Ectopic mTERT expression in mouse embryonic stem cells does not affect differentiation but confers resistance to differentiation- and stress-induced p53-dependent apoptosis

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Summary

The fundamental role of telomerase is to protect telomere ends and to maintain telomere length during replication; hence, telomerase expression is high in stem cells but reduced upon differentiation. Recent studies indicate that telomerase might play other roles besides telomere maintenance. We have investigated the role of telomerase in cellular differentiation and death. Here, we show that ectopic expression of mouse telomerase catalytic subunit (mTERT) does not affect embryonic stem (ES) cell proliferation or differentiation in vitro, but protects ES cells against cell death during differentiation. Ectopic mTERT expression also confers resistance to apoptosis induced by oxidative stress and other genotoxic insults. This resistance depends on the catalytic activity of mTERT. Stress-signal-induced p53 accumulation and consequent p53-dependent apoptotic target gene expression was not affected by mTERT overexpression. However, although chemical inhibition of p53 by α-pifithrin reduced stress-induced apoptosis in vector-expressing cells, it did not significantly affect apoptosis in mTERT-expressing cells. Moreover, overexpression of mTERT in *p53*$^{-/-}$ ES cells did not confer further resistance to genotoxic insults, suggesting that mTERT might exert its protective effect by antagonizing the p53 pathway. Altogether, our findings indicate that ectopic mTERT expression in ES cells does not affect differentiation but confers resistance to apoptosis, and suggest that this strategy might be used in improving the efficiency of stem-cell therapies.

Key words: Apoptosis, Differentiation, ES cells, mTERT, p53, Stress signals

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Introduction

Telomerase is a ribonucleoprotein complex comprising the catalytic subunit (TERT) and an RNA component (TR) that adds TTAGGG repeats to chromosome ends, thus maintaining the length of telomeres (Collins and Mitchell, 2002). Telomerase is present in human stem cells and progenitors but is hardly detectable in the vast majority of differentiated somatic tissues (Urquidi et al., 2000). However, telomerase is reactivated during tumorigenesis, preventing telomere erosion and immortalization (Kim et al., 1994). By contrast, telomerase activity is more readily detectable in mouse cells and tissues, which generally contain longer telomeres than humans (Kipling and Cooke, 1990; Martin-Rivera et al., 1998; Greenberg et al., 1998). Moreover, murine embryonic stem (ES) cells have been shown to exhibit high telomerase activity (Sharma et al., 1995).

The potential role of telomerase in regulating cell growth has been evaluated by overexpression and gene-ablation experiments. Overexpression of human TERT (hTERT) in various cell lines resulted in resistance to several forms of apoptosis and in extended cellular life-span (Fu et al., 2000; Akiyama et al., 2002; Gorbunova et al., 2002; Luiten et al., 2003). By contrast, ablation of telomerase activity by antisense approaches or by expression of the dominant-negative hTERT resulted in enhanced sensitivity to cell death and consequently, growth inhibition (Fu et al., 1999; Misawa et al., 2002; Yuan and Mei, 2002; Nakajima et al., 2003; Teng et al., 2003; Liu et al., 2004). However, limited experiments have been performed with mouse TERT (mTERT). Overexpression of mTERT in transgenic mice resulted in susceptibility to several forms of tumour (Oh et al., 2001; Artandi et al., 2002; Gonzalez-Suarez et al., 2002). Gene knockout experiments revealed that the *Terc*$^{-/-}$ and *mTERT*$^{-/-}$ mice had no obvious abnormalities in early generations, although significant telomere shortening was observed in successive generations, resulting in increased apoptosis and malignancies arising from genomic instability (Rudolph et al., 1999; Yuan et al., 1999). It was also demonstrated that telomerase can add telomeric repeats to chromosome breaks upon irradiation, suggesting that telomerase might have additional roles beyond telomere

maintenance (Slijepcevic and Bryant, 1998). Together, these data suggest a crucial role for TERT in regulating cellular growth, although the mechanistic basis for some of these observations is not completely understood.

Telomerase activity is regulated during differentiation, being reduced after ES-cell differentiation and during placental differentiation (Sharma et al., 1995; Armstrong et al., 2000; Rama et al., 2001). Accordingly, tumours arising out of progenitor cells lose their tumorigenicity upon differentiation, during which the telomerase activity is also dramatically reduced (Xu et al., 1999; Koyanagi et al., 2000). Although ectopic overexpression of hTERT in human mesenchymal cells and leukaemic cells was shown not to affect differentiation (Kobune et al., 2003; Yamada et al., 2003), terminal differentiation of human HaCaT cells was blocked by constitutive hTERT expression (Cerezo et al., 2003). These data raise the possibility that telomerase activity must be tightly regulated during cellular differentiation, although it appears to be cell-type specific. We have therefore evaluated whether ectopic expression of mTERT in pluripotent mouse ES cells would affect their differentiation capacity, as well as their response to differentiation and stress-induced apoptosis.

Materials and Methods

Cell lines and plasmids

CCE and *p53*$^{-/-}$ (P1.1) murine ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and 2000 international units (IU) leukaemia inhibitory factor (LIF), as described (Sabapathy et al., 1997). Differentiation of ES cells was induced by adding all-trans-retinoic acid to culture medium without LIF. A *Not*I/*Hin*dIII fragment containing the coding sequence of mTERT (Martin-Rivera et al., 1998) was subcloned into pcDNA vector (Invitrogen). Catalytically inactive mTERT (also cloned into pcDNA vector) is a mutant form of mTERT in which amino acids 1186 and 1187 (both aspartic acid) were changed to alanine (F. Ishikawa, Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University, Japan). For electroporation, plasmids were linearized with *Sca*I and purified by phenol-chloroform extraction, precipitated and dissolved in sterile water. 30 μg linearized plasmid was used to transfect 1×10^7 ES cells. For CCE cells, stable ES clones were selected and maintained in 250 μg ml^{-1} G418, and single colonies were isolated and analysed. For *p53*$^{-/-}$ ES cells, linearized plasmid was co-transfected with pLoxPuro (Arakawa et al., 2001) at a ratio of 5:1 and selected with 1 μg ml^{-1} puromycin.

Semiquantitative RT-PCR

For the semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), total RNA was isolated by TRIZOL reagent (Invitrogen) from ES cells. First-strand cDNA was synthesized by SuperScript II (Invitrogen) and PCR was carried out with Taq DNA polymerase (Qiagen). Primer sequences and PCR conditions used were as described previously by others (Sabapathy et al., 1997; Nozawa et al., 2001; Mitsui et al., 2003).

TRAP assays

The telomeric repeats amplification protocol (TRAP) assay was performed using TRAPeze telomerase detection kit (Intergen). Briefly, approximately 1×10^6 cells were lysed in CHAPS lysis buffer and the protein concentration was determined. Enzymatic reaction and PCR were performed according to the protocols provided by manufacturer. The PCR product was resolved by 10% polyacrylamide-gel electrophoresis (PAGE) in TBE buffer and visualized by staining with ethidium bromide.

Determination of ECMA-7 expression

Anti-ECMA-7 antibody was obtained from culture supernatants of ECMA-7 hybridomas (Sabapathy et al., 1997). For detection of ECMA-7 antigen, ES cells were harvested and washed with PBS once and then incubated with anti-ECMA-7 antibody for 30 minutes at room temperature. Cells were washed once with 0.5% Tween 20, 1% bovine serum albumen (BSA) in PBS and incubated with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG antibody for 30 minutes at room temperature. Cells were washed once and resuspended in PBS for flow cytometry.

Cell-death assays

Two independent cell-death assays were used in this study: propidium iodide (PI) exclusion and annexin-V staining. For PI exclusion, cells were harvested and washed once in PBS. Cell suspension was briefly incubated with 50 μg ml^{-1} PI and immediately analysed by flow cytometry to detected PI-positive cells, which represent the dead population. To detect annexin-V-positive cells, ES cells were incubated with FITC-conjugated annexin-V (BD Biosciences) and 50 μg ml^{-1} PI in binding buffer (10 mM HEPES, pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) for 15 minutes in the dark at room temperature. Cells were analysed by flow cytometry immediately after incubation.

Western-blot analysis

ES cells were harvested and washed with PBS once and then lysed in lysis buffer [50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1% sodium-dodecyl-sulfate (SDS), 1% Triton X-100 and protease inhibitors]. To detect mTERT, nuclear extract was used as described previously (Shats et al., 2003). Cells were briefly lysed with hypertonic buffer and nuclei were precipitated. 50 μg protein from whole-cell lysate or 100 μg nuclear extract was used in 10% SDS-PAGE and 7% SDS-PAGE, respectively, and then transferred to PVDF membrane and incubated with anti-p53 antibody (CM5; Novocastra), anti-telomerase antibody, anti-topoisomerase-I antibody (Santa Cruz Biotechnology) or anti-actin antibody (Sigma). Blots were incubated with ECL western-blotting detection reagent (Amersham) and chemiluminescence was detected with Biomax MR X-ray film (Kodak).

Cleaved caspase-3 staining

Cleaved caspase-3 was measured using an FITC-conjugated antibody against active caspase-3 apoptosis kit (BD Biosciences). Briefly, cells were harvested and washed with PBS and resuspended in Cytofix/Cytoperm™ solution for 20 minutes on ice. Cells were then incubated with antibody at room temperature for 30 minutes. After incubation, cells were washed with Perm/wash™ buffer once and analysed by flow cytometry.

Proliferation assays

ES cells were incubated with 10 μM bromodeoxyuridine (BrdU) for 30 minutes, washed with PBS once and fixed in 70% ethanol overnight. 2 N HCl/0.5% Triton X-100 solution was used to denature DNA then neutralized with 0.1 M $Ba_2B_4O_7\cdot10\ H_2O$, pH 8.5 solution. Cells were then incubated with FITC-conjugated anti-BrdU antibody

(BD Biosciences) diluted in 0.5% Tween 20, 1% BSA in PBS. Cells were washed once with 0.5% Tween 20, 1% BSA in PBS, pelleted and resuspended in 50 μg ml^{-1} PI in PBS for flow cytometry.

Results

Generation of *mTERT*-overexpressing ES cells

Feeder-independent ES cells (CCE cells) (Robertson et al., 1986) were used to generate mTERT-overexpressing cells. CCE cells electroporated with a *mTERT* cDNA expression vector or an empty vector (pcDNA) were selected on G418 and single colonies were isolated. Expression of mTERT was assayed by RT-PCR and colonies expressing relatively high levels of mTERT compared with pcDNA-transfected cells were selected (Fig. 1A). Western-blot analysis also revealed that *mTERT* overexpression resulted in increased mTERT protein level compared with vector-expressing cells (Fig. 1B). The telomerase enzymatic activity of the cells was determined by TRAP assay, which confirmed the increase in telomerase activity in *mTERT*-transfected clones (Fig. 1C). It should be noted that vector-transfected ES cells also show telomerase activity correlating with high basal mTERT protein production, confirming previous results describing the presence of relatively high telomerase activity in undifferentiated ES cells (Sharma et al., 1995). However, the *mTERT*-overexpressing clones displayed significantly more telomerase activity than their pcDNA-transfected counterparts. All subsequent experiments were performed using two independent mTERT-overexpressing ES-cell clones, which gave similar and consistent results, excluding any effects caused by integration sites variations.

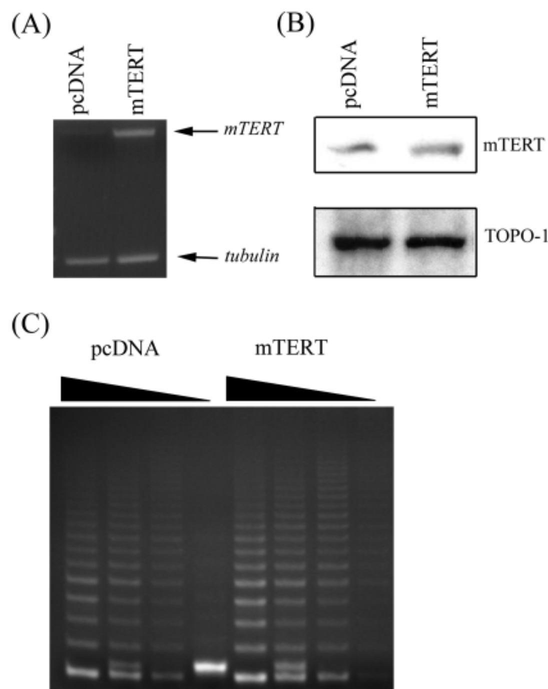

Fig. 1. Generation of *mTERT*-overexpressing ES cells. (A) Expression of telomerase was determined by RT-PCR in vector-transfected and *mTERT*-expressing ES cells. 3 μg total RNA was reverse-transcribed into cDNA and two sets of primers were used to amplify the genes encoding mTERT and tubulin simultaneously. (B) Protein level of mTERT was determined by western-blot analysis using 100 μg nuclear extract from vector-transfected and *mTERT*-expressing ES cells. Topoisomerase-I levels were also determined, which represent loading in each lane. (C) Telomerase activity of vector-transfected and *mTERT*-expressing ES cells was determined by a TRAP assay. Decreasing amounts of protein (50 ng, 10 ng, 2 ng and 0.4 ng) were used in the assay.

Differentiation of *mTERT*-overexpressing ES cells

Because telomerase activity is downregulated upon differentiation of ES cells (Sharma et al., 1995), we next evaluated whether enforced overexpression of *mTERT* would affect the differentiation potential of these cells. To this end, vector-transfected and *mTERT*-overexpressing ES-cell clones were induced to differentiate with 0.1 μM (data not shown) and 0.3 μM (Fig. 2A) retinoic acid. The progress of differentiation was monitored both by morphological appearance and by determining the amount of ECMA-7 stem-cell-specific antigen expression, which is specifically expressed in undifferentiated ES cells (Kemler, 1980). Retinoic-acid-induced differentiation caused similar changes in the morphological characteristics of both vector-transfected and *mTERT*-overexpressing cells, resulting in a flattened appearance after 6 days (Fig. 2A). Similar results were obtained with other methods of differentiation [i.e. dimethyl-sulfoxide (DMSO)-induced differentiation] (data not shown). RT-PCR and immunoblot analysis indicated that expression of exogenous *mTERT* was maintained in differentiated *mTERT*-expressing cells, excluding the possibility that the expression of exogenous *mTERT* was not shut down during differentiation (Fig. 2B,C). Becase there were no obvious defects in the differentiation process, we determined whether the rate of differentiation was affected in *mTERT*-overexpressing ES cells by following the loss of ECMA-7 expression over 6 days. Flow-cytometric analysis revealed that the pcDNA-transfected ES cells lost a significant amount of ECMA-7 expression 2 days after differentiation induction, and the cells did not express any ECMA-7 by 4 days after differentiation induction (Fig. 2D, left). *mTERT*-overexpressing cells also lost ECMA-7 expression at a similar rate (Fig. 2D, right), suggesting that the rate of differentiation was unaffected by *mTERT* overexpression.

We also evaluated the differentiation capacity of *mTERT*-overexpressing ES cells by analysing several molecular changes associated with differentiation. Expression of *rex 1*, a stem cell marker that has been shown to be downregulated during differentiation (Rogers et al., 1991), was reduced in vector-transfected and *mTERT*-overexpressing ES cells upon differentiation (Fig. 2E). Moreover, expression of *α foetal protein* (*afp*), *gata 4*, *goosecoid* and *pax 6*, which are differentiation markers of primitive endoderm, mesoderm and neuroectoderm, respectively (Blum et al., 1992; Arceci et al., 1993; Abe et al., 1996; Renoncourt et al., 1998), were upregulated to equal extents in vector-transfected and *mTERT*-expressing ES cells upon differentiation (Fig. 2E). Taken together, these data suggest that *mTERT* overexpression does not affect ES-cell differentiation.

Ectopic *mTERT* expression affects cell growth under physiological conditions

While monitoring alterations in differentiation rates, we

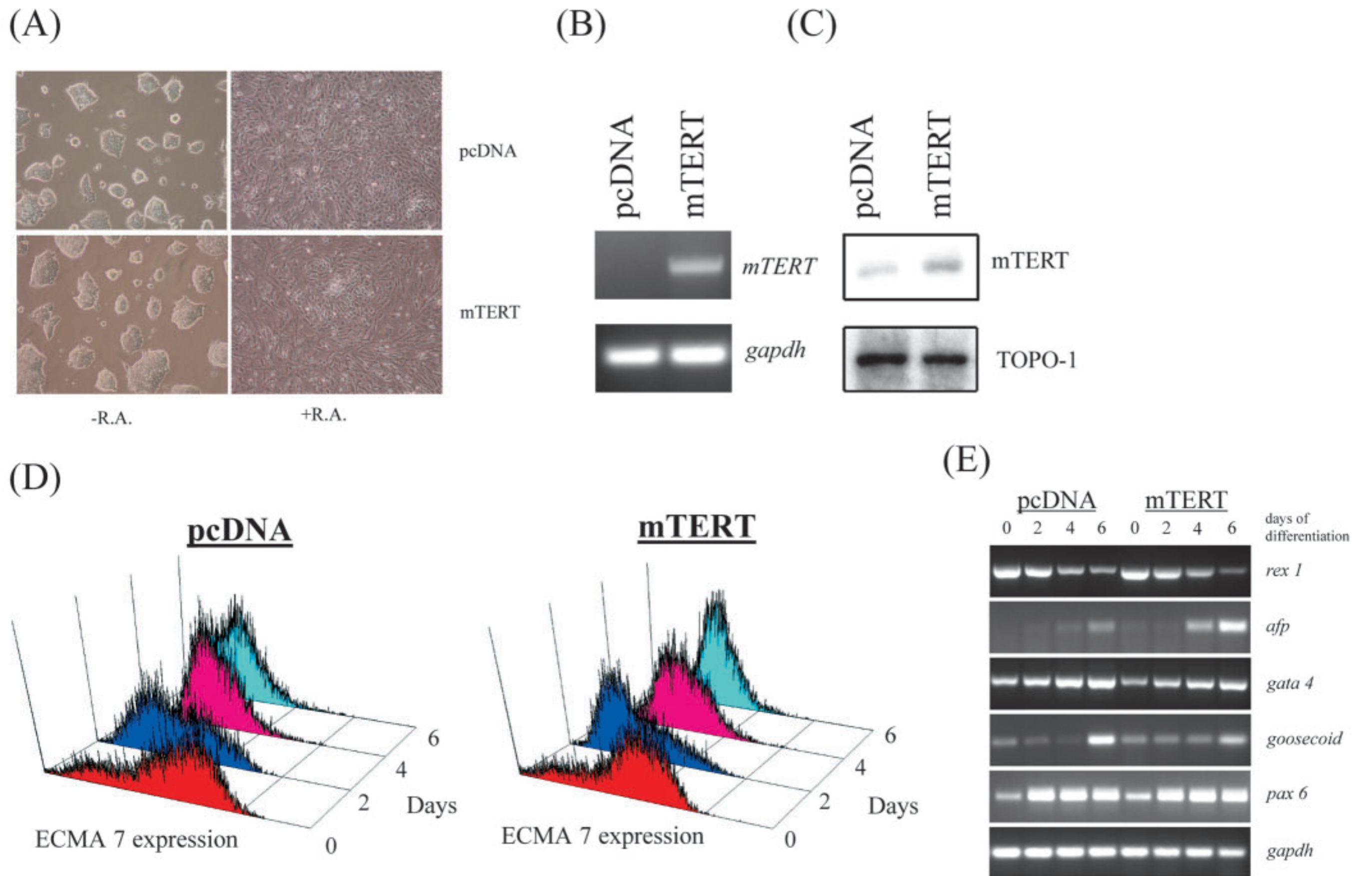

Fig. 2. Differentiation properties of *mTERT*-overexpressing ES cells. (A) Differentiation of ES cells was induced with 0.3 μM retinoic acid for 6 days. Cell morphology of non-induced and induced cells was monitored with phase-contrast microscopy. (B) Expression of *mTERT* in differentiated ES cells was determined by RT-PCR in vector-transfected and *mTERT*-expressing ES cells. (C) Protein level of mTERT in differentiated ES cells was determined by western-blot analysis using 100 μg nuclear extract from vector-transfected and *mTERT*-expressing ES cells. Topoisomerase-I levels were also determined, which represent loading in each lane. (D) Vector-transfected or *mTERT*-expressing ES cells were induced to differentiate with 0.3 μM retinoic acid for 2 days, 4 days and 6 days. The loss of surface expression of a stem-cell-specific antigen (ECMA-7) was monitored over time by flow cytometry. (E) Expression of stem-cell and differentiation markers was determined by RT-PCR. ES cells were induced to differentiate with retinoic acid for 6 days. Cells were harvested at different days of differentiation and RNA was extracted for RT-PCR analysis.

noticed that colonies of *mTERT*-overexpressing cells were much larger than those of the vector-transfected cells (data not shown). We therefore determined whether *mTERT* overexpression would alter the growth properties of undifferentiated ES cells. Analysis of cumulative cell numbers over several days indicated that *mTERT*-overexpressing cells had a growth advantage, which was obvious after 3 days in culture (Fig. 3A). In order to elucidate the cause of the observed differences, we investigated whether there were differences in the proliferation or basal cell death rates of these cells. Asynchronous cultures of cells were labelled with BrdU to monitor proliferating cells and analysed by flow cytometry after staining with anti-BrdU antibody. The amounts of BrdU-labelled cells in both the vector-transfected and the *mTERT*-expressing populations were not significantly different (65.68±0.71% and 66.07±2.32%, respectively) (Fig. 3B).

We therefore determined whether there were differences in apoptosis. Analysis of basal cell death rates revealed that there was a small but statistically significant difference between the two populations. The basal level of cell death in vector-transfected cells was 3.19±0.2%, whereas the levels in the *mTERT*-overexpressing population was 2.11±0.24%, indicating that *mTERT* overexpression conferred resistance to apoptosis ($P<0.05$ by an unpaired Student's t test) (Fig. 3C). Because this effect was small, we further investigated whether the cell-death rates were altered upon differentiation, during which differentiation-associated apoptosis ensues (Castro-Obregon and Covarrubias, 1996). Both vector-transfected and *mTERT*-overexpressing ES cells were differentiated and the cumulative cell-death rate was monitored over the first 3 days of retinoic-acid-induced differentiation by the PI exclusion method. Differentiation-induced cell death was markedly reduced in the *mTERT*-overexpressing cells over the course of differentiation ($P<0.05$ by an unpaired Student's t-test) (Fig. 3D). These findings together suggest that, although ectopic *mTERT* expression does not alter the proliferation potential of ES cells, it does affect the basal cell-death rates both in the undifferentiated state and during differentiation, thereby conferring a growth advantage on *mTERT*-overexpressing ES cells.

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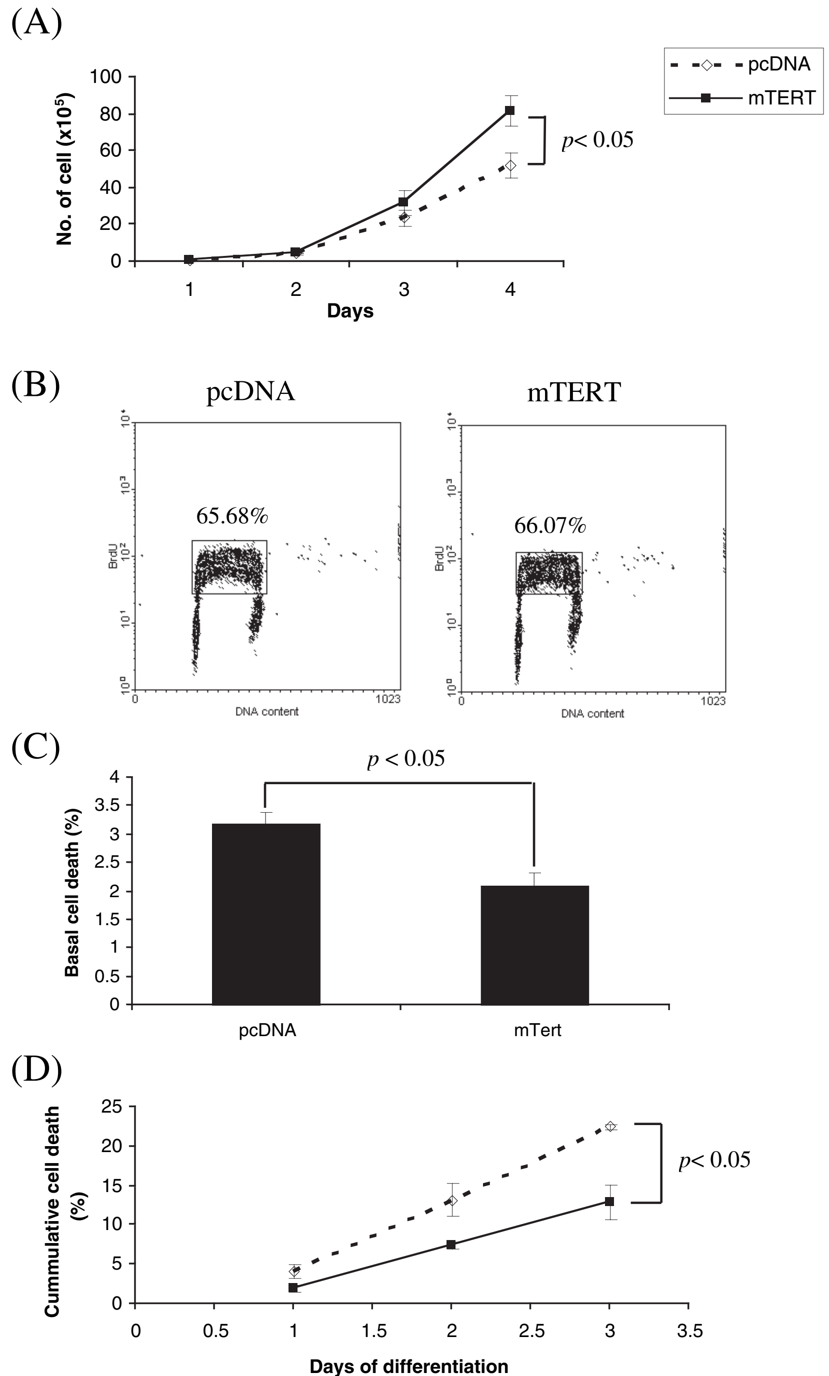

Fig. 3. Ectopic *mTERT* expression affects cell growth under physiological conditions. (A) Growth curve of pcDNA-transfected and *mTERT*-expressing ES cells. Representative experiment is shown. Data represent means±s.d. from one of the experiments in duplicate. (*) represents $P<0.05$ in a one-tailed unpaired Student's *t* test. (B) Proliferating cells were identified by labelling with BrdU for 30 minutes and followed by staining with FITC-conjugated anti-BrdU antibody. Representative flow-cytometry results are shown. The gated area represents the $BrdU^+$ proliferating cells. (C) Basal cell death was determined in pcDNA-transfected and *mTERT*-overexpressing ES cells by PI exclusion. Data represent means±s.d. from one of the experiments in triplicate. (*) represents $P<0.05$ in a one-tailed unpaired Student's *t* test. (D) Undifferentiated ES cells were induced to differentiate with 0.3 μM retinoic acid for 3 days and the cumulative cell death over the course of differentiation was determined as described in Fig. 3C. Data represent means±s.d. from one of the three independent experiments in duplicate. (*) indicates $P<0.05$ in a one-tailed unpaired Student's *t* test.

mTERT overexpression protects ES cells from stress-induced apoptosis

Overexpression of *hTERT* protects several human cell lines against stress-induced cell death (Lu et al., 2001; Gorbunova et al., 2002). We found that *mTERT* overexpression also conferred a survival advantage upon retinoic-acid-induced differentiation, during which oxidative radicals are produced (Castro-Obregon and Covarrubias, 1996), and so we explored the possibility that these cells are also resistant to other forms of cellular insults, including oxidative stress. Hydrogen peroxide (H_2O_2) was used as a source of oxidative stress, and cell death was determined upon treatment with various doses of H_2O_2. *mTERT*-overexpressing cells were significantly resistant to H_2O_2-mediated cell death compared with vector-transfected cells over all doses of H_2O_2 tested (Fig. 4A). This suggests that mTERT can protect cells from oxidative stress and probably explains the reduced cell-death rates observed during retinoic-acid-induced differentiation (Fig. 3D).

In addition to oxidative stress, we investigated whether *mTERT* overexpression could also protect ES cells against other forms of genotoxic insult. To this end, cell death after treatment with various doses of chemotherapeutic agents such as cisplatin (Fig. 4B) and doxorubicin (Fig. 4C) was determined. Like H_2O_2, overexpression of *mTERT* conferred a significant survival advantage against both cisplatin and doxorubicin, compared with vector-transfected cells (Fig. 4B,C).

We extended the analysis on cell death by analysing the proportion of annexin-V staining by flow cytometry, which identify cells undergoing apoptosis (compared to necrosis), upon genotoxic insults. Doxorubicin (0.6 μg ml^{-1}) and H_2O_2 (1 mM) treatment resulted in accumulation of annexin-V-positive pcDNA-transfected cells (control vs doxorubicin and H_2O_2: 4% vs 44.5% and 16.4%, respectively) (Fig. 5, left). However, the *mTERT*-overexpressing cells were markedly resistant to apoptosis induced by both agents (control vs doxorubicin and H_2O_2: 3.4% vs 17.6% and 6.5%, respectively) (Fig. 5, right). A similar trend in resistance to apoptosis was observed on treatment of cells with various doses of stress agents (data not shown). Together, these findings suggest that *mTERT* overexpression in ES cells confers resistance to apoptosis.

Production of catalytically inactive mTERT does not protect ES cells from apoptosis

It has been shown that hTERT protects breast-cancer cells from

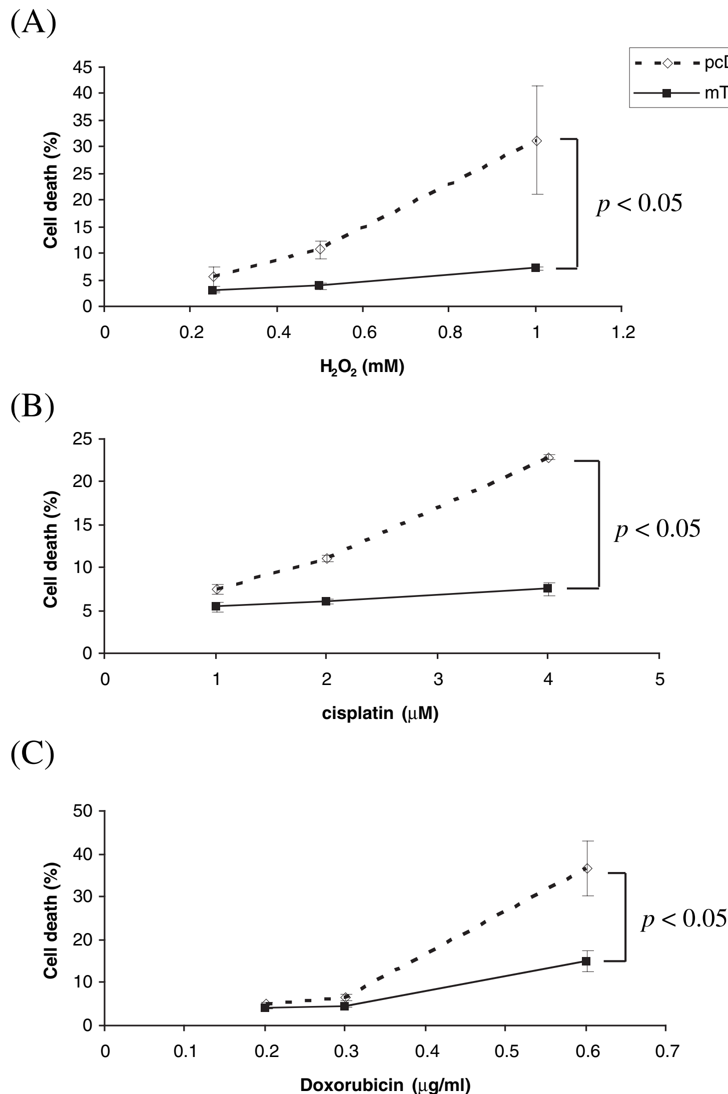

Fig. 4. *mTERT*-overexpressing ES cells are more resistant to stress-induced cell death. Undifferentiated ES cells were treated with various doses of H_2O_2 for 24 hours (A) and with cisplatin (B) or doxorubicin (C) for 12 hours, and cell death was determined by PI exclusion. Data represent means±s.d. from one of the three independent experiments in duplicate. (*) indicates P<0.05 in a one-tailed unpaired Student's t test.

apoptosis independently of its catalytic activity (Cao et al., 2002). However, the enzymatic activity was required to protect HeLa cells from apoptosis (Zhang et al., 2003), suggesting that dependence on the telomerase enzymatic activity to suppress apoptosis might be cell-type specific. Therefore, we further investigated whether the catalytic activity of mTERT is required to protect ES cells against apoptosis. We generated stable ES cells overproducing the catalytically inactive mTERT, in which the aspartic residues at positions 1186 and 1187 were changed to alanines (F. Ishikawa, personal communication). The expression of the inactive mTERT was confirmed by RT-PCR analysis (Fig. 6A). Despite increased mTERT production, these ES cells did not show an increase in the telomerase enzyme activity compared with vector-transfected ES cells (Fig. 6B), confirming that the exogenous mTERT is indeed catalytically inactive. We then tested whether overproduction of catalytically inactive mTERT can protect ES cells from genotoxic insults. Cell death of inactive-mTERT-producing cells was comparable to that of vector-transfected cells (vector-transfected vs inactive-mTERT-producing ES cells: 37.1±0.57% vs 49.9±0.57% at 0.5 µg ml^{-1} doxorubicin and 68.8±3.54% vs 69.65±0.78% at 1 µg ml^{-1} doxorubicin) (Fig. 6C), whereas production of catalytically active mTERT drastically reduced cell death after doxorubicin treatment (Fig. 6C). Similar results were obtained with annexin-V staining (data not shown), confirming that production of inactive mTERT cannot protect ES cells from apoptosis and indicate that the enzyme activity of mTERT is required for its anti-apoptotic properties in ES cells.

Role of p53 in mTERT-mediated resistance to cell death

ES cells express high levels of p53 that are reduced upon differentiation and undergo p53-dependent cell death during differentiation (Sabapathy et al., 1997). Moreover, all the genotoxic drugs used in the study induce apoptosis in a p53-dependent manner (de Vries et al., 2002; Siddik, 2003). Hence, we investigated whether mTERT-mediated resistance to cell death occurs by inhibiting p53-dependent cell death. We noticed that endogenous p53 was upregulated by all genotoxic insults to equal extents in vector and *mTERT*-overexpressing cells (Fig. 7A), suggesting that the induction of p53 is not affected by *mTERT* overexpression. Consistently, treatment with these genotoxic agents resulted in the upregulation of several p53-dependent apoptotic target genes, such as *noxa* and *puma*, in both vector and *mTERT*-overexpressing ES cells (Fig. 7B). There was no difference in the levels of *bax* expression during apoptosis in ES cells (Fig. 7B). The upregulation of p53 and its target genes suggested that p53 function is not compromised by *mTERT* overexpression. However, cleavage of caspase-3, the hallmark of apoptotic execution and a downstream event subsequent to p53 activation via the mitochondrial pathway (Schuler and Green, 2001; Wang, 2001), was compromised in *mTERT*-overexpressing cells compared with vector-transfected cells upon doxorubicin treatment (vector-transfected vs *mTERT*-overexpressing cells: 46.19% vs 16.28%) (Fig. 7C). Similar results were obtained with cisplatin treatment (data not shown). These findings suggested that telomerase might act downstream of or independent of p53 to inhibit apoptosis.

We therefore investigated whether mTERT overexpression could affect p53-mediated apoptosis. To this end, we assayed cell death in the absence or presence of pifithrin-α (PFTα), a chemical inhibitor of p53 (Komarov et al., 1999) (Fig. 8A). Cells were incubated with PFTα and subsequently treated with cisplatin to induce cell death. The proportion of cisplatin-induced cell death in vector-transfected ES cells incubated with PFTα was reduced and was strikingly similar to control (DMSO incubated) *mTERT*-overexpressing ES cells (PFTα-treated vector cells vs DMSO-treated *mTERT*-overexpressing

Journal of Cell Science

Journal of Cell Science

cells: 13.14±0.67% vs 13.23±0.12% at 5 μM cisplatin and 18.55±0.28% vs 18.17±0.19% at 10 μM cisplatin) (Fig. 8A), indicating that *mTERT* overexpression was as effective as inhibiting p53. In addition, incubation of *mTERT*-overexpressing ES cells with PFTα before cisplatin treatment only slightly reduced the apoptotic rates (DMSO- vs PFTα-treated: 13.23±0.23% vs 10.22±0.21% at 5 μM cisplatin and 18.17±0.19% vs 12.65±1.28% at 10 μM cisplatin) (Fig. 8A). These results suggest that the anti-apoptotic effect of *mTERT* overexpression probably occurs through the inhibition of the p53-dependent cell death pathway.

Recently, it was shown that PFTα also suppress cellular functions other than the inhibition of p53 (Komarova et al., 2003; Rocha et al., 2003). Therefore, we generated *mTERT*-overexpressing *p53*$^{-/-}$ ES cells to confirm the role of p53 in mTERT-mediated anti-apoptotic effects. RT-PCR analysis confirmed that the *p53*$^{-/-}$ (P1.1) ES cells do not express *p53* (Fig. 8B) and that *mTERT*-overexpressing *p53*$^{-/-}$ ES cells expressed significantly higher levels of *mTERT* mRNA (Fig. 8C). We then tested whether the exogenous *mTERT* expression conferred anti-apoptotic effects on *p53*$^{-/-}$ ES cells. Similar to PFTα treatment, cell death of *mTERT*-overexpressing CCE ES cells (*p53*$^{+/+}$) was only slightly higher than that of vector-transfected *p53*$^{-/-}$ ES cells, confirming that *mTERT* overexpression was almost as effective as knocking out *p53* in terms of anti-apoptotic capacity, especially at lower doses of doxorubicin (*mTERT*-overexpressing CCE ES cells vs vector-transfected *p53*$^{-/-}$ ES cells: 27.55±0.97% vs 18.76±1.35% at 1 μg ml^{-1} doxorubicin and 39.75±1.35% vs 20.84±3.03% at 2 μg ml^{-1} doxorubicin) (Fig. 8D). Moreover, production of exogenous mTERT or inactive mTERT in *p53*$^{-/-}$ ES cells did not confer further resistance to doxorubicin treatment (vector-transfected vs mTERT-producing vs inactive-mTERT-producing *p53*$^{-/-}$ ES cells: 18.76±1.35% vs 25.06±3.44% vs 24.72±3.12% at 1 μg ml^{-1} doxorubicin and 20.84±3.03% vs 25.11±7.89% vs 20.22±8.36% at 2 μg ml^{-1} doxorubicin, respectively) (Fig. 8D). Together, the data suggest that the survival advantage conferred by exogenous *mTERT* expression is through the suppression of p53-dependent apoptosis.

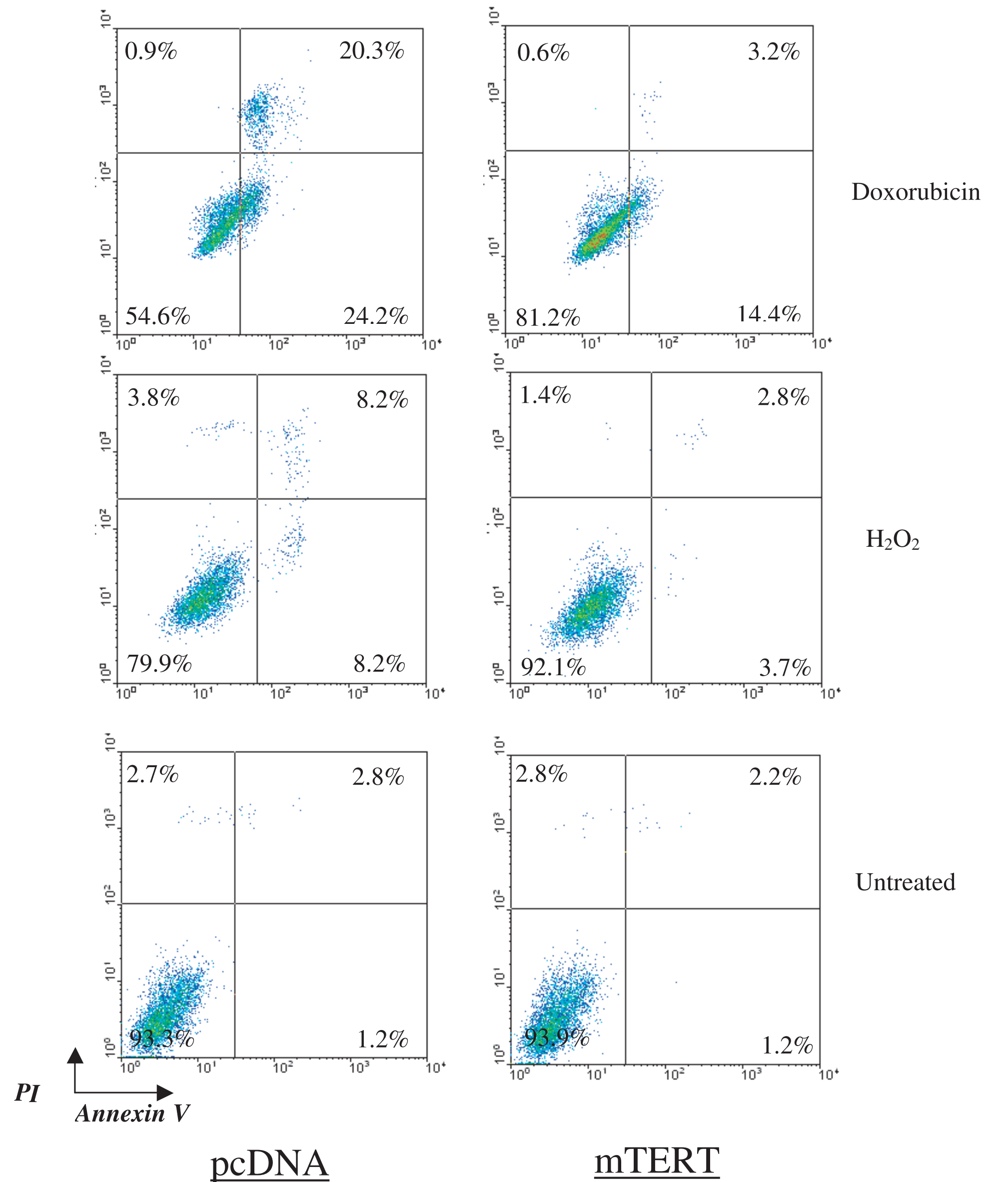

Fig. 5. *mTERT* overexpression protects ES cells from stress-induced apoptosis. Vector-transfected (left) and *mTERT*-overexpressing (right) ES cells were treated with 0.6 μg ml^{-1} doxorubicin (top) or 1 mM H_2O_2 (middle) for 12 hours. Untreated controls are shown at the bottom. Apoptosis was determined by staining cells with FITC-conjugated annexin-V and PI, and analysed by flow cytometry. Representative results are shown from one of the experiments in duplicate.

Discussion

The results presented here indicate that ectopic overexpression of *mTERT* does not affect the differentiation capacity or the rate of differentiation of mouse ES cells. However, constitutive *mTERT* expression confers a survival advantage, especially during differentiation and stress-induced apoptosis. Furthermore, the mTERT-mediated anti-apoptotic effect appears to be dependent on its catalytic activity and is due to the inhibition of the p53-dependent apoptotic pathway.

Our data indicate that enforced *mTERT* expression does not affect the initial differentiation process, consistent with recent findings in human mesenchymal stem cells and leukaemic stem

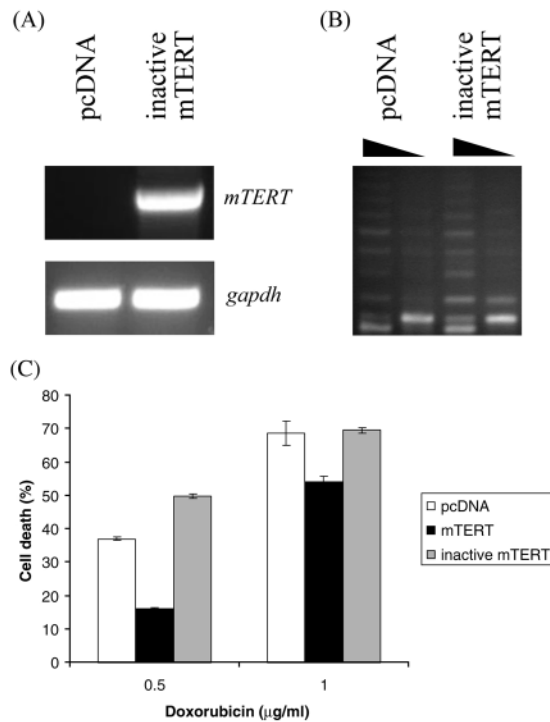

Fig. 6. Production of catalytically inactive mTERT does not protect ES cells from apoptosis. (A) Expression of a gene encoding catalytically inactive mTERT was determined by RT-PCR in vector-transfected and inactive-mTERT-producing ES cells. 3 μg RNA was extracted from undifferentiated ES cells and reverse transcribed into cDNA for PCR. (B) Telomerase activity of vector-transfected and inactive-mTERT-producing ES cells was determined by a TRAP assay. Decreasing amounts of protein (50 ng and 5 ng) were used in the assay. (C) The indicated undifferentiated ES cells were treated with various doses of doxorubicin for 12 hours, and cell death was determined by PI exclusion. Data represent means±s.d. from one of the three independent experiments in duplicate.

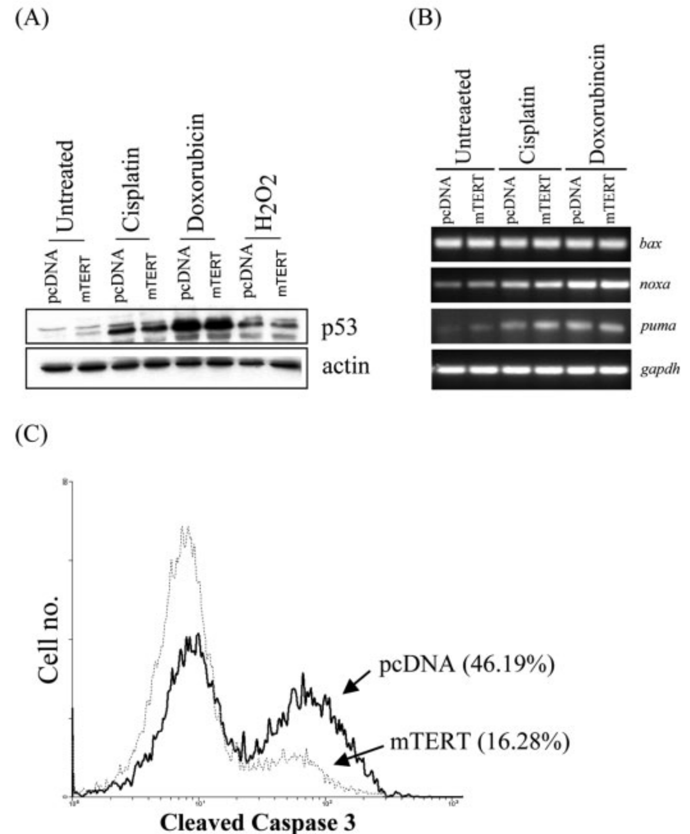

Fig. 7. Role of p53 in mTERT-mediated resistance to cell death. (A) p53 protein levels of untreated or cells treated with 5 μM cisplatin, 0.3 μg ml^{-1} doxorubicin and 0.5 mM H_2O_2 were determined by western blotting. Actin levels were determined to show loading. (B) Expression of p53 target genes (*bax*, *noxa* and *puma*) in vector-transfected and *mTERT*-expressing ES cells was determined by RT-PCR. Total RNA was extracted from untreated cells and ES cells treated with 5 μM cisplatin or 0.3 μg ml^{-1} doxorubicin and reverse transcribed into cDNA for PCR of p53 target genes. (C) Activation of caspase-3 was determined after doxorubicin treatment (0.3 μg ml^{-1}) of pcDNA-transfected and *mTERT*-overexpressing cells by staining with FITC-conjugated cleaved caspase-3-specific antibody and flow cytometry.

cells, in which differentiation was not affected by exogenous telomerase (Kobune et al., 2003; Yamada et al., 2003). These data suggest that the decrease in telomerase activity observed during differentiation in several cell systems is probably a consequence of the differentiation process, during which the pluripotence of the stem cells is lost. It is thus conceivable that differentiated cells do not require to maintain telomerase activity because their telomeres need not be protected any further upon loss of 'stem-ness' and, hence, differentiation results in a reduction in telomerase activity. However, we cannot exclude the possibility that terminal differentiation, leading to quiescence of cells, could be affected by the overexpression of *TERT*.

Oxidative-stress-associated cell death occurs during ES-cell differentiation by retinoic acid (Castro-Obregon and Covarrubias, 1996). Consistently, we found that *mTERT*-expressing ES cells were more resistant to differentiation and oxidative-stress-induced cell death. Moreover, exogenous telomerase expression also conferred resistance to other genotoxic-stress-induced cell death in these cells. These findings are consistent with other studies showing that suppression of telomerase results in cell death in neurons (Zhu et al., 2000), PC12 cells (Fu et al., 1999), breast-cancer cells (Cao et al., 2002) and cervical cancer cells (Kushner et al., 2000), whereas ectopic telomerase expression protected PC12 cells (Lu et al., 2001) and fibroblasts (Gorbunova et al., 2002) from cell death. However, the mechanism by which telomerase protects cells against cell death remains controversial. Cao et al. (Cao et al., 2002) demonstrated that hTERT regulates cell survival independent of its enzymatic activity in breast cancer cells, whereas Zhang et al. (Zhang et al., 2003) showed that the catalytic activity and the binding to 14-3-3, a downstream target of p53, are essential for its anti-apoptotic function in HeLa cells. Our results also indicate that the catalytic activity of mTERT is required for its anti-apoptotic function, suggesting that the mechanisms of TERT-mediated anti-apoptosis could be different in different cell types.

ES cells are atypical in that they express high levels of p53,

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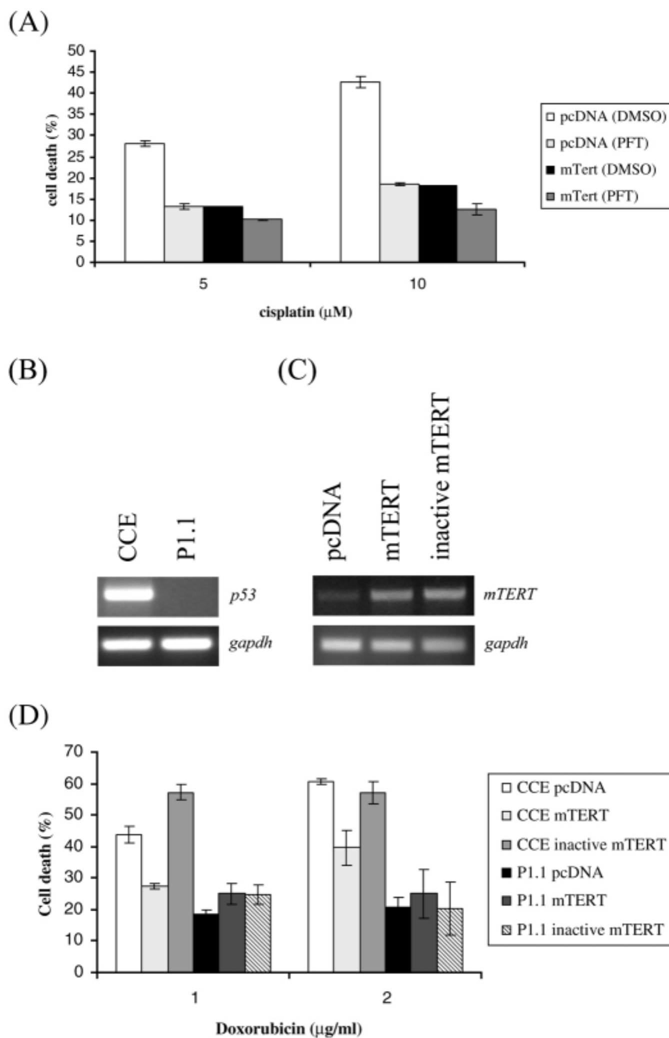

Fig. 8. Effect of p53 inhibition and deficiency on mTERT-mediated resistance to cell death. (A) Pifithrin α (PFTα) (10 μM) was added to cells 24 hours before cisplatin treatment in order to inhibit p53. Cell death was then determined upon treatment with various doses of cisplatin in the presence of PFTα by PI exclusion. Data represent means±s.d. from one of the experiments in duplicate. (B) Expression of p53 in undifferentiated CCE ES cells (*p53*$^{+/+}$) and P1.1 ES cells (*p53*$^{-/-}$) was determined by RT-PCR. 3 μg RNA was extracted from undifferentiated ES cells and reverse transcribed into cDNA for PCR. (C) Expression of *mTERT* in the indicated undifferentiated ES cells was determined by RT-PCR as described above. (D) Undifferentiated CCE ES cells (*p53*$^{+/+}$) and P1.1 ES cells (*p53*$^{-/-}$) were treated with various doses of doxorubicin for 12 hours, and cell death was determined by PI exclusion. Data represent means±s.d. from one of the experiments in duplicate.

which is crucial for cell death during differentiation or caused by genotoxic insults (Sabapathy et al., 1997). Our data indicate that, although mTERT protects ES cells from apoptosis, its production did not affect p53 levels and the induction of p53-dependent apoptotic target genes. However, chemical inhibition of p53 function by PFTα did not significantly affect the apoptotic rates in *mTERT*-overexpressing cells. Moreover, *mTERT* overexpression did not confer a survival advantage on *p53*$^{-/-}$ ES cells, suggesting that the mTERT-mediated protective effect might come about by antagonizing the p53 pathway, probably through targets downstream of p53. Consistently, Wiman and colleagues have recently found that hTERT antagonizes p53-mediated apoptosis (Rahman et al., 2004). Taken together, these data indicate that telomerase expression confers resistance to apoptosis through inhibition of the p53 pathway.

This study also highlights the differences between cell types with respect to the ability of telomerase to regulate cellular proliferation. Exogenous telomerase was shown to increase proliferation in somatic lens epithelial cells (Xiang et al., 2002), whereas *mTERT* overexpression did not affect ES-cell proliferation. This might be due to the differences in the regulation of cell-cycle progression between these cell types. *hTERT* overexpression was shown to increase cellular proliferation rates by regulating phosphorylation of the retinoblastoma protein (Rb) (Xiang et al., 2002). Unlike somatic cells, cell-cycle progression in ES cells is regulated by total Rb protein levels rather than the phosphorylation status of Rb (Savatier et al., 1994), which probably accounts for the lack of a proliferation advantage of *mTERT*-overexpressing ES cells.

Human cancers, many of which are derived from progenitor populations, exhibit increased telomerase activity and are often associated with poor prognosis (Kim et al., 1994; Ohali et al., 2003; Ulaner et al., 2003). Moreover, inhibition of telomerase activity often results in sensitivity to drug-induced apoptosis (Kondo et al., 1998; Misawa et al., 2002; Yuan and Mei, 2002). The data presented here demonstrate that resistance to drug-induced cell death is a direct consequence of increased telomerase activity, suggesting that reactivation of telomerase during tumorigenesis might contribute directly to survival advantage and chemotherapeutic drug resistance. Therefore, it is conceivable that therapeutic drugs that have the ability to reduce telomerase activity would be of clinical relevance in TERT-overproducing tumours. In this respect, histone-deacetylase inhibitors, which have been shown to decrease cellular telomerase activity (Suenaga et al., 2002), have also been demonstrated to have antitumour properties (Camphausen et al., 2004; Kuefer et al., 2004). Thus, future therapeutic strategies can be aimed at reducing the telomerase activity to combat cancers.

This study highlights the anti-apoptotic role of mTERT in ES cells through a mechanism involving the inhibition of p53-dependent apoptosis. In addition, telomerase downregulation appears to be a consequential event during cellular differentiation, because loss of telomerase activity in knockout mice and *mTERT* overexpression in ES cells appear not to affect the differentiation process. Thus, these findings can be extended to stem-cell therapies, because the overexpression of *TERT* has no negative effects on cellular differentiation and should be useful for increasing the viability and replicative potential of stem cells.

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