

## Immortalized mouse mammary cells *in vivo* do not exhibit increased telomerase activity

Cheng Jiang<sup>1</sup>, Lily Juo<sup>2</sup>, Thenaa K.Said<sup>2</sup>, Henry Thompson<sup>1</sup> and Daniel Medina<sup>2,3</sup>

<sup>1</sup>AMC Cancer Research Center, Denver, CO 80214, USA and <sup>2</sup>Baylor College of Medicine, Department of Cell Biology, One Baylor Plaza, Houston, TX 77030, USA

<sup>3</sup>To whom correspondence should be addressed

**The acquisition of immortalization is an early and carefully documented event in mouse mammary tumorigenesis. Activation of telomerase activity is one hypothesis to explain the acquisition of immortalization. We examined the activity of telomerase in well-defined immortalized, non-tumor cell populations of mouse mammary tissue *in vivo*. The results indicated that normal virgin and mid-pregnant mammary gland had low to moderate levels of telomerase activity, respectively. In comparison with the levels detected in pregnant mammary gland, telomerase activity was elevated in mammary tumors *in situ* and in established mammary cell lines *in vitro*, both tumorigenic and non-tumorigenic; however, hyperplastic alveolar preneoplastic mammary outgrowths and non-tumorigenic ductal outgrowths, both *in vivo* immortalized populations, had telomerase activity <12% of the levels detected in normal pregnant mammary gland. These results suggest that elevated telomerase activity is not necessary for the immortalization phenotype in *in vivo* mouse mammary cell populations and that elevated telomerase activity occurs as a late event in mammary tumorigenesis. Furthermore, the data suggest that low levels of telomerase activity are characteristic for mouse mammary epithelial cells and not sufficient for immortalization. These data suggest that other factors play more important roles in the induction and/or maintenance of the immortalization state in mammary cell populations.**

### Introduction

Most cancers are considered to be immortal cell populations. The telomere hypothesis of cellular immortalization is the favorite hypothesis to explain the acquisition of immortalization (1–4). The hypothesis states that telomere size shortens progressively as a consequence of chromosome replication. The end result of telomere shortening is senescence. In order for cancer cells to escape senescence and evolve, the enzyme telomerase is activated and/or increased to stabilize and increase telomere length (2). This hypothesis is supported by the observation that telomerase is activated in tumor virus induced-immortalization of eukaryotic cells (1,4) and that numerous established cell lines and tumors of human origin

\***Abbreviations:** EL, extended lifespan; TM, transformed mammary; PBS, phosphate buffered saline; PCNA, polymorphic cell nuclear antigen; CHAPS, 3-[(cholamidopropyl)-dimethylammonium]-1-propane sulfonate; TRAP, telomeric repeat amplification protocol; PCR, polymerase chain reaction; ITAS, internal telomerase assay standard; TBE, Tris-buffer-EDTA; BrdU, bromodeoxyuridine.

exhibit increased telomerase activity compared with their normal cells (3). As normal human somatic cells (with some exceptions) exhibit no or extremely low levels of telomerase activity, the results observed in human cell lines and cancer are striking and provide strong support for these hypotheses (3,5,6,7). In the data by Kim *et al.* (3), 98 of 100 immortal human cell populations exhibited positive telomerase activity. This result has been supported and extended by numerous investigators (8–14). In contrast, there are a few reports in the literature where specific neoplasias (15–17), and immortalized cell lines (18) are not associated with increased telomerase activity. Additionally, telomerase activity has been detected in sun-damaged human skin, skin psoriasis and dermatitis, all non-neoplastic conditions (14) and at low levels in hepatitis (12).

The status of elevated telomerase activity of human cancers *in vivo* is consistent among different studies. The status of telomerase activity in pre-malignant human tissues *in vivo* has not been as thoroughly examined. Data in the literature suggest that pre-malignant lesions of pancreas, prostate, colon (adenomatous polyps), stomach, head and neck (erythroplakia) and benign prostatic hyperplasia do not exhibit elevated telomerase activity (8,11,13,19–21). In other experiments pre-malignant lesions of head and neck, designated as either dysplasia or oral leukoplakia, did exhibit elevated telomerase activity in 100% (7/7) and 38% (10/26) of the lesions, respectively (22,23). The mortal or immortalized status of these lesions is unknown. If telomerase function does correlate with immortalization phenotype in human tissues, then one has to conclude that immortalization is a late event in the development of human cancer (2). However, as stated clearly by others (5,24), the data on telomerase activity and immortalization in human tissue is primarily correlative, albeit an extremely strong correlation.

The status of telomerase activity in mouse tumor systems is complicated by the demonstrable levels of telomerase activity in many normal mouse organs (25–27). The considerable background levels of telomerase activity in normal mouse organs requires careful quantification of telomerase in tumor cells. Several reports have demonstrated elevated telomerase activity in viral oncogene and carcinogen-induced mouse skin carcinomas (26–28), carcinogen-induced rat colon tumors (29), viral oncogene-induced pancreatic tumors (28), and mammary tumors from *neu* transgenic mice (26). As in human pre-malignant lesions, elevated telomerase activity was not observed in either pancreatic islet hyperplasia, pre-malignant early stage skin hyperplasia or early stage papillomas, although late stage papillomas did exhibit elevated telomerase activity (26–28). It is worth noting that the immortalized status of mouse skin papillomas is not well studied.

In order to critically evaluate the causal relationship between telomerase and immortalization, well-defined immortalized, non-tumorigenic cell populations *in vivo* are useful. Two recent reports concluded that immortalization of human fibroblasts *in vitro* could occur in the absence of telomerase activation

but with telomere stabilization (30,31). In these studies, the criteria of immortalization was an *in vitro* criteria and not verified by *in vivo* growth. *In vitro* cell lines are not optimal models because the lack of *in vivo* tumorigenicity of these cell lines is not frequently ascertained and also can be due to culture-induced changes independent of intrinsic tumorigenicity. The mouse mammary system is one of the few models where the acquisition and criteria of immortality in *in vivo* tissues has been studied and carefully documented (32,33). Immortalization has been defined as infinite division potential and is based on orthotopic transplantation of normal or transformed mammary cells into syngeneic mice. The results of such experiments have demonstrated that normal mammary epithelial cells have a finite life span of five to six transplant generations whereas preneoplastic and neoplastic cell populations can be serially transplanted indefinitely (33,34). Recent experiments have demonstrated that the immortalized phenotype can occur independently of hyperplastic or tumorigenic capability (35). Such *in vivo* cell populations, termed extended lifespan (EL<sup>\*</sup>), resemble normal mammary ducts morphologically and functionally and are absolutely ovarian hormone dependent for growth and morphogenesis. The p53 status of the EL lines is wild-type (Medina and Butel, unpublished observations).

We have examined the EL ductal outgrowth lines along with established mammary epithelial cell lines *in vitro* and normal, hyperplastic and neoplastic mammary tissue *in vivo* for telomerase activity to test the hypothesis that elevated telomerase activity is necessary and sufficient for immortalization.

## Materials and methods

### Cells and tissues

All cell lines and tissues were of mammary epithelial origin in Balb/c mice. The biological and tumorigenic properties of the cell lines and tissues have been described previously (35–39). Briefly, the model system examined herein involves mammary tissues that represent different stages in the development of mammary neoplasia. In this model, normal cells give rise to immortalized ductal outgrowths (termed EL), which are ovarian hormone-dependent and weakly tumorigenic and also to immortalized alveolar outgrowths (termed TM for transformed mammary) which are primarily ovarian hormone-independent and tumorigenic. The TM outgrowths represent a heterogeneous group of lesions that have been classified into three stages based on their respective tumorigenic potentials. Stage I lesions are non or weakly tumorigenic, stage II lesions have a moderate (20–70%) tumorigenic potential with a long mean latent period for tumor formation (10–12 months) and stage III lesions have a high (>90%) tumorigenic potential with a short mean tumor latent period (≤5 months). The EL and TM cell populations were derived from normal mature virgin mammary gland and arose ‘spontaneously’ as a consequence of growth in cell culture. Normal mammary tissues were mature virgin gland (12–16, and 56 weeks of age) and mid-pregnant mammary gland (11–14 days). The immortalized ductal lines EL11 and EL12 were from transplant generations 28–37, preneoplastic hyperplastic outgrowth lines TM2L, TM2H, TM3 and TM40 represented hyperplasias from different stages and were from transplant generations 28–33, 37, 22–27 and 9–15 respectively. Tumors were primary tumors arising from the different hyperplasias.

### Cell culture

Virgin mammary gland, EL11 and EL12 ductal tissues were also examined before and after primary culture in three-dimensional collagen gels. The epithelial cells were cultured as described previously (36) and examined at day 0 and day 7 of culture in order to examine the telomerase activity in enriched epithelial cells of normal and immortalized mammary ductal cells.

### Proliferation assays

BrdU was purchased from Sigma Chemical Co. (St Louis, MO), and was dissolved in sterile phosphate buffered saline (PBS) at 20 mg/ml. Two-hour pulse-labeling experiments were carried out using 4–6 animals per group. Each animal received 70 µg/g body weight bromodeoxyuridine (BrdU) i.p. The animals were killed, mammary glands were harvested, flattened and fixed in methacarn (methanol:chloroform:acetic acid; 60:30:10) overnight and post-

fixed in acetone. Paraffin-embedded tissue samples were cut in 4 µm thick sections, stained with hematoxylin and eosin for general histological study or used for immunohistochemical staining.

Tissue sections were deparaffinized and blocked as described in (40). BrdU immunohistochemistry was performed using the cell proliferation kit from Amersham following the manufacturer’s protocol. The total number of cells counted was 1200 cells per section. For PCNA staining, the procedure was followed as described in (40).

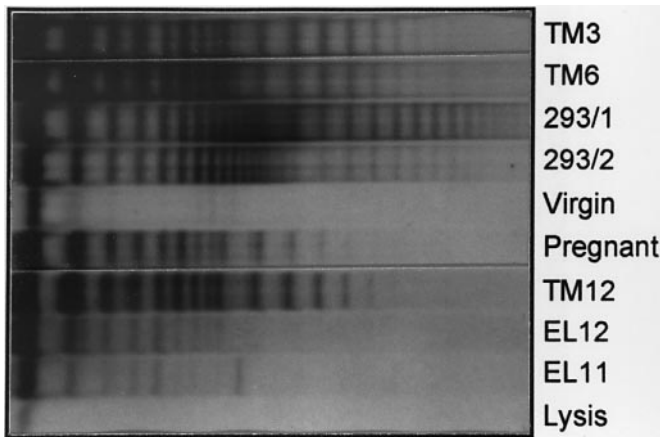
### Telomerase assay

Telomerase was assayed by two versions of the telomeric repeat amplification protocol (TRAP) procedure described initially by Kim *et al.* (3) with modification of Wright *et al.* (41). In our initial studies, each sample was washed with 500 µl of ice-cold wash buffer (10 mM HEPES–KOH (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM dithiothreitol) and pelleted at 10 000 r.p.m. for 5 min at 4°C. The washing buffer was removed and 300 µl of ice-cold lysis buffer was added (10 mM Tris–HCl (pH 7.5), 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM phenyl-methylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The samples were homogenized by hand with a glass grinder and placed on ice for 30 min, and then centrifuged at 16 000 g for 20 min at 4°C. The supernatants were collected into 1.5 ml tubes and flash-frozen in a dry-ice–ethanol bath. Samples were stored at –80°C. The protein concentration of the cell and tissue extracts were determined with the BCA Protein Assay kit (Pierce) and samples were diluted to a concentration of 1 mg/ml protein. The telomerase assay was performed in two steps; first, a telomerase extension of an oligonucleotide primer (TS) which served as the substrate for telomerase and second, polymerase chain reaction (PCR) amplification with the oligonucleotide primer pair TS 5′-AATCCGTCGAGCAGAGTT-3′ (forward), and CX5′-(CCCTTA)3CCCTAA-3′ (reverse). The PCR buffer contained 20 mM Tris–HCl (pH 8.3); 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.005% Tween-20 and 1 mM EGTA. Aliquots of 10 µl of 5XPCR buffer (TRAP buffer) were mixed with 0.1 µg (1 µl) of TS primer, 50 µM each dNTPS (1 µl), bovine serum albumin (0.1 mg/ml), 1 µg of T4g protein (1 µl), 0.4 µl of (α-P32) dCTP (3000 Ci/mmol), 1 µl of a cell extract and 0.5 µl of ITAS DNA (1×10<sup>-18</sup> g/µl) (added as an internal control). Diethyl pyrocarbonate-treated water was added to bring a total volume of 48.5 µl per assay. The reaction mixture was incubated for 30 min at 22°C for telomerase-mediated extension of the TS primer. Following incubation, 0.1 µg of the CX primer (1 µl) and 2 µl of Taq DNA polymerase (Perkin–Elmer) were added to each tube. The samples were denatured (94°C, 3 min) and subjected to PCR amplification in a thermal cycler for 31 cycles (94°C for 30 s, 50°C for 30 s and 72°C for 45 s). As a positive control, 1 µl of a cell extract from a sample with known telomerase activity (TM-6 mammary tumor cell line) and as a negative control, 1 µl of lysis buffer without cell extract, were used. In addition to the positive and negative samples run in each experiment, experiments assessed the importance of sample size, heat and RNase treatment. Heat and RNase treatment were run on initial experiments using pregnant, virgin and tumor tissue, and, in each case, eliminated telomerase activity. To determine the optimal protein load per sample, for the initial set of assays, pregnant, virgin, EL11 tissue, EL11 and EL12 cultured cells (day 0 and day 7) and TM6 tumor cell line were examined over a range of 0.03 µg to 3 µg. From these experiments, we concluded that 1 µg protein was a reproducible and satisfactory sample size.

In the second set of assays, the same and additional tissue lysates were examined utilizing the TRAPeze™ Telomerase Detection kit (Oncor, Gaithersburg, MD). This kit eliminates the need for a wax barrier hot start and incorporates primers for amplification of a 36-p internal positive standard that provides a positive control for accurate quantitation of telomerase activity with a linear range. Using this kit, we examined a subset of samples over a range of 0.1 µg to 5 µg and picked 2 µg to examine all the samples in two gels run at the same time.

### Electrophoresis and quantification

In the initial experiments, the PCR products were analyzed by electrophoresis of 25 µl aliquots of reaction mixtures on 10% non-denaturing, 0.4 mm thick acrylamide gels, run in 0.5×TBE buffer until the xylene-cyanol had migrated 15 cm from the bottom of the gel. In the second set of experiments utilizing the TRAPeze™ kit, the samples were electrophoresed on 12% polyacrylamide gels, run in 1×TBE buffer until the bromophenol blue marker had migrated 9 cm from the loading wells. The gels were then dried, exposed for 16–20 h to Kodak Biomax film (Eastman Kodak Company). The signal intensity was measured by densitometry. Telomerase activity was determined by adding the intensity of all the bands minus the background observed on the negative control lanes. The signal intensities of the bands from the experimental samples were compared relative to the signal intensity of the bands from the positive controls that were run with each experiment.

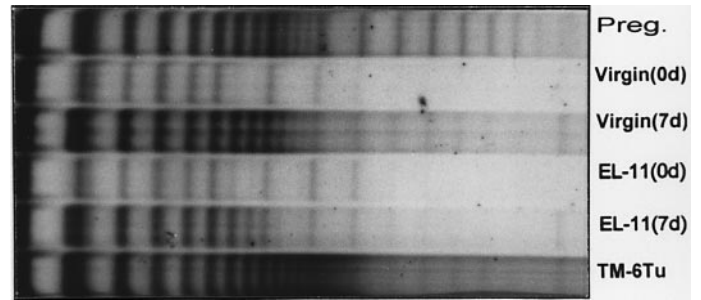


**Fig. 1.** Telomerase activity in normal (virgin, pregnant), immortal, non-tumorigenic (EL11, EL12) and neoplastic mammary tissues (TM12) and in neoplastic cell lines (TM3, TM6, 293).

## Results

Telomerase activity in normal and neoplastic mammary tissues and in two cell lines *in vitro* derived from mammary preneoplasias is illustrated in Figure 1. RNase treatment abolished the activity by 99%. Telomerase activity was present in normal pregnant mammary glands, present weakly in virgin gland (18% of pregnant gland), and at high levels in a TM12 primary mammary tumor (1.7 times greater than pregnant) and in the two preneoplastic cell lines (TM3, TM6; 2.1 times greater than pregnant gland). The 293 human kidney carcinoma cell line was an additional positive control. Levels of telomerase activity in mammary fat pads containing EL11 and EL12 ductal outgrowths gave telomerase activities less than pregnant gland (43% less) and slightly higher than virgin mammary gland.

The epithelial cell compartment of the normal mammary ducts as well as the EL11 and EL12 ductal outgrowths in the mammary fat pad contribute in the order of 10–15% of the total protein mass due to the large contribution of the adipose stroma. In order to rule out the possibility that the low level of telomerase activity was an artifact that reflected the low percentage of ductal epithelial cells, the epithelial component of the mammary gland that contained normal ducts, EL11 and EL12 ductal outgrowths were enriched by enzymatic digestion of the gland and only the epithelial cell pellet was collected. The telomerase activity of the pellets was examined at day of collection (day 0) in three experiments and after 7 days of culture (day 7) in a collagen gel in two of the three experiments. The samples at day 7 of culture represented an increase of epithelial cells caused by active proliferation in the collagen gel with growth factors. Figure 2 illustrates the results of one of these experiments. Telomerase activity was evident in the pregnant mammary gland, it was lower in day-0 virgin epithelial cells (26% of pregnant gland) and day-0 EL11 epithelial cells (18% of pregnant gland), and highest in the TM6 tumor cell line (1.25 times greater than pregnant gland). Culturing the virgin and EL11 ductal outgrowths for 7 days increased the telomerase activity 3.2 times in each case. A repeat of the 7-day culture protocol reproduced the same increase in telomerase activity in both cell populations. The mean telomerase activity of the three zero day experiments for both virgin and EL11 cells was 48% of the pregnant gland. The results demonstrated



**Fig. 2.** Telomerase activity in pregnant mammary tissue and in mammary epithelial cells of virgin and EL11 tissues. Day 0 represents the epithelial cell pellet prior to cell culture and day 7 represents after 7 days of culture in a collagen gel.

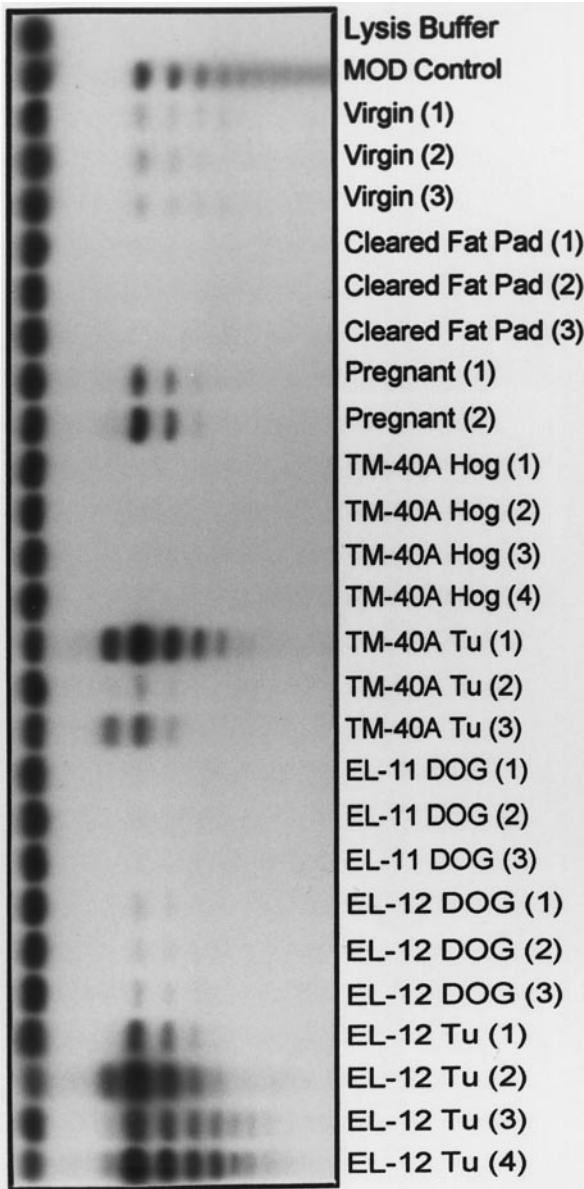
that telomerase activity was not increased in immortalized, non-tumorigenic mammary epithelial cells.

In order to more carefully quantitate the extent of telomerase activity, we utilized the TRAPEze™ Telomerase Detection kit, which contains an internal standard at 36 bp. Figures 3 and 4 illustrate telomerase activity in normal mammary gland, ductal and alveolar hyperplasias and their derivative tumors. The results show that the cleared mammary fat pad, devoid of epithelia parenchyma, has no detectable activity compared with the low activity in virgin and moderate activity in pregnant gland. The activity in virgin gland was 27% of that in the pregnant gland. Of the six separate ductal and 15 separate hyperplastic outgrowth samples, the mean telomerase activity was 12% of the virgin duct and 12% of the pregnant gland, respectively. The two samples of hyperplasias with modest telomerase activities, were detected in two transplant generations of TM2H and TM3H outgrowths that produced palpable tumors within 4 months after transplantation. In contrast, 15 of 16 tumors exhibited elevated telomerase activity with a mean activity 3.7 times greater than the pregnant gland. The specificity of the assay was confirmed by heat inactivation of telomerase enzyme. Figure 5 shows the titration of the telomerase activity over 0.1 to 5 µg of protein and demonstrates that telomerase activity was not artificially missed or exaggerated in any of the samples. A summary of the relative levels of telomerase activity in the mouse mammary tissues is illustrated in Figure 6. The results indicate telomerase activity in the immortalized non-tumor populations is ≤12% of that detected in normal, mortal pregnant mammary gland.

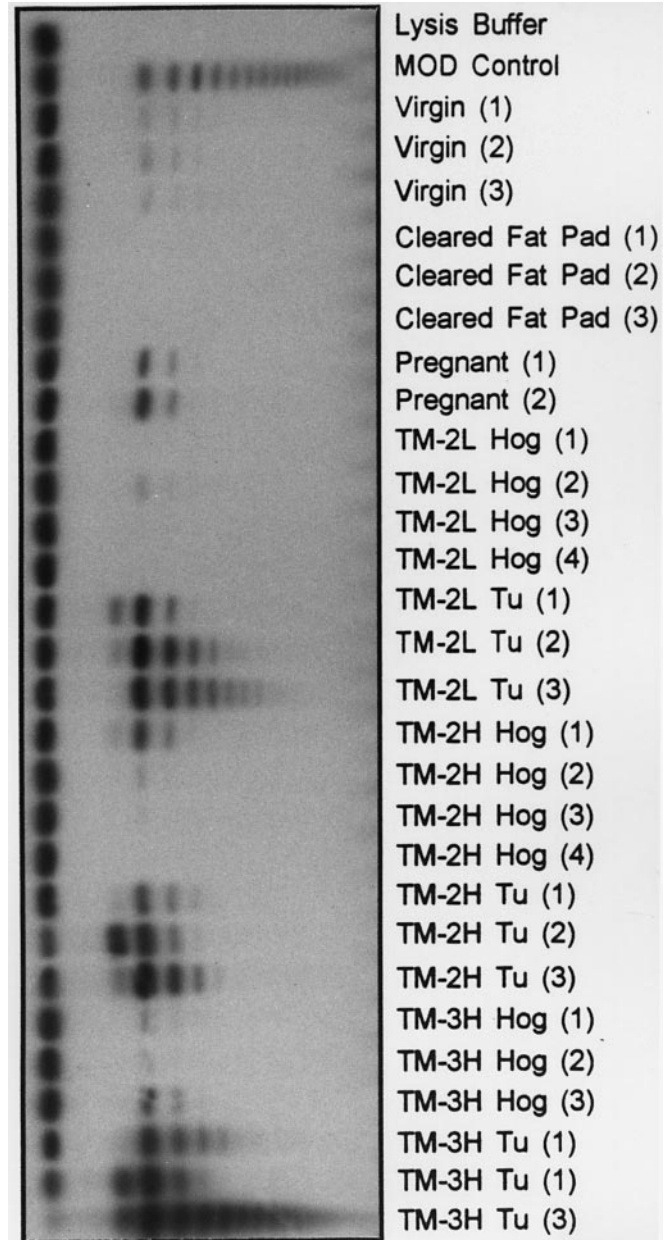
The relationship between proliferation status and telomerase activity is illustrated in Table I. The pregnant mammary gland has significant proliferative activity as measured by BrdU labeling and PCNA-labeling indices. It is of note that the percentage of normal cells (pregnant) that are at some stage in the cell cycle, as reflected by PCNA-labeling, is equal to or greater than the hyperplasias but less than primary tumors. The normal virgin duct and immortalized duct show PCNA-labeling indices 6.2 and 24% respectively (42). However, the high BrdU- and PCNA-labeling indices and the low telomerase activities of stage II and III hyperplasias suggest there is little direct correlation between the two cellular processes (Table I).

## Discussion

The experiments reported herein examined telomerase activity in a well-characterized model of multistage murine mammary tumorigenesis *in vivo*. This model is characterized by the presence of discrete and well-defined *in vivo* cell populations



**Fig. 3.** Telomerase activity in multiple samples of mouse mammary tissues *in situ*. Each lane contains a separate tissue sample. The bottom band in each lane represents the 36 bp internal positive control. HOG refers to alveolar hyperplastic outgrowths, DOG refers to ductal outgrowths and Tu refers to tumor. TM40A is a stage II hyperplasia and EL11 and EL12 are separate immortalized ductal outgrowths. The MOD is a mouse mammary cell line used as a known telomerase positive control.

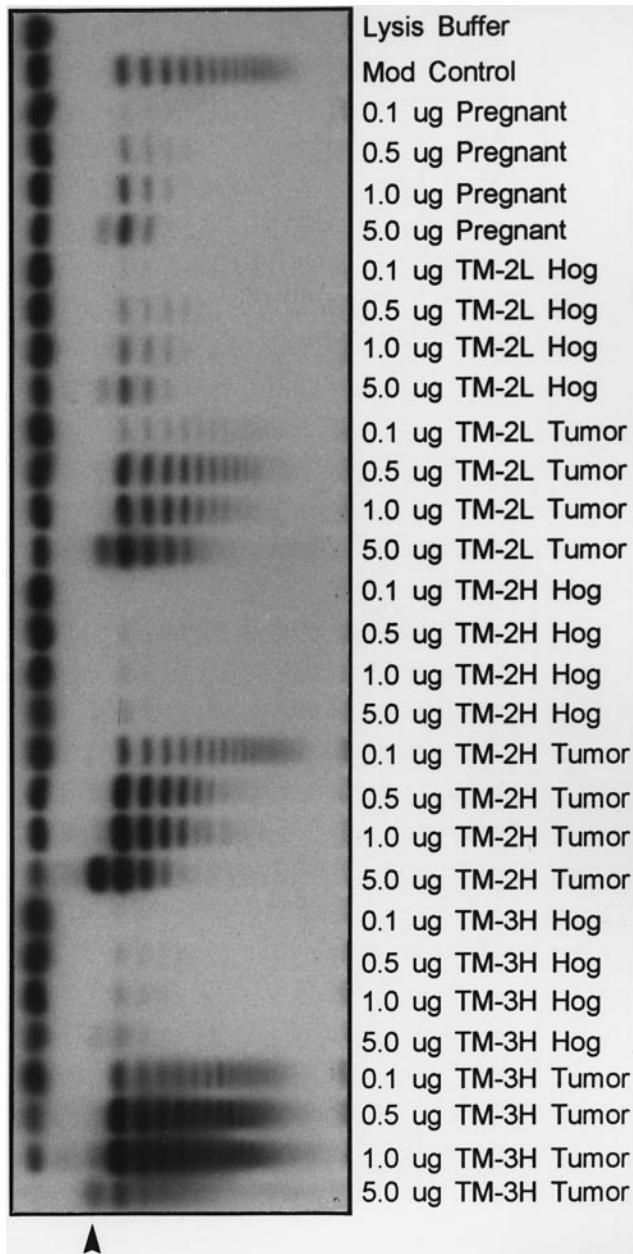


**Fig. 4.** Telomerase activities in multiple samples of mouse mammary hyperplasias and tumors *in vivo*. The TM2L and TM3H hyperplastic outgrowth lines are stage I and stage II lesions respectively, and the TM2H line is a stage III lesion.

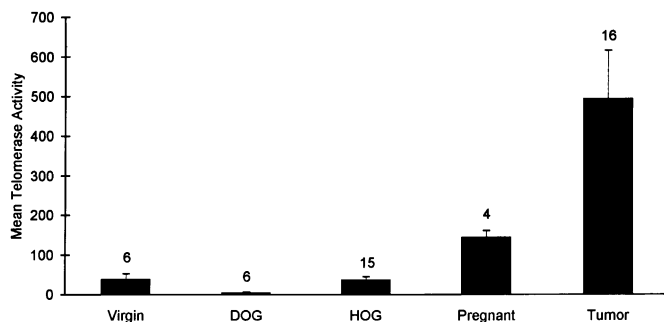
representing four stages in the evolution of normal to neoplastic mammary cells. The experiments specifically addressed the question of whether increased telomerase activity was detectable in immortalized non-tumorigenic mammary ductal cells. The results show that enhanced telomerase activity does not occur in these immortalized cell populations *in vivo*. To our knowledge, this is the first examination of telomerase activity in a known cell population of immortalized, non-tumorigenic cells *in vivo*.

The results raise the question of whether this system is the exception to the rule that telomerase activity is necessary and sufficient for cell immortalization. There is extensive data that immortal tumorigenic cell lines *in vitro* exhibit enhanced telomerase activity (3,5,6). There is also substantial data that

immortal, non-tumorigenic cell lines *in vitro* exhibit telomerase activity. However, the relevance of *in vitro* cell lines to growth, immortalization and tumorigenicity can be complicated by selection artifacts found in cultured cells. It is not known whether immortalization *in vitro* measures the same biological phenotype as immortalization *in vivo*. Establishment of a cell line *in vitro* essentially selects for a stem cell population grown under the most elemental conditions; i.e. cells in isolation grown in two dimensions under a minimal set of growth factors. However, immortalization of epithelial cells *in vivo* requires cells to grow in a normal micro-environment in a three-dimensional organization and subject to the influences of neighboring cells and complex hormonal and growth factors. Growth *in vivo* is thus a more stringent test for immortalization and mimics the event that occurs in the evolution of neoplasia.



**Fig. 5.** Representative samples of each mammary stage were titrated for telomerase activity over a range of protein input from 0.1 µg to 5 µg. In some cases, a heavy protein load led to the appearance of a non-specific band (arrow).



**Fig. 6.** Summary of telomerase activity in mouse mammary tissues and cell lines. The data are expressed as the mean ± SEM. The numbers above each bar represent the number of individual cell populations sampled for this analysis.

**Table I.** Proliferation measurements of mammary tissues

Tissue	Proliferation index (%)	
	BrdU	PCNA
Pregnant	9.4 ± 1.4 (3)	46.5 ± 2.6 (4)
TM hyperplasia I, II	7.4 ± 2.4 (14)	24.3 ± 5.6 (13)
TM hyperplasia III	11.4 ± 2.3 (13)	50.3 ± 2.7 (2)
TM tumors	11.4 ± 3.4 (7)	81.3 ± 11.7 (7)

So far, the only system that *in vivo* immortalization has been carefully examined in is the mouse mammary system (32–34,39). The mammary system fulfils the requirements to test vigorously the role of telomerase activation in immortalization and tumorigenesis. The current data suggest that increased telomerase activity is not necessary for the immortalization phenotype in mouse mammary cells as demonstrated by the normal levels of telomerase activity in both immortalized, non-tumorigenic mammary ductal outgrowths and in immortalized, preneoplastic mammary outgrowths.

A second question is whether the low level of telomerase activity present in normal mammary cells is necessary for immortalization. This is a more difficult question to answer by just measuring telomerase activity. Two facts are worth noting. First, the low level of nascent telomerase activity is clearly not sufficient for immortalization as it is well established that virgin and pregnant mammary epithelial cells are mortal cell populations with a life span measured as six transplant generations (32,33). The absence of telomerase activity in the cleared fat pads argues that the telomerase activity measured in the gland is of epithelial, not stromal origin. Secondly, it is curious that the level of telomerase activity in the ductal and alveolar outgrowths was actually less than in their respective normal cell controls. This result argues that even the low level of telomerase activity is not maintained or necessary for an immortalized phenotype. The low levels of telomerase activity may reflect other physiological influences on normal cell function such as hormonal regulation of telomerase activity. Recent results have suggested that varying levels of telomerase activity in normal human endometrium and rat prostate reflect hormonal status of the host (43–45). In addition, telomerase activity can be downregulated in some types of leukemias exposed to differentiation-inducing agents (46,47).

The data reported herein support the emerging data in the literature that telomerase activation in cell population *in vivo* is a late stage event. Thus, the data in mammary cells is consistent with the results in transgenic mouse models of epidermal carcinogenesis and pancreatic carcinogenesis (28), and in carcinogen-induced mouse epidermal carcinogenesis (27). It is also consistent with results published regarding pre-malignant lesions in human cancer (11,19,23). It is important to note that in none of the above organ systems could the immortalized phenotype be rigorously assessed, therefore the relationship between telomerase activation and immortality could not be directly addressed in these systems.

The data are also consistent with reports in the literature that normal mouse mammary tissues exhibit significant telomerase activity (26,48). An apparent discrepancy is the recent report by Broccoli *et al.* (48), which reported little telomerase activity in normal virgin gland and *wnt-1* hyperplastic mammary glands. The very low levels in normal gland reflect the low

epithelial cell component of the virgin mammary gland and are not necessarily a direct measurement of intrinsic telomerase activity in mammary epithelial cells. The low levels in hyperplastic gland are similar to the results reported with the TM hyperplastic lines. The telomerase activity in the TM hyperplasias is 12% of the activity of tumors; this level is similar to the telomerase activity of *wnt-1* hyperplasias, which was 14% of telomerase activity in *wnt-1* mammary tumors (48).

The correlation between telomerase activity and the proliferation status of the cells is ambiguous and complex (3,4,41,49). Some conditions that increase proliferation may also be associated with increase in telomerase activity, but these are distinct from immortalization. Thus, the high levels of telomerase activity in 11 to 14 day pregnant mammary gland may be linked to either the proliferative state of the cells and/or to hormonal stimulation of the gland. Also, the simple process of culturing mammary cells *in vitro* enhances telomerase activity in normal mammary cells as evidenced herein and by Chadeneau *et al.* (26), in immortal mammary cells and established non-tumorigenic cell lines. Alternatively, immortalized cell populations with very low telomerase activities may have proliferation indices that vary from low to high. Thus, proliferation indices of the TM hyperplastic glands are similar to those of the mid-pregnant gland, although they are lower than those observed in tumors. At the other extreme, immortalized ductal outgrowths have very low telomerase activities, with PCNA-labeling indices that are 50% of that found in pregnant gland (38). With respect to tumors, it is likely that the high level of telomerase activity reflects multiple levels of dysregulation of proliferation controls. This possibility is evidenced in the results of Broccoli *et al.* (48) who have shown that the levels of telomerase RNA are slightly elevated (two times) in tumors compared with hyperplasias but telomerase activity is elevated eight times. Similarly, the levels of cell-cycle regulatory proteins and their kinase-associated activities are elevated much higher in these mammary tumors than is suggested by an examination of proliferation indices (40).

It is important to note that the data do not address the question of telomere length as the critical determinant in the acquisition of immortalization. It is conceivable that telomere maintenance in mouse mammary cells is regulated by mechanisms other than the telomerase enzyme. Examples of potential alternative mechanisms have been discussed by Bryan *et al.* (30).

In summary, the results demonstrate that elevated telomerase activity is not obligatory for the establishment of the immortalized state in mouse mammary cells *in vivo*. One has to consider either that the mouse mammary system is an exception to the rule or that telomerase activity reflects altered growth regulation of the tumor cell and is not causal to the acquisition of immortalization. If the latter, attention should be focused on other mechanisms, as emphasized by other investigators (30,50). The role of telomerase in chromosomal segregation in mitosis is one example of a different role for telomerase (50). If the former, then it is necessary to test the telomerase hypothesis of immortalization in carefully defined *in vivo* systems. Regardless of the answer, it is still important to recognize that telomerase activation is a very frequent event in neoplastic cells of human and murine origin and thus provides an appropriate and inviting target for chemotherapeutic or chemopreventive intervention.

## Acknowledgements

We gratefully acknowledge the technical assistance of Frances Kittrell in the preparation of the cell suspensions and to Dr Raghu Sinha for graphic art. We are grateful for the assistance of Dr Marcello Aldaz for constructive input into the methodology of the telomerase assay and interpretation of the data. This research was supported by NCI grant CA63137 and DOD grant DAMD17-94-I-4274.

## References

1. Counter,C.M., Avilion,A.A., LeFeuvre,C.E., Stewart,N.G., Greider,C.W., Harley,C.B. and Bacchetti,S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.*, **11**, 1921–1929.
2. Shay,J.W., Wright,W.E. and Werbin,H. (1993) Toward a molecular understanding of human breast cancer: a hypothesis. *Breast Cancer Res. Treat.*, **25**, 83–94.
3. Kim,N.W., Piatyszek,M.A., Prowse,K.R. *et al.* (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011–2015.
4. Counter,C.M., Botelho,F.M., Wang,P., Harley,C.B. and Bacchetti,S. (1994) Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein–Barr virus-transformed human B lymphocytes. *J. Virol.*, **68**, 3410–3414.
5. Rhyu,M.S. (1995) Telomeres, telomerase, and immortality. *J. Natl Cancer Inst.*, **87**, 884–894.
6. Bacchetti,S. and Counter,C.M. (1995) Telomeres and telomerase in human cancer (Review). *Int. J. Oncol.*, **7**, 423–432.
7. Harle-Bachor,C. and Boukamp,P. (1996) Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. *Proc. Natl Acad. Sci. USA*, **93**, 6476–6481.
8. Hiyama,E., Yokoyama,T., Tatsumoto,N. *et al.* (1995) Telomerase activity in gastric cancer. *Cancer Res.*, **55**, 3258–3262.
9. Hiyama,E., Gollahon,L., Kataoka,T., Kuroi,K., Yokoyama,T., Gazdar,A.F., Hiyama,K., Piatyszek,M.A. and Shay,J.W. (1996) Telomerase activity in human breast tumors. *J. Natl Cancer Inst.*, **88**, 116–122.
10. Harley,C.B. and Villeponteau,B. (1995) Telomeres and telomerase in aging and cancer. *Curr. Opin. Genet. Dev.*, **5**, 249–255.
11. Chadeneau,C., Hay,K., Hirte,H.W., Gallinger,S. and Bacchetti,S. (1995) Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.*, **55**, 2533–2536.
12. Tahara,H., Nakanishi,T., Kitamoto,M., Nakashio,R., Shay,J.W., Tahara,E., Kajiyama,G. and Ide,T. (1995) Telomerase activity in human liver tissues: Comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res.*, **55**, 2734–2736.
13. Sommerfeld,H.-J., Meeker,A.K., Piatyszek,M.A., Bova,G.S., Shay,J.W. and Coffey,D.S. (1996) Telomerase activity: A prevalent marker of malignant human prostate tissue. *Cancer Res.*, **56**, 218–222.
14. Taylor,R.S., Ramirez,R.D., Ogoshi,M., Chaffins,M., Piatyszek,M.A. and Shay,J.W. (1996) Detection of telomerase activity in malignant and nonmalignant skin conditions. *J. Invest. Dermatol.*, **106**, 759–765.
15. Rogalla,P., Kazmierczak,B., Rohen,C., Trams,G., Bartnitzke,S. and Bullerdiek,J. (1994) Two human breast cancer cell lines showing decreasing telomeric repeat length during early *in vitro* passaging. *Cancer Genet. Cytogenet.*, **77**, 19–25.
16. Hiyama,E., Ishioka,S., Yamakido,M., Inai,K., Gazdar,A.F., Piatyszek,M.A. and Shay,J.W. (1995) Telomerase activity in small-cell and non-small-cell lung cancers. *J. Natl Cancer Inst.*, **87**, 895–901.
17. Gupta,J., Han,L.-P., Wang,P., Gallie,B.L. and Bacchetti,S. (1996) Development of retinoblastoma in the absence of telomerase activity. *J. Natl Cancer Inst.*, **88**, 1152–1157.
18. Rogan,E.M., Bryan,T.M., Hukku,B. *et al.* (1995) Alterations in *p53* and *p16<sup>INK4</sup>* expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell. Biol.*, **15**, 4745–4753.
19. Edington,K.G., Loughran,O.P., Berry,I.J. and Parkinson,E.K. (1995) Cellular immortality: A late event in the progression of human squamous cell carcinoma of the head and neck associated with *p53* alteration and a high frequency of allele loss. *Mol. Carcinogenesis*, **13**, 254–265.
20. Hiyama,E., Kodama,T., Shinbara,K., Iwao,T., Itoh,M., Hiyama,K., Shay,J.W., Matsuura,Y. and Yokoyama,T. (1997) Telomerase activity is detected in pancreatic cancer but not in benign tumors. *Cancer Res.*, **57**, 326–331.
21. Lin,Y., Uemura,H., Fujinami,K., Hosaka,M., Harada,M. and Kubota,Y.

- (1997) Telomerase activity in primary prostate cancer. *J. Urol.*, **157**, 1161–1165.
22. Mao, L., El-Naggar, A.K., Fan, Y.-H., Lee, J.S., Lippman, S.M., Kayser, S., Lotan, R. and Hong, W.K. (1996) Telomerase activity in head and neck squamous cell carcinoma and adjacent tissues. *Cancer Res.*, **56**, 5600–5604.
23. Mutinangura, A., Supiyaphun, P., Trirekapan, S., Sriuranpong, V., Sakuntabhai, A., Yenrudi, S. and Voravud, N. (1996) Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. *Cancer Res.*, **56**, 3530–3533.
24. deLange, T. (1994) Activation of telomerase in a human tumor. *Proc. Natl Acad. Sci. USA*, **91**, 2882–2885.
25. Prowse, K.R. and Greider, C.W. (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl Acad. Sci. USA*, **92**, 4818–4822.
26. Chadeneau, C., Siegel, P., Harley, C.B., Muller, W.J. and Bacchetti, S. (1995) Telomerase activity in normal and malignant murine tissues. *Oncogene*, **11**, 893–898.
27. Bednarek, A., Budunova, I., Slaga, T.J. and Aldaz, C.M. (1995) Increased telomerase activity in mouse skin premalignant progression. *Cancer Res.*, **55**, 4566–4569.
28. Blasco, M.A., Rizen, M., Greider, C.W. and Hanahan, D. (1996) Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nature Genetics*, **12**, 200–204.
29. Yoshimi, N., Ino, N., Suzui, M., Hara, A., Kei, N., Sato, S. and Mori, H. (1996) Telomerase activity of normal tissues and neoplasms in rat colon carcinogenesis induced by methylazoxymethanol acetate and its difference from that of human colonic tissues. *Mol. Carcinogenesis*, **16**, 1–5.
30. Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S. and Reddel, R.R. (1995) Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.*, **14**, 4240–4248.
31. Small, M.B., Hubbard, K., Pardin, J.R., Marcus, A.M., Dhanaraj, S.N. and Sethi, K.A. (1996) Maintenance of telomeres in SV40-transformed pre-immortal and immortal human fibroblasts. *J. Cell. Physiol.*, **168**, 727–736.
32. Daniel, C.W. (1972) Aging of cells during serial propagation *in vivo*. *Adv. Gerontological Res.*, **4**, 167–200.
33. Daniel, C.W., Aidells, B.D., Medina, D. and Faulkin, L.J. Jr (1975) Unlimited division potential of precancerous mouse mammary cells after spontaneous or carcinogene-induced transformation. *Fed. Proc.*, **34**, 64–67.
34. Medina, D. (1996) The mammary gland: A unique organ for the study of development and tumorigenesis. *J. Mamm. Gland Biol. Neoplas.*, **1**, 5–19.
35. Medina, D. and Kittrell, F.S. (1993) Immortalization phenotype dissociated from the preneoplastic phenotype in mouse mammary epithelial outgrowths *in vivo*. *Carcinogenesis*, **14**, 25–28.
36. Kittrell, F.S., Oborn, C.J. and Medina, D. (1992) Development of mammary preneoplasias *in vivo* from mouse mammary epithelial cells *in vitro*. *Cancer Res.*, **52**, 1924–1932.
37. Medina, D., Kittrell, F.S., Liu, Y.-J. and Schwartz, M. (1993) Morphological and functional properties of TM preneoplastic mammary outgrowths. *Cancer Res.*, **53**, 663–667.
38. Medina, D., Kittrell, F.S., Oborn, C.J. and Schwartz, M. (1993) Growth factor dependency and gene expression in preneoplastic mouse mammary epithelial cells. *Cancer Res.*, **53**, 668–674.
39. Medina, D. (1996) Preneoplasia in mammary tumorigenesis. In Dickson, R.B. and Lippman, M.E. (eds) *Mammary Tumor Cell Cycle, Differentiation and Metastases*. Kluwer Academic Publishers, Norwell, MA, pp. 37–69.
40. Said, T.K. and Medina, D. (1995) Cell cyclins and cyclin dependent kinase activities in mouse mammary tumor development. *Carcinogenesis*, **16**, 823–830.
41. Wright, W.E., Shay, J.W. and Piatyszek, M.A. (1995) Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucl. Acids Res.*, **23**, 3794–3795.
42. Said, T.K., Bonnette, S. and Medina, D. (1996) Immortal, nontumorigenic mouse mammary outgrowths express high levels of cyclin B1/cdc2 kinase. *Cell Prolif.*, **29**, 623–639.
43. Kyo, S., Takakura, M., Kohama, T. and Inoue, M. (1997) Telomerase activity in human endometrium. *Cancer Res.*, **57**, 610–614.
44. Saito, T., Schneider, A., Martel, N., Mizumoto, H., Bulgay-Moerschel, M., Kudo, R. and Nakazawa, H. (1997) Proliferation-associated regulation of telomerase activity in human endometrium and its potential implication in early cancer diagnosis. *Biochem. Biophys. Res. Commun.*, **23**, 610–614.
45. Meeker, A.K., Sommerfield, H.J. and Coffey, D.S. (1996) Telomerase is activated in the prostate and seminal vesicles of the castrated rat. *Endocrinol.*, **137**, 5743–5746.
46. Xu, D., Gruber, A., Peterson, C. and Pisa, P. (1996) Suppressing of telomerase activity in HL60 cells after treatment with differentiating agents. *Leukemia*, **10**, 1354–1357.
47. Bestilny, L.J., Brown, C.B., Miura, Y., Robertson, L.D. and Riabowol, K.T. (1996) Selective inhibition of telomerase activity during terminal differentiation of immortal cell lines. *Cancer Res.*, **56**, 3796–3802.
48. Broccoli, D., Godley, L.A., Donehower, L.A., Varmus, H.E. and DeLange, T. (1996) Telomerase activation in mouse mammary tumors: Lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol. Cell. Biol.*, **16**, 3765–3772.
49. Holt, S.E., Shay, J.W. and Wright, W.E. (1996) Refining the telomere–telomerase hypothesis of aging and cancer. *Nature Biotechnol.*, **14**, 836–839.
50. Kirk, K.E., Harmon, B.P., Reichardt, I.K., Sedat, J.W. and Blackburn, E.H. (1997) Block in anaphase chromosome separation caused by a telomerase template mutation. *Science*, **275**, 1478–1481.

Received on April 22, 1997; revised on July 8, 1997; accepted on July 17, 1997