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Telomerase inhibition by peptide nucleic acids reverses 'immortality' of transformed human cells

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Telomerase activity, the ability to add telomeric repeats to the ends of chromosomes, has been detected in most immortal cell lines including tumor cells, but is low or absent in most diploid, mortal cells such as those of somatic tissues. Peptide nucleic acids (PNAs), analogs of DNA or RNA which bind to complementary nucleic acids with very high affinity, were co-electroporated into immortal human cells along with a selectable plasmid. Introduction of PNAs inverse-complementary to telomerase RNA effectively inhibited telomerase activity in intact cells, shortened telomeres, reduced colony size, and arrested cell proliferation after a lag period of 5–30 cell generations, consistent with suppression of their 'immortality'. Electroporation of selection plasmid alone had no effect, while PNAs of altered sequence were markedly less effective in each assay. This constitutes the first demonstration of cell growth arrest through telomerase inhibition, upon treatment of intact cells with an exogenous compound which can be efficiently delivered *in vivo*. The phenotype of telomerase-inhibited transformed cells differs from senescence of normal diploid fibroblasts, but rather resembles the crisis state of incompletely transformed cells.

Keywords: telomere; telomerase; peptide nucleic acid; cell transformation; immortality; cancer

Introduction

Diploid cells in culture have a limited proliferative capacity (Hayflick, 1977) which is characteristic of each cell type and donor species. Telomerase activity, the ability to add telomeric repeats (TTAGGG in mammals) to the ends of chromosomes, is undetectable in most diploid, 'mortal' cell types and strains (Counter *et al*., 1992; Kim *et al*., 1994), but has been observed in a majority of 'immortal' cell lines–aneuploid cells, whether tumor-derived or transformed *in vitro*, which have unlimited division potential. Transgenic expression of a telomerase protein gene in human diploid fibroblasts extends their replicative lifespans (Bodnar *et al*., 1998), indicating that the ability to extend chromosome ends may be the primary factor limiting cell division in normal somatic cells. Since telomerase activity is observed in most neoplastic cells and tissues yet is low or absent in somatic tissues (Kim *et al*., 1994; Broccoli *et al*., 1995; Shay and Bacchetti, 1997), inhibitors of telomerase activity hold promise as anti-cancer agents which might attenuate the growth of tumors while having little or no effect on normal tissues. Telomerase-negative mice, although unimpaired initially (Blasco *et al*., 1997), by generation six showed hypoproliferative defects in gonads, lymphoid and hematopoietic cells (Lee *et al*., 1998). At least some transgenic clones derived from two human cell lines, expressing RNA complementary to full-length telomerase RNA, showed reduced cell proliferation and shortened telomeres (Feng *et al*., 1995; Kondo *et al*., 1998b). Here we report that cellular immortality can also be reversed upon exogenous addition of sequence-specific telomerase inhibitors to cultured human cells.

Peptide nucleic acids (PNAs) are analogs of RNA and DNA in which the pentose-phosphate backbone is replaced by an oligomer of N-(2-aminoethyl)glycine. These molecules are resistant to biodegradation (Demidov *et al*., 1994) and anneal by Watson:Crick base-pairing to complementary RNA or DNA sequences with much higher affinity than the corresponding phosphodiester oligomers (Nielsen *et al*., 1991; Egholm *et al*., 1993). Human telomerase is inhibited *in vitro* by short PNA oligonucleotides complementary to the telomere-templating portion of telomerase RNA (Norton *et al*., 1996), and in particular those which maintained base-pairing to cytidines in this region (Hamilton, 1997). In the present study we compared the short- and long-term *in vivo* effects of 11- or 13-mer PNA molecules, which either conserved or disrupted complementarity to cytidines 50–52 and 56 of the telomerase-RNA active site.

PNAs are potentially quite versatile molecules, which can be readily modified by additions to their termini, in particular of charged peptides to facilitate their uptake by living cells and/or targeting to nuclei. In order to selectively study just those cells taking up PNAs, we employed co-electroporation of a selectable plasmid, along with short PNAs modified by addition of a 3′-terminal lysine residue, under conditions which allow transgene expression in 40–70% of target cells (Shammas *et al*., 1997). Coordinate uptake of multiple

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nucleic acid species by electroporated cells is a well-established procedure, both for stable (Reid *et al*., 1991; Chu *et al*., 1987) and transient (Chu *et al*., 1987; Xia *et al*., 1997) transfections, enabling selection of a subpopulation of susceptible cells which have internalized exogenous nucleic acids. Lacking such selection, untransfected cells would have a proliferative advantage over telomerase-inhibited cells and would soon predominate in the culture. We also attempted to minimize the number of cell divisions required for the detection of a telomerase-deficient 'phenotype' by choosing recipient cell lines with unusually short telomeres: the SV40-transformed cell lines AT-SV1 (Ataxia telangiectasia, GM05849) and Cys-SV (cystinosis, GM02894), which have telomere terminal restriction fragments of median length 2.2 kbp (kilobase pairs) and 3.3 kbp, respectively (Xia *et al*., 1996). We found that introduction into these immortal human cells, of peptide nucleic acids complementary to telomerase RNA, effectively inhibits telomerase activity, shortens telomeres, and after a substantial lag period arrests cell proliferation, indicating suppression of cellular 'immortality'.

Results

Design of peptide nucleic acid (PNA) inhibitors of human telomerase

We synthesized PNAs complementary to telomerase RNA, and control (mismatched) PNAs, and determined their abilities to inhibit human telomerase activity *in vitro* by a standard 'TRAP' assay (Wright *et al*., 1995) near the midpoint of its linear range (800 cell-equivalents per assay; see Holt *et al*., 1996). Concentrations resulting in 50% inhibition of telomerase activity in HeLa cell nuclear extract at 37°C (IC_{50} values), interpolated from dilution series for the PNAs, are shown in Table 1.

The underlined part of each sequence in Table 1 corresponds to telomere repeat units (nearly two hexanucleotide repeats in the tel-RNA), believed to be essential to alignment of tel-RNA with existing telomere termini, and to provide a template for telomerase synthesis of added DNA repeats (Blackburn, 1992); PNA sequences noncomplementary to telomerase RNA are shown in bold. The first complementary 'Tel-PNA' 11-mer (i) effectively inhibits telomerase activity in human cell extracts and permeabilized cells, with IC_{50} values at 37°C of 10 and 70 nM, respectively, and a T_m for binding to a complementary DNA of 71°C (Norton *et al*., 1996; Hamilton *et al*., 1997). Substitution of A for one (Hamilton *et al*., 1997) or two G's (oligomer ii, above), in the 5′-GGG, decreased the T_m for complementary PNA:DNA duplex by $\geqslant 30$°C, and reduced telomerase inhibition *in vitro* by at least 100-fold.

Inhibition by PNAs of telomerase activity in human cells

We next asked whether the same PNAs were capable of inhibiting telomerase activity following transfection into intact human cells. For this purpose, an SV40-immortalized human cell line (AT-SV1), shown

Table 1 Telomerase-RNA-complementary PNA oligonucleotides and controls

Description of sequence	*Sequence*	*IC_{50}*
Telomerase-RNA (tel-RNA), sequence of RNA strand:	3′-CAAUCCCAAUCUGUUU-5′	
11-mer PNAs:		
i. Inverse-complement of telomerase-RNA sequence:	5′-GGGTTAGACAA-lys	10 nM
ii. Double mutant (MM1) of tel-RNA inverse-complement:	5′-AAGTTAGACAA-lys	1 μM
iii. Mutant (MM2) of tel-RNA inverse-complement:	5′-GTTAG**A**GTTAG-lys	>33 μM
13-mer PNAs:		
iv. Inverse-complement of telomerase-RNA sequence:	5′-TAGGGTTAGACAA-lys	1 nM
v. Permutation (MM3) of tel-RNA inverse-complement:	5′-TAG**AC**TTAGGGAA-lys	1 μM

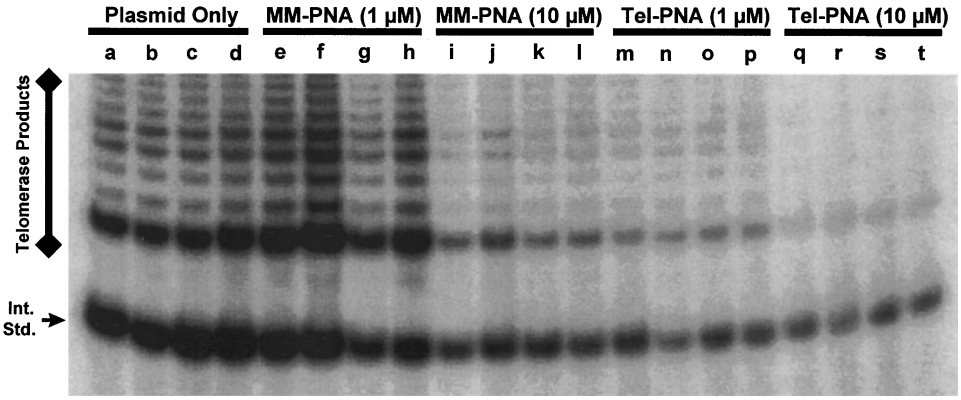

Figure 1 Assay of telomerase activity extracted from electroporated human cells. Replicate TRAPeze assays (Oncor; $n=4$) are shown for telomerase activity (Wright *et al*., 1995) extracted from AT–SV1 cells immediately upon lysis, 3 days after electroporation (Shammas *et al*., 1997; Chu *et al*., 1987) with a selectable neomycin-resistance plasmid, ±PNA at 1 or 10 μM. Telomerase extension products form a 'ladder' of 6-bp increments, reflecting stepwise addition of telomeric repeat units (Blackburn, 1992). These products, and a synthetic PCR target added as an internal control, were amplified by polymerase chain reaction (Wright *et al*., 1995; Holt *et al*., 1996) with a ^{32}P end-labeled primer, analysed by acrylamide gel electrophoresis and autoradiography

previously to express telomerase activity (Xia *et al*., 1996), was electroporated with a selectable marker plasmid (pSV2neo, 3 nM) either alone or together with a telomerase-complementary PNA or a mismatched-control PNA, each at 1 or 10 μM. Cells were grown under selective conditions for 3 days, and then harvested and frozen in aliquots for telomerase assay. Assays conducted at 25°C (Figure 1), or 37°C, indicate telomerase inhibition by Tel-PNA at 1 or 10 μM (sequence iv, above), and substantially less inhibition by a mismatched control PNA (ii, above). The mismatched PNA was more inhibitory at the lower temperature (Table 2), suggesting that mismatched PNAs form stable duplexes with telomerase RNA under less stringent conditions.

Fate of PNA oligonucleotides transfected into human cells in culture

We assessed the time-course of PNA retention following electroporation into the AT-SV1 cell line. The PNA utilized had sequence (i), above, with tetramethyl rhodamine attached through a 5′-glycine. Rhodamine-PNA at 10 μM was co-electroporated with a selectable plasmid (pCEP4) and the recipient cells were selected continuously in 50 μg/ml hygromycin. One day after electroporation, before substantial antibiotic selection had occurred, $\geqslant$60% of attached cells displayed rhodamine fluorescence (not shown). By 3 days post-electroporation, almost all

Table 2 Telomerase inhibition by PNAs electroporated into intact human cells

Condition	*Activity in TRAP assay (% of positive control*±s.e.) *25°C*	*37°C*
No PNA	100±2	100±2
1 μM MM-1 (ii)	100±2	100±2
10 μM MM-1 (ii)	79±3	100±2
1 μM Tel–PNA (iv)	70±3	78±2
10 μM Tel–PNA (iv)	37±2	38±3

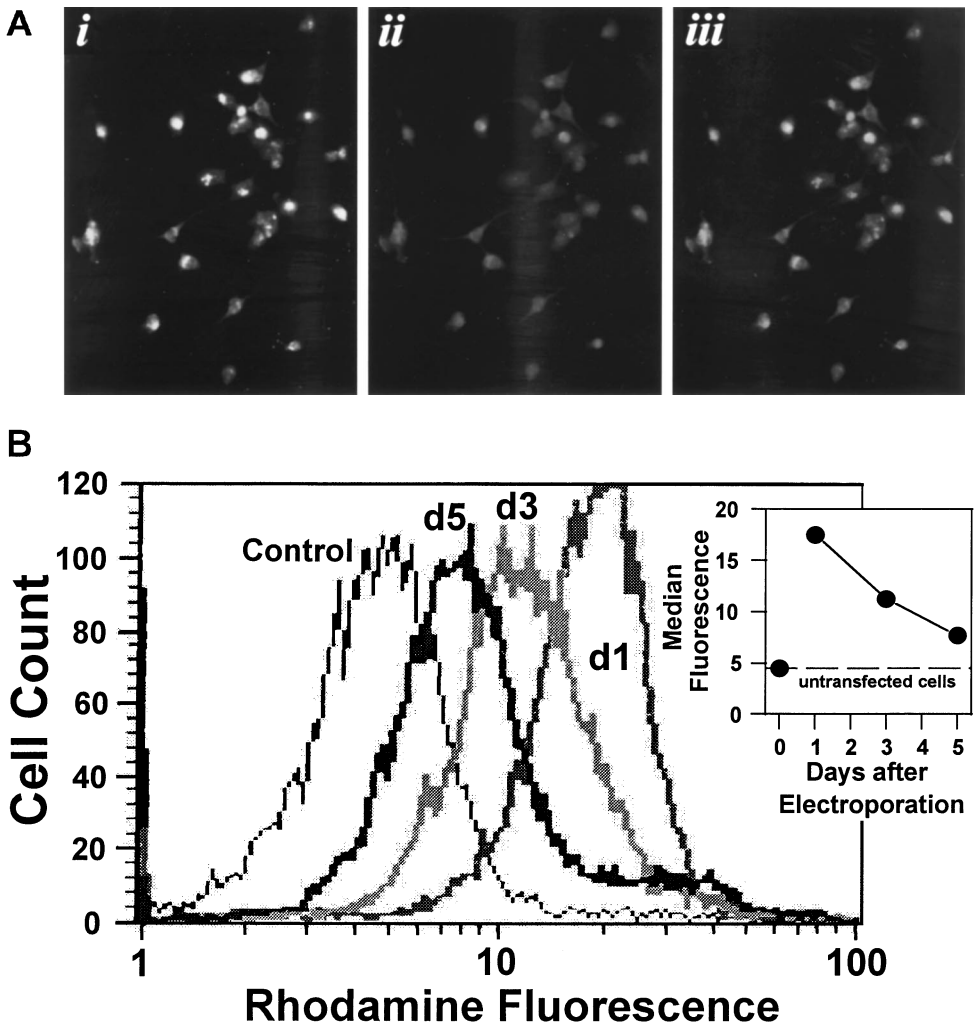

Figure 2 Rhodamine-PNA fluorescence following electroporation into human cells. (**A**) AT–SV1 cells were fixed 72 h after electroporation with 10 μM rhodamine-PNA, and counterstained with phalloidin – which binds to actin filaments – labeled with fluorescein isothiocyanate (FITC; Sigma). A representative field is shown, viewed by rhodamine fluorescence (panel i: excitation peak 550 nm, emission 570 nm), or FITC fluorescence (panel ii: excitation peak 490 nm, emission 520 nm). Nuclear phalloidin fluorescence, seen in a few cells, may be crossover from exceptionally bright rhodamine fluorescence. (**B**) Distribution of rhodamine fluorescence per cell, shown on a logarithmic scale, were assessed by cell flow analysis at days 1, 3 and 5 after electroporation of 10 μM rhodamine-PNA into AT-SV1 cells. Fluorescence of unelectroporated cells is shown as 'Control'. (Inset) Log-linear decline, with time after electroporation, of median rhodamine fluorescence per cell

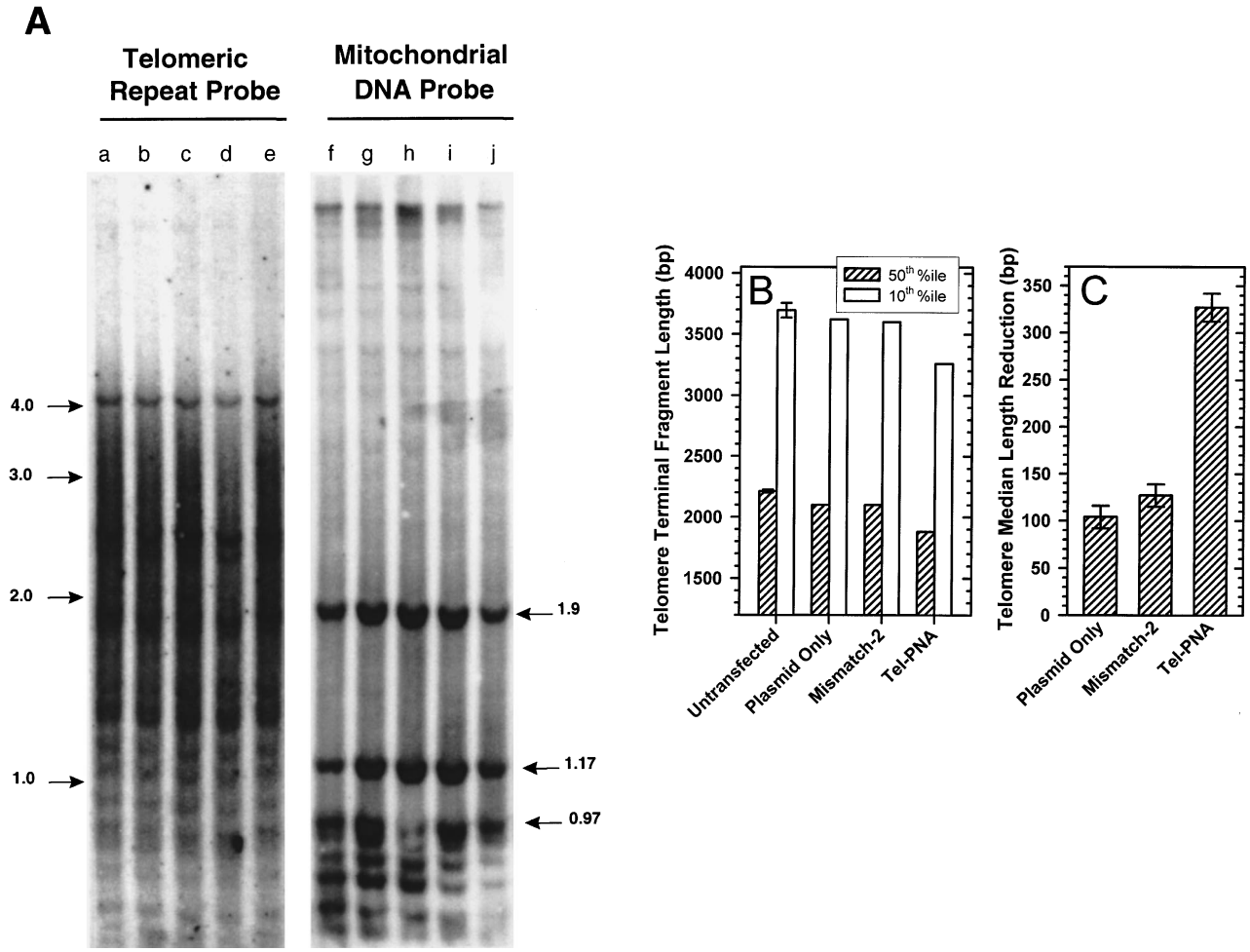

Figure 3 Reduction in telomere length following cell electroporation with PNA complementary to telomerase RNA. (**A**) Telomere terminal restriction fragments (left) and mitochondrial DNA fragments (right), revealed by successive probings of a single Southern-blot filter with $\gamma[^{32}P]$-$(TTAGGG)_4$ telomeric probe, or mitochondrial ^{32}P-DNA labeled by octanucleotide-primed synthesis from cloned, full-length human mtDNA. Each lane contains 5 μg human genomic DNA digested in two successive 1 h incubations, each with 30 units *Hin*fI endonuclease, prior to agarose gel electrophoresis. DNA was isolated from untransfected AT-SV1 cells (lanes a, e, f, j), or from cells receiving selection plasmid only (lanes b, g), plasmid plus Mismatch-2 PNA (iii) (lanes c, h), or plasmid plus Match Tel-PNA (ii) (lanes d, i). (**B**) Tenth and 50th percentile lengths, calculated from fragment size distributions in scanned lanes (Molecular Dynamics Beta-Imager 2000). Error bars indicate s.e. for quadruplicate lanes. (**c**) Median length reductions, relative to untransfected controls; histogram heights and error bars indicate mean $\pm$ s.e. of median telomere lengths determined from three separate gels and dual hybridizations

surviving cells showed rhodamine fluorescence (Figure 2A, i: compare panels i and ii), which was predominantly nuclear with subnuclear foci apparent in some cells. Somewhat weaker fluorescence, similarly localized to nuclei, was also observed after 5 days (not shown). Extracts prepared from electroporated cells at day 3 were analysed by high pressure liquid chromatography (HPLC). Recovered material containing rhodamine (based on absorbance monitored at 546 nm) ran as a single sharp peak at the same position as the initial rhodamine-PNA (data not shown), indicating that little or no degradation had occurred.

The amount of rhodamine fluor retained per cell was measured using a fluorescence-activated cell flow analyzer (FACScan, Becton-Dickinson, San José, CA, USA), on days 1, 3 and 5 following electroporation (Figure 2B). The half-life of intracellular rhodamine label, estimated from median fluorescence per cell (Figure 2B inset), is 2.0 days.

Effect of PNAs on telomere length

AT-SV1 cells were electroporated for three cycles (11–16 cell generations) with a selection plasmid plus Tel-PNA or mismatch PNA, or plasmid alone (see Materials and methods for details). We then measured the lengths of terminal restriction fragments (Harley *et al*., 1990), generated by digesting genomic DNA with telomere-sparing restriction endonucleases, and detected by Southern-blot hybridization to end-labeled telomeric-repeat oligonucleotides as described previously (Xia *et al*., 1996). To control for lane-to-lane variation in fragment migration, each filter was rehybridized to mitochondrial DNA probes. Median lengths of telomere-sequence fragments were then calculated for each lane, with respect to the largest *Hin*f1 mitochondrial DNA fragments. Figure 3A,B, shows data from one experiment, and Figure 3C summarizes median telomere lengths from three assays. Note in Figure 3A a reduction in both

intensity and length of diffusely-migrating telomeric fragments, in lane d (Tel-PNA) relative to the other lanes. Telomeres shortened by 327 ± 15 bp (base pairs; mean $\pm$ s.e. for three measurements) following three electroporation cycles with Tel-PNA, *vs* 104–127 bp after electroporation with mismatched PNA or selection plasmid alone. Thus, a small reduction in telomere length arose from recurrent electroporation, which can produce nicks in DNA and is optimally efficient under conditions of substantial cell lethality (Chu *et al*., 1987), whereas a threefold greater effect was specific to Tel-PNA.

Growth inhibition after electroporation of telomerase-PNAs into immortal human cells

The effect of telomerase-complementary PNAs on cell growth potential was examined in the AT-SV1 cell line, for which the median length of telomeric fragments was 2.2 kbp (Xia *et al*., 1996). Cells were co-electroporated with PNAs and a selection plasmid, either pCEP4(*hyg*) or pSV2neo, and then grown for 7 days in medium supplemented with the corresponding selective agent (hygromycin or G418) to eliminate cells which failed to take up the foreign molecules. Surviving cells were then harvested, counted, and a portion was replated after electroporation with the same PNA and the other selection plasmid, alternating the two selection modalities to impede accumulation of stable transformants. Cell numbers, assessed at the end of each electroporation cycle, remained quite stable for the first three cycles (11–16 cell generations), and then declined precipitously over the next two cycles to $\sim$2% of the previous cell number (Figure 4A). Mismatched PNAs were considerably less effective in growth inhibition than the Tel-PNA sequence complementary to telomerase RNA (Figure 4A and additional data not shown).

Nuclear labeling indices for AT-SV1 cells were determined as described previously (Shammas *et al*., 1997), by *in situ* reaction of cells with antibody to BUdR-containing DNA (Amersham), following 18 h incorporation of this thymidine analog. The results (Figure 4B) indicate that neither PNA had any effect on DNA replication in surviving (substrate-attached) cells. The fraction of labeled nuclei ranged from 83–96%, quite far from levels of 5–15% expected for diploid fibroblasts undergoing replicative senescence (Cristofalo and Sharf, 1973) but consistent with the

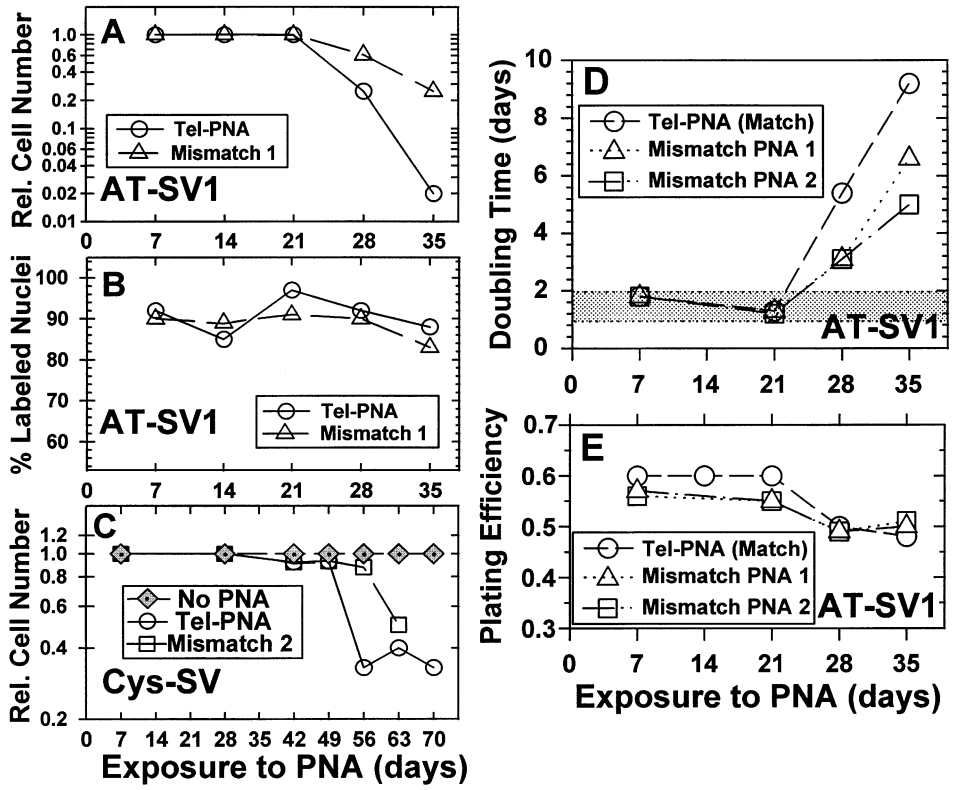

Figure 4 Limited replication potential in immortalized human cell lines electroporated with telomerase-inhibitory PNAs. (**A**) Cell numbers are shown on a logarithmic scale, relative to untransfected cultures, at successive passages of AT-SV1 cells. At the start of each 7-day electroporation cycle, 7×10^6 cells were electroporated (Shammas *et al*., 1997; Chu *et al*., 1987) with 1 μM Tel-PNA or a control PNA (Mismatch 1), together with a selection plasmid (20 μg, pSV2neo or pCEP4[hyg^R] in alternate cycles), or with selection plasmid alone. Following electroporation, cells were plated in multiple flasks at 0.5×10^6 cells each, and grown in medium containing G418 or hygromycin for 7 days ($\sim$5 MPD for control cultures, which by then had achieved 7×10^6 cells/flask). Cells were detached with trypsin/EDTA and counted. (**B**) Cells at the completion of each cycle, as above, were tested for 18 h BUdR incorporation, monitored with anti-BUdR and fluorescent second antibody (17). (**C**) As **A**, except that the Cys-SV cell line was used in place of AT-SV1. (**D**) Growth rates were calculated for AT-SV1 cells, from 4-point determinations of cell numbers on days 1, 3, 5 and 7 of each electroporation cycle; this experiment was independent of that in **A** above. Doubling times are calculated as slopes of regression lines for $\log_2$ (cell number) *vs*. time, for which linear correlation coefficients, r, were $\geqslant$0.96 at cycle 1, $\geqslant$0.99 at cycle 3, $\geqslant$0.93 at cycle 4, and $\geqslant$0.97 at cycle 5. (**E**) Survival and plating efficiency were determined for each culture, as the fraction of electroporated cells which attached within 24 h. The ordinate thus reflects combined effects of cell survival of eletroporation, and cell attachment upon plating. Symbols: $\diamond$, selection plasmid only; $\triangle$, Mismatch 1 control PNA; $\square$, Mismatch 2 PNA; $\bigcirc$, Tel-PNA (each PNA, 1 μM)

onset of crisis in SV40-transformed cells (Ozer *et al*., 1996; Rubelj *et al*., 1997).

A very similar decline in cell proliferation was observed for the Cys-SV cell line (median telomere length 3.3 kbp; Xia *et al*., 1996), except that the lag phase prior to a pronounced growth decrease was substantially longer – six transfection cycles or 22–32 cell generations (Figure 4C). As with AT-SV1, mismatched PNA elicited a diminished or later-onset effect. Overall, we observed a marked arrest of 'immortal' cell proliferation by PNAs in seven or nine experiments. In the two exceptions, an initial decline in cell number was followed by unabated cell growth which we tentatively attribute to expansion of cells failing to take up PNA, but stably transfected with both selection plasmids (and thus not susceptible to antibiotic killing).

Growth kinetics for AT-SV1 cells, assessed by measuring cell numbers at four points in each transfection cycle, confirmed the results of single-point assays. Cell doubling time remained nearly constant (1.3–1.9 days) for three cycles or 11–16 cell doublings, and then increased to 9.2 days over the next two cycles (Figure 4D) in cells receiving PNA complementary to the telomerase RNA sequence (Tel-PNA). These results, and an attenuated effect of two Mismatch PNAs, closely parallel the decline in cell number observed in separate experiments (Figure 4A). SV40-transformed human cells normally have doubling times of 0.9–2.0 days (shaded rectangle in Figure 4D), under a range of growth conditions. The impairment in growth rate and in cell numbers, for cells receiving telomere-sequence PNAs, cannot be a consequence of differential mortality or plating efficiency following electroporation, since plating efficiency/survival for the three PNAs over five transfection cycles (Tel-PNA, 55.6 ± 2.7[s.e.]%; Mismatch-1, 54.6 ± 2.2%; Mismatch-2, 52.4 ± 1.5%) did not differ significantly from one another or from electroporations with selection plasmid alone (54.8 ± 2.7%, not shown), although each declined slightly with cycle number (Figure 4E).

Colony size reduction in immortal but not mortal cells exposed to Tel-PNA

We next measured the distribution of colony sizes, a sensitive and robust measure of cell replicative potential (Smith *et al*., 1978; Harley and Goldstein, 1978), for immortal AT-SV1 and mortal HSC172 human cells electroporated a single time with 10 μM PNAs (accompanied by pSV2neo as a selection marker), just prior to low-density plating in G418-containing medium. Although repeated electroporation is not feasible in this protocol, essentially all G418-resistant colonies (stably transfected with plasmid pSV2neo) would be expected to have taken up PNA due to the ~3400-fold molar excess of PNA over selection plasmid. For the immortal AT–SV1 recipient cell line, colony sizes were not significantly different between cells receiving Match Tel–PNA (iv), Mismatch (v), or plasmid alone (no PNA) at 1 week post-electroporation (Figure 5A). By 2 weeks, colonies arising from cells exposed to the matching Tel–PNA were reduced slightly (17%) in mean size below those treated with plasmid alone ($P < 0.005$; Figure 5B).

Three weeks after electroporation with matching Tel–PNA, colony sizes (mean ± s.e. in mm) in two experiments were 1.55 ± 0.04 and 1.41 ± 0.09, but reached 2.40 ± 0.11 and 2.00 ± 0.14 for cells receiving mismatched PNA, *vs* 2.05 ± 0.10 and 2.53 ± 0.18 for cells receiving only the selection plasmid. In each experiment, introduction of Match Tel-PNA resulted in smaller colonies than either control (each $P < 10^{-6}$, by Fisher's *t*-test). Most strikingly, cells receiving the Match PNA formed very few colonies of >1.5 mm diameter by 2 weeks (Figure 5B), or exceeding 2.5 mm at 3 weeks – close to the mean size for no-PNA colonies (Figure 5C) – indicating a marked impairment in maximal replicative potential (Smith *et al*., 1978; Harley and Goldstein, 1978). In contrast, fibroblasts of the untransformed (mortal) HSC172 strain formed colonies with essentially the same size distribution (Figure 5D), whether electroporated with Match Tel-PNA (mean ± s.e. 3.3 ± 0.1 cm) or plasmid alone (3.4 ± 0.2 cm). Similar results were seen for HSC172 cells at earlier times after plating (not shown). HSC172 forms larger colonies than AT-SV1, reflecting the much greater substrate-attachment surface of diploid fibroblasts, especially as they senesce, relative to immortal cells.

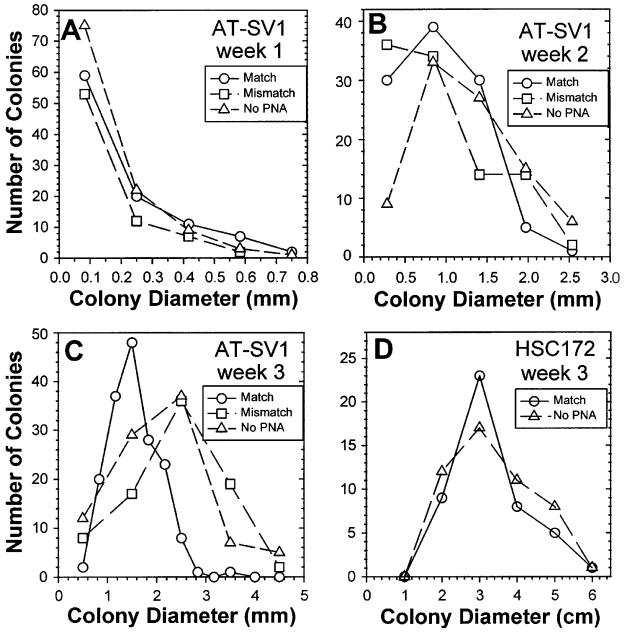

Figure 5 Colony size distributions of immortal or mortal cells electroporated with PNA. AT–SV1 (**A**–**C**) and HSC172 (**D**) cells were electroporated with either the Match 13-mer (iv in Table 1) or the corresponding Mismatch PNA (v), each at 10 μM, accompanied by pSV2-neo (~3 nM) as a selection plasmid, or with pSV2neo alone ('No PNA'). Cells were immediately plated at low-density in regular growth medium, and were fixed, stained and photographed after 7 (**A**), 14 (**B**), or 21 (**C**, **D**) days of growth in G418-containing medium. Colony diameters were measured 'blind' by naïve scorers, on enlarged laser prints of entire flasks or dishes, measuring only colonies which were circular or clearly composed of intersecting circular colonies

Discussion

Immediate in vivo *effects of PNAs targeted to telomerase-RNA*

Rhodamine-tagged PNA, complementary to telomerase RNA, was localized in the nuclei of recipient cells

within 24 h of electroporation, and remained visible there through day 5 (Figure 2A and data not shown). Based on the exponential decline of rhodamine-PNA signal in these cells (Figure 2B), ~9% of the PNA initially taken up would remain after 7 days, the interval between repeated electroporations with PNA. The half-life observed for rhodamine-PNA after electroporation into AT-SV1 cells (2.0 days; see Figure 2B inset) does not differ significantly from the doubling time, 1.9±0.1 days, measured for these recipient cells under identical electroporation and culture conditions (Figure 4D, 7-day time point). Since HPLC analyses indicate that the internalized rhodamine-PNA remained intact, our data are consistent with its loss solely by dilution, due to cell proliferation. This supports and extends a previous report that PNAs are not degraded by human serum or cell extracts (Demidov *et al*., 1994), presumably because they are not recognized as substrates by proteases or nucleases.

Inhibition of telomerase activity was observed in extracts obtained from cells 3 days following electroporation, dependent on both PNA length and complementarity to telomerase RNA (Table 2). This strongly implies that telomerase inhibition involves base-pairing of the PNA with the target RNA. Whereas our previous demonstration that PNAs complementary to telomerase RNA can inhibit telomerase activity *in vitro* when added directly to telomerase extracts (Norton *et al*., 1996; Hamilton *et al*., 1997), the present observations (Table 1 and Figure 1) indicate that PNAs can inhibit telomerase in intact cells. Although PNA binding to telomerase RNA could in principle occur after cell lysis, this seems unlikely given that cells had been washed at least six times since PNA addition, and any residual free PNA within nuclei would have been diluted by at least a further 2000-fold in the assay.

Telomere attrition in PNA-inhibited cells

The observed net telomere length reduction attributable to treatment with matching Tel-PNA – 210 bp over 11–16 cell generations, or ~15 bp/generation – was determined with high precision and reproducibility, using mitochondrial DNA restriction fragments as internal size standards. This rate of loss is close to that expected from incomplete replication of chromosome ends, in the absence of exonuclease trimming (Zakian, 1997). It represents the rate of telomere shortening in telomerase-inhibited immortal cells, and is clearly less than the telomere attrition rate (50–100 bp per division) estimated for mortal human fibroblasts lacking detectable telomerase activity (Harley *et al*., 1990). The difference may be attributable to differing exonuclease levels in these two cell types (see Zakian, 1997), or may indicate that AT-SV1 telomeres, at 2.1–2.2 kbp, can tolerate only a very limited further reduction in length before limiting cell viability, consistent with earlier estimates that the minimum telomere terminal-fragment length for human cells is ~2 kbp (Harley *et al*., 1990). It is also possible that there is residual telomerase activity in these cells, not inhibited by Tel-PNA.

PNA-induced growth arrest is distinct from cell toxicity

Growth arrest of cells taking up Tel–PNA does not appear to be a consequence of nonspecific PNA toxicity, for the following reasons: (a) Sequences with reduced complementarity to telomerase RNA were 2- to 10-fold less inhibitory (Figure 4), whereas PNA toxicity would not be expected to be sequence dependent. (b) No effect on cell growth or morphology was observed during a lag phase of 5–16 cell generations for AT–SV1 cells, or 22–32 generations for Cys-SV cells which have longer telomeres (Xia *et al*., 1996), again inconsistent with toxicity (see Figure 4). The appearance of an extended lag phase prior to cell growth impairment contrasts with the rapid inhibition of cell division seen after addition of an oligonucleotide designed to target telomerase for destruction by RNase L (Kondo *et al*., 1998a). This difference may reflect a cell-type-specific response, or the two distinct inhibitory mechanisms utilized: irreversible inactivation of telomerase *vs* competitive inhibition of the intact enzyme. (c) Even during the period of growth inhibition, plating efficiency was only slightly attenuated (Figure 4E), and DNA synthesis was unimpaired (Figure 4B), features quite atypical of response to cellular toxins. (d) Colony size was reduced only for immortal cells receiving PNA complementary to telomerase RNA, but not for mortal cells under the same conditions (Figure 5). The last control is particularly noteworthy, since diploid human cells are generally more susceptible to suboptimal culture conditions than are transformed cells, and this vulnerability is especially evident during low-density plating for colony formation. The colony size assay, since it produced results concordant with much more labor-intensive studies of cell growth potential and kinetics, appears quite promising as a screening procedure for exogenous inhibitors of telomerase.

The nature of cell mortality associated with telomerase inhibition

SV40-transformed cells, repeatedly transfected with telomerase-targeted PNAs, appeared in most respects indistinguishable from untransfected cells during the initial electroporation cycles. In seven of nine experiments, however, cell growth then declined sharply, suggesting a reversal of 'immortalization'. Rather than undergo cell senescence, these cells appeared to enter crisis (see below), much as reported for HeLa cell clones stably transfected with a plasmid expressing antisense telomerase RNA (Feng *et al*., 1995), and for a human glioblastoma line stably transfected with the same anti-telomerase plasmid, although a subset of differentiated cells emerged from crisis (Kondo *et al*., 1998b). Crisis, the eventual fate of all cells expressing SV40 T-antigen but not immortalized (Ozer *et al*., 1996; Rubelj *et al*., 1997), is a period of active cell replication, offset by a comparable rate of cell death and/or detachment – with characteristics similar or identical to apoptosis. Immortal cells may emerge following crisis, or occasionally appear even before crisis, as very rare clonal events reflecting further mutations in one of at least four complementation groups (Pereira-Smith and Smith, 1988). In

contrast, senescence is the normal state of late-passage diploid cells in culture (Hayflick, 1977; Martin *et al*., 1970), and is characterized by pronounced increases in nuclear and cell volume, cell attachment area, and interdivision time (Hayflick, 1977), with a corresponding drop in the fraction of cells engaging in replicative DNA synthesis (Cristofalo and Sharf, 1973).

The cells growth-arrested after prolonged exposure to telomerase PNAs displayed several characteristics indicative of crisis rather than cellular senescence: pervasive cell detachment and death, despite normal labeling indices for cells remaining attached (83–96%; Figure 4B), and 'rounding' or retraction of substrate-anchoring cell membrane, accompanied by the appearance in many cells of nuclear fragmentation (not shown), as is commonly seen in apoptosis. These observations are consistent with a dual-barrier model of cellular senescence, in which T antigen overcomes the first barrier by reducing or obviating the normal cell's requirement for mitogenic stimuli (autocrine and paracrine growth factors, ligand binding by fibronectin receptor, etc.), allowing cells to proliferate until cell death or crisis ensues (Wright *et al*., 1989). Mutations overcoming the second obstacle are rare (Shay and Wright, 1989; Cheng *et al*., 1997), and may involve reactivation of telomerase expression, or other mechanisms of overcoming the telomere-extension problem such as activation of inter-telomere recombination (Wang and Zakian, 1990). The observation that SV40-immortalized cells, when telomerase-inhibited, appear to enter crisis in a manner indistinguishable from pre-immortal cells expressing SV40 T-antigen, argues that telomerase activation is the sole or principal 'second phase' mechanism for completion of immortal transformation in these cells. This may involve, directly or indirectly, inactivation of the SEN6 locus (Banga *et al*., 1997) – reintroduction of which suppresses growth of cells immortalized by T-antigen (Sandu *et al*, 1994).

Conclusions and future prospects

Our results demonstrate that exogenous addition of inhibitors of human telomerase can reverse cell immortality. The PNA inhibitors used in these studies are both potent and selective. Methods have recently been described that allow spontaneous uptake of PNAs into mammalian cells (Simmons *et al*., 1997; Pooga *et al*., 1998), and permit targeting to intracellular mRNA species (Pooga *et al*., 1998), suggesting that PNAs may themselves be useful lead compounds for therapy. In addition, 2′-O-meRNA oligonucleotides, a type of oligomer already being used in clinical trials, have also been shown to be effective *in vitro* inhibitors of strand elongation by telomerase (Pitts and Corey, 1998). Several other compounds have also been demonstrated to inhibit telomerase *in vitro* (Fletcher *et al*., 1996; Pai *et al*., 1998; Federoff *et al*., 1998; Wheelhouse *et al*., 1998), but their efficacies *in vivo*, and in particular their abilities to reduce telomere length and cell proliferation, have not been reported (Sharma *et al*., 1997). Our observations of markedly decreased cell proliferation following telomerase-specific PNA introduction, in two immortal cell lines and by three assay systems, support proceeding to long-term experiments applying this pharmacologically well-characterized class of compounds to resolve the question of whether telomerase is a useful target for chemotherapy of human cancer.

Materials and methods

Cell culture

Cell lines AT-SV1 (Ataxia telangiectasia, GM05849) and Cys-SV (cystinosis, GM02894) were obtained from the Genetic Mutant Cell Repository (National Institute for General Medical Sciences), maintained by the Coriell Institute for Medical Research, Camden, NJ, USA. Cell culture procedures were carried out as described by Moerman and Goldstein (1986), growing cells in 60- or 100-mm dishes (Corning) or T175 flasks (Sarstedt), in Eagle's minimal essential medium (MEM) plus 10% fetal bovine serum (HyClone). Cultures are maintained at 5% CO_2 and 90–95% relative humidity, 37°C, in a Stericult 200 incubator (Forma), and cells are counted after EDTA/trypsin harvest by resistive pulse detection (Coulter, models Z_F and Z2).

PNA synthesis

Peptide nucleic acids (PNAs) were synthesized manually as described previously (Norton *et al*., 1995) using monomers obtained from PerSeptive Biosystems (Framingham, MA, USA).

Electroporation and growth of cells

Because cells which escaped transfection might have a proliferative advantage over telomerase-inhibited cells, human cells were co-electroporated with PNA and a selectable marker plasmid (pCEP4 [InVitrogen] or pSV2neo). Untransfected cells were then eliminated by selection with either hygromycin, or the neomycin analog G418, respectively. Although pCEP4 contains the Epstein-Barr virus *oriP* and *EBNA-1* genes, which allow episomal maintenance of plasmid at 10–20 copies per cell (Cachianes *et al*., 1993), pCEP4 and pSV2neo were used in alternate cycles in order to impede the accumulation of stable integrants for either plasmid, which would be indifferent to selection for the integrated marker in subsequent cycles. AT-SV1 or Cys-SV cells were grown to 70–80% confluence as above, washed three times with PBS (phosphate-buffered saline), and detached with EDTA/trypsin. Cells (7×10^6 per electroporation) were rinsed in low-salt electroporation buffer (Chu *et al*., 1987), resuspended in 0.8 ml buffer at 0°C, and electroporated (Gene-Pulser, Bio-Rad, Richmond, CA, USA) at 180 V, 960 μF, in a pre-cooled electroporation cuvette (Bio-Rad) with 20 μg of plasmid pSV2neo or pCEP4 (InVitrogen) and 1 or 10 μM PNA oligonucleotides – pretreated to 65°C for 5 min to dissociate aggregates. Cells were then restored to isotonicity and mixed with growth medium for plating in 60-mm dishes and T-175 flasks. Following 24 h recovery, either G418 (200 μg/ml, to select pSV2neo) or hygromycin (50 μg/ml, to select pCEP4) was added to cells in regular growth medium. The level of each antibiotic was sufficient to reduce the numbers of susceptible (untransfected) AT-SV1 cells by at least 98% on day 5, and by >99.5% on day 7 after electroporation. Cells in one set of 60-mm dishes were harvested at days 1, 3, 5 or 7 after electroporation, and counted to determine growth kinetics. Cells in a second set of 60-mm dishes were harvested similarly at days 1, 3, 5 or 7 after electroporation, counted, washed three times with PBS, and stored at −80°C as cell pellets for telomerase activity assays. Cells in flasks were harvested at day 7 for counting, DNA preparation, and the next cycle of electroporation and selection.

Assay of telomerase activity

Telomerase activity was assayed essentially as described (Holt *et al*., 1996) using the TRAPeze™ telomerase detection kit (Oncor, Gaithersburg, MD, USA). AT–SV1 cells electroporated with a selection plasmid alone or with plasmid and PNA were lysed as recommended by Oncor, and 800 cell-equivalents of lysate per assay were assessed for telomerase activity. The linear range for this assay extends from 10 to 4000 cell-equivalents per reaction (Holt *et al*., 1996). TRAPeze reaction mixture (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, and 0.01% g BSA, 50 mM of each dNTP, and primer mix supplied in the kit) was added directly to the mixture of PNA and cell extract. This reaction was incubated 30 min at 25°C to allow telomerase elongation of 'TS' primer. After elongation and amplification of telomerase products, reactions were run on 10% nondenaturing polyacrylamide gels. Telomerase activity was quantitated by phosphorimager analysis (Molecular Dynamics Beta-Imager 2000, Sunnyvale, CA, USA), comparing the ratio of telomerase products to an internal standard for each lysate as described (Hamilton *et al*., 1997). Telomerase activity from cells electroporated with plasmid only was assigned a value of 100%.

Estimation of telomere length

Genomic DNA was isolated from cells (Shmookler Reis and Goldstein, 1983) and telomere length was estimated as described (Xia *et al*., 1996). DNA (6 μg) was digested twice in succession, each time with a sixfold excess of restriction enzyme *Hin*fI (New England Biolabs), then electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane (Xia *et al*., 1996). Telomeric DNA was detected by hybridization of the membrane with γ-^{32}P-labeled $(TTAGGG)_4$ probe. Autoradiographic signal was quantitated using a scanning phosphorimager (Molecular Dynamics). The $(TTAGGG)_4$ probe was then stripped off (Sambrook *et al*., 1989) and the membrane was rehybridized with a cloned mitochondrial DNA probe, labeled with α-^{32}P-dCTP (Megaprime Labeling Kit, Amersham).

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References

Banga SS, Kim S-H, Hubbard K, Dasgupta T, Jha KK, Patsalis P, Hauptschein R, Gamberi B, Dalla-Favera R, Kraemer P and Ozer HL. (1997). *Oncogene,* **14,** 313–321.
Blackburn EH. (1992). *Annu. Rev. Biochem.,* **61,** 113–129.
Blasco MA, Lee H-W, Hande MP, Samper E, Lansdorp PM, DePinho RA and Greider CW. (1997). *Cell,* **91,** 25–34.
Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shay JW, Lichsteiner S and Wright WE. (1998). *Science,* **279,** 349–352.
Broccoli D, Young JW and de Lange T. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 9082–9086.
Cachianes G, Ho C, Weber RF, Williams SR, Goeddel DV, Leung DW. (1993). *Biotechniques,* **15,** 255–259.
Cheng RZ, Shammas MA, Li J and Shmookler Reis RJ. (1997). *Exp. Cell Res.,* **234,** 300–312.
Chu G, Hayakawa H and Berg P. (1987). *Nucleic Acids Res.,* **15,** 1311–1326.
Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S. (1992). *EMBO J.,* **11,** 1921–1929.
Cristofalo VJ and Sharf BB. (1973). *Exp. Cell Res.,* **76,** 419–427.
Demidov VV, Potaman VN, Frank-Kamenetskii MD, Egholm M, Buchardt O, Sonnichsen SH and Nielsen PE. (1994). *Biochem. Pharmacol.,* **48,** 1310–1313.
Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, Berg RH, Kim SK, Norden B and Nielsen PE. (1993). *Nature,* **365,** 566–568.
Fedoroff OY, Salazar M, Han H, Chemeris VV, Kerwin SM and Hurley LH. (1998). *Biochemistry,* **37,** 12367–12374.
Feng J, Funk WD, Wang S-S, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW and Villeponteau B. (1995). *Science,* **269,** 1236–1241.
Fletcher TM, Salazar M and Chen S-F. (1996). *Biochemistry,* **35,** 15611–15617.
Hamilton SE, Pitts AE, Katipally RR, Jia X, Rutter JP, Davies BA, Shay JW, Wright WE and Corey DR. (1997). *Biochemistry,* **36,** 11873–11880.
Harley CB and Goldstein S. (1978). *J. Cell. Physiol.,* **97,** 509–516.
Harley CB, Futcher BA and Greider CW. (1990). *Nature,* **345,** 458–460.
Hayflick L. (1977). *Handbook of the Biology of Aging*. Finch CE and Hayflick L (eds). Van Nostrand Reinhold Co.: NY, pp. 159–186.
Holt SE, Norton JC, Wright WE and Shay JW. (1996). *Meth. Cell Sci.,* **18,** 237–248.
Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW. (1994). *Science,* **266,** 2011–2015.
Kondo S, Kondo Y, Li G, Silverman RH and Cowell JK. (1998a). *Oncogene,* **16,** 3323–3330.
Kondo S, Tanaka Y, Kondo Y, Hitomi M, Barnett GH, Ishizaka Y, Liu J, Haqqi T, Nishiyama A, Villeponteau B, Cowell JK and Barna BP. (1998b). *FASEB J.,* **12,** 801–811.
Lee H-W, Blasco MA, Gottlieb GJ, Horner JW II, Greider CW and DePinho RA. (1998). *Nature,* **392,** 569–574.
Martin GM, Sprague C and Epstein C. (1970). *Lab. Invest.,* **23,** 86–91.
Moerman EJ and Goldstein S. (1986). *Methods in Diabetes Research, Vol. II, Clinical Methods*. Clarke WL, *et al*. (eds). John Wiley & Sons: NY. pp. 283–312.
Nielsen PE, Egholm M, Berg RH and Buchardt O. (1991). *Science,* **254,** 1497–1500.
Norton JC, Piatyszek MA, Wright WE, Shay JW and Corey DR. (1996). *Nature Biotech.,* **14,** 615–619.
Norton JC, Waggenspack JH, Varnum E and Corey DR. (1995). *Bioorg. Med. Chem.,* **3,** 437–445.
Ozer HL, Banga SS, Dasgupta T, Houghton, J, Hubbard K, Jha KK, Kim SH, Lenahan M, Pang Z, Pardinas JR and Patsalis PC. (1996). *Exp. Gerontol.,* **31,** 303–310.
Pai RB, Pai SB, Kukhanova M, Dutschman GE, Guo X and Cheng YC. (1998). *Cancer Res.,* **58,** 1909–1913.
Pereira-Smith OM and Smith JR. (1988). *Proc. Natl. Acad. Sci. USA,* **85,** 6042–6046.
Pitts AE and Corey DR. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 11549–11554.
Pooga M, Soomets U, Hällbrink M, Valkna A, Saar K, Rezaei K, Kahl U, Hao J-X, Xu X-J, Wiesenfeld-Hallin Z, Hökfelt T, Bartfai T and Langel Ü. (1998). *Nature Biotechnol.,* **16,** 857–861.
Reid LH, Shesely EG, Kim HS and Smithies O. (1991). *Mol. Cell. Biol.,* **11,** 2769–2777.

Rubelj I, Venable SF, Lednicky J, Butel JS, Bilyeu T, Darlington G, Surmacz E, Campisi J and Pereira-Smith OM. (1997). *J. Gerontol.,* **52,** pp. B229–B234.

Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular Cloning; A Laboratory Manual*, Laboratory Press: Cold Spring Harbor, NY. pp. 9.47–9.58.

Sandhu AK, Hubbard K, Kaur GP, Jha KK, Ozer HL and Athwal RS. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 5498–5502.

Shammas MA, Xia SJ, Shmookler Reis RJ. (1997). *Genetics,* **146,** 1417–1428.

Sharma S, Raymond E, Soda H, Sun D, Hilsenbeck SG, Sharma A, Izbicka E, Windle B and Von Hoff DD. (1997). *Ann. Oncol.,* **8,** 1063–1074.

Shay JW and Bacchetti S. (1997). *Eur. J. Cancer,* **33,** 787–791.

Shay JW and Wright WE. (1989). *Exp. Cell Res.,* **184,** 109–118.

Shmookler Reis RJ and Goldstein S. (1983). *J. Biol. Chem.,* **258,** 9078–9085.

Simmons CG, Pitts AE, Mayfield LD, Shay JW and Corey DR. (1997). *Bioorg. Med. Chem. Lett.,* **7,** 3001–3006.

Smith JR, Pereira-Smith OM and Schneider EL. (1978). *Proc. Natl. Acad. Sci. USA,* **75,** 1353–1356.

Wang S-S and Zakian VA. (1990). *Nature,* **345,** 456–458.

Wheelhouse RT, Sun D, Han H, Han FX and Hurley LH. (1998). *J. Amer. Chem. Soc.,* **120,** 3261–3262.

Wright WE, Pereira-Smith OM and Shay JW. (1989). *Mol. Cell. Biol.,* **9,** 3088–3092.

Wright WE, Shay JW and Piatyszek MA. (1995). *Nucl. Acids Res.,* **23,** 3794–3795.

Xia SJ, Shammas MA and Shmookler Reis RJ. (1996). *Mutat. Res.,* **364,** 1–11.

Xia SJ, Shammas MA and Shmookler Reis RJ. (1997). *Mol. Cell. Biol.,* **17,** 7151–7158.

Zakian VA. (1997). *Cell,* **91,** 1–3.