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## RESEARCH COMMUNICATION

# Myc activates telomerase

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**Telomere maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase enzyme is itself a marker for tumor cells, but the genetic alterations that activate the enzyme during neoplastic transformation have remained a mystery. Here, we show that Myc induces telomerase in both normal human mammary epithelial cells (HMECs) and normal human diploid fibroblasts. Myc increases expression of hEST2 (hTRT/TP2), the limiting subunit of telomerase, and both Myc and hEST2 can extend the life span of HMECs. The ability of Myc to activate telomerase may contribute to its ability to promote tumor formation.**

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Telomerase activity is largely absent from somatic cells in vivo and from normal human cells in culture (Counter et al. 1992). As these cells proliferate, telomeric repeats are progressively lost as a result of incomplete replication of chromosome ends during each division cycle (Watson 1972; Olovnikov 1973; Harley et al. 1990; Hastie et al. 1990). Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life span. According to this model, erosion of chromosome ends triggers cellular senescence (Harley et al. 1990; for review, see Harley 1991). Bypass of senescence can be accomplished by negation of tumor suppressor pathways (e.g., p53 and Rb/p16). This allows continued proliferation (extended life span) that is accompanied by further telomere loss (Counter et al. 1992). Indefinite proliferation in the absence of a telomere maintenance strategy would eventually result in a complete loss of telomeres and in destabilization of chromosomes (Singer and Gottschling 1994). Because this situation is probably incompatible with survival, cells with an indeterminate life span must adopt strategies for telomere conservation. As predicted, cells that emerge from extended life span as immortal cell lines often activate the telomerase enzyme (Counter et al. 1992; Kim et al. 1994).

Cells that are programmed for continuous proliferation generally maintain telomere length. For example,

many stem cell populations possess telomerase activity (Counter et al. 1992; Kim et al. 1994). Telomerase is also induced in mitogen-stimulated lymphocytes and is detected in mitotically active regions of hair follicles and intestinal crypts (for review, see Greider 1998). The association of telomerase with cell proliferation has led to the hypothesis that telomere maintenance is simply a housekeeping function. However, proliferating normal cells in culture generally lack telomerase activity (Counter et al. 1992; Kim et al. 1994).

Stabilization of telomeric repeats may be a prerequisite for tumorigenesis (Counter et al. 1992). Consistent with this notion, telomerase is activated in a high percentage of late-stage human tumors and is present in most tumor-derived cell lines in culture (Counter et al. 1992, 1994; Kim et al. 1994; Shay and Wright 1996). To test the role of telomere maintenance in tumorigenesis, we surveyed known oncogenes for their ability to activate telomerase.

## Results and Discussion

### *Myc activates telomerase*

Normal human mammary epithelial cells (HMECs) lack telomerase, whereas immortal HMEC-derivatives and breast tumor cell lines are almost universally telomerase-positive (Shay et al. 1995; Bryan and Reddel 1997; Shay and Bacchetti 1997). Introduction of HPV-16 E6 protein into primary HMECs stimulates telomerase activity, suggesting that, in these cells, a single genetic event can potentiate the enzyme (Shay et al. 1993; Klingelutz et al. 1996; Fig. 1A,C and 2). Therefore, we asked whether increased expression of other cellular or viral oncogenes could induce telomerase in HMECs. Ectopic expression of *mdm-2* failed to induce telomerase, consistent with the observation that activation of telomerase by E6 is separable from the ability of E6 to promote the degradation of p53 (Klingelutz et al. 1996; Fig. 2). Several other cellular and viral oncoproteins, including E7, activated Ras (V12), cyclin D1, *cdc25C*, and *cdc25A*, also failed to induce telomerase (Fig 2). However, introduction of a c-Myc expression cassette stimulated telomerase activity in HMECs (Figs. 1A and 2). Enzyme activity was elevated within one passage after transduction of HMECs with a retrovirus that directs Myc expression (Fig. 1C). The Myc-expressing populations displayed levels of telomerase activity that approximated those seen in breast carcinoma cell lines (Fig. 1A; e.g., T47D).

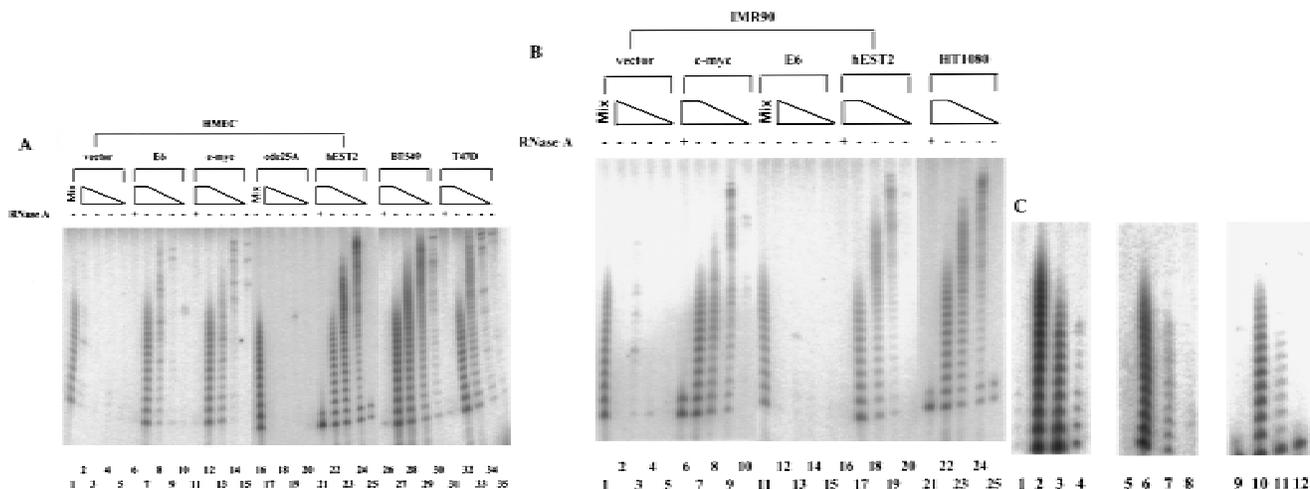
Introduction of E6 into normal human diploid fibroblasts failed to activate telomerase (Klingelutz et al. 1996; Shay et al. 1993; Figs. 1B,C and 2). Similar results were observed for E1A (Fig. 2), activated Ras (V12, not shown), or a dominant-negative p53 allele (Fig. 2). However, telomerase was induced by transduction of either IMR-90 (Figs. 1B,C and 2) or WI-38 cells (Fig. 1C) with a retrovirus that directs c-Myc expression. As with HMECs, activity was apparent immediately after drug

[Key Words: Myc; telomerase; hEST2; tumorigenesis]

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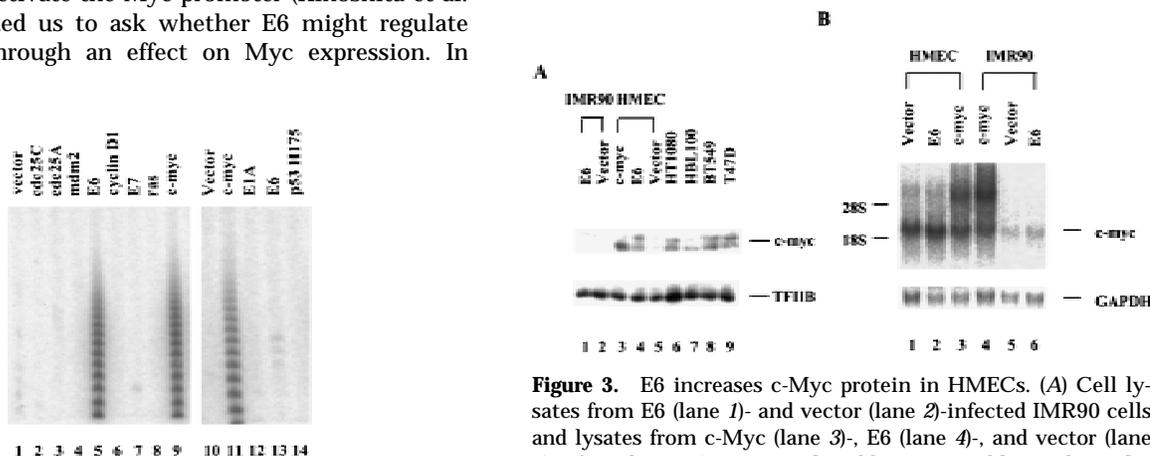


**Figure 1.** Myc activates telomerase. (A) Primary HMECs at passage 12 were infected with empty vector (lanes 1–5), E6 (lanes 6–10), c-Myc (lanes 11–15), cdc25A (lanes 16–20), or hEST2 (lanes 21–25) viruses. Breast cancer cell lines BT549 (lanes 26–30) and T47D (lanes 31–35) were included for comparison. TRAP assays contained lysates from 10,000 (lanes 2,6,7,11,12,17,21,22,26,27,31,32), 1000 (lanes 3,8,13,18,23,28,33), 100 (lanes 4,9,14,19,24,29,34), or 10 (lanes 5,10,15,20,25,30,35) cells. (– and +) Absence or presence of RNase A, respectively. (Mix; lanes 1,16) To exclude the presence of inhibitors in apparently negative lysates, lysate from 10,000 of the indicated cells was mixed with lysate from 10,000 c-Myc-expressing cells. (B) IMR90 cells at passage 14 were infected with empty vector (lanes 1–5), c-Myc (lanes 6–10), E6 (lanes 11–15), or hEST2 (lanes 16–20) viruses. HT1080 cells (lanes 21–25) were included for comparison. TRAP assays were performed with decreasing cell equivalents as in A. (C) HMEC (lanes 1–4), IMR90 (lanes 5–8), or WI38 (lanes 9–12) cells were infected with empty vector (lanes 1,5,9), hEST2 (lanes 2,6,10), c-Myc (lanes 3,7,11), or E6 viruses (lanes 4,8,12). Cells were selected for ~5 days with puromycin or hygromycin and then lysed for telomerase assay. Each lane corresponds to 10,000 cells.

selection (Fig. 1C). The Myc-expressing cells contained levels of telomerase activity comparable to those seen in a telomerase-positive fibrosarcoma cell line, HT1080 (Fig. 1B).

Although c-Myc expression elevates telomerase in both normal epithelial cells and in normal fibroblasts, the HPV-16 E6 protein has been shown to affect telomerase only in epithelial cells (Klingelutz et al. 1996). Therefore, we questioned the basis of cell-type specific telomerase activation by E6. A recent report suggesting that E6 can activate the Myc promoter (Kinoshita et al. 1997) prompted us to ask whether E6 might regulate telomerase through an effect on Myc expression. In

HMECs, expression of E6 induced Myc to levels approaching those achieved upon transduction of HMECs with a Myc retrovirus (Fig. 3A). Surprisingly, E6-induced alterations in Myc protein did not reflect changes in the abundance of *myc* mRNA (Fig. 3B). Therefore, Myc expression must be controlled post-transcriptionally by E6 in HMECs. In contrast, Myc levels remained unaltered following expression of E6 in IMR-90 cells wherein E6 is incapable of activating telomerase (Fig. 3A). Although E6



**Figure 2.** Oncogene activation of telomerase. HMECs (lanes 1–9) or IMR90 cells (lanes 10–14) were infected with viruses that direct the expression of the indicated oncogenes (lanes 2–9,11–14) or empty vector (lanes 1,10). Cell extracts were analyzed by TRAP assay.

**Figure 3.** E6 increases c-Myc protein in HMECs. (A) Cell lysates from E6 (lane 1)- and vector (lane 2)-infected IMR90 cells and lysates from c-Myc (lane 3)-, E6 (lane 4)-, and vector (lane 5)-infected HMECs were analyzed by Western blot with a polyclonal Myc antibody. Tumor cell lines, HT1080 (lane 6), HBL100 (lane 7), BT549 (lane 8), and T47D (lane 9), were included for comparison. The expression of TFIIIB was used to normalize loading. (B) Northern analysis of Myc RNA levels in total RNA. GAPDH was probed as a loading control.

may regulate telomerase by other mechanisms, this result is consistent with a model in which E6 regulates telomerase in HMECs by altering the abundance of Myc.

### Myc induces hEST2

The presence of the mRNA encoding hEST2, the catalytic subunit of telomerase, strictly correlates with telomerase activity. The hEST2 message is undetectable in normal tissue and in normal cell lines but is present in immortal and tumor-derived cell lines (Harrington et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). Moreover, hEST2 expression and telomerase are suppressed concomitantly when cells are induced to differentiate (Meyerson et al. 1997). These results suggest that

availability of hEST2 may limit telomerase activity, as was demonstrated recently in a number of normal cell lines (Weinrich et al. 1997; Bodnar et al. 1998). Expression of hEST2 could also induce telomerase in HMECs and WI38 and IMR-90 cells (Fig. 1A–C). Activity was apparent immediately following selection of hEST2-expressing cells (Fig. 1C), and the level of telomerase activity observed in hEST2-expressing populations consistently exceeded that observed in cell populations containing c-Myc (Fig. 1A–C).

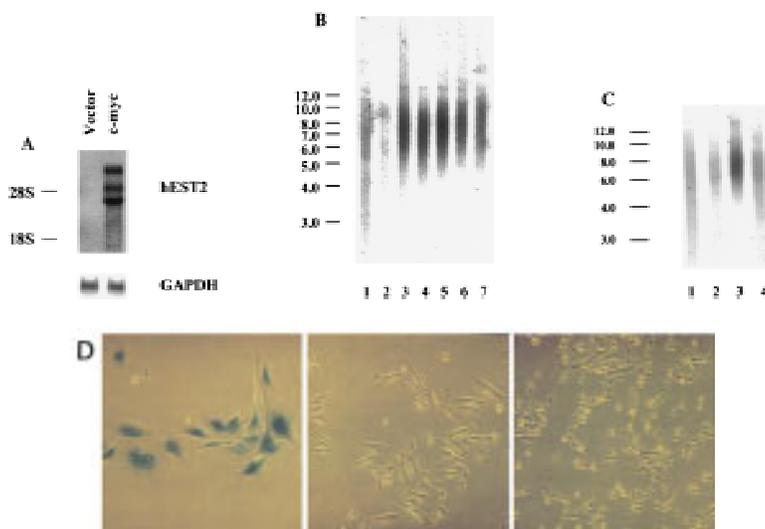
Because increased expression of hEST2 was sufficient to activate telomerase in both HMECs and in IMR-90 cells (Fig. 1), we asked whether Myc activates telomerase through an effect on hEST2. As expected, hEST2 mRNA was not detectable in normal HMECs, but was induced

at least 50-fold following transduction with a Myc retrovirus (Fig. 4A). Thus, Myc regulates telomerase by controlling the expression of a limiting telomerase subunit. Because Myc enhances the expression of responsive genes, its action on hEST2 could be either direct or indirect.

### hEST2 increases replicative life span in HMECs but not in IMR-90 cells

Preservation of telomeric repeats requires either the telomerase enzyme or the activation of an alternative pathway for telomere maintenance (Kim et al. 1994; Broccoli et al. 1995; Strahl and Blackburn 1996; Wright et al. 1996; Bryan and Reddel 1997). In addition, telomere length can be controlled by telomere-binding proteins (van Steensel and de Lange 1997). To determine whether activation of telomerase in HMECs is sufficient to stabilize telomere length, we followed telomeric restriction fragment (TRF) size as HMECs proliferated either in the presence or absence of telomerase activity. In normal HMECs, telomere length and the abundance of telomeric sequences diminished slightly as cells underwent multiple rounds of division (Fig. 4B). Activation of telomerase by expression of hEST2 not only prevented telomere shrinkage but also increased both the overall abundance of telomeric sequences and the average length of telomeres (Fig. 4B). In Myc-expressing cells, however, the abundance of telomeric sequences was intermediate between that observed in cells expressing hEST2 and that observed in control cells (Fig. 4C). Telomere lengths followed a similar pattern (Fig. 4C).

Generally, in tumors and in immortal cell lines, telomeres are short but stable (Hastie et al. 1990). Comparison of TRF levels in Myc-expressing HMECs to those in early-passage HMECs suggested that Myc probably stabilized telomeres rather than promoted an increase in TRFs as occurs in hEST2-expressing



**Figure 4.** Myc regulates hEST2 and extends cellular life span in HMECs. (A) hEST2 Northern analysis of poly(A)<sup>+</sup> RNA from normal HMECs and from HMECs that had been infected with a Myc retrovirus. A Northern blot with GAPDH was performed as a loading control. (B) Genomic DNA (3  $\mu$ g) from early-passage HMECs (passage 12, lane 1), late-passage HMECs (passage 26, lane 2), and hEST2-expressing HMECs [infected at passage 12 and cultured for 3 (lane 3), 6 (lane 4), 8 (lane 5), 10 (lane 6), or 14 (lane 7) additional passages] was digested with *Rsa*I and *Hinf*I. Fragments were separated on a 0.8% agarose gel, and telomeric restriction fragments were visualized with a <sup>32</sup>P-labeled human telomeric sequence (TTAGGG)<sub>3</sub> probe. (C) Genomic DNA (3  $\mu$ g) from early-passage HMECs (passage 12, lane 1), vector-infected HMEC (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane 2), hEST2-expressing HMECs (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane 3), and Myc-expressing cells (infected at passage 12 and cultured for six additional passages or ~18 PD, lane 4) were digested with *Rsa*I and *Hinf*I. Fragments were probed with a telomeric probe as described in B. TRF intensity was quantitated on a Fuji BAS2000 PhosphorImager. Normalizing vector-containing HMECs (lane 2) to 100 units of intensity, both early passage HMECs (lane 1) and Myc-expressing HMECs (lane 4) gave ~150 units of intensity and hEST2-expressing HMECs (lane 3) gave ~200 units of intensity. (D) HMECs transduced with empty vector (left), hEST2 (middle), or c-Myc viruses (right) were grown to a PDL of ~56–60. At this PDL, vector cells adopted a senescent morphology and ceased growth. Cells expressing c-Myc and hEST2 continued to proliferate. To assess the percentage of senescent cells in the population, each culture was stained for senescence-associated  $\beta$ -galactosidase. Greater than 95% of the vector-containing cells were  $\beta$ -galactosidase positive whereas <10% of cells expressing hEST2 or Myc were stained.

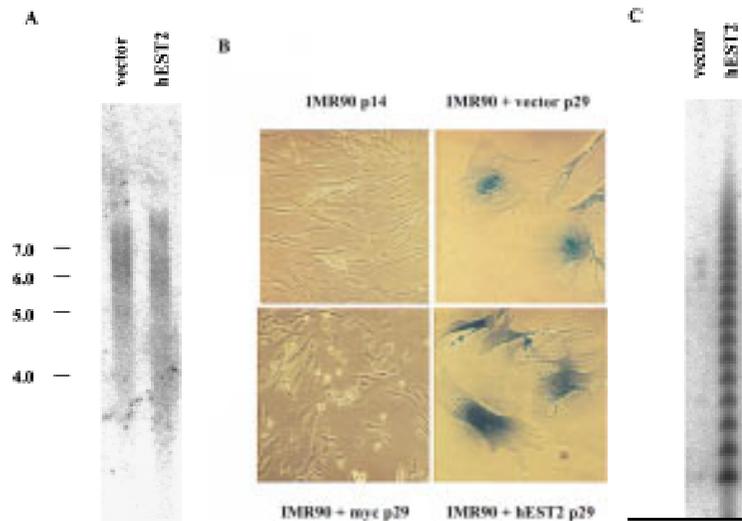
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HMECs (Fig. 4C). Thus, alterations in telomere dynamics after Myc transduction mimic the situation in tumors. Telomerase was ~10-fold more active in hEST2-expressing cells than in Myc-expressing cells. Thus, differences in telomerase activity likely reflect differences in hEST2 expression as the abundance of viral hEST2 mRNA greatly exceeded native hEST2 mRNA levels present in either Myc-expressing HMECs or in any of the tumor cell lines tested to date.

Telomere length has been proposed as the counting mechanism that determines the replicative life span of a cell (Harley 1990; Harley et al. 1991). At a population doubling level (PDL) of ~55–60, vector-containing HMECs ceased proliferation, adopted a senescent morphology (for review, see Stein and Dulic 1995), and stained positive for senescence-associated  $\beta$ -galactosidase (Dimri et al. 1995; Fig. 4D). These cells also showed increased expression of PAI, another senescence marker (Goldstein et al. 1994; data not shown). In contrast, normal HMECs that had received either hEST2 or c-Myc at early passage displayed an extended life span. Cells expressing either c-Myc or hEST2 continued to proliferate beyond the normal senescence point and did not show evident  $\beta$ -galactosidase staining or increased PAI expression (Fig. 4D; data not shown). In both c-Myc and hEST2-expressing cell populations, <10% of cells showed any senescence-associated phenotype at the point at which vector-infected cells senesced. Furthermore, neither population has shown any accumulation of senescent cells during subsequent growth. At present, hEST2- and Myc-expressing populations are a minimum of 40 population doublings (PD) beyond the normal senescence point.

In IMR-90 cells, the consequences of telomerase activation differed from those observed in HMECs. Although hEST2 induced telomerase activity to high levels in early-passage (p14) IMR-90 cells (Fig. 1B,C), this activity was not accompanied by an increase in either the abundance or the length of telomeric sequences (Fig. 5A). To examine the consequences of telomerase activation in normal fibroblasts, vector-containing IMR-90 cells and telomerase-positive hEST2-expressing IMR-90 cells were cultured continuously until the replicative life span of the normal IMR-90 cells was exhausted. Consistent with the idea that telomeres but not telomerase activity per se regulate replicative senescence, hEST2 expression failed to alter the life span of IMR-90 cells. These cells entered replicative senescence within two passages of the point at which normal IMR-90 cells senesced (Fig. 5B). Even after >1 month of maintenance, not a single cell from a population of  $>10^6$  cells escaped the senescence block. Senescence was not attributable to a loss of telomerase as the arrested hEST2-expressing IMR-90 population maintained activity (Fig. 5C).

In contrast, IMR-90 cells engineered to express c-Myc display an extended life span (Fig. 5B), even though these



**Figure 5.** Telomerase activation does not affect life span in IMR-90 cells. (A) TRF length of senescent vector-containing IMR-90 and hEST2-expressing IMR-90 cells was analyzed as in Fig. 4. (B) Early-passage IMR-90 cells (passage 14) were infected with empty vector, a hEST2 retrovirus, or a Myc retrovirus as indicated. Cells were passaged until the vector-infected cells reached senescence (~15 additional passages). At this time, hEST2 cells also senesced, but Myc-expressing IMR-90 cells continued to proliferate. Shown are senescence-associated  $\beta$ -galactosidase stains of early-passage IMR-90 cells, senescent vector-containing IMR-90 cells, senescent hEST2-expressing IMR-90 cells and Myc-expressing IMR-90 cells that have bypassed the senescence point and entered extended life span. (C) Telomerase assays of lysates derived from senescent vector-containing IMR-90 and hEST2-expressing IMR-90 populations. Each lane corresponds to 10,000 cells.

cells do not show an obvious stabilization of telomeres. At present Myc-expressing IMR-90 populations have grown for >17 passages (~68 PD) beyond the normal senescence point. Therefore, Myc can extend the life span of a cell even when telomerase activation fails to do so.

These results indicate that the ability of telomerase to extend life span is not universal. Telomerase-positive cells may senesce and still maintain telomerase activity. The mechanisms that regulate the ability of telomerase to extend telomeres and life span may provide an additional level of control over indefinite proliferation and thus tumorigenesis. Furthermore, telomerase-negative cells may adopt alternative strategies for telomere maintenance [alternative lengthening of telomeres (ALT); Bryan and Reddel 1997] and, therefore, achieve immortality without activation of telomerase. The long-term strategy for telomere maintenance adopted by an individual cell will likely depend on the constellation of genetic alterations that such a cell acquires along the pathway to immortality and possibly neoplastic transformation.

The *myc* oncogene is activated by overexpression, gene amplification, translocation, and possibly mutation in a wide variety of different tumor types (Alitalo et al. 1987). Because Myc can elevate telomerase in normal epithelial and fibroblast cells to a level approximating that observed in tumor cell lines, increased Myc activity could account for the presence of telomerase in many



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