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Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype

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Expression of the human telomerase catalytic component, hTERT, in normal human somatic cells can reconstitute telomerase activity and extend their replicative lifespan[1,2]. We report here that at twice the normal number of population doublings, telomerase-expressing human skin fibroblasts (BJ-hTERT) and retinal pigment epithelial cells (RPE-hTERT) retain normal growth control in response to serum deprivation, high cell density, G1 or G2 phase blockers and spindle inhibitors. In addition, we observed no cell growth in soft agar and detected no tumour formation *in vivo*. Thus, we find that telomerase expression in normal cells does not appear to induce changes associated with a malignant phenotype.

Normal cells are contact inhibited and depend on serum for continued proliferation. We monitored the cell-cycle distribution of parental and *TERT*-expressing cells and showed that both were arrested by contact and serum starvation (Table 1). Moreover, serum induction stimulated the starved cells to resume cycling. In contrast, near-senescent BJ fibroblasts and 340-RPE cells exhibited very low cycling fractions in all conditions. We noted that the RPE-hTERT clones responded more slowly to serum starvation. As RPE cultures can be phenotypically heterogeneous[3], differences in the magnitude and kinetics of the response may be due to clonal variations.

pRb phosphorylation is required for progression through S phase. pRb activity is regulated by proteins such as CDK4, cyclin D1 and p16 (ref. 4). In both parental and *TERT*-expressing cells, we observed that pRb was predominantly hyperphosphorylated in subconfluent, proliferating cultures but became hypophosphorylated in confluent cultures (Fig. 1). Furthermore, we found that the level of pRb proteins was downregulated at confluence. In contrast, p16 expression did not change with cell density, irrespective of whether *TERT* was expressed or not. Although p16 is induced in senescent cells, its induction in short-term cell-cycle arrest appears to depend on the cell types examined and the status of other cell-cycle regulators[5]. We noted that one of the RPE-hTERT clones, T58, has undetectable levels

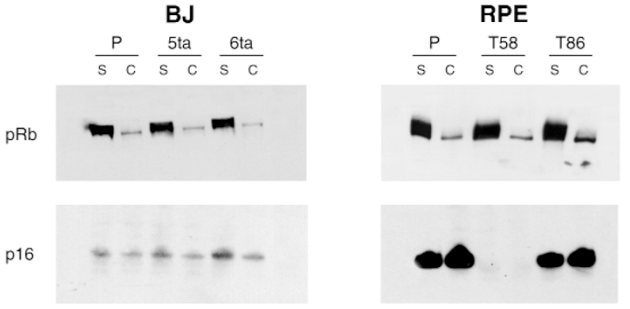

Fig. 1 Expression of pRb and p16 as a function of cell density. BJ parental cells (P; PD 36–39), BJ-hTERT clones 5ta (PD 104–106) and 6ta (PD 108–110), 340-RPE parental cells (P; PD 33–38) and RPE-hTERT clones T58 (PD 99–100) and T86 (PD 98–105) were maintained as either subconfluent cultures (S) or confluent cultures for 72 h (C). pRb and p16 expression was assayed in each sample using western-blot analysis.

Table 1 • hTERT clones respond normally to cell density and serum induction

	% cells in S phase[a]			
	Subconfluence[b]	Confluence[b]	0% serum[c]	10% serum[c]
Young BJ (PD 20)	17.1	3.5	3.6	20.8
Old BJ (PD 88–94)	3.5	2.2	3.6	6.0
BJ 5ta (PD 120)	16.3	1.4	2.9	29.5
BJ 6ta (PD 110)	14.5	1.1	2.8	34.8
Young 340-RPE (PD 21–23)	10.7	3.7	5.3	17.9
Old 340-RPE (PD 54–55)	1.9	4.8	2.2	4.0
340-RPE T58 (PD 114–122)	11.8	3.6	9.6 (7.0)	16.7
340-RPE T86 (PD 115–125)	20.7	4.2	11.4 (4.8)	18.2

[a]The proportion of cells in S phase was analysed by FACS following PI staining. Values represent the average of duplicate cultures analysed from two separate experiments. [b]Cells were grown in 10% serum at subconfluence or maintained at confluence for 10 (BJ cells) or 25 d (340-RPE cells) before analysis. [c]Cells were maintained in 0% serum for 3 d (or 7 d for the numbers in parentheses) and the cultures were at subconfluence throughout the experimental period. Parallel cultures were then switched to 10% serum and analysed after 24 h.

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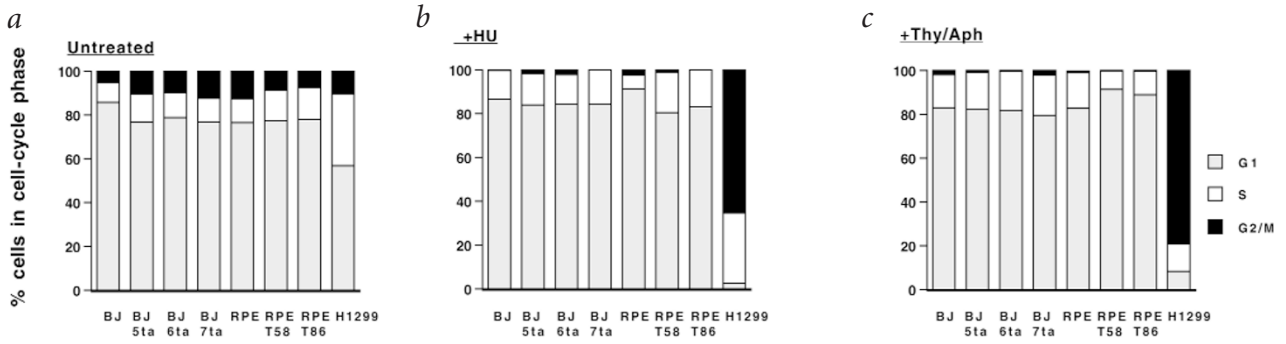

Fig. 2 *TERT*-expressing clones arrest appropriately in response to G1/S blockers. Exponentially growing BJ and 340-RPE cells, their hTERT clones and H1299 cells were treated with hydroxyurea (HU) or a combination of thymidine and aphidicolin (Thy/Aph) for 72 h and analysed by flow cytometry following staining with propidium iodide. The percentage of cells in G1, S and G2/M phases is shown for untreated cells (***a***), HU (***b***) and Thy/Aph (***c***) treated cells. Data represent the average of three separate experiments.

of p16 expression, despite an intact pRb response. In contrast, the parental line and T86 clone have abundant p16 levels. This may be due to clonal variation in p16 levels in the parental RPE cells before *TERT* transfection. Nevertheless, our data indicate that *TERT*-expressing cells do not have an altered pRb or p16 response to cell-cycle checkpoint arrests compared with parental non-*TERT* transfected cells.

We examined the integrity of several cell-cycle checkpoints in *TERT*-expressing cells. Treatment with hydroxyurea (HU) induces a p53-independent early S-phase arrest[6], whereas thymidine (Thy)/aphidicolin (Aph) arrests cells at the G1/S boundary[7]. Both parental and *TERT*-expressing cells appropriately arrested in response to both S-phase blockers (Fig. 2). We observed an increase in the proportion of cells in either G1 or S phase, and a decrease in those in G2/M phase. Preliminary data showed that pRb was also appropriately hypophosphorylated upon HU treatment (data not shown). In contrast, H1299 tumour cells accumulated in the G2/M phase, presumably due to a delay in cell-cycle progression caused by DNA breakage resulting from a disruption of nucleotide pools.

The antimetabolite PALA causes a predominant, p53-dependent, G1- and sometimes G2-phase arrest[6,8–10]. These cell-cycle checkpoints also require intact pRb function[8]. γ-irradiation and X-rays, on the other hand, induce DNA damage and activate a p53-dependent G1 and a p53-independent G2 arrest[11]. In response to PALA, γ-irradiation or X-rays, we observed an increase in the G1/S ratios in both parental and *TERT*-expressing cells, whereas that of the p53-deficient fibroblasts WS1E6 remained low[6] (0.83; Fig. 3, data not shown for X-irradiation). The greater increase in the G1/S ratio in response to PALA treatment observed in RPE-hTERT clones (2.9 to 23 or 75) relative to that in the parental 340-RPE cells (3.2 to 11.5) could be due to

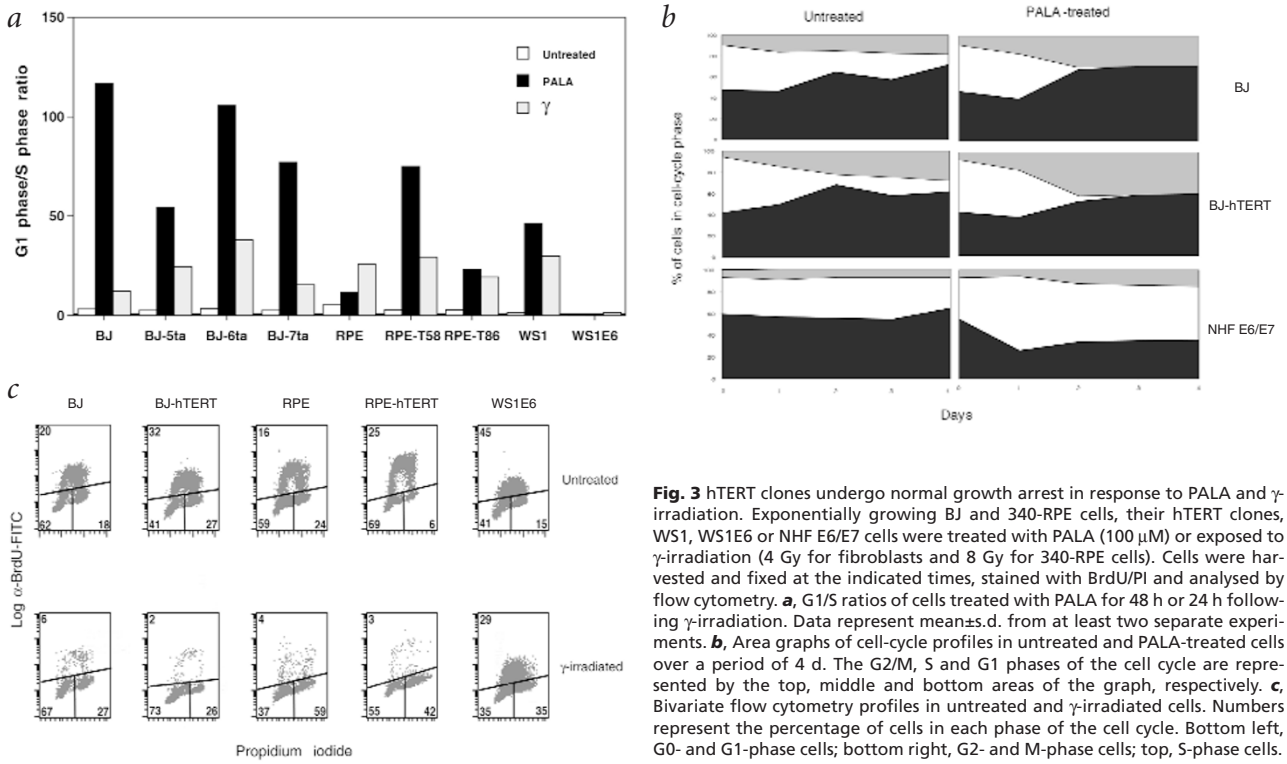

Fig. 3 hTERT clones undergo normal growth arrest in response to PALA and γ-irradiation. Exponentially growing BJ and 340-RPE cells, their hTERT clones, WS1, WS1E6 or NHF E6/E7 cells were treated with PALA (100 μM) or exposed to γ-irradiation (4 Gy for fibroblasts and 8 Gy for 340-RPE cells). Cells were harvested and fixed at the indicated times, stained with BrdU/PI and analysed by flow cytometry. ***a***, G1/S ratios of cells treated with PALA for 48 h or 24 h following γ-irradiation. Data represent mean±s.d. from at least two separate experiments. ***b***, Area graphs of cell-cycle profiles in untreated and PALA-treated cells over a period of 4 d. The G2/M, S and G1 phases of the cell cycle are represented by the top, middle and bottom areas of the graph, respectively. ***c***, Bivariate flow cytometry profiles in untreated and γ-irradiated cells. Numbers represent the percentage of cells in each phase of the cell cycle. Bottom left, G0- and G1-phase cells; bottom right, G2- and M-phase cells; top, S-phase cells.

Table 2 • hTERT clones are anchorage dependent and not tumorigenic *in vivo*

Cell types	Colony numbers[a]	% tumour incidence (n)[b]
MCF7	2,244 ± 138	ND
MDA-MB-435S	1,389 ± 120	ND
HT1080	104 ± 85	100 (10)[c]
293	373 ± 86	100 (10)[c]
SW26i	0	0 (10)
BJ parental	0	0 (24)
BJ hTERT clones (n=3)		
5ta	0	0 (24)
6ta	0	0 (48)[d]
7ta	0	0 (24)
340-RPE parental	0	ND
340-RPE hTERT clones (n=12)	0	ND

[a]Cells (5×10^4) were plated in soft agar in duplicate and colonies composed of 40 or more cells were scored after 2–3 weeks for MCF7, MDA-MB-4356, HT1080 and 293 cells and 6 weeks for the other cell types. Results represent mean ±s.d. from two separate experiments. [b]Cells were injected subcutaneously into nude mice with matrigel to enhance *in vivo* growth. '% tumour incidence' represents the proportion of mice developing xenografts that are larger than those in control mice (n=2) injected with matrigel alone. Mean graft size of control mice was 40.5 mg at 2 months, whereas xenograft size in mice injected with tumour cells typically reached 1 g or more in the same time frame. n, number of animals treated. [c]All except one mouse were sacrificed before the 2-month time point because tumours had exceeded 1 g. [d]One mouse developed an abcess at the site of injection and another mouse was found to develop an abdominal hernia at day 28. Both were euthanized at day 35. ND, not done.

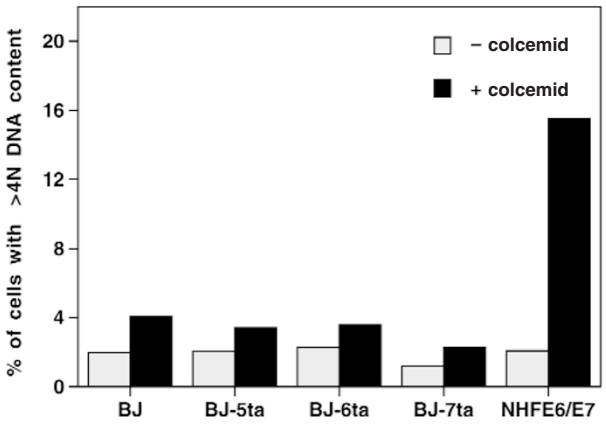

Fig. 4 hTERT clones arrest appropriately in response to the spindle inhibitor colcemid. Exponentially growing BJ cells, BJ-hTERT clones (5ta, 6ta, 7ta) and NHF E6/E7 cells were treated with colcemid (50 ng/ml) for 72 h, stained with BrdU/PI and analysed by flow cytometry. The percentage of cells with greater than 4N DNA content was shown in untreated and treated cultures. Data represent the average value from two or more independent experiments.

the clonal origin of these cells. A time-course analysis further confirmed that BJ parental and *TERT*-expressing cells showed similar kinetics of cell-cycle arrest (Fig. 3*b*).

Microtubule destabilizing agents, such as colcemid and nocodazole, arrest cells with 4N DNA content[12,13], whereas transformed cells often re-replicate, causing the accumulation of cells with greater than 4N DNA content. Re-replication has been linked to deficiencies in p53, pRb, p21, or p16 function[12]. We did not observe any accumulation of cells with greater than 4N DNA content in parental or *TERT*-expressing cells in response to colcemid (data not shown for 340-RPE cells) or nocodazole (data not shown). In contrast, colcemid induced significant re-replication in E6/E7-infected NHF cells with mutations including p53 and pRb deficiency (Fig. 4).

Transformed cells, unlike normal cells, can grow independently of anchorage and may form tumours *in vivo*[14,15]. Neither parental nor hTERT clones (3 BJ and 12 340-RPE clones) formed colonies in soft agar (Table 2), indicating maintenance of anchorage-dependent growth. Moreover, parental BJ and hTERT clones did not form tumours in nude mice after 2 months (Table 2). No tumour growth was detected for up to 5.5 months, or when 10 times the amount of cells (10^7 cells/mouse) were used (data not shown). In contrast, 100% of mice injected with HT1080 or 293 cells developed tumours. Thus, under the conditions examined here, hTERT does not induce changes that allow tumour growth *in vivo*.

Another characteristic of normal cells is a diploid karyotype. The hTERT clones used in the present study (3 BJ and 2 340-RPE clones at population doublings (PD) 101–142) were diploid by G-banding (data not shown). One BJ and one RPE hTERT clone contained additional genetic material on chromosome 6 and the X chromosome, respectively, but both clones maintained normal behaviour compared with parental cells. To date, BJ- and RPE-hTERT clones have reached more than 200 population doublings and some polyploid or tetraploid cells have been observed. Preliminary data suggests that these cells maintain functional cell-cycle checkpoints and anchorage-dependent growth. Further analysis will be required to determine whether the acquisition of an abnormal karyotype is a consequence of telomerase expression or of long-term culture *per se*[16–18].

A causal relationship between telomerase expression, telomere maintenance and replicative lifespan was recently reported in BJ and 340-RPE cells[1]. Cells such as germ cells or stem cells contain telomerase activity[19–21], have extensive replicative potential and yet are functionally normal, indicating telomerase expression *per se* is not oncogenic. This is further supported by our findings that telomerase expression in normal somatic cells does not cause abnormal growth control or oncogenic transformation. The availability of primary human cells with greatly extended or immortal lifespan in the absence of mutations in cell-cycle checkpoint genes and genetic instability will serve as valuable research tools as well as provide therapeutic opportunities for age-related diseases.

Methods

Cells. Normal RPE cells 340-RPE, BJ fibroblasts and their *TERT*-transfected clones (RPE-hTERT clones, T58 and T86; BJ-hTERT clones, 5ta, 6ta and 7ta) were maintained as described[1]. For all experiments, BJ and 340-RPE parental cells were used at PD 20–63 and PD 20–39, respectively (maximal replicative lifespan: BJ, PD 85–95; 340-RPE, PD 55–57). BJ- and RPE-hTERT clones were used at PD 101–142. hTERT clones were compared with the parental population because clones containing control vector had senesced, mostly by PD 75 for BJ and PD 55 for 340-RPE, soon after transfection and subsequent cell expansion. Other cells used in the present study included normal human embryonic skin fibroblasts WS1 or normal foreskin fibroblasts NHF; E6- and/or E7-infected fibroblast cell strains WS1E6 and NHF E6/E7 (refs 6,8,9); SV40-transformed cell line SW26i; adenovirus-transformed embryonic kidney epithelial cells 293; tumour cell lines HT1080 fibrosarcoma cells, H1299 lung carcinoma cells, MCF7 and MDA-MB-435S breast cancer cells.

Western-blot analysis. Cells were lysed with 1% SDS in 10 mM Tris, pH 7.5, and protein extracts (pRb, 10 µg; p16, 20 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. We stained the blots with Ponceau S solution (Sigma) to monitor the amounts of protein loaded. After a blocking step using 5% fat-free dry milk, we incubated the membrane with a monoclonal antibody against either pRb or p16 (1–2 µg/ml; PharMingen) for 1 h at RT. The antibody to pRb recognizes both hyper- and hypo-phosphorylated forms of the protein. The immunoblots were then incubated with goat anti-mouse IgG conjugated to peroxidase (1:7,500; Vector) for 1 h and visualized with enhanced chemiluminescence detection reagents (Super Signal Substrate, Pierce).

Chemicals, X-ray and γ-irradiation. We exposed asynchronous cultures to cell-cycle blockers for 72 h at the following concentrations: hydroxyurea (1–5 mM), thymidine and aphidicolin (1–5 mM and 1–5 μg/ml, respectively), colcemid (50–2,000 ng/ml) and nocodazole (15–50 ng/ml). PALA (100 μM; National Cancer Institute) was added to cells for up to 4 d (refs 6,8–10). Irradiation with 15–25 Gy of X-rays was delivered at 250 cGy/min at RT using a Torrex 150D X-ray machine (EG & G Astrophysic Research; 140 kVp, 5 mA; half-value layer; 1.0 mm Cu). γ-irradiation was performed at RT with a ^{60}Co γ-irradiator (Gammabeam 150-C) at a distance of 40 cm at approximately 2.2 Gy/min. We irradiated fibroblasts with 1, 4, or 8 Gy of γ-irradiation and 340-RPE cells with 4, 8, or 12 Gy.

Cell-cycle analysis. To monitor cell-cycle distribution, cells were fixed in methanol and stained with propidium iodide (PI) solution containing RNase and analysed for DNA content using a Coulter EPICS Elite ESP flow cytometer (Coulter Electronics). We determined the percentage of cells in G1, S and G2/M at the time of harvest using a Multicycle AV computer system (Phoenix Flow Systems). To monitor the progression through the cell cycle, we performed BrdU-PI double staining as described[6,8,9]. Briefly, we prepared nuclear pellets by lysing cells with HCl, incubating with anti-BrdU-FITC (Becton Dickinson) and counterstaining with PI solution containing RNase. Samples were analysed on a Beckton Dickinson FACScan.

Clonogenic soft agar assay. Cells were resuspended at 1×10^4 cells/ml in growth medium containing 0.36% agar (Difco). Cell suspension (5 ml) was added to 60-mm plates (Costar) precoated with 0.9% solid agar (5 ml). We counted colonies composed of 40 or more cells after 2–6 weeks.

***In vivo* tumorigenic assay**. Cell suspensions were mixed 1:1 with matrigel (Collaborative Biomedical Products) before injection. Matrigel was used to provide additional growth stimulation[22,23] (G.M. Coviello-McLaughlin and C.-P.C., pers. comm.). Cells (1 or 10×10^6) or matrigel alone were injected subcutaneously in the left scapular region of nude mice. We measured cell/matrigel xenografts, matrigel boluses and body weights immediately following injection and twice each week until the termination of the study at 2 or 5.5 months post-inoculation, or until xenografts reached 1 g in measured volume. Parallel cultures of the hTERT clones were maintained *in vitro* without drug selection and were shown to maintain telomerase expression.

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