 72 h after transfection with hTERT-specific small interfering RNAs (siRNAs). Within the same time frame, hTERT depletion facilitated the induction of apoptotic cell death by cisplatin, etoposide, mitomycin C and reactive oxygen species, yet failed to sensitize cells to death induction via the CD95 death receptor. Experiments performed with p53 knockout cells or chemical p53 inhibitors revealed that p53 was not involved in the chemosensitizing effect of hTERT knockdown. However, the proapoptotic Bcl-2 family protein Bax was involved in cell death induction by hTERT siRNAs. Depletion of hTERT facilitated the conformational activation of Bax induced by genotoxic agents. Moreover, Bax knockout abolished the chemosensitizing effect of hTERT siRNAs. Inhibition of mitochondrial membrane permeabilization by overexpression of Bcl-2 or expression of the cytomegalovirus-encoded protein vMIA (viral mitochondrial inhibitor of apoptosis), which acts as a specific Bax inhibitor, prevented the induction of cell death by the combination of hTERT depletion and chemotherapeutic agents. Altogether, our data indicate that hTERT inhibition may constitute a promising strategy for facilitating the induction of the mitochondrial pathway of apoptosis.
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Introduction

Cancer cells characteristically replicate without limit, provide their own growth signals, ignore growth-inhibitory signals, sustain angiogenesis, invade through basal membranes and capillary walls, proliferate in unnatural locations and avoid cell death (Hanahan and Weinberg, 2000). Oncogenic cell death inhibition is achieved at several levels. Cancer cells frequently inactivate the p53 pathway that links the DNA-damage response to mitochondrial apoptosis, either through transcriptional effects of p53 or owing to a physical interaction of p53 with proapoptotic members of the Bcl-2 family (Vogelstein *et al*., 2000; Vousden and Lu, 2002; Perfettini *et al*., 2004). Similarly, cancer cells often inactivate the intrinsic pathway of apoptosis that relies on mitochondrial membrane permeabilization (MMP). This inhibition is typically achieved by the overexpression of MMP inhibitors (such a as Bcl-2) or by the loss of MMP inducers (such as Bax) (Ionov *et al*., 2000; Debatin *et al*., 2002; Danial and Korsmeyer, 2004; Green and Kroemer, 2004). Moreover, some cancer cells become resistant to apoptosis induction via the extrinsic pathway of apoptosis that is triggered by a special class of plasma membrane receptors, the death receptors (Peter and Krammer, 2003). This oncogenic apoptosis resistance can be achieved through alterations in the composition of the death-inducing signaling complex (DISC) that usually triggers caspase activation upstream or independent of MMP (Schulze-Bergkamen and Krammer, 2004).

Unlimited replication of tumor cells is usually achieved because tumor cells express telomerase, an enzyme that can add telomeric repeats onto chromosome terminal repeats, and thus avoids the 'telomere problem', a biological clock that limits the lifespan of normal, telomerase-negative somatic cells owing to progressive erosion of telomeres (DePinho, 2000; Blackburn, 2001). Telomerase is a ribonucleoprotein composed by a catalytic protein subunit (human telomerase reverse transcriptase (hTERT)) and an RNA moiety (TERC). Several oncogenes including *c-Myc* and *c-Jun* induce hTERT expression (Takakura *et al*., 2005), whereas the tumor suppressor *p53* reduces hTERT expression (Xu *et al*., 2000). Cancer cells thus overexpress hTERT, and the intensity of hTERT expression

is an independent negative prognostic factor in non-small-cell lung carcinoma (Lantuejoul *et al*., 2005). As hTERT expression is characteristic of cancer cells, some gene therapeutic approaches are based on the use of hTERT promoter, which leads to the expression of target genes in malignant cells with telomerase activity, while normal sparing normal cells lacking telomerase (Komata *et al*., 2002; Lin *et al*., 2002; Kawashima *et al*., 2004; Ito *et al*., 2005; Jacob *et al*., 2005).

Importantly, several groups have found that cancer cells are somehow 'addicted' to hTERT expression, meaning that downmodulation of hTERT by antisense oligonucleotides or small interfering RNAs (siRNAs) compromises cell survival. This effect involves alterations in the transcriptome (Smith *et al*., 2003; Li *et al*., 2005) as well as in chromatin structure (Masutomi *et al*., 2005) that are unrelated to the shortening of telomeres, suggesting that hTERT may have other functions than telomere maintenance. Typically, targeting of hTERT and hTERC has similar effects on telomerase activity, but only the downregulation of hTERT causes a rapid decline in cell growth, suggesting an enzymatic activity-independent mechanism by which hTERT maintains tumor cell survival and proliferation (Folini *et al*., 2005). Moreover, the inhibition of hTERT expression can trigger apoptosis before a significant effect on the mean telomere length is obtained. Similarly, it has been found that transfection-enforced overexpression of hTERT

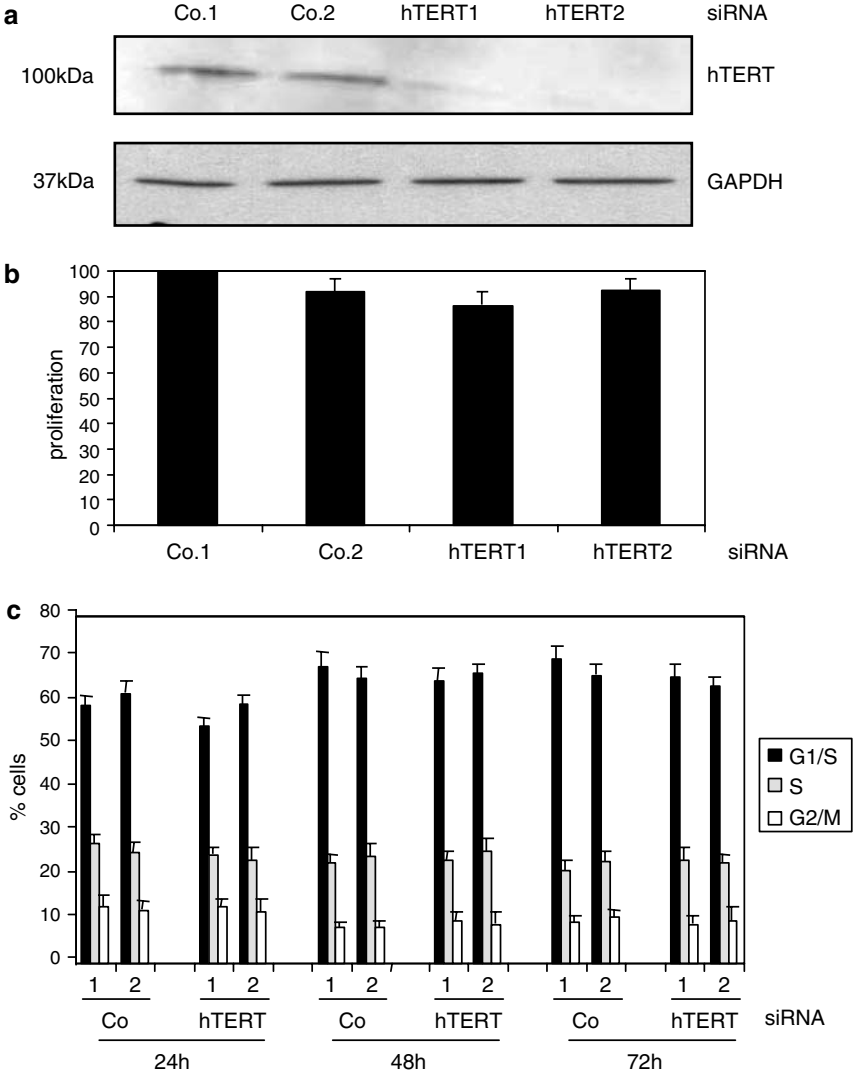

Figure 1 Knockdown of the hTERT gene without acute toxicity. (**a**) Knockdown of hTERT at the protein level. HeLa cells were transfected with the indicated small interfering RNAs, and the downmodulation of the hTERT protein was determined by immunoblot 48 h after transfection. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. (**b**) Normal survival of cells subjected to acute hTERT depletion. After the indicated period, cell cultures transfected with hTERT-specific or control small interfering RNAs (siRNAs) were subjected to the determination of overall dehydrogenase activity using a tetrazolium-based assay. (**c**) Normal cell cycle progression after hTERT depletion. Cells transfected with the hTERT or control siRNAs were subjected to cell cycle analysis, as indicated in Materials and methods. Data are shown for hTERT siRNA1 and co-siRNA1. Similar results were obtained for hTERT siRNA2 and co-siRNA2 (not shown). Results are means ± s.d. of triplicates. All experiments were repeated at least two times, yielding similar results.

can reduce stress-induced cell death (Lu *et al*., 2001; Saretzki *et al*., 2001; Gorbunova *et al*., 2003; Luiten *et al*., 2003; Kang *et al*., 2004). According to one group, hTERT that lacks reverse transcriptase activity loses its cytoprotective activity (Zhang *et al*., 2003), but according to another group, hTERT mutants that lack telomerase activity still can suppress cell death induction, for instance by DNA-damaging agents (Rahman *et al*., 2005). However, these results were mostly based on the non-physiological overexpression of hTERT.

Based on the above premises, we decided to re-investigate the role of hTERT in apoptosis control. To address this conundrum, we took advantage of siRNAs that cause acute hTERT depletion in short-term transfection assays that avoid artifacts linked to a long-term suppression of hTERT (and that could cause genomic instability). Using this approach, we found that acute hTERT inhibition facilitates apoptosis induction through the mitochondrial pathway. Our results unravel a novel role of hTERT as an endogenous inhibitor of mitochondrial apoptosis.

Results and discussion

Chemosensitizing effect of hTERT-specific small interfering RNAs

Reportedly, the knockdown of hTERT can have toxic effects, leading to a proliferative arrest or even cell death (Li *et al*., 2005). Using retroviral techniques for stable hTERT inhibition, this toxic effect is usually observed 5–10 days post knockdown, depending on the cell line, including for HeLa cells (10 days) (Li *et al*., 2005) and HCT116 cells (5 days) (Li *et al*., 2004). We have re-examined the putative toxic effect of hTERT knockdown in HeLa cells, in which transfection with two distinct hTERT-specific siRNA (hTERT1 and hTERT2) led to the disappearance of hTERT protein within 48 h (Figure 1a). This acute hTERT depletion did not have any deleterious effect of cell survival (Figure 1b) up to 72 h post-transfection and did not affect cell cycle progression, as indicated by comparison with two control siRNAs targeting two irrelevant genes (luciferase and emerin, respectively) (Figure 1c). In addition, acute hTERT depletion did not enhance the rate of cell death as measured by a variety of distinct methods, both in HeLa cells (Figures 2–4) and in HCT116 cells (see below). Although knockdown of hTERT had no cytotoxic activity on its own, hTERT did sensitize HeLa cells to the DNA-damaging chemotherapeutic agent cisplatin. Thus, HeLa cells lacking hTERT expression were more sensitive to cell death induction by cisplatin than their hTERT-expressing counterparts. This chemosensitizing effect of hTERT depletion was observed using a variety of different cytofluorometric methods for the evaluation of apoptosis, namely phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane (measured with an Annexin V-fluorescein isothiocyanate (FITC) conjugate; Figure 2), plasma membrane permeabilization (determined with the vital dye propidium iodide (PI); Figures 2 and 4), chromatin degradation leading to DNA loss (determined with the DNA intercalating agent ethidium bromide, Figure 3), as well as the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$; measured with $DiOC_3(6)$; Figure 4). Cell death defined as loss of viability occurred after the cells manifested biochemical changes that are classically associated with apoptotic cell death (Zamzami *et al*., 1995; Castedo *et al*., 1996), namely PS surface exposure (Figure 2) and $\Delta\Psi_m$ dissipation (Figure 4).

Taken together, these data support the contention that acute hTERT depletion, although non-toxic on its

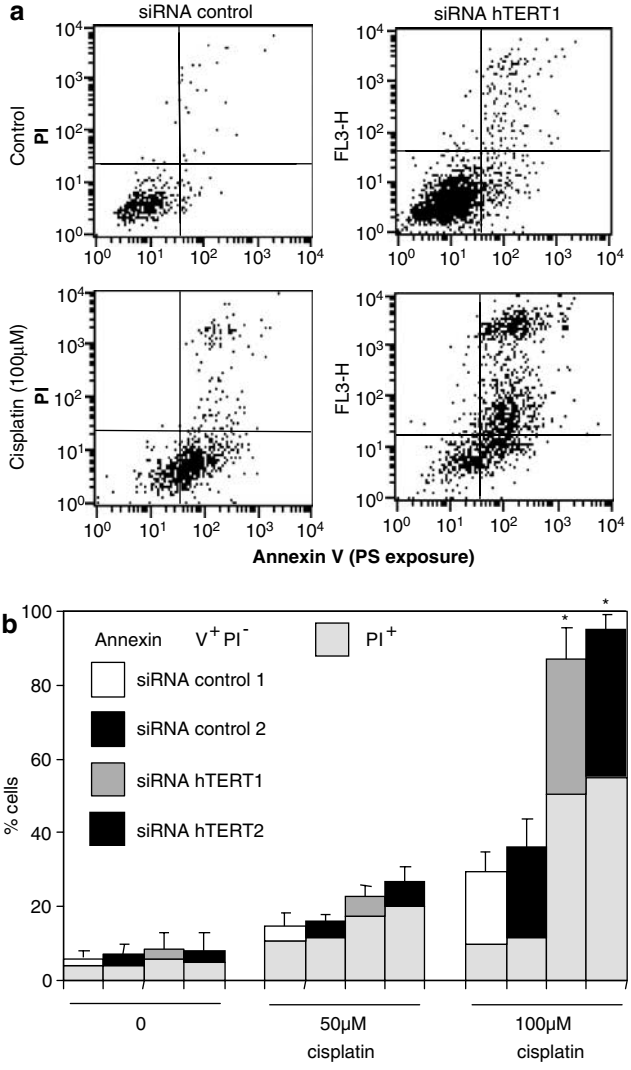

Figure 2 Knockdown of hTERT sensitizes to apoptosis induction by cisplatin. HeLa cells were subjected to the transfection of the indicated small interfering RNAs and were then (48 h later) treated with the indicated concentrations of cisplatin for 24 h, followed by staining with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate for the detection of dying (Annexin V^+ PI^-) or dead (AnnexinV^+PI^+) cells. Representative fluorescence-activated cell sorting pictograms are shown in (**a**) and mean data $\pm$s.d. ($n=3$) are shown in (**b**). Asterisks indicate a significant ($P<0.01$, Student's *t*-test) chemosensitizing effect of hTERT-depleted cells as compared to control cells exposed to similar conditions.

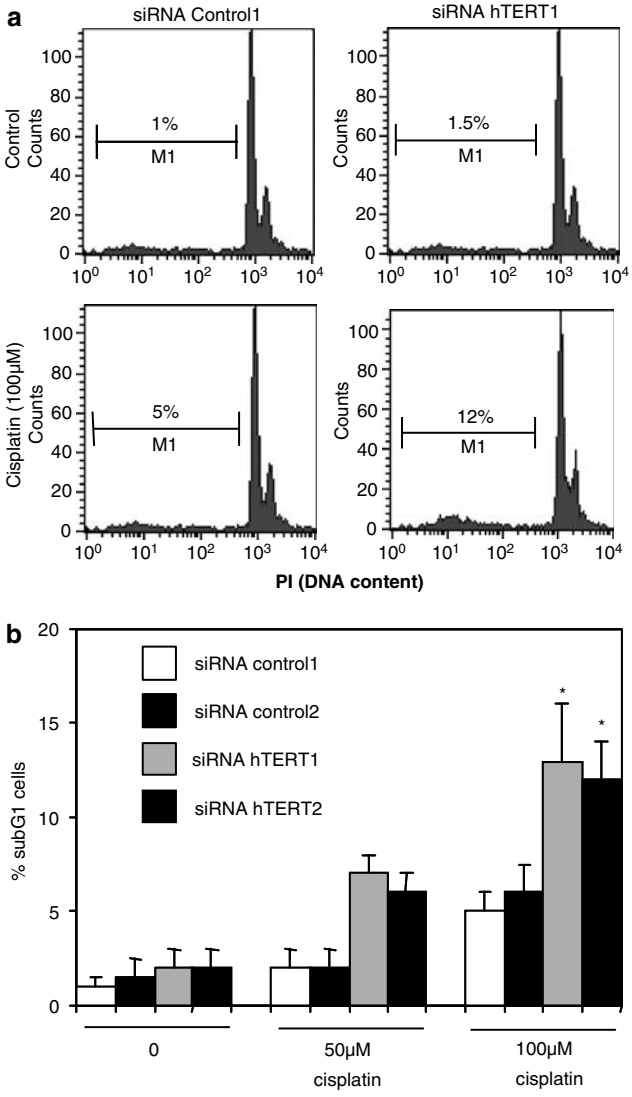

Figure 3 Knockdown of hTERT sensitizes to nuclear apoptosis induction by cisplatin. HeLa cells were subjected to transfection with the indicated small interfering RNAs as in Figure 2 and were treated with cisplatin for 24 h, followed by permeabilization and fluorescence-activated cell sorting determination of propidium iodide-labeled DNA. Representative histograms are shown in (**a**), and statistical analyses in (**b**) were performed as in Figure 2.

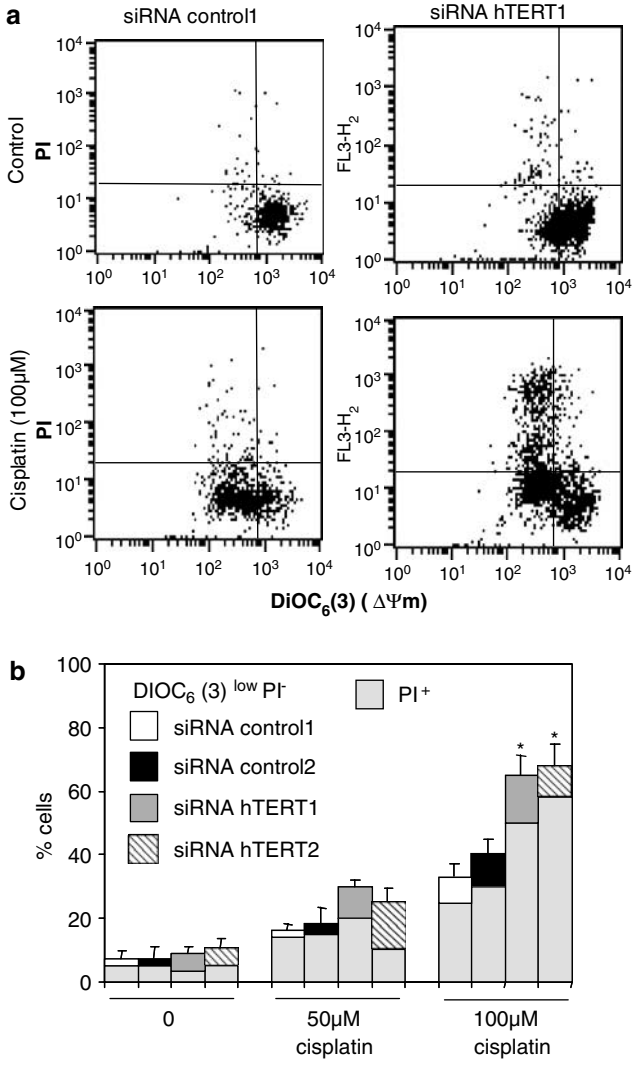

Figure 4 Knockdown of hTERT sensitizes to mitochondrial apoptosis. HeLa cells were transfected with different small interfering RNAs (siRNAs) and were then (48 h later) treated with cisplatin for 24 h (as in Figures 2 and 3), followed by staining with propidium iodide and the mitochondrial transmembrane potential-sensitive dye dihexyloxacarbocyanine iodide. Representative fluorescence-activated cell sorting pictograms are shown in (**a**) and mean data ±s.d. ($n=3$) are shown in (**b**). Asterisks indicate a significant ($P<0.01$, Student's *t*-test) chemosensitizing effect of the hTERT siRNAs.

own, facilitates the induction of apoptotic cell death by DNA damage.

Chemosensitization by hTERT depletion relies on the intrinsic pathways of apoptosis and occurs in a p53-independent fashion

To determine the mechanisms of hTERT-siRNA-facilitated apoptosis, we first determined the range of the chemosensitizing effect of hTERT depletion. Importantly, the chemosensitizing effect of hTERT depletion was observed for a variety of other chemotherapeutic agents including etoposide (VP16) and mitomycin C (Figure 5). The death-sensitizing effect of hTERT depletion was also detectable when cells were treated with the pro-oxidant *tert*-butylhydroperoxide, yet was not found when the cells were killed by treatment with an agonist anti-CD95 antibody (Figure 5). Thus, hTERT depletion can sensitize cells to killing by a variety of agents that activate the intrinsic (mitochondrial) cell death pathway, yet fails to sensitize to the extrinsic (death receptor-mediated) pathway (Figure 6a and b). Additional experiments showed that apoptosis induced by anti-CD95 through caspase 8 activation was blocked by Bcl-2 overexpression and that hTERT depletion did not overcome the antiapoptotic action of Bcl-2 (Figure 6a and b). Hence, the apoptosis-modulatory activities of hTERT and Bcl-2 are clearly distinct.

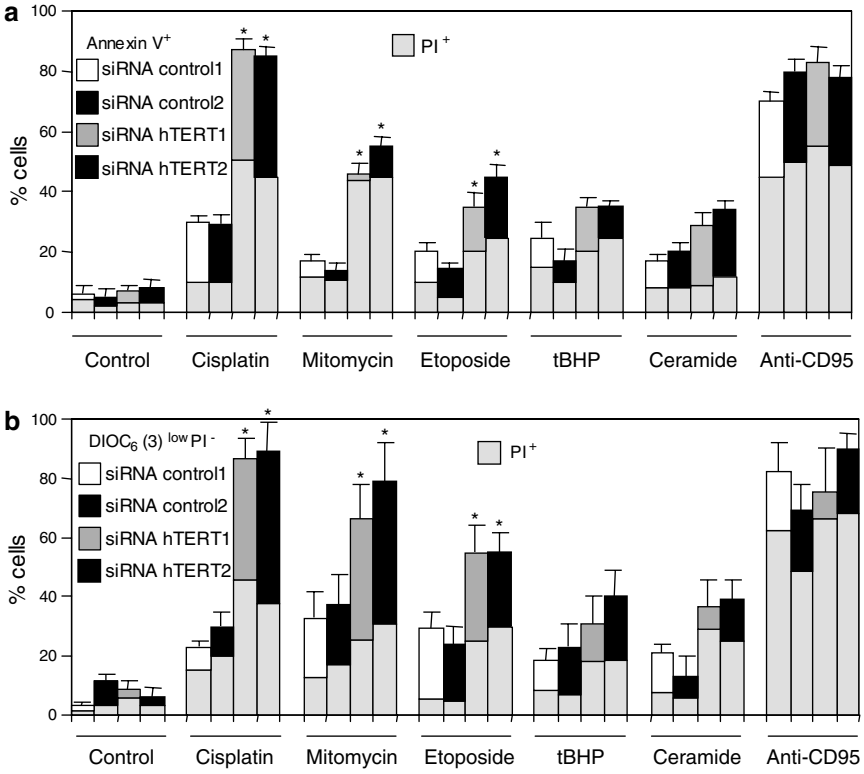

Figure 5 Broad chemosensitizing effect of the hTERT knockdown. HeLa cells depleted from hTERT as in Figures 1–4 were treated with cisplatin, mitomycin, etoposide, *tert*-butylhydroperoxide (tBHP), anti-CD95 or C-2 ceramide for 24 h and the percentage of dying or dead cells was determined using the combined Annexin V/propidium iodide (PI) staining (**a**), as in Figure 2, or the combined dihexyloxacarbocyanine iodide/PI staining (**b**), as in Figure 3. Asterisks indicate statistically significant cytoprotective effects of the two hTERT small interfering RNAs.

Several reports have suggested a privileged crosstalk between hTERT and p53 (Cao *et al*., 2002; Rahman *et al*., 2005) Therefore, we evaluated the chemosensitizing effect of hTERT in the presence of cyclic pifithrin-α, a chemical inhibitor of p53-mediated transactivation (Komarov *et al*., 1999). Cyclic pifithrin-α did not abolish the chemosensitizing effect of hTERT depletion, independently of the test that was measured to determine cell death induction, be it staining with Annexin V (Figure 7a) or with $DiOC_3(6)$ (Figure 7b). Similar results were obtained when the chemosensitizing effect of hTERT was comparatively assessed on wild-type HCT116 cells (that express p53) and p53 knockout cells. Although the absence of p53 attenuated apoptosis induction by cisplatin, it did not prevent increased cell death stimulated by the combination of hTERT depletion plus cisplatin, as compared to either of these manipulations alone (Figure 8).

Altogether, it appears that hTERT knockdown sensitizes to cell death induction through the intrinsic pathway, and does so in a p53-independent fashion.

Role of mitochondria and caspases in chemosensitization by hTERT depletion

To further characterize the mechanisms through which hTERT siRNAs can facilitate cell death induction, we assessed the effect of the combination therapy (hTERT depletion plus cisplatin) on HCT116 cells lacking expression of the proapoptotic Bcl-2 family protein Bax. As shown in Figure 8, Bax was required for apoptosis induction by hTERT depletion plus cisplatin, at all levels (PS exposure, $\Delta\Psi_m$, loss and plasma membrane permeabilizaton). Next, we determined the implication of Bax in this setting by a completely different approach, namely by assessing a conformational change in Bax that can be detected with a monoclonal antibody specific for active, membrane-inserted Bax (Wolter *et al*., 1997). Using this approach, we found that Bax activation was enhanced by the combination of hTERT depletion and cisplatin treatment as compared to either hTERT siRNA or cisplatin alone (Figure 9). To confirm the critical role of mitochondria in cell death induction, we took advantage of cells that overexpress the mitochondrion-stabilizing protein Bcl-2 or the structurally unrelated cytomegalovirus-encoded vMIA (viral mitochondrial inhibitor of apoptosis), which localizes to mitochondria where it neutralizes Bax (Poncet *et al*., 2004; Goldmacher, 2005). Both Bcl-2 and vMIA inhibited cell death induction by hTERT siRNA plus cisplatin, and thus prevented PS exposure (Figure 10a) and $\Delta\Psi_m$ loss (Figure 10b).

In strict contrast with Bcl-2 and vMIA, caspase inhibition by *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoro-

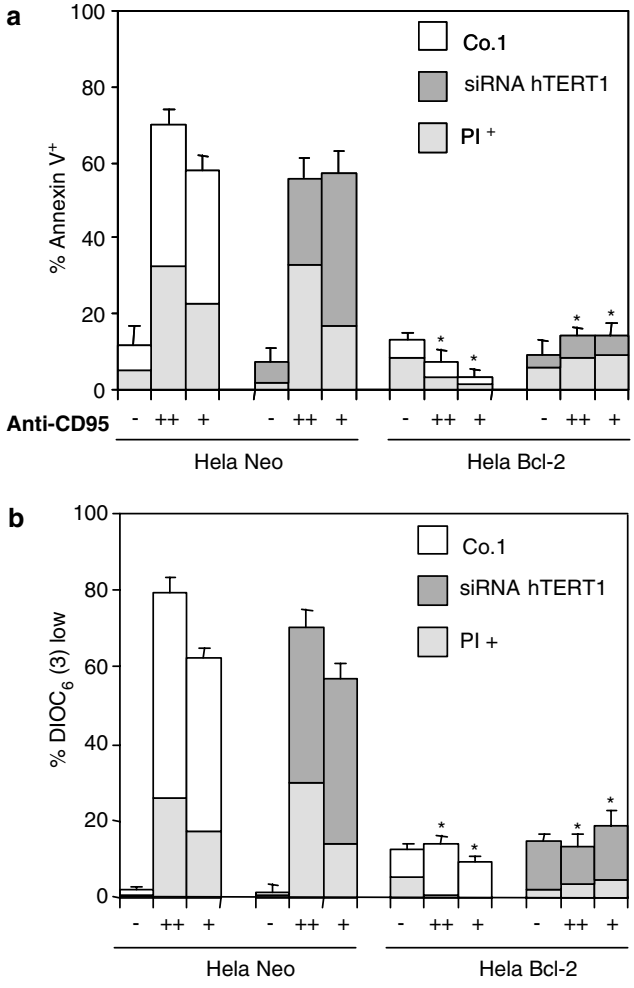

Figure 6 Failure of the hTERT knockdown to sensitize to apoptosis induced by anti-CD95. HeLa cells stably expressing vector only (Neo) or Bcl-2 were transfected with hTERT small interfering RNAs and were then (48 h later) treated with anti-CD95 (+250 pg/ml; ++500 pg/ml) for 12 h, followed by staining with Annexin V/propidium iodide (PI) (**a**), as in Figure 2, or with dihexyloxacarbocyanine iodide/PI (**b**), as in Figure 3. Asterisks indicate significant cytoprotective effects of Bcl-2.

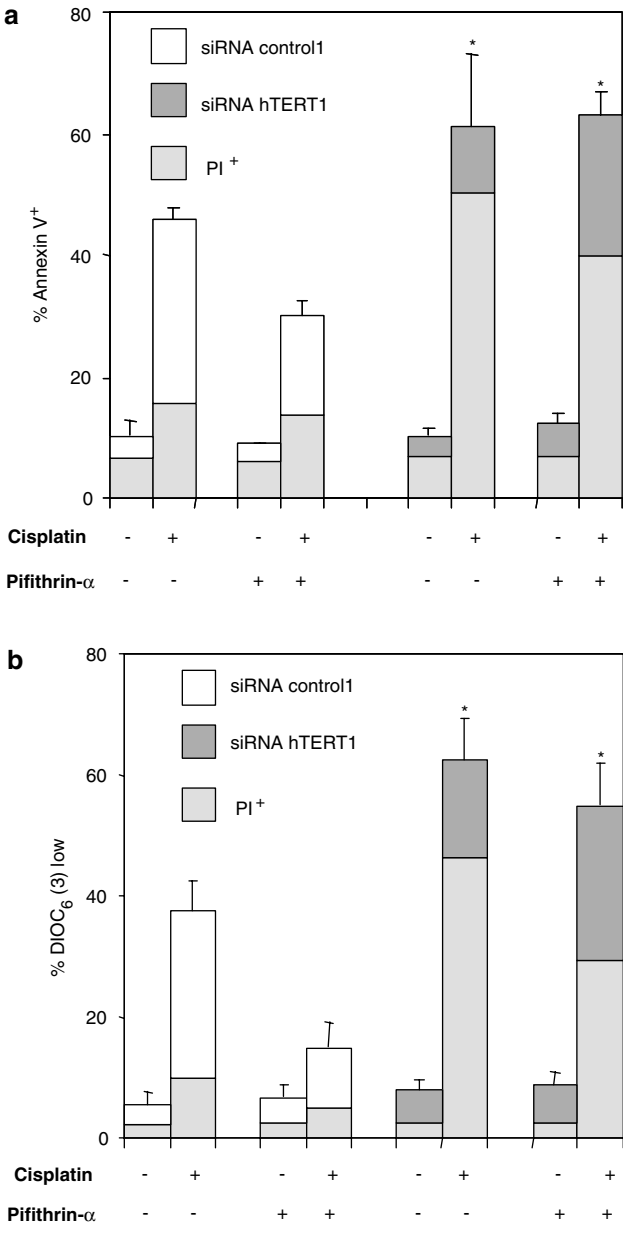

Figure 7 p53 independence of the hTERT small interfering RNA (siRNA)-mediated chemosensitizing effect. HeLa cells transfected with hTERT1 siRNA or control1 siRNA were treated with 100 μM cisplatin alone or in the presence of 30 μM cyclic pifithrin-α for 24 h. Fluorescence-activated cell sorting analyses (X $\pm$ s.d., $n=3$) of cell death parameters were performed after Annexin V/propidium iodide (PI) (**a**) or dihexyloxacarbocyanine iodide/PI staining (**b**).

methylketone (Z-VAD-fmk) failed to stabilize $\Delta\Psi_m$. Thus, cells treated with hTERT siRNA and cisplatin manifested the $\Delta\Psi_m$ dissipation irrespective of the addition of Z-VAD-fmk (Figure 11b). However, as an internal control of its efficacy, Z-VAD-fmk was able to retard PS exposure (Figure 11a) and plasma membrane permeabilization (Figure 11b). These data indicate that caspase activation is dispensable for $\Delta\Psi_m$ dissipation and must occur at a post-mitochondrial step of the cell death cascade. Moreover, these data demonstrate that the depletion of hTERT facilitates the $\Delta\Psi_m$ dissipation in a caspase-independent fashion.

Altogether, these results support the fundamental role of Bax-mediated MMP in the chemosensitization conferred by hTERT depletion, and delineate a molecular order of the cell death cascade (MMP upstream of caspases) facilitated by hTERT inhibition.

Concluding remarks

It has been suspected for long that hTERT may contribute to tumorigenesis by a telomere length-independent mechanism because mutant hTERT, incapable of maintaining telomere length, can transform cell lines *in vitro* (Stewart *et al*., 2002). As shown, here, hTERT functions as an endogenous inhibitor of the mitochondrial pathway of apoptosis, in addition to its cardinal importance in tumor cell immortalization. This

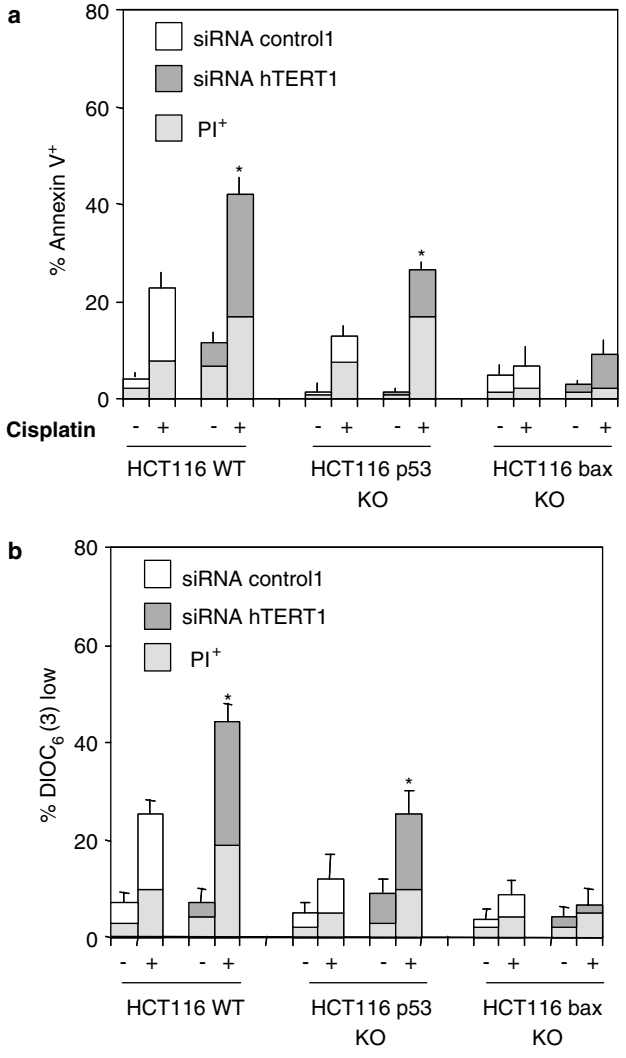

Figure 8 p53 independence but Bax dependence of the hTERT small interfering RNA (siRNA)-mediated chemosensitizing effect. Control, p53-deficient or Bax-deficient HCT116 cells were transfected with control or hTERT-specific siRNAs and were then (48 h later) cultured for 24 h in the absence or presence of 100 μM cisplatin. Fluorescence-activated cell sorting analyses (X$\pm$s.d., $n=3$) of cell death parameters were performed after Annexin V/propidium iodide (PI) (**a**) or dihexyloxacarbocyanine iodide/PI staining (**b**).

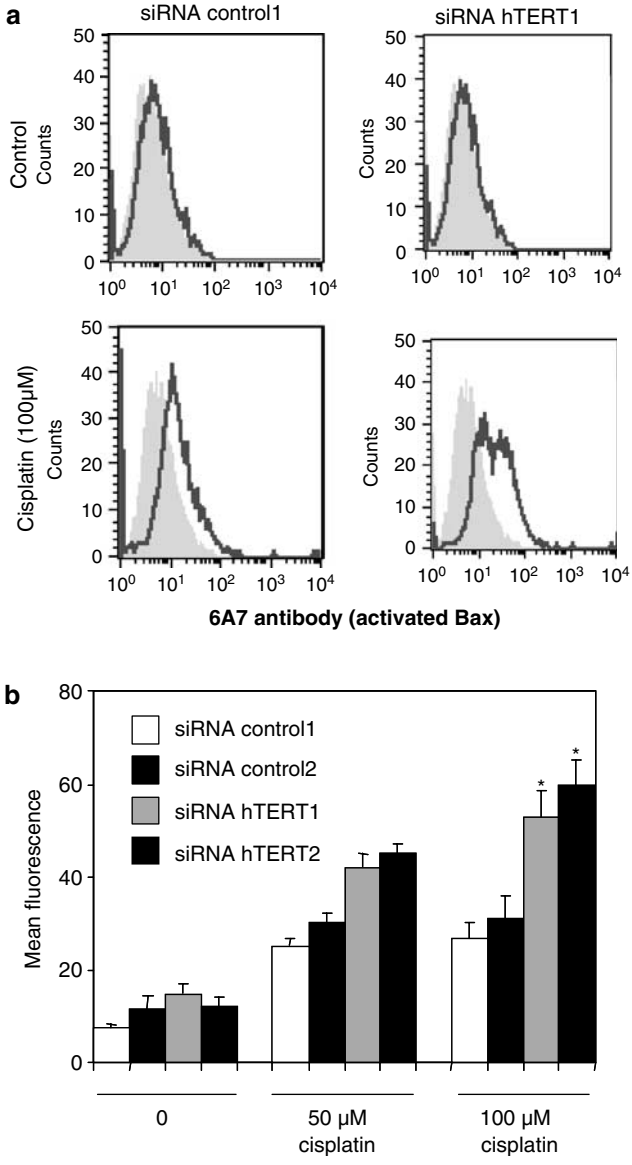

Figure 9 hTERT small interfering RNA (siRNA) facilitates the conformational activation of Bax. HeLa cells were transfected with control or hTERT-specific siRNAs and then cultured either in the absence or presence of cisplatin (100 μM, 24 h). Then, the cells were permeabilized with a neutral detergent, stained with a monoclonal antibody specific for the N-terminus of Bax (that is only accessible when Bax is activated and membrane-inserted) and subjected to fluorescence-activated cell sorting (FACS) analysis. Representative FACS diagrams are shown in (**a**) and a statistical analysis of the data is provided in (**b**). Results are means$\pm$s.d. of triplicates and arrows refer to significant effects of the hTERT1 siRNA.

contention is based on the observation that down-modulation of hTERT by siRNAs has no toxic effects by itself (Figure 1), yet sensitizes cancer cells to apoptosis induction by DNA-damaging agents as well as by reactive oxygen species (Figure 2). Although suppression of hTERT sensitizes to the mitochondrial pathway of apoptosis, it has no such effect on CD95-induced cell death. The chemosensitizing effect is obtained immediately, as soon as the hTERT protein expression level is reduced (within 48–72 h). This time frame excludes the possibility that telomere erosion participates in the chemosensitizing effect.

As to the mechanisms that are involved in the sensitization to cell death induction by hTERT down-modulation, our data clearly show that p53 is not required for this effect (Figures 7 and 8). In contrast, our results point to increased activation of the Bax protein, which triggers the mitochondrial pathway of apoptosis, and Bax is required for apoptosis facilitated by hTERT inhibition (Figures 9 and 10). We did not find any effect of hTERT depletion on the expression level of Bax itself or on that of Bax-regulatory proteins such as Bak, Bcl-2,

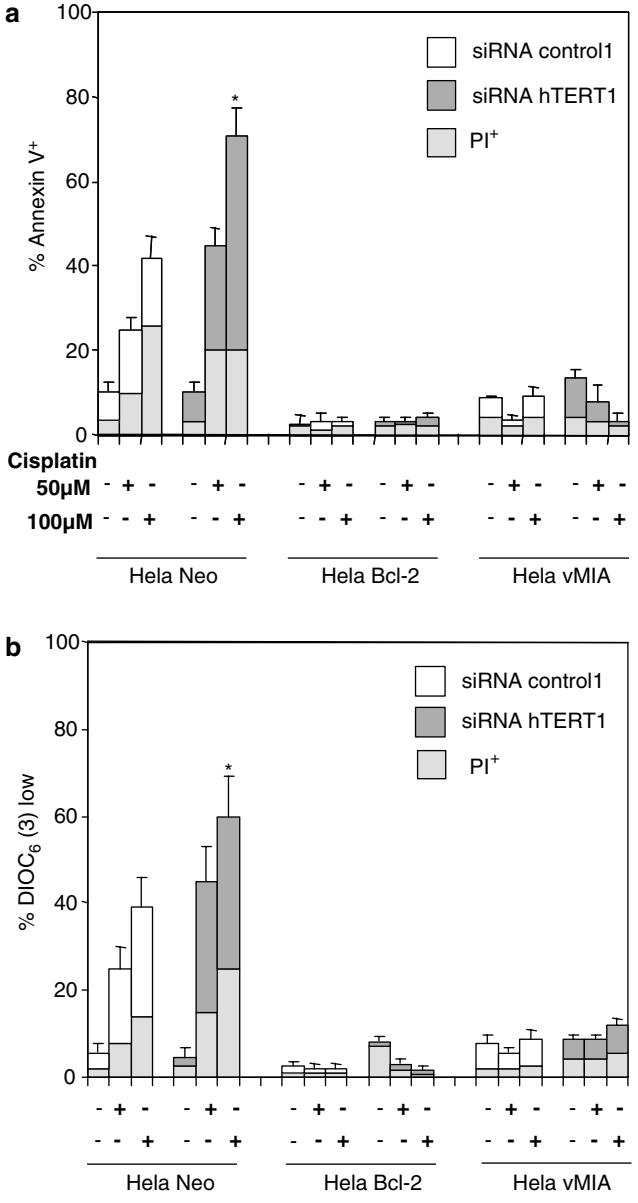

Figure 10 Mitochondrial stabilization by Bcl-2 or vMIA abolishes hTERT small interfering RNA (siRNA)-mediated chemosensitizing. HeLa cells transfected with vector only (Neo), Bcl-2 or vMIA were subjected to sequential siRNA-mediated hTERT depletion and cisplatin treatment, followed by fluorescence-activated cell sorting analyses of death-associated mitochondrial transmembrane potential dissipation and loss of cellular viability.

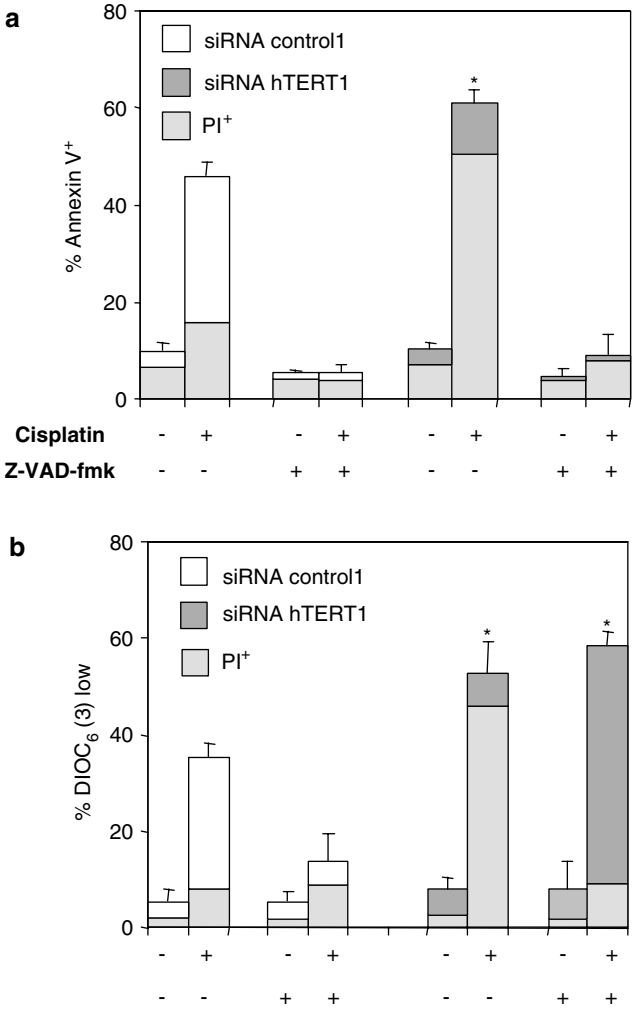

Figure 11 Lack of caspase involvement in hTERT small interfering RNA (siRNA)-mediated sensitization to mitochondrial apoptosis. HeLa cells subjected to the standard treatment with control or hTERT-specific siRNAs were exposed to an *vitro* chemotherapy with cisplatin (100 μM), alone or in combination with the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (50 μM), stained with AnnexinV/propidium iodide (PI) (**a**) or dihexyloxacarbocyanine iodide/PI (**b**) and analysed by cytofluorometry. Results are representative of three independent experiments.

Bcl-X_L, Puma/Bbc3, Noxa, Bim, Bid or Bad (not shown), suggesting that post-translational (rather than transcriptional or translational) modifications in the Bax interactome account for the enhanced activation of Bax in hTERT-depleted cells. Thus, the mechanism through which Bax is activated in this pathway remains an open question for future investigation. Santos *et al*. (2004) have shown that hTERT is targeted to the mitochondria and could sensitize cells to oxidative stress. Moreover, Haendeler *et al*. (2003, 2004) have published evidence suggesting that oxidative stress favors the nuclear export of hTERT, which in turn might contribute to the antiapoptotic activity of hTERT. Thus, a direct effect of hTERT on cytoplasmic, presumably mitochondrial, targets appears plausible, and a possible overlap between the hTERT and the Bax interactomes should be investigated in the future.

Our data suggest that inhibition of hTERT expression by siRNA may constitute a valid strategy of chemosensitization. Future investigation will reveal whether hTERT-specific siRNAs with improved *in vivo* pharmacokinetics may enter the clinics. Intriguingly, there are a number of drugs used in experimental chemotherapy that are able to downmodulate hTERT expression at the

mRNA or protein levels. This applies to rapamycin (Zhou *et al*., 2003), histone deacetylase inhibitors (Wu *et al*., 2005), imatinib mesylate (Gleevec) (Uziel *et al*., 2005), the Bcl-2/Bcl-XL-specific antisense oligonucleotide 4625 (Del Bufalo *et al*., 2005), as well as vitamin D3 (Jiang *et al*., 2004). It remains to be determined, however, to which extent hTERT downmodulation may explain the cytotoxic and chemosensitizing effects of such drugs.

Irrespective of these incognita, it appears that hTERT may constitute an ideal target for a double-hit anticancer strategy. Whereas inhibition of the catalytic telomerase activity should limit the lifespan of tumor cells and hence exert a long-term effect obtained by chronic therapy, acute inhibition of the second antiapoptotic function can yield an immediate chemosensitizing effect, including in tumors in which the p53 pathway is subverted. However, tumors in which the Bax-dependent mitochondrial pathway of apoptosis induction has been invalidated by loss of expression of Bax or overexpression of Bcl-2 are refractory to chemosensitization by hTERT inhibition.

Materials and methods

Cell lines, culture conditions and transfection

Wild-type HeLa cells, or HeLa transfected with the pcDNA3.1 vector encoding the neomycin resistance gene (Neo), Bcl-2 cells or the cytomegaloviurs-encoded vMIA (Goldmacher *et al*., 1999; Belzacq *et al*., 2001) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM of pyruvate, 10 mM Hepes and 100 U/ml penicillin/streptomycin at 37°C under 5% CO_2. Wild-type, p53 knockout (Bunz *et al*., 1999) and Bax knockout HCT116 cells (Zhang *et al*., 2000) were cultured in the same conditions, with the only difference that DMEM was replaced by McCoy's 5A medium.

RNA interference

Selected oligonucleotides RNAs were synthesized, purified and annealed into siRNAs duplexes (Proligo, Boulder, CO, USA). RNA interference of hTERT expression was performed using specific siRNA sequences for targeting hTERT mRNA, named hTERT siRNA1 (sense strand, 5′-rgrArArCrGrgrgrgrCr CrUrgrgrArArCrCrArUArTT-3′) and hTERT siRNA2 (sense strand, 5′-rUrUrUrCrArUrCrArgrgrAgArgrUrUrUrgrgr ATT-3′) (Masutomi *et al*., 2003). For transfection, cells were cultured in six-well plates (1.5×10^5 cells/well) for 24 h and then transfected with 1 μg siRNA formulated into liposomes with 3 μl oligofectamine (Invitrogen, Carlsbad, CA, USA). Controls were obtained by transfection with two siRNA controls, luciferase (control siRNA1) (Lewis *et al*., 2002) and emerin (control siRNA2) (Harborth *et al*., 2001). Two days after transfection, cells were treated with distinct cytotoxic drugs.

Cell death induction and inhibition

Cells were cultured for 24 h with different doses of drugs: cisplatin, mitomycin, etoposide, anti-CD95, C2-ceramide, cyclosporine A and *tert*-butyl-hydroperoxidate (Sigma-Aldrich, Taufhirchen, Germany). In some experiments, cells were incubated with 100 μM Z-VAD-fmk (Bachem, Torrance, CA, USA) and 30 μM cyclic pifithrine-α (Sigma).

Flow cytometry

We used 40 nM 3,3′ dihexyloxacarbocyanine iodide ($DIOC_6(3)$) for $\Delta\Psi_m$ quantification, 1 μg/ml PI for determination of cell viability and Annexin V conjugated with FITC (Bender Medsystems) for assessment of PS exposure (Castedo *et al*., 2002a). The cellular DNA content was quantified on ethanol-fixed cells that were stained with PI (Nicoletti *et al*., 1991). To study Bax activation, cells ($5–1 \times 10^5$ cells per condition) were treated with 50 or 25 μM cisplatin for 24 h. Cells were fixed in 0.25% paraformaldehyde in PBS for 5 min, washed in PBS three times and incubated with 1:50 anti-Bax antibody (clone, 6A7, BD Bioscience) in 100 μg/ml digitonin diluted in PBS–1% BSA for 30 min (Castedo *et al*., 2002b). After three washes in PBS–1% BSA, cells were incubated with 1:100 FTIC-conjugated secondary anti-mouse antibody diluted in PBS–1% BSA for 30 min. After two washes, cells were resuspended in PBS and analysed on a fluorescence-activated cell sorting Vantage cytofluorometer (Becton Dickinson, Heidelberg, Germany).

Determination of cell proliferation

Cell proliferation was determined by seeding 2500 cells per well in 96-well incubation plates using the WST-1 colorimetric assay (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. Briefly, at 24, 48 and 72 h, cells were incubated for 3 h with WST1 and absorbance (A) was measured in each well in an automatic scanning photometer at a wavelength of 570 nm. Each experimental point was determined in triplicate. The percentage of cell proliferation was calculated according to the formula $P = (A$ in treated cells$/A$ in control cells$) \times 100$, after background subtraction.

Immunoblot

Cells were lysed for 15 min in 50 mM Hepes, 150 mM NaCl, 5 mM EDTA, plus 0.1% NP-40, supplemented with a protease inhibitor cocktail (1 mM dithiothreitol and 1 mM phenylmethylsulphonly fluoride; Roche Molecular Biochemicals, Mannheim, Germany), and centrifuged at 12 000 r.p.m. for 20 min to remove debris. Total protein content was determined with the Bio-Rad DC kit. A 50 μg portion of protein was loaded on a 10% SDS–polyacryamide gel electrophoresis. Anti-hTERT antibody (Novocastra, Newcastle on Tyne, UK) was used to determined hTERT protein, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA, USA) was used as a loading control. Immonoblot analyses were developed using the enhanced chemoluminescence-based detection kit (Pierce, Rockford, IL, USA).

Abbreviations

$\Delta\Psi_m$, mitochondrial transmembrane potential; DAPI, 4′,6-diaminidino-2-phenylindole; DiOC6(3), 3,3′ dihexyloxacarbocyanine iodide; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, mitochondrial membrane permeabilization; PI, propidium iodide; PS, phosphatidylserine; siRNA, small interfering RNA; Z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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