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The objectives of this study are 1) to identify factors that regulate the growth and differentiation of organoids formed by two types of normal human breast epithelial cells (HBEC) in Matrigel; 2) to characterize the expression and function of estrogen receptors (ER) in normal and *in vitro* neoplastically transformed HBEC; and 3) to determine if a HBEC type with stem cell characteristics (Type I) is more susceptible to telomerase activation and immortalization. The major results are 1) Type I HBEC in conjunction with Type II cells are capable of forming ductal and end bud-like structures in Matrigel which preserve the undifferentiated state of HBEC for a long time; 2) Type I normal HBEC and their neoplastically transformed clones express a variant ER *in vitro* on plastic while expressing a wild type ER in tumors developed in nude mice or grown *in vitro* in Matrigel; 3) high susceptibility of Type I HBEC to telomerase activation and immortalization; and 4) the lifespan of HBEC can be effectively extended by co-transfection with a dominant-negative mutant p53 and the human c-myc. These findings indicate Type I stem cells as targets for carcinogenesis and inactivation of p53 and activation of telomerase as major events in initial stage of breast carcinogenesis.

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Chia-Cheng Chang Aug 27 '99

PI - Signature Date

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Grant Number DAMD17-96-1-6099
Chia-Cheng Chang, Ph.D.

IV. Table of Contents

V. INTRODUCTION

We have previously developed a culture method to grow two morphologically distinguishable types of normal human breast epithelial cells (HBEC) from reduction mammoplasty (1). These two types of cells are substantially different in many phenotypes (1-3) as listed in Table 1. The most significant characteristics of Type I HBEC related to breast carcinogenesis are (a) the expression of estrogen receptors (ER); (b) stem cell features (i.e., ability of Type I cells to differentiate into Type II cells and to form budding/ductal structures on Matrigel); and (c) the high susceptibility to neoplastic transformation by oncogenic stimulus (i.e., SV40 large T-antigen induced anchorage independent growth and high frequency of immortalization) (1, 4). Since breast cancers are very likely to be derived from stem cells and ER-positive HBEC, the major objectives of this project are (a) to develop and characterize an *in vitro* organoid system using Type I and Type II HBEC for analyzing factors affecting normal and abnormal growth and differentiation of human mammary gland and for studying the mechanism of carcinogenesis; and (b) to characterize the structure/function and regulation of estrogen receptors expressed in normal and *in vitro* neoplastically transformed cell lines.

The mechanism that Type I HBEC were more susceptible to neoplastic transformation is not known. In general, tumor cells are known to express telomerase that maintains telomere length for continuous growth whereas

normal cells lack the telomerase activity (5). A recent report, however, concludes that telomerase activity is a biomarker of cell proliferation but not malignant transformation in human cells including breast epithelial cells (6). Since we have the putative human breast epithelial stem cells and have developed and preserved a series of SV40 transformed Type I and Type II HBEC at different passages, we are in a good position to determine whether human breast epithelial stem cells express telomerase and whether the innate level of telomerase activity in Type I HBEC is sufficient for immortalization or readily activated during neoplastic transformation. This became an additional task for this project (Task 9).

One of the task (Task 5) proposed to study the expression of ER in ionizing radiation transformed HBEC. Although we have shown that ionizing radiation is capable of extending the lifespan of Type II HBEC (7) and converting a SV40 immortalized Type I HBEC line into tumorigenic cells (8), we were unsuccessful in immortalizing Type I cells by x-rays. Therefore, we decided to use co-transfection with a dominant-negative p53 and a human c-myc. To our surprise, the technique appears to be very effective in extending the lifespan of HBEC. Most importantly, the resulting transformed clones expressed the ER and grew in soft agar. This represents a recent breakthrough in this study.

VI. BODY

A. Characterization of Budding and Ductal Structures Formed by Normal HBEC in Matrigel (Task 1)

The results of this study was reported in great detail in last year's Annual Report in regard to 1) conditions to form budding/ductal structures; 2) the types of organoids formed by the two types of HBEC; and 3) the alternate growth of HBEC as monolayer on plastic and organoid in Matrigel to preserve the undifferentiated state of HBEC for extended time. This project is largely completed except for the immunofluorescent staining of the organoids to reveal gene expression. A new Research Associate (Dr. Mei-hui Tai) experienced in this technique is now working to finish this remaining characterization. Recently, she has worked out the technique to section the organoid developed in Matrigel for immunostaining. Using this technique, she has shown that the centers of the spherical balls formed by Type II HBEC in Matrigel are not hollow as they appear. The structure (Fig. 1) is actually similar to the squamous metaplasia developed by rat mammary organoid in Matrigel (9). The technique will be applied to budding/ductal structures to reveal their structure and gene expression by immunostaining. Part of the results is included in a paper to be published in *Cancer Research* (4). A manuscript to report the bulk of the study is in preparation.

B. The Effects of Hormones and Growth Factors on Growth and Differentiation of Budding/Ductal Structures Formed by Normal HBEC on Matrigel (Task 2)

We have done one experiment comparing budding/ductal structure formation in MSU-1 medium deleting one of the three major growth factor and hormones (EGF, insulin and hydrocortisone) and in the complete medium. The results from this study indicate that these three chemicals are essential for budding/ductal growth. Due to the priority to start and finish other more competitive projects, a systematic study of growth factors and hormones on mammary organoid development has been delayed until the coming year.

C. Comparison of Estrogen Receptor (ER) Expression in Two Types of Normal HBEC Grown on Plastic and in Matrigel (Task 3)

In the first year annual report, we reported an important finding in regard to the regulation of ER expression by extracellular matrix components, i.e., Matrigel is able to mimic the *in vivo* condition in inducing the expression of the wild type ER (66 kd) and a 51 kd variant ER in neoplastically transformed Type I HBEC which, similar to Type I normal HBEC, expressed only the 48 kd variant ER when grown on plastic. While this observation was reproducible, we did not have time to complete the work in the past year because the Research Associate, who worked on this project, has spent most of his time in developing a quantitative telomerase assay and to finish the telomerase study (Task 9). Besides the reproducibility of the major finding, we also demonstrated that the structure of the 51 kd variant ER expressed *in vivo* (tumor in

nude mice) and in Matrigel culture is clearly different from the 48 kd variant ER expressed in cells grown on plastic, i.e., an anti-ER antibody recognizing the N-terminal portion of ER can detect both the wild type ER and the 51 kd ER but not the 48 kd ER (Fig. 18 in 1998 Annual Report).

The preliminary results have been presented at the Annual Meeting of the American Association for Cancer Research (Sun, W. and C.C. Chang, 1998. Matrigel mimics the *in vivo* condition in activating the expression of the wild type estrogen receptor in a human breast epithelial cell type. *Proc. Am. Assoc. Cancer Res.* 39:407). Now our study on telomerase (Task 9) is completed and in press. My Research Associate is working on the project again and is expected to finish the study soon.

D. Estrogen Receptor Expression in SV40 Large T-antigen Transformed Human Breast Epithelial Cells (Task 4)

This task has been completed. A detailed report was presented in the 1997 Annual Report. A paper was published on this work (Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang, 1997. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* 18:251-257).

E. Estrogen Receptor Expression in Ionizing Radiation Transformed HBEC (Task 5)

We have characterized a series of immortal, weakly tumorigenic and highly tumorigenic cell lines derived from Type I HBEC following sequential treatment with SV40 large T-antigen, x-rays and neu oncogene (Kang, K.S. et al. 1998. Involvement of tyrosine phosphorylation of $p185^{C\text{-}erbB2/neu}$ in tumorigenicity induced by x-rays and neu oncogene in human breast epithelial cells. *Molecular Carcinogenesis* 21:225-233. Reprint submitted in 1998 Annual Report). This paper (8) is one of the few reported successes in the creation of human tumour cells *in vitro* as cited by a recent paper (10) by Dr. Robert A. Weinberg. These immortal or tumorigenic cells, when grown on plastic, expressed the 48 kd variant ER (3). The tumorigenic cell lines expressed the wild type ER *in vivo* or in Matrigel as described in the previous section.

The original proposal plans to characterize the expression of ER in HBEC immortalized by x-rays. These immortal cells are to be produced by a different project supported by a different grant from NIEHS. Now since that project only produced extended life clones (7) but not immortalized HBEC, we decided to immortalize our cells by co-transfection with a dominant-negative mutant p53 and a human c-myc. The results are described in the following.

Transfection of Human Breast Epithelial Cells by Co-Transfection with a Mutant p53 and the Human c-myc.

Introduction

Human breast epithelial cells have been immortalized by various methods using SV40 large T-antigen (1), human papilloma virus 16 E6 or E7 (11), radiation (12), mutant p53 (13-15) and the catalytic component of telomerase, hTERT (16). In these studies, cell types difference (early vs. late passage; pre-MO vs. post-MO) clearly affects the outcome of immortalization by different agents (11, 16). Except the use of our Type I HBEC (1, 3, 4), none of the immortalized cell lines has been reported to express ER and to be capable of anchorage independent growth (AIG). The immortalization of HBEC by radiation was rare and requires many repeated treatments (2 Gy x 15) (12). The frequency of immortalization with mutant p53 was also very low. By using a dominant-negative mutant p53 (del 239 p53) reported to immortalize HBEC (13), we were unsuccessful in immortalizing our HBEC in several experiments.

Recently, we co-transfected our cells with a different mutant p53 and the human c-myc. The experiments produced 12 independent clones with extended lifespan which are likely to become immortal. A preliminary characterization showed that these clones express ER and are capable of AIG.

Materials and Methods

Cell culture and plasmids. The method to develop the primary cultures of two types of HBEC from reduction mammoplasty is as described previously (1). A temperature-sensitive mutant p53 (val

substitutes for ala at amino acid 145), originally cloned by Moshe Oren into a plasmid containing the G418-resistant gene, was obtained from Dr. Peter M. Glazer of Yale University. The human c-myc plasmid (pSVHu-c-myc) cloned by Jay Morgenstern was provided by Dr. R.A. Weinberg.

Transfection and selection of transformants. The first passage primary cultures were subcultured 1-2 times before replated for transfection. 40 and 25 plates (9 cm) of Type II HBEC (HME21) (approximately 1 x 10^6/plate) were used for transfection in two separate experiments respectively. In addition, 8 plates of Type I HBEC were also similarly transfected. The transformation of these cells by co-transfection with the mutant p53 and human c-myc was achieved by lipofectin-mediated transfection (1). The transformants were selected by G418 (50 μg/ml) 3 days after transfection.

Characterization of transformed clones. The G418-resistant colonies from the co-transfection experiments were isolated and propagated. The initial characterization of these clones includes 1) the determination of the lifespan by calculating the cumulative population doubling level (cpdl); 2) the ability to grow in soft agar (AIG); 3) the expression of p53 and ER; and 4) the capability of gap junctional intercellular communication.

Results

Isolation of transformed clones. For Type II HBEC, the first experiment yielded 8 colonies in 40 plates. These are independent clones since each of

them was isolated from a different plate. The second experiment produced three G418-resistant colonies, two of them were from the same plate. Therefore, the frequency of transfectants is very low. For Type I cells, no G418-resistant proliferating colony was found.

Preliminary characterization of transformed colonies. Unlike our previous observation with few G418-resistant colonies isolated after transfection with del 239 mutant p53 (13), the colony morphology of this new batch of G418-resistant colonies are very similar to Type I cells rather than Type II cells (Fig. 2). Most importantly, all of them have extended lifespan although their rate of population doubling varies. Up to this date, all of these clones are actively proliferating even after 52 cpdl (Table 2). All of the 5 clones examined have a high level of p53 expression as shown by immunostaining (Fig. 3). Unexpectedly, all 3 clones examined for the ability to grow in soft agar were capable of AIG (Fig. 4). Four out of five clones tested for the expression of ER showed the expression of a variant ER (~48 kd) by Western blotting similar to Type I HBEC grown on plastic (Fig. 5). However, 4 clones tested for gap junctional intercellular communication by the scrape loading/dye transfer technique showed the ability of cell-cell communication (Fig. 6) similar to the parental Type II cells. At the early stage (20-30 cpdl), the telomerase activities of 8 clones examined have not been activated and are similar to the level of activity of the parental cells (Fig. 7).

Discussion

Although the frequency of extended life clones from these co-transfections of mutant p53 and c-myc is low, the resulting clones are very interesting. Not only do they have high proliferating potential, they showed many of the phenotypes of Type I HBEC similar to those SV40 large T-antigen transformed Type I cells. These include colony morphology, AIG and the expression of ER. However, they do retain the ability of GJIC. The mechanism for the appearance of the unexpected phenotypes in these clones are not clear. It is possible that the target cells for the transfection are a small population of residual Type I cells or early progenitor cells, newly derived from Type I cells, which have the ability to dedifferentiate into Type I cells.

The mechanism for the transformation is clearly related to the expression of the transfected mutant p53. Whether the transfected c-myc plays a role remains to be determined after its expression is known. Although this is an ongoing experiment, judging from the high proliferating ability of the clones, they are very likely to become immortal spontaneously. If not, these clones should be able to be immortalized by further transfection with the telomerase gene, hTERT. The immortalized clones should be very useful for carcinogenesis study since they are capable of AIG and expressing the ER. Besides the discovery of an effective method to transform HBEC, the significance of this study is to provide further evidence that defective p53 is the focus of breast carcinogenesis. This concept is also supported by the

functions of 3 hereditary breast cancer syndromes, i.e., p53 mutation in Li-Fraumeni Syndrome, the Ataxia telangiectasia gene product ATM that phosphorylates and activates p53 and the BRCA1 that activate the expression of p21, a gene also regulated by p53.

F. Mechanism of the Expression of ER in Type II HBEC by SV40 Large T-antigen (Task 6)

The normal Type II HBEC did not express the ER. The SV40 transformed Type II cells, similar to Type I cells, expressed the 48 kd variant ER when grown on plastic (3). The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen may induce the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast carcinoma cells with SV40. These cells expressing the large T-antigen resulted from SV40 transfection, however, remain ER-negative (3). Alternatively, in the Type II HBEC population, there might exist a small population of ER-positive transitional cells, newly differentiated from Type I cells, which are the target cells for SV40 transformation. Indeed, we have observed a morphologically distinguishable atypical HBEC type which attached on plastic early similar to Type II cells after trypsinization and subculture. These cells are also very likely to be derived from Type I cells as they may be found in some Type I cell colony. Preliminary study shows that these cells have mixed phenotypes of Type I and Type II cells (i.e., not growth-inhibited by FBS, proficient in gap junctional intercellular communication

and ER-positive). It is possible that a small minority of these cells were present in Type II cell population and they are the target cells transformable by SV40. We have done one experiment to transfect 2 plates of atypical HBEC with a plasmid carrying the wild type SV40 large T-antigen (PRNS-1) from Dr. Johng S. Rhim). The experiment, however, failed to produce the transformed colony. We will repeat the experiment in the future.

In light of the results from the mutant p53 experiment and as discussed previously, the expression of ER in transformed Type II cells could be the result of dedifferentiation of progenitor cells that are being transformed.

G. The Biological Functions of Estrogen Receptors Expressed in Normal HBEC (Task 7)

Only Type I HBEC expressed the ER. When grown on plastic, these cells expressed the 48 kd variant ER which appears to be non-functional in DNA-binding (3) and not responsive to estrogen for cell growth (2). What is not known is whether the wild type ER is expressed in budding/ductal structures formed in Matrigel and responsive to estrogen for growth. This will be studied in the coming year. The techniques for sectioning of organoids in Matrigel has been developed in the past year. This will be used in conjunction with immunostaining for this study.

H. The Biological Functions of Estrogen Receptors Expressed in SV40 Large T-antigen and X-ray Transformed HBEC (Task 8)

We have developed and characterized SV40 and x-ray transformed Type I HBEC lines (3, 8). These cells expressed a 48 kd ER *in vitro* on plastic and the wild type ER and a 51 kd ER in tumors formed in nude mice and in cells embedded in Matrigel (see report on Task 3 and reference 3). The 48 kd ER expressed in cells on plastic appears not to respond to estrogen to stimulate cell growth (Fig. 19 in 1998 Annual Report). Additional experiments show that these neoplastically transformed Type I HBEC (M13SV1R2N1) (8) also did not respond to estrogen for growth on plastic plates coated with different extracellular matrix components (collagen I, IV, fibronectin and laminin) (Biocoat Cellware, Collaborative Biomedical Products) (Fig. 8). The Matrigel also seems not to modify the estrogen response for growth when these cells were inoculated on top, in between of two layers (overlay) or mixed (embedded) with Matrigel (Fig. 9). This grant did not seek approval for animal experiments. Previous experiments carried out under a different grant (NIEHS ES07256) show that M13SV1R2N1 formed tumors in both male and female mice (8) and in ovariectomized nude mice with or without estrogen pellet (Table 3).

All these experiments indicate that the *in vitro* transformed cells are non-responsive to estrogen for growth *in vitro* and do not require estrogen

for tumor growth despite the expression of the wild type ER. Therefore, we suspect the non-responsiveness of these cells to estrogen could be the result of their transformation by SV40 large T-antigen. To test this hypothesis, we have transfected the ER-positive, estrogen-dependent breast cancer cells MCF-7. The results, indeed, show that the response of SV40 large T-antigen expressing MCF-7 cells have partially reduced their response to estrogen for cell growth *in vitro* (Fig. 10). This result will be confirmed using more clones.

I. Correlation of High Susceptibility of a Normal HBEC Type with Stem Cell Characteristics to Neoplastic Transformation with its High Potential for Telomerase Activation (Task 9)

This Task has been completed. A detailed report was presented in the 1998 Annual Report. A manuscript has been accepted for publication in *Cancer Research* (W. Sun, K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization) (manuscript included in Appendices).

Figure Legends

Figure 1 The structure of spherical balls formed by Type II normal HBEC on Matrigel as revealed by sectioning. The structure is similar to squamous metaplasia developed by rat mammary organoid in Matrigel (9).

Figure 2 The morphology of p53 ts mutant/c-myc transformed colony.

Figure 3 The expression of p53 in ts mutant p53/c-myc transformed clones.

Figure 4 Anchorage independent growth of M21-22 (top) and M21-24 (bottom).

Figure 5 The expression of estrogen receptor in 5 ts mutant p53/c-myc transformed clones (M21-22, 23, 24, 82 and M21-B2-2) analyzed by Western blotting. R2N1 transformed by SV40, x-rays and neu oncogene is used as a positive control (CC, from cell culture; TT, from tumor tissue).

Figure 6 Gap junctional intercellular communication in M21-22 studied by the scrape loading/dye transfer technique.

Figure 7 The telomerase activity of ts mutant p53/c-myc transformed clones (lane 1-8) at early passage (20-30 cpdl) studied by the TRAP assay. Lane 9-11 are immortal, weakly tumorigenic and highly tumorigenic cell lines respectively derived from Type I HBEC following SV40, x-rays and neu oncogene treatment.

Figure 8 The growth of M13SV1R2N1 on different extracellular matrix component coated plate in medium with or without estradiol (10 nm). Cell numbers were determined by quantitative measure of DNA in cells harvested from each plate.

Figure 9 The growth of M13SV1R2N1 in Matrigel in the presence and absence of estradiol (10 nm). Cell numbers were quantified by the MTT assay (*Biotechniques* 24: 1038-1043, 1998).

Figure 10 The effect of estradiol on growth of MCF-7 and a MCF-7 clone expressing SV40 large T-antigen.

Figure 1.

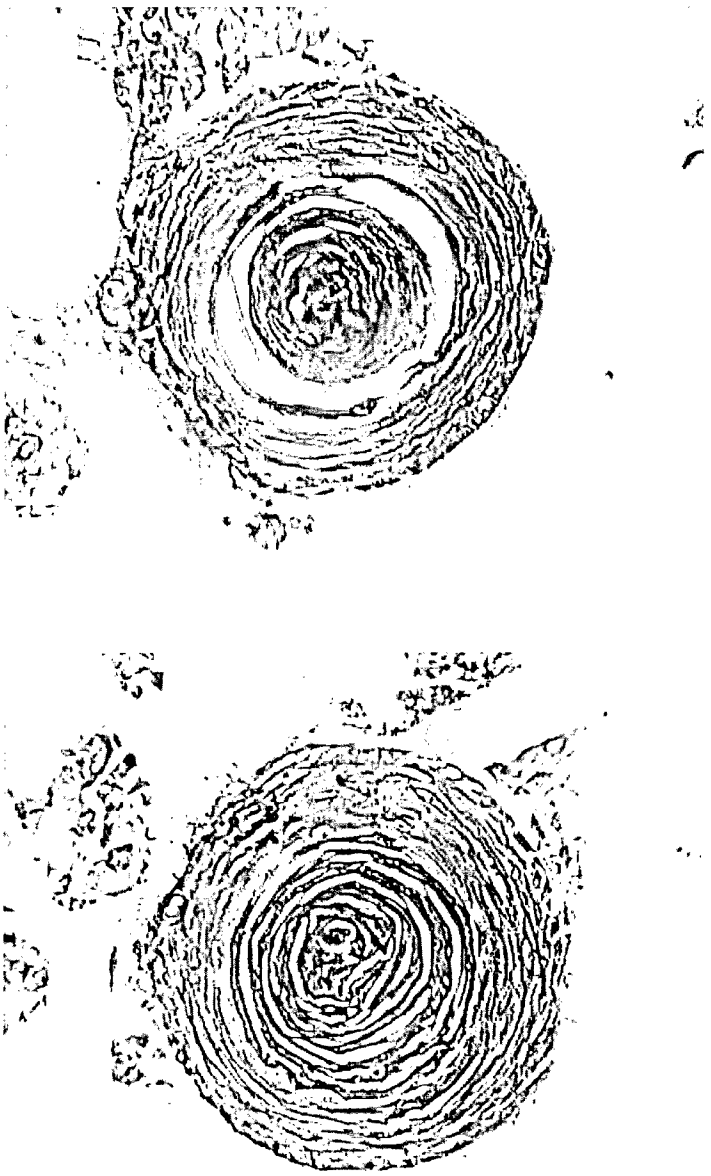

Figure 2.

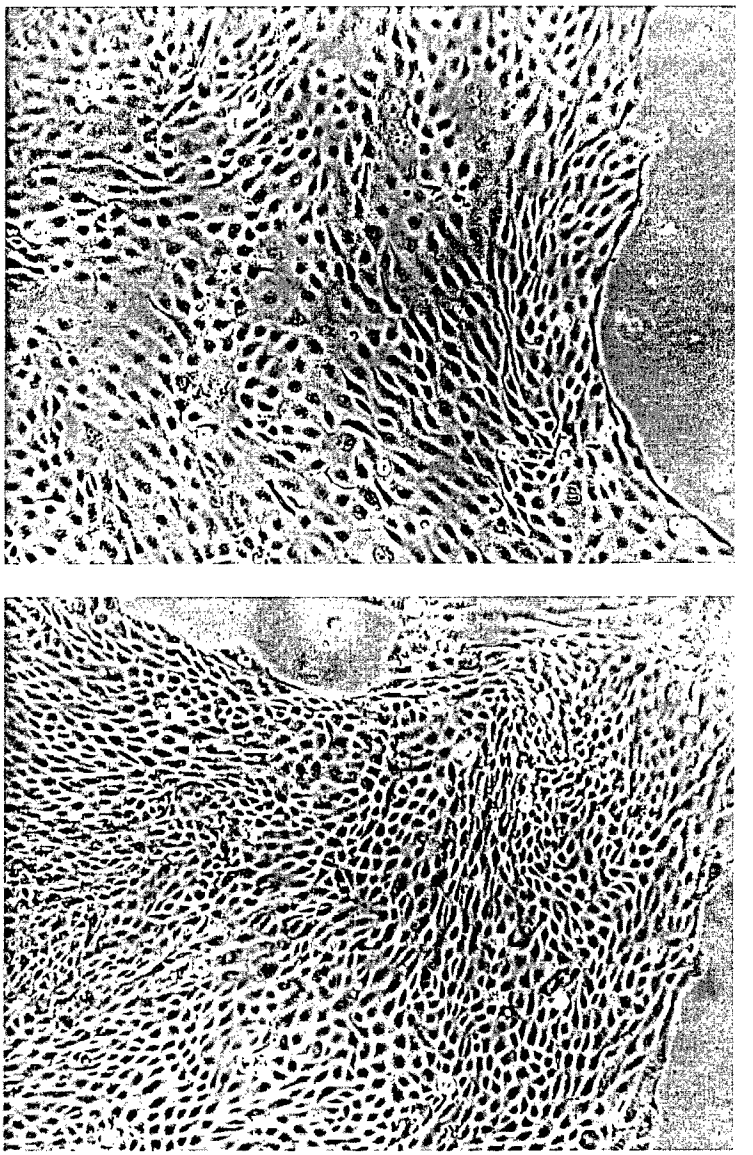

Figure 3.

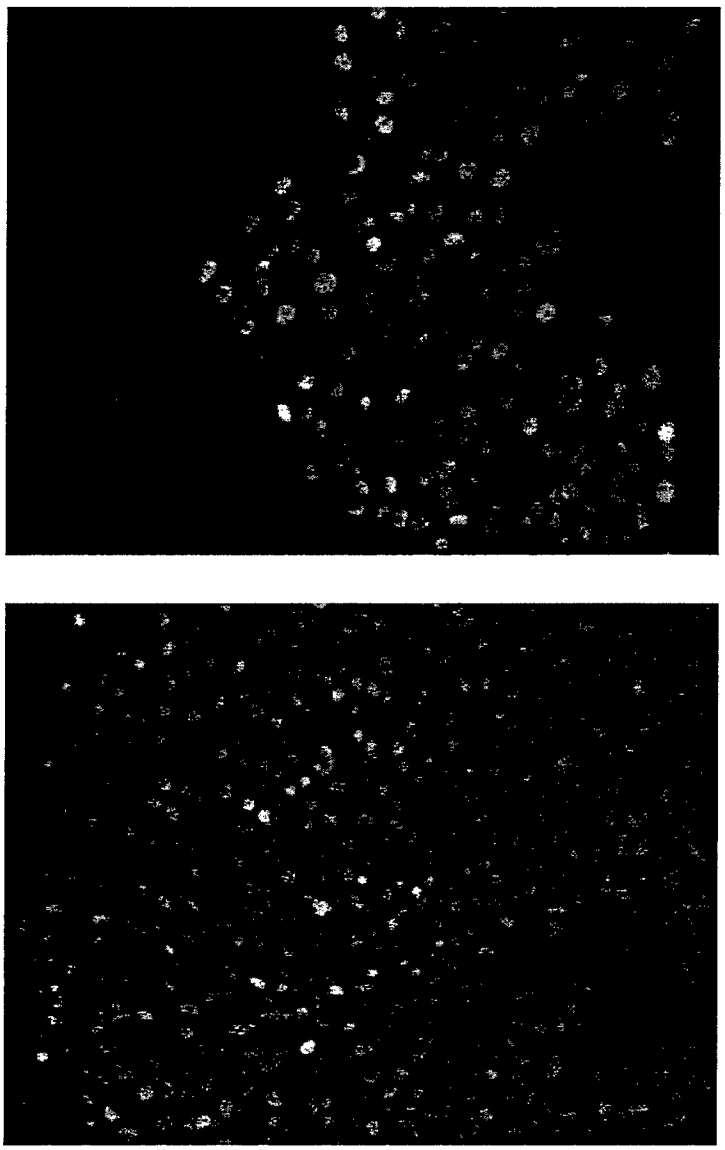

Figure 4.

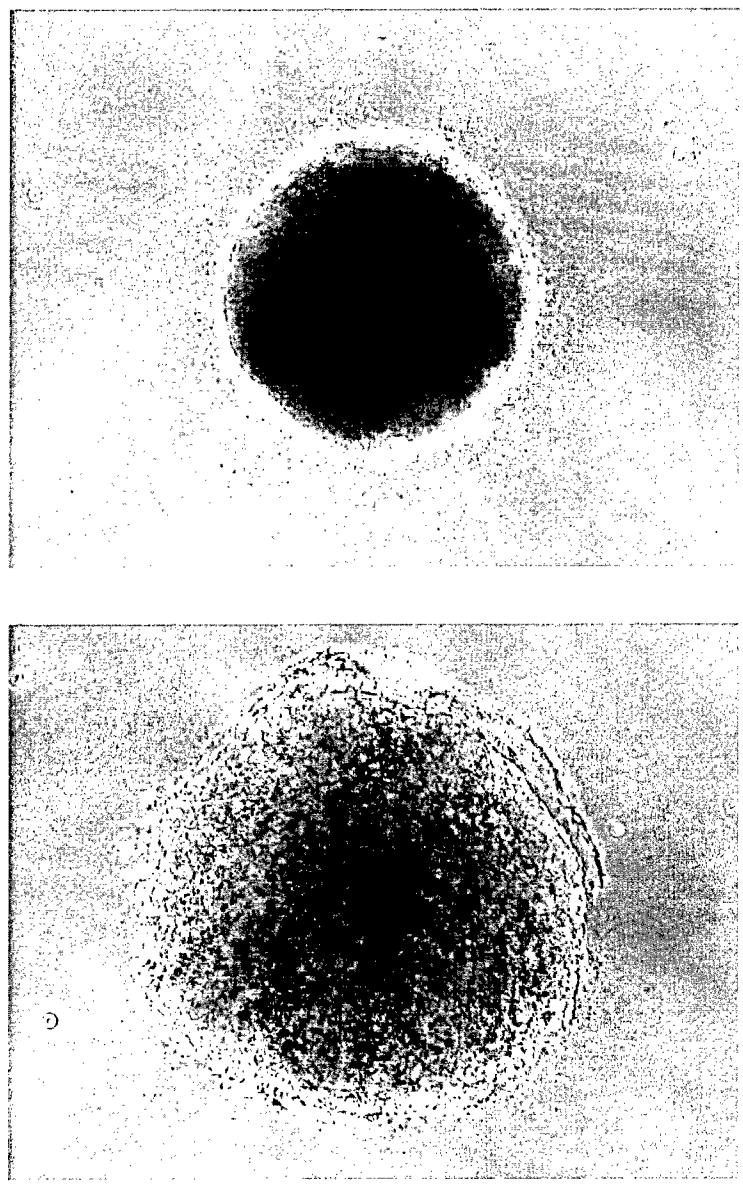

Figure 5.

ER-alpha expression in ts mp53/c-myc transformed HEBC clones

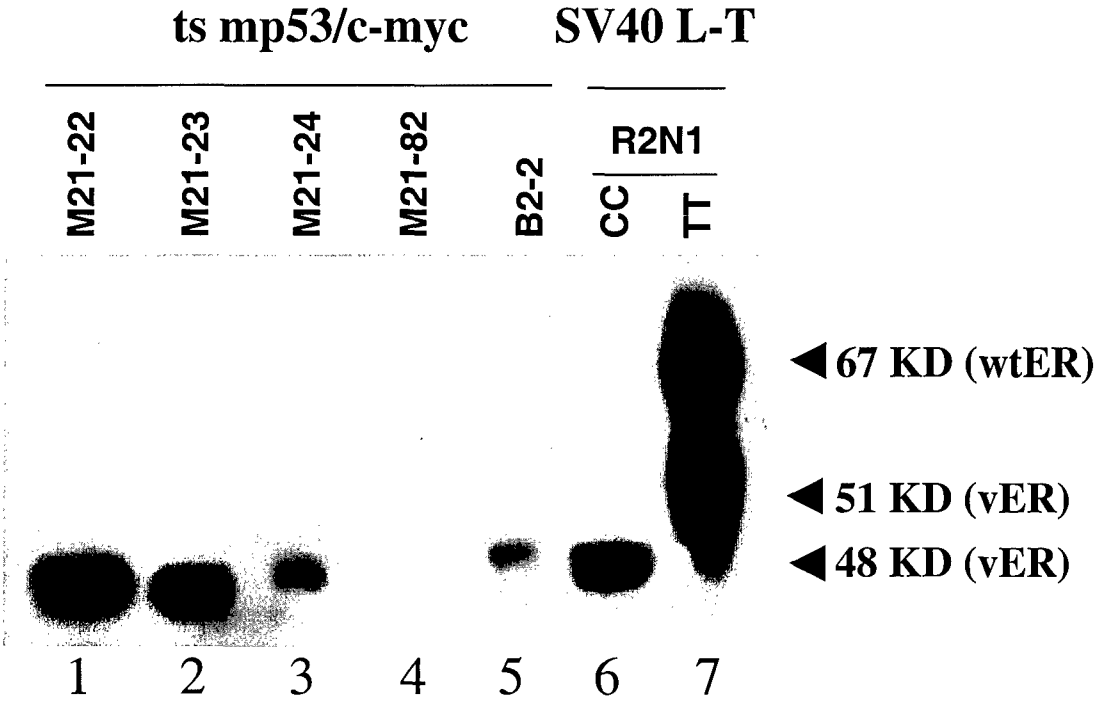

Figure 6.

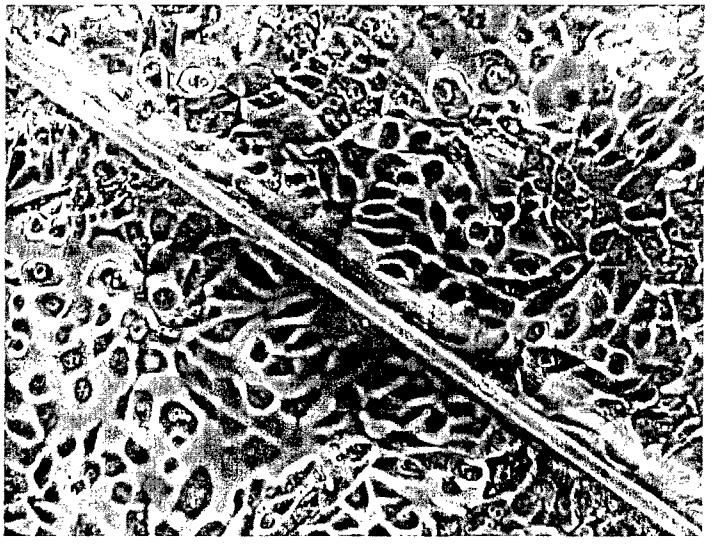

Figure 7.

Telomerase activity in ts mp53/c-Myc transformed HBEC clones at early passage

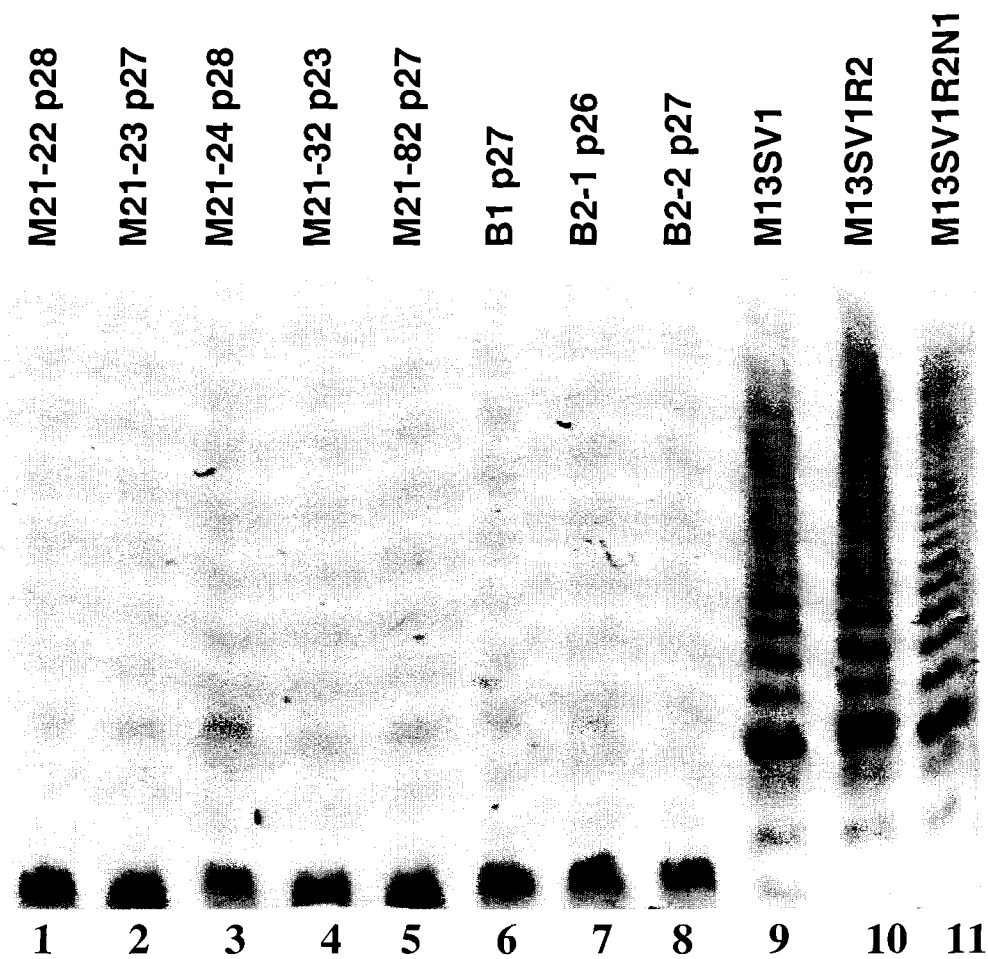

Figure 8

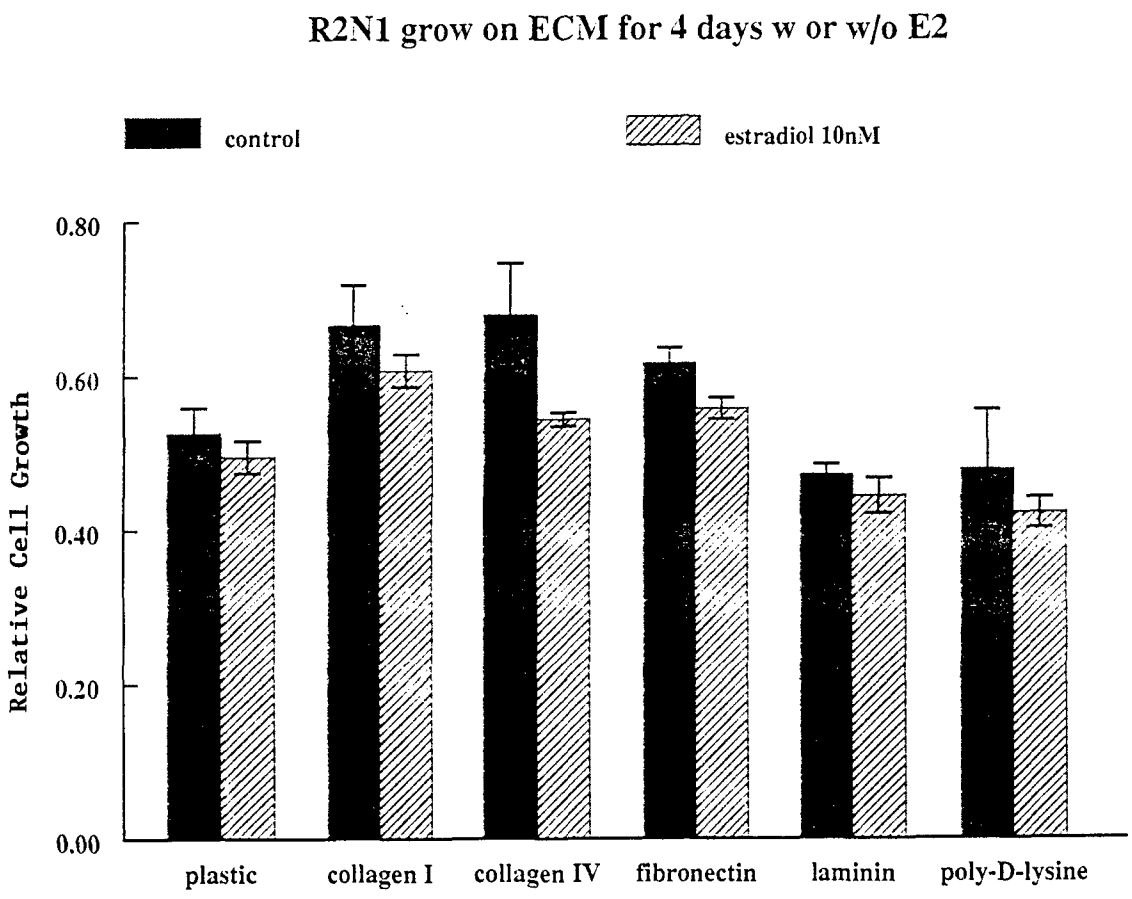
R2N1 grow on ECM for 4 days w or w/o E2
control
estradiol 10nM
Relative Cell Growth
0.80
0.60
0.40
0.20
0.00
plastic
collagen I
collagen IV
fibronectin
laminin
poly-D-lysine

Figure 9

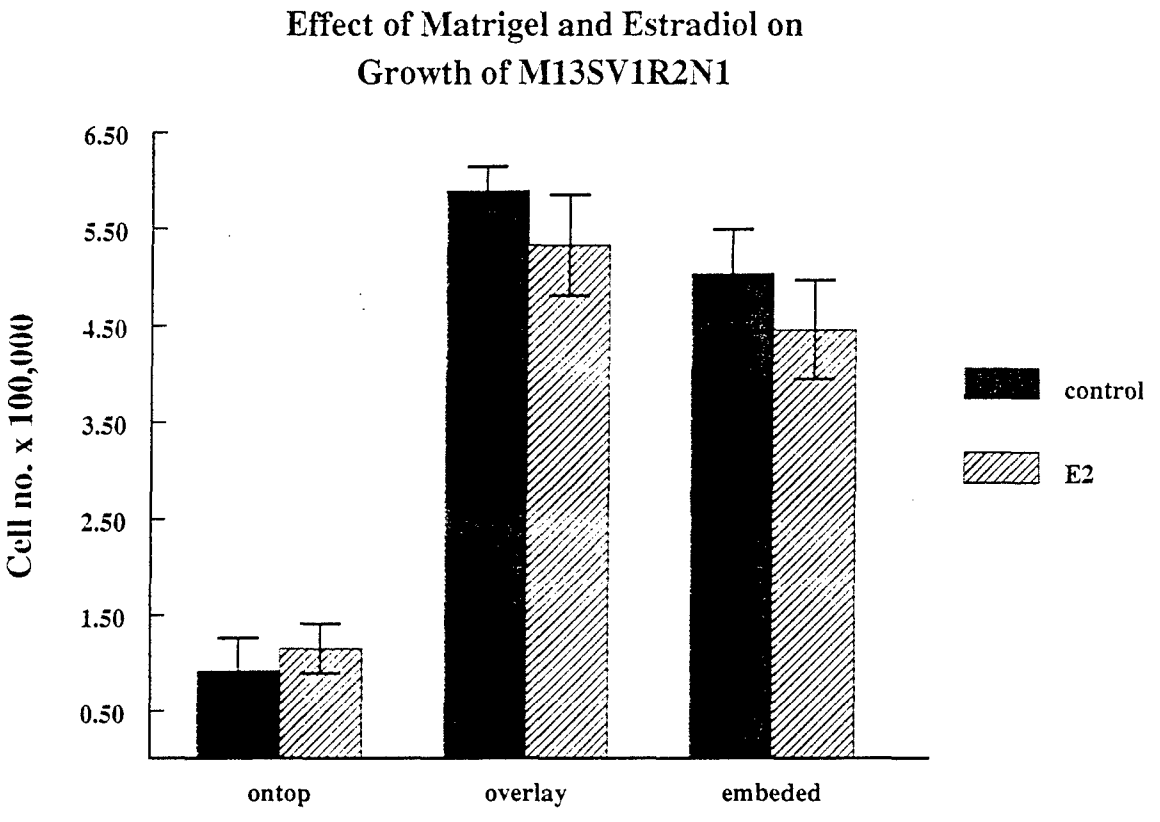
Effect of Matrigel and Estradiol on
Growth of M13SV1R2N1
Cell no. x 100,000
6.50
5.50
4.50
3.50
2.50
1.50
0.50
ontop
overlay
embeded
control
E2

Figure 10

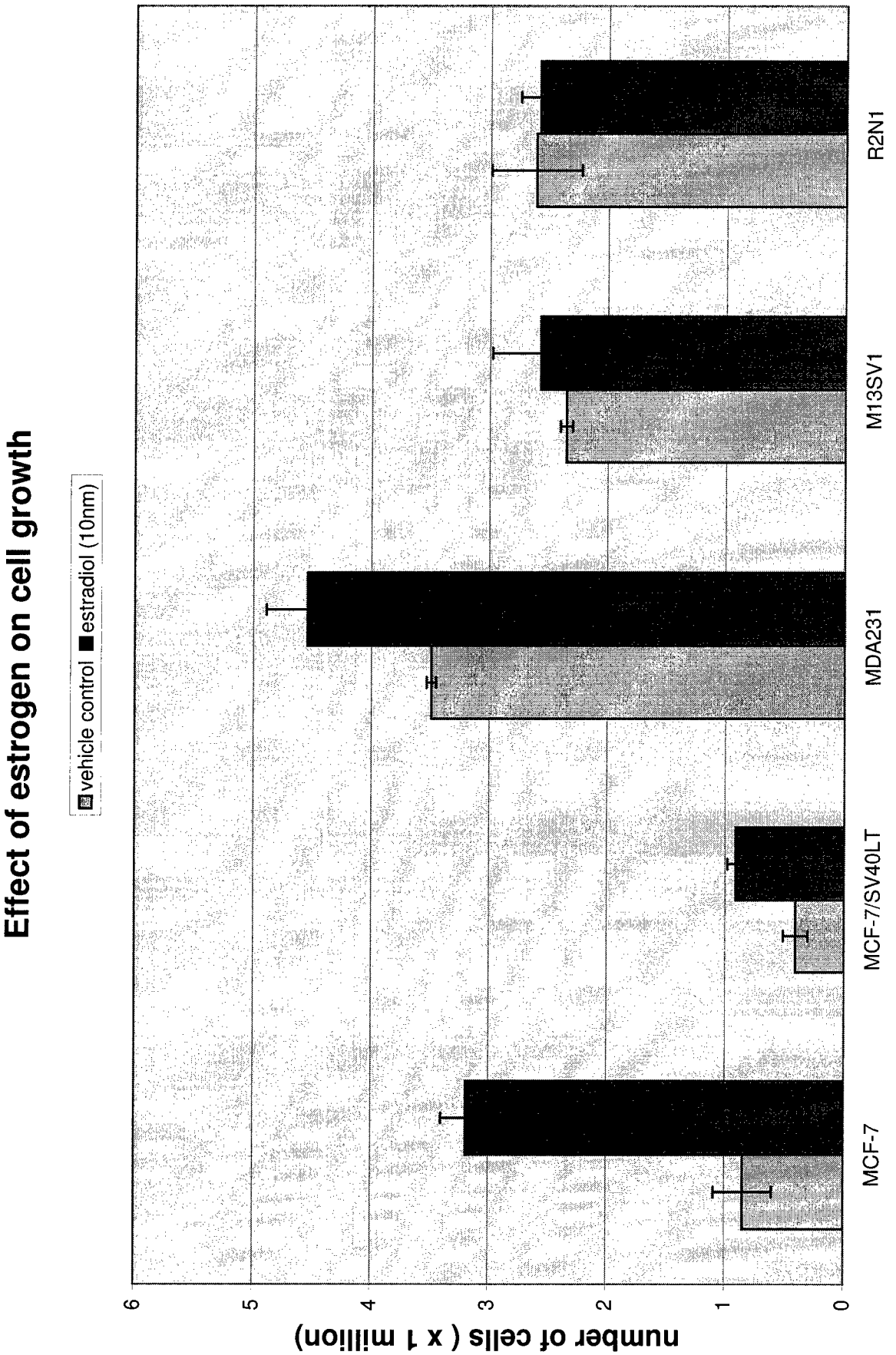
Effect of estrogen on cell growth
vehicle control
estradiol (10nm)
number of cells (x 1 million)
0
1
2
3
4
5
6
MCF-7
MCF-7/SV40LT
MDA231
M13SV1
R2N1

Table 1. Major phenotypic differences between Type I and Type II HBEC

	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobble-stone appearance
Colony morphology	Boundary smooth and restricted	Boundary not smooth
Attachment on plastic surface after trypsinization	Late	Early
Effect of FBS	Growth promotion	Growth inhibition
Gap junctional intercellular communication	Deficient	Efficient
Expression of:		
Connexin 26	–	+
Connexin 43	–	+
Epithelial membrane antigen	+	–
Cytokeratin 18	+	–
Cytokeratin 19	+	–
Cytokeratin 14	–	+
α6 Integrin	–	+
Estrogen receptor	+	–
Effect of cAMP (induced by cholera toxin, forskolin)	Induces Type I cells to change into Type II cells	
Organoid on Matrigel	Budding/ductal structure Acini	Hollow balls elongated cell mass
Response to SV40 large T-antigen:		
Anchorage independent growth	+	–
Spontaneous immortalization	High frequency	Low frequency

Table 2. Extended Lifespan Clones Isolated from HME21 Transfected by m p53/c-myc

Exp. 1

	cpdl	AIG	ER	GJIC
M21-22	52	+	+	+
M21-23	35		+	
M21-24	43	+	+	+
M21-32	38			
M21-41	27			
M21-73	32			
M21-82	33		–	+
M21-104	30			

Exp. 2

	cpdl	AIG	ER	GJIC
M21-B1	32			
M21-B2-1	31			
M21-B2-2	42	+	+	+

cpdl = cumulative population doubling level; AIG = anchorage independent growth; ER = estrogen receptor; GJIC = gap junctional intercellular communication

Table 3.

Tumors developed by M13SV1R2N1 cells in ovariectomized athymic mice

No. of Mice (No. of Tumors)	Estrogen Pellet (2 mg)	Average Tumor Weight (mg)
6 (12)	–	470± 87
5 (10)	+	427± 136

VII. KEY RESEARCH ACCOMPLISHMENTS

1. The discovery that normal Type I human breast epithelial cells (HBEC) and their neoplastically transformed derivatives express a variant ER (~48 kd) when grown *in vitro* on plastic and that tumors formed by these cells in nude mice expressed a wild type ER (~66 kd) and a different variant ER (~51 kd).
2. Demonstrated that Matrigel is able to mimic the *in vivo* condition to activate the expression of the wild type ER.
3. Obtained further evidence that Type I HBEC have stem cell characteristics, i.e., the ability to form budding/ductal structures and to maintain the undifferentiated state in Matrigel for a long time, in addition to previous observations that Type I cells are capable of differentiating into other types of cells.
4. The demonstration that Type I cells are more susceptible to telomerase activation and immortalization following transfection by SV40 large T-antigen (i.e., Type I cells are target cells for breast carcinogenesis).
5. The development of an effective method to extend the lifespan (bypassing senescence) or to immortalize HBEC which express the ER and are capable of anchorage independent growth by co-transfection with a dominant-negative p53 and human c-myc.

VIII. REPORTABLE OUTCOMES

A. Published Papers

1. Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang, 1997. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* 18: 251-257.
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3. Tanimoto, A., C.Y. Kao, C.C. Chang, Y. Sasaguri and R. Padmanabhan, 1998. Deregulation of cdc2 gene expression correlates with overexpression of a 110 kDa CCAAT box binding factor in transformed cells. *Carcinogenesis*, 19: 1735-1741.
4. Hsieh, C.Y. and C.C. Chang, 1999. Stem cell differentiation and reduction as a potential mechanism for chemoprevention of breast cancer. *Chinese Pharm. J.* 51: 15-30.

B. Manuscripts in Press

1. Sun, W., K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell

characteristics to telomerase activation and immortalization. *Cancer Res.*

2. Chang, C.C., C.J. Liu, W. Sun, M. Saitoh and J.E. Trosko. Roles of ionizing radiation in neoplastic transformation of human breast epithelial cells. *Radiation Res.*

C. Abstracts, Presentations

1. Sun, W. and C.C. Chang, 1998. Matrigel mimics the *in vivo* condition in activating the expression of the wild type estrogen receptor in a human breast epithelial cell type. *Proc. Am. Assoc. Cancer Res.* 39: 407. New Orleans, LA. March 28-April 1.
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3. Sun, W., K.S. Kang, J.E. Trosko and C.C. Chang, 1999. A normal human breast epithelial cell (HBEC) type with stem cell characteristics is more susceptible to telomerase activation and immortalization. *Proc. Am. Assoc. Cancer Res.* 40: 635. AACR Annual Meeting, Philadelphia, PA, April 10-14.

4. Chang, C.C., C.Y. Hsieh, W. Sun, C.J. Liu, M. Saitoh and J.E. Trosko, 1999. Role of ionizing radiation in neoplastic transformation of human breast epithelial cells. 11th International Congress of Radiation Research, Dublin, Ireland, July 18-23. *Radiation Res.* Vol. 1, Congress Abstracts p. 74.

5. Chang, C.C., W. Sun, K.S. Kang and J.E. Trosko, 1999. A normal human breast epithelial cell (HBEC) type with stem cell characteristics is more susceptible to telomerase activation and immortalization. 11th International Congress of Radiation Research Satellite meeting: In Vitro Transformation, Cork City, Ireland, July 24-25.

D. Graduate Student and Postdoctoral Trainee Supported by This Award

Graduate student:
Angela Cruz
Maki Saitoh

Postdoctoral trainee:
Kyung-Sun Kang
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Ching-Yi Hsieh
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IX. CONCLUSIONS

As described in Section VI – E, the co-transfection with a temperature-sensitive mutant p53 and the human c-myc appears to be a very effective method to transform normal HBEC. The phenotypes of the transformed

clones such as AIG and ER expression are similar to our Type I HBEC transformed by SV40. It should be noted that except our SV40 transformed Type I HBEC, AIG and ER expression have not been reported by other immortalized HBEC in literature. This newly-developed method and the transformed clones should be very useful for carcinogenesis study. The fact that HBEC can be easily transformed by dominant-negative mutant p53 in addition to the correlation of the 3 hereditary breast cancer syndromes to the defective p53 (i.e., p53 mutation in Li-Fraumeni Syndrome, the Ataxia telangiectasia gene product ATM that phosphorylates and activates p53 and the BRCA1 that activates the expression pf $p21^{WAF1}$, a gene also regulated by p53) strongly suggests p53 as a major focus for breast carcinogenesis.

Our tumorigenic Type I cells transformed by sequential treatment with SV40, x-rays and neu oncogene did not require estrogen for cell growth or tumor development. This could be due to the expression of SV40 large T-antigen as shown by the reduced sensitivity to estrogen for growth in T-antigen expressing MCF-7 cells. To confirm this hypothesis, we are testing whether Type I HBEC transformed by other means such as mutant p53 could be estrogen-dependent similar to MCF-7 cells.

Our study of telomerase activity in normal and neoplastically transformed HBEC clarifies two issues regarding the role of telomerase in breast carcinogenesis. First, both normal Type I and Type II HBEC did express a low level of telomerase activity. However, this low level of telomerase activity may not be sufficient for neoplastic transformation.

Second, in all the SV40 immortalized HBEC clones assayed, the telomerase activity was invariably greatly activated at mid- or late passage, in contrast to senescent clones which never showed telomerase activation. Furthermore, the ability of Type I HBEC to become immortal at high frequency (11/11) appears to be correlated with their high potential for telomerase activation. Therefore, telomerase activation could be a major biomarker and mechanism for the transition from extended lifespan to immortalization, contrary to a previous report (6) that concludes that telomerase activity is a biomarker of cell proliferation but not malignant transformation. The high potential of Type I HBEC to activate telomerase activity also provides a mechanism why Type I cells might be target cells for neoplastic transformation.

Our characterization of organoids formed by the two types of HBEC on Matrigel provides strong evidence that Type I HBEC are breast epithelial stem cells. The Type I cells not only are capable of differentiating into other cell type (1) and forming budding/ductal structures on Matrigel, the organoids thus formed are also able to preserve HBEC for a long time. The alternate cycling of the two types of HBEC as monolayer culture on plastic and as organoid in Matrigel could be a way to preserve and amplify human breast epithelial stem cells for other research on human breast epithelial stem cells. This organoid system should be useful as an *in vitro* model for analyzing factors that affect normal and abnormal growth and differentiation of human mammary gland and for studying the mechanism of carcinogenesis.

Our finding of the differential expression of ER *in vitro* and *in vivo* indicates the importance of cellular environment in regulating the expression of ER. We further demonstrated that the *in vivo* effect may come from extracellular matrix components as shown by the ability of Matrigel to mimic the *in vivo* condition in inducing the expression of wild type ER. This might provide a new strategy to control the ER expression and the growth of estrogen-dependent breast cancer by modulating the structure and function of extracellular matrix.

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XI. APPENDICES

Reprints of published papers and manuscripts in press:

1. Tanimoto, A., C.Y. Kao, C.C. Chang, Y. Sasaguri and R. Padmanabhan. Deregulation of cdc2 gene expression correlates with overexpression of a 110 kDa CCAAT box binding factor in transformed cells. *Carcinogenesis*, 19: 1735-1741, 1998.
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3. Sun, W., K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Res.* (in press).
4. Chang, C.C., C.J. Liu, W. Sun, M. Saitoh and J.E. Trosko. Roles of ionizing radiation in neoplastic transformation of human breast epithelial cells. *Radiation Res.* (in press).

High Susceptibility of a Human Breast Epithelial Cell Type with Stem Cell Characteristics to Telomerase Activation and Immortalization

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Runnig Title: High immortalization rate of a breast epithelial cell type

Abbreviations: HBEC, human breast epithelial cell; E.L., extended lifespan; SV40, simian virus 40; cpdl, cumulative population doubling level; TRAP, telomeric repeat amplification protocol; AIG, anchorage independent growth.

ABSTRACT

Tumor cells have been hypothesized to be derived primarily from stem cells and remained in a relatively undifferentiated state. Here, we have studied whether a normal human breast epithelial cell (HBEC) type with stem cell characteristics (Type I) is more susceptible to telomerase activation and immortalization than a cell type with basal cell phenotypes (Type II). After SV40 large T-antigen transfection, telomerase activation was found in all of the Type I HBEC clones (11/11) but only in 10% of the Type II clones (1/10) examined. Furthermore, all and only the clones with telomerase activation have become immortal. These results indicate that high susceptibility to telomerase activation may be a feature of stem cells and a basis that they are target cells for carcinogenesis.

INTRODUCTION

Cancer cells are generally recognized as being in a relatively undifferentiated state. This undifferentiated state of tumor cells could either be due to dedifferentiation of a differentiated cell or to blocked differentiation of stem cells which give rise to tumor cells (1). The latter view is similar to earlier concept of cancer as a disease of cell differentiation (2) or of "oncogeny as blocked or partially blocked ontogeny" theory (3).

In human breast cancer, there is evidence that stem cell proliferation and differentiation play an important role in carcinogenesis. First, epidemiological studies indicate that the lifetime risk of developing breast cancer in child-bearing women seems to be linearly related to the age at which a women has her first full-term pregnancy (4), and that breast cancer risk is higher in those who are nulliparous or late parous (5; 6). Second, in the study of radiation effects of the atomic bomb survivors in Japan, the frequency of ionizing radiation-induced breast cancers was found to be inversely correlated with age of women at the time of exposure, indicating a higher breast cancer risk for the undifferentiated mammary gland (7). Although the role of stem cells in cancer is implicated, whether stem cells are more susceptible to neoplastic transformation has not been experimentally examined.

Normal somatic cells have a finite life span. Telomere shortening as a consequence of chromosome end-replication problem has been proposed as a

mitotic clock for cellular senescence (8). Telomerase, a ribonucleoprotein complex with reverse transcriptase activity which use a RNA template to add TTAGGG hexonucleotide repeat onto the end of chromosomes, is capable of maintaining the telomere length and replicative activity of cells (9). Telomerase activation is an important mechanism in maintaining telomere length stability and acquiring immortalization, which is a pivotal step in carcinogenesis(10-13).

We have developed a cell culture method to grow two types of normal human breast epithelial cells (HBEC) from reduction mammoplasty (14). One type of cells (Type II HBEC), similar to those commercially available, shows basal epithelial cell markers, while the new cell type (Type I HBEC) expresses luminal epithelial phenotypes and estrogen receptors (14; 15). Importantly, Type I HBEC also shows stem cell characteristics (i.e. the ability to differentiate into other cell type by cyclic AMP-inducing agents and to form budding/ductal structures in Matrigel). Therefore, it offers an opportunity to examine whether these cell with stem cell characteristics are more susceptible to telomerase activation and immortalization, a distinct mechanism that might potentiate stem cell as target for neoplastic transformation.

MATERIALS AND METHODS

Cell Culture and Mammary Organoids Formation

The cell culture and method to develop the two types of normal HBEC culture from reduction mammoplasty are as described previously (14-16). The two types of HBECs are morphologically distinguishable and substantially different in many phenotypes (14). In contrast to Type II cells, the major features of type I HBEC are the deficiency in gap junctional intrercellular communication (GJIC), the growth promotion by fetal bovine serum and the expression of estrogen receptors and luminal epithelial cell markers (i.e. epithelial membrane antigen and cytokeratin 18) (14-16).

Growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) was used to study mammary organoid structure formation with Type I and Type II HBECs. Approximately, $1x10^6$ cells were plated on 35 mm culture dishes or 2-chamber Lab-Tek culture slides with a layer of Matrigel. Matrigel remains solid in the 37°C humidifed inculator where cells are allowed to aggregate for one day. After cells attached to Matrigel, medium can be changed or a second layer of Matrigel can be placed on top of the first layer. Culture medium was changed once every two days.

Development of SV40 Large T-antigen Transformed Type I and Type II HBEC.

The normal HBECs were transfected either with SV40 DNA (GIBCO-BRL, Gaithersburg, MD) (M13SV1 derived from the primary HBEC, HME13) or with a plasmid carrying an origin-defective SV40 genome expressing a wild type large T-antigen (PRNS-1, a gift from Johng S. Rhim of the National Cancer Institute) (M15SV1-11 and M15SV21-30 were derived from Type I and Type II HME 15 respectively) by lipofectin (GIBCO-BRL, Gaithersburg, MD). The actively proliferating colonies were selected in the background of senescent or near senescent cells (M13SV1 from Type I HBEC) or by their resistance to G418 (0.4 mg/ml for M15SV1-11 from Type I HBEC and 0.15 mg/ml for M15SV 21-30 from Type II HBEC). The proliferation potential of transformed clones were determined by their total cumulative population doubling levels (cpdl) using the formula, cpdl = ln(Nf/Ni)/ln 2, where Ni and Nf are initial and final cell numbers, respectively, and ln is the natural log. The initial cell number was $2x10^5$ for each propagation.

During the course of determining potential cpdl for each SV40 transformed cell line, the populations of cells at different cpdl were preserved in liquid nitrogen. For telomerase assay, the cells at early (22-30 cpdl), middle (50-60 cpdl) and late (100-110 cpdl) passages were grown and harvested to prepare cell lysates.

Polymerase Chain Reaction (PCR)-Based Telomerase Assay.

Cells grown to about 50-70% confluency were harvested by trypsinization. After cell counts, the cells were centrifuged to remove trypsin

solution. The cell pellet for each culture was washed with 10 ml phosphate buffered saline (PBS) and then centrifuged to remove PBS. Cells were then suspended at 1 x 10^6 cells per ml in PBS and aliquoted to eppendoff tubes. After cells were centrifuged and PBS carefully removed, the cell pellets were stored at –85°C. For the telomerase assay, the cell pellet was thawed and resuspended in 200 ul of 1x CHAPS lysis buffer/10^6 cells and left on ice for 30 minutes. The samples were spun in a microcentrifuge at 12,000 g for 20 minutes at 4°C. The cell lysate for each sample was aliquoted to several new tubes and stored at –85°C. The original lysate represents the concentration of 5,000 cells/ul. Further dilution of cell lysate was adjusted based on the level of telomerase activity for individual cell line. Telomerase activity was examined by the telomeric repeat amplification protocol (TRAP) assay (17) utilizing TRAPeze™ Telomerase Detection Kit (Oncor, Gaithersburg, MD). This protocol includes primers of a 36 base pairs (bp) internal positive standard for amplification, thus providing a positive control for accurate quantitation of telomerase activity within a linear range. Each analysis included a negative control (CHAPS-lysis buffer instead of cell lysate), heat-inactivated control (sample incubated at 85°C for 10 minutes prior to the assay) and a positive control (breast carcinoma cell line MCF-7). The products of TRAP assay were resolved by electrophoresis in a nondenaturing 12% polyacrylamide gel electrophoresis (PAGE) in a buffer containing 54 mM Tris-HCI (pH8.0), 54 mM boric acid and 1.2 mM EDTA. The gel was stained

with Syber Green (Molecular Probes, Inc., Eugene, OR), and visualized by 302 nm UV transilluminator. Images were captured and analyzed by AlphaImager™ (Alpha Innotech Corporation, San Leandro, CA).

RESULTS

Stem Cell Characteristics of Type I HBEC as Indicated by Organoid Formation and Growth

Previously, we have shown that Type I HBEC have the ability to differentiate into Type II HBEC by cyclic AMP-inducing agents (cholera toxin and forskolin) (14; 18). Additional evidence that Type I cells have stem cell characteristics came from the study of organoid formation and growth on Matrigel. When type I and Type II cells were plated separatedly on top of Matrigel or in between two layers of Matrigel, Type I cells characteristically formed acinar structures which are formed by luminal epithelial cells as shown previously (19) and some limited budding structure formation (Fig. 1B), while Type II cells with basal epithelial phenotypes formed hollow spheres (Fig 1D). When the two Types of cells were plated together on Matrigel, they formed ductal and terminal end bud-like structure (Fig 1F). Since mammary stem cells are known to be present in the end bud for ductal morphogenesis and elongation(20; 21), the ability of Type I HBEC to form these structures strongly indicates that Type I HBEC population contains mammary epithelial stem cells which are capable of giving rise to luminal and basal epithelial cells.

High Susceptibility of Type I HBECs to Immortalization

Both Type I and Type II HBECs were transfected with an origin-defective SV40 genome expressing the wild type large T-antigen (PRNS-1) and selected by G418. Eleven independent clones were isolated from ~3 x 10^6 Type I HBEC, while ten clones were derived from ~4 x 10^6 Type II HBEC. All these Type I and Type II clones were able to acquire extended lifespan (i.e. more than 30 cumulative population doubling level, cpdl; normal HBEC never grew more than 20 cpdl). Therefore, the ability of SV40 large T-antigen transformed Type I and Type II HBEC clones to acquire extended lifespan appears to be comparable. However, the frequencies that E.L. clones became immortal are significantly different between Type I and Type II HBEC derived clones. All of the eleven (100%) E.L. clones derived from Type I cells have become immortal (i.e. actively proliferating after more than 100 cpdl). In comparison, only one out of ten (10%) E.L. clones derived from Type II cells has become immortal. Excluding the immortal clone, the average lifespan of E.L. clones from Type II HBEC was 43 ± 5 cpdl. It is clear that Type I HBECs with stem cell characteristics were more susceptible to immortalization following SV40 large-T antigen transfection.

Telomerase Activity in Primary HBECs.

To study the potential mechanisms underlying the high susceptibility of Type I HBECs to immortalization, we first studied the telomerase activities in primary Type I and Type II HBECs without SV40 transfection.

As shown in Fig. 2A, telomerase activity was present in both Type I (lane 2-4) and Type II (lane 6-8) HBECs. The level of activity, however, was weak compared to the breast carcinoma cell line, MCF-7 (lane 14). At 1/10 of the cells used in the assay (250 vs. 2,500), the telomerase activity in MCF-7 was significantly higher than Type I and Type II HBECs. In contrast, telomerase activity was undetectable in human breast stromal fibroblasts at early passage (lane 10-12). To exclude the possibility that low level of telomerase activity from primary HBECs may be due to the existence of potential telomerase inhibitors, assays with mixed cell lysates from both HBEC and MCF-7 were carried out (Fig. 2B). Since HBECs did not affect the telomerase activity of MCF-7 cells in the lysate mixtures (lane 2 and lane 5), no telomerase inhibitor was detected from either Type I or Type II HBECs.

The Weak Telomerase Activity in Normal HBEC is Insufficient for Immortalization

Since a low level of telomerase activity was found in primary HBECs, it is important to study whether further telomerase activation is required for these cells to become immortal (to overcome M2 crisis). We have investigated the telomerase activity during the course of cell passage toward crisis or immortalization (Fig. 3). Same level of telomerase activity was found in early passage (before 50 cpdl) as compared to that of normal HBECs. There was a dramatic increase of the activity around 50 to 60 cpdl for clones that became

immortal, and the high activity remained thereafter. In contrast, clones without telomerase activation always stopped proliferating at crisis, shown later in Fig. 4B. So, the low level of telomerase activity detected in normal HBEC seems inadequate to sustain cell grow beyond crisis period.

High Susceptibility of Type I HBEC to Telomerase Activation.

Cell samples collected in three time points which represent the stage of early passage (extended lifespan, ~30 cpdl), the mid-passage (around potential crisis period, ~ 50-60 cpdl) and the late passage (immortalization, > 100 cpdl) respectively were assayed. All of the eleven Type I HBEC derived clones showed a significant increase of telomerase activity at mid or late passage (part of the results are shown in Fig. 4A). However, among Type II HBEC derived clones, only the one became immortal has shown activated telomerase activity. Five of the nine Type II clones that did not become immortal were randomly selected for telomerase activity assay. All these clones completely lost their activity at mid-passage (Fig. 4B). As summarized in Table 1, all of Type I and Type II clones with an activated telomerase activity have become immortal eventually. These results suggest that high immortalization rate of Type I HBEC clones is very likely due to their high capacity of telomerase activation in M2 phase.

DISCUSSION

The major findings of this study are : a) Type I HBEC were more susceptible to immortalization (11/11) than Type II HBEC (1/10) following SV40 large T-antigen transfection; b) both normal Type I and Type II HBECs had low level of telomerase activity which was insufficient for maintaining cell proliferation capacity unless it could be activated; c) high capacity of telomerase activation for Type I HBEC had resulted in more efficient immortalization compared to Type II HBEC. Since Type I HBECs have stem cell characteristics, these results suggest a putative mechanism that stem cells are very likely to be the target cells for neoplastic transformation.

We have previously shown that Type I and Type II HBEC differ substantially in their response to an oncogenic (SV40) stimulus, i.e., Type I cells had the ability of anchorage independent growth (AIG^+), whereas SV40 transformed Type II cells totally lack the ability to grow in soft agar (AIG^-)(14). We were able to confirm that SV40 transformed Type I HBEC clones obtained in this study are capable of AIG (8/8, our unpublished results). Therefore, this study provides additional and stronger evidence that Type I HBEC are more susceptible to the initial neoplastic transformation by SV40 to acquire two major and common tumor cell phenotypes, i.e., AIG^+ and immortality.

Previous studies have shown that candidate stem cells from the adult tissues such as human epidermal cells from the basal layer (22) or

hematopoietic progenitor cells (23-26) have telomerase activity. The specific cells with telomerase activity in these cell populations assayed, however, were not well characterized. In this regard, the characteristics of our two types of HBEC are well defined: Type II cells with basal epithelial cell phenotypes and Type I cells with luminal and stem cell characteristics. Besides differences in gene expression (i.e., cytokeratin, α6 integrin, connexins and estrogen receptor), the clear difference in cell and colony morphology between the two ensures that we have pure populations of either cell types.

While it is reasonable to know that the Type I cells with stem cell characteristics have telomerase activity, the presence of telomerase activity in Type II HBEC appears difficult to reconcile with previous reports that normal breast tissues (17) and normal HBEC (27) do not have telomerase activity. It is possible that our Type II cells are newly derived from Type I cells. Therefore, it could be considered as progenitor cells for basal epithelial cells. The low telomerase activity in normal HBEC and early passage SV40 transformed HBEC was not due to lower proportion of cycling cells since all cell cultures were harvested at near log phase of cell growth. It excludes the possibility that low level of telomerase activity are due to low rate of cell growth. While this low level of telomerase activity appears not sufficient for unlimited growth for SV40 transfected cells.

The ability of Type I HBEC to form budding /ductal structures strongly support the presence of mammary epithelial stem cells. The similarity of phenotypes between breast carcinoma cells and Type I cells (i.e. deficiecny in GJIC, expression of estrogen receptors and luminal epithelial cell markers) further indicates that breast cancers could be derived from Type I cells, as a result of blocked differentiation, consisting with the oncogeny as blocked ontogeny theory of carcinogenesis (3).

The high susceptibility of SV40 transfected Type I cells to immortalization may provide a basis for the idea that stem cells are major targets for neoplastic transformation. In turn, the high susceptibility to telomerase activation appears to be a mechanism that Type I cells become immortal more efficient than Type II HBEC. However, the mechanism that telomerase is more easily activated in Type I cells remains unknown, which is the focus of our future study. In human tumor cells, the activity of telomerase has been shown to be cell cycle-dependent, with highest level detected in S-phase and lowest level was found in cells arrested at G_2/M (28). Overexpression of cyclin D1 and/or cyclin E was a typical feature of breast cancers with high telomerase activity (29). It is likely that telomerase expression may be partly under the same control that regulates G_1 to S phase transition. In this respect, our previous observation that the expression of Cyclin D_1 was higher in Type I HBEC than Type II cells (15) may be relevant.

A previous report concludes that telomerase activity may not be a biomarker for malignant transformation since it is present in both normal and tumor cells (30). This conclusion may be misleading since the comparison was made between tumor cells and unrelated normal cells and the quantitative difference was not taken into consideration. Using a well-characterized quantitative assay, we found that, although both normal and immortal or tumorigenic cells have telomerase activity, the activities in immortal or tumorigenic cells are dramatically higher than that in normal cells. Indeed, the transition from low to high telomerase activity may be an indicator when the immortal cells are present in the population during the course of immortalization.

This study also has implications concerning the mechanism of carcinogenic initiation of breast epithelial cells. Since the function of SV40 large T-antigen is to inactivate p53 and pRb and to induce CCAAT box binding factor that transactivates cell cycle regulating genes such as cdc2 (31), alteration in cell cycle regulation appears to be the major event in transforming a normal HBEC to a cell with extended lifespan. The subsequent conversion of a cell with extended lifespan to an immortal cell clearly involves the activation of telomerase, as shown in this study. This is consistent with a recent report that both Rb/$P16^{INK4a}$ inactivation and telomerase activity are required to immortalize human breast epithelial cells (27).

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FIGURE LEGENDS

Figure 1. HBEC colonies formed on plastic and organoids on Matrigel formed by two types of normal HBECs. Type I and Type II colonies developed on plastic are morphologically distinguishable. On Matrigel, Type II cells typically formed hollow spheres (H.S.), while Type I cells formed limited number of bud-like (B.) structure. The combination of Type I and Type II cells in 1:2 or 1:3 ratios can generate many budding (B.)/ductal (D.) structure in 2-3 weeks.

Figure 2. Telomerase activity in Type I and Type II HBECs. **A.** Telomerase activity was detected in cell lysates derived from different cell numbers as shown by PCR-based telomeric repeat amplification protocol (TRAP) as described in Materials and Methods. Lane 1 and lanes 5, 9, 13, 15 represent negative controls without cell lysate and heat inactivated controls, respectively. Type I HBEC, Type II HBEC and fibroblasts used in this assay were all derived from mammary tissue of one patient. As a positive control, the breast carcinoma cell line, MCF-7, showed a high level of telomerase activity (lane 14, 250 cells). Low levels of telomerase activity was detected in both normal Type I (lanes 2, 3, 4) and Type II (lanes 6, 7, 8) HBECs; the activity in fibroblasts was undetectable (lanes 10, 11, 12). **B.** Absence of telomerase inhibitor in Type I and Type II HBECs. Telomerase activity from a mixture of telomerase-positive MCF-7 cell lysate and HBEC lysate was examined to clarify that low levels of

telomerase activity in both types of HBECs were not due to the presence of telomerase inhibitors in these cells. Lane 3 and lane 6 show the telomerase activity in cell lysates derived from 100 MCF-7 cells, whereas lane 2 and lane 5 show the telomerase activities in the cell lysate mixtures of 100 Type I or 100 Type II HBEC with 100 MCF-7 cells, respectively. No significant difference of telomerase activity was found between MCF-7 lysate and the mixtures.

Figure 3. Telomerase activation during the course of immortalization. A detailed analysis of telomerase activity at the expanded range of cpdl for a SV40 transformed Type I HBEC line (M15SV8). The elevation of telomerase activity was found at 50-60 cpdl for both cell lines. Cell lysate derived from 500 cells was used for telomerase assay for each sample.

Figure 4. Difference of telomerase activation between Type I and Type II HBECs derived clones. **A.** Telomerase activity in SV40 transformed Type I HBEC derived cell lines. Five SV40 transformed cell lines (M15SV2, M15SV7, M15SV9, M15SV10, and M15SV11) which became immortal were examined for telomerase activity at low passage (L, ~22-30 cpdl), middle passage (M, ~50-60 cpdl), and high passage (H, >100 cpdl). In all these cell lines, telomerase activities were elevated at mid- or late passage. Cell lysate derived from 500 cells was used for telomerase assay for each

sample. **B.** Telomerase activity in SV40 transformed Type II HBEC. The majority of SV40 transfected Type II clones (nine out of ten) did not become immortal beyond extended lifespan. Five (M15SV21, 24, 26, 27, 29) among these nine clones were analyzed for telomerase activity at low passage (L, ~25 cpdl) and mid-passage (M, ~40 to 50 cpdl). Telomerase activities in these clones diminished from low to middle passage when they were approaching senescence. The only immortalized Type II HBEC (M15SV30) showed telomerase activation at middle passage (lane 11). Cell lysate derived from 500 cells was used for telomerase assay for each sample.

Table 1 Telomerase activity in SV40 large T-antigen transformed Type I and Type II HBEC at different cumulative population doubling level (cpdl).

Figure 1

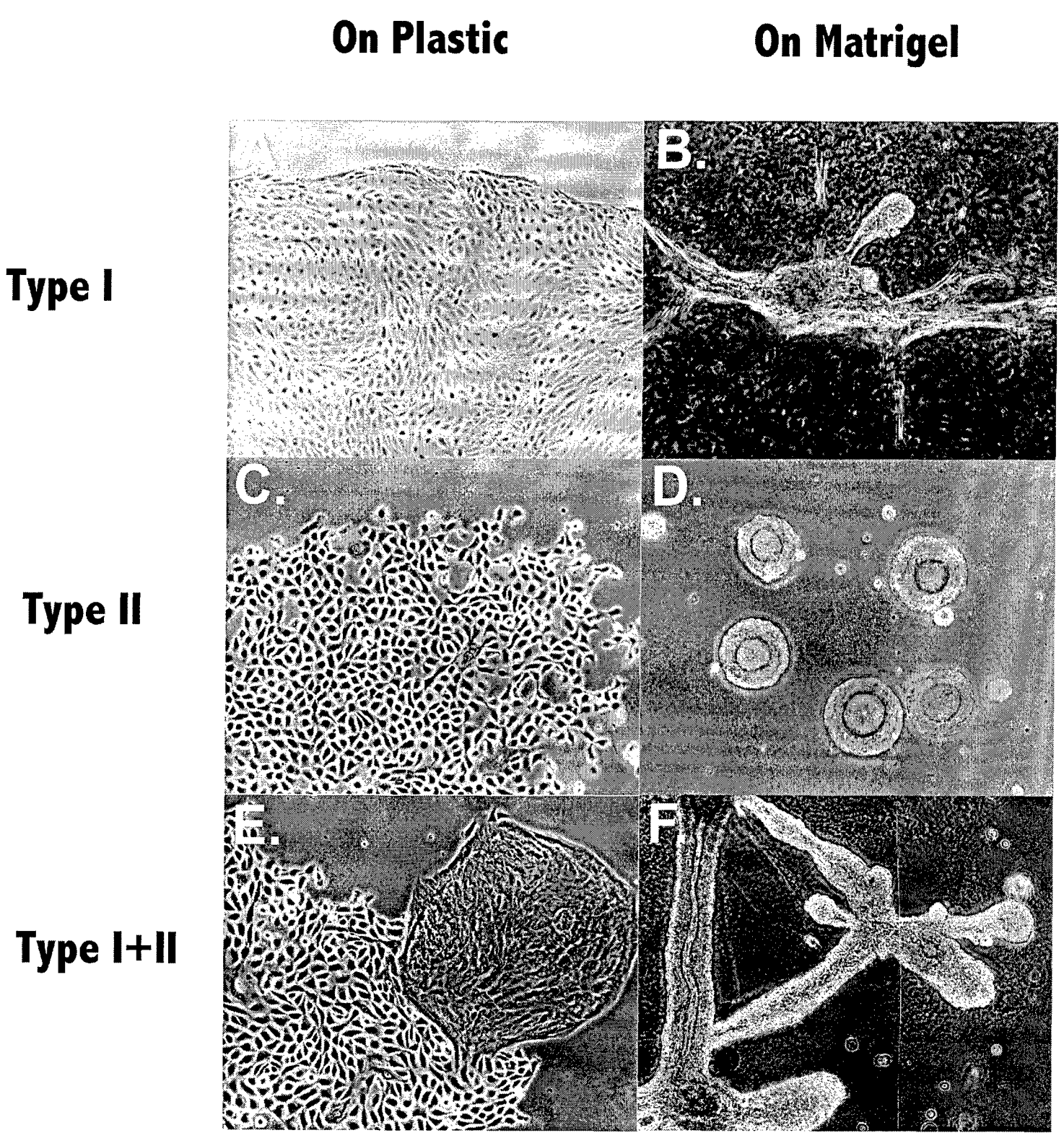
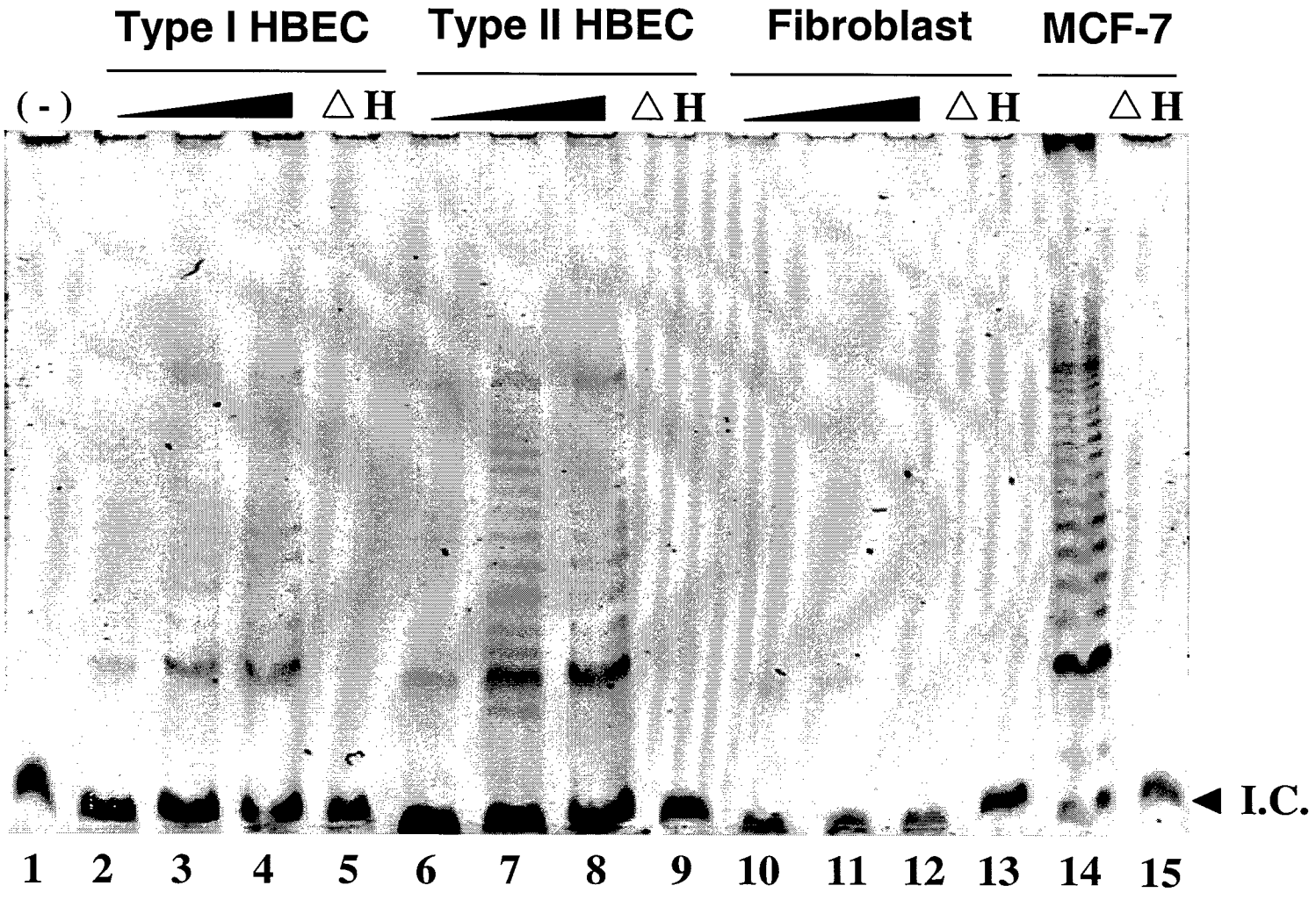
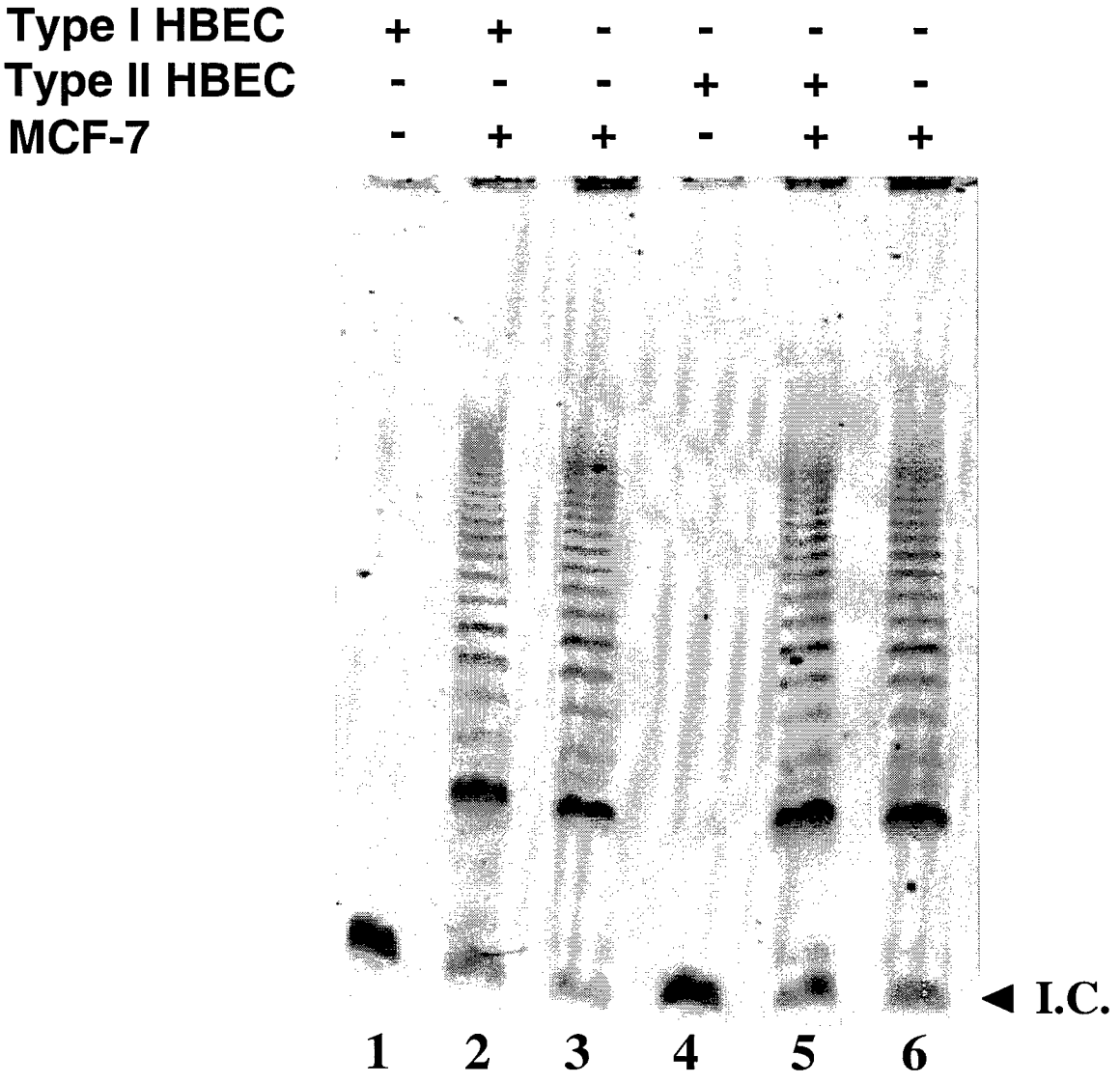
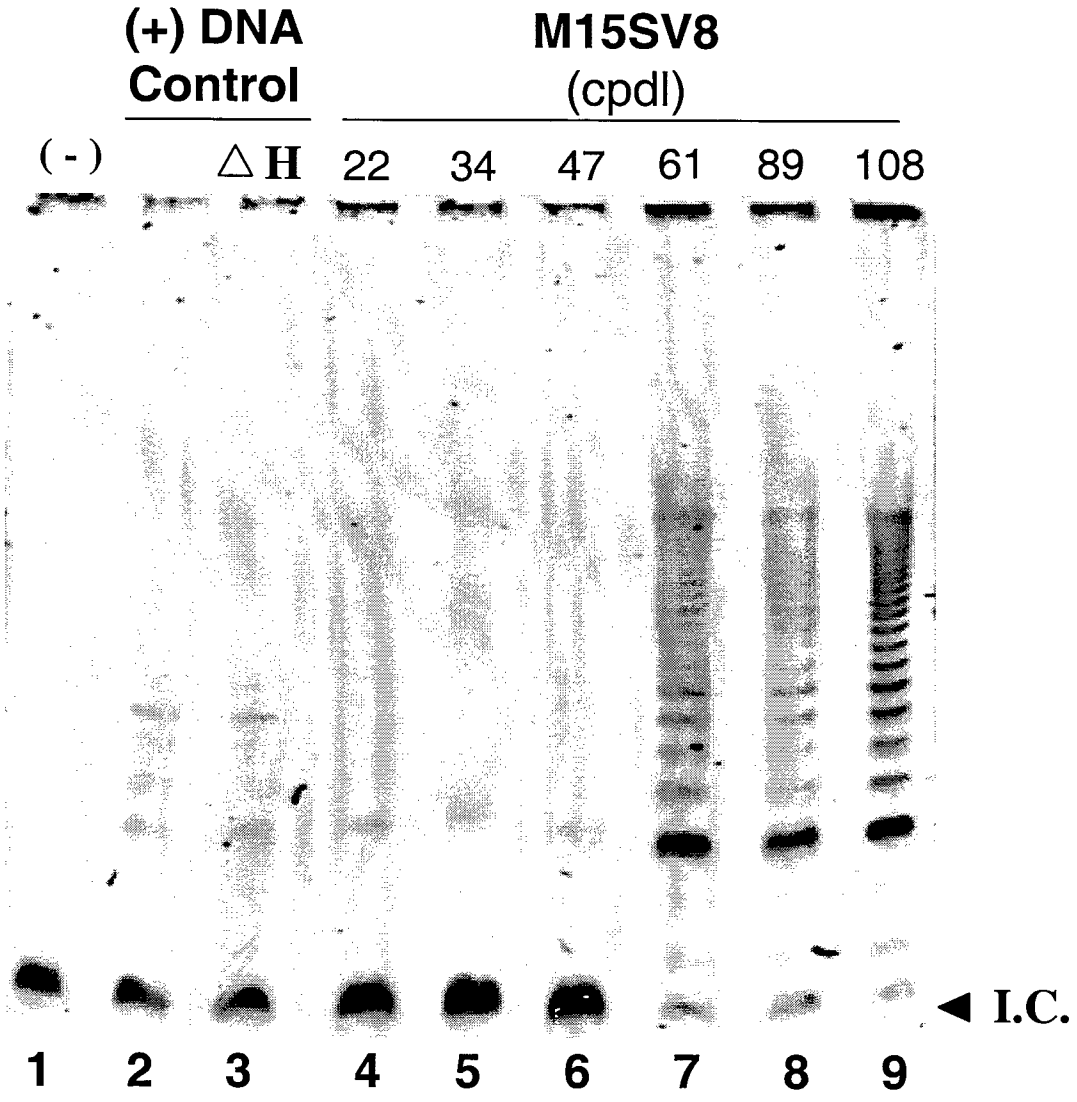


Figure 2A


Type I HBEC
Type II HBEC
Fibroblast
MCF-7
(-)
△H
△H
△H
△H
◀ I.C.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Type I HBEC + + - - - -
Type II HBEC - - - + + -
MCF-7 - + + - + +
◄ I.C.
1 2 3 4 5 6


(+) DNA Control
M15SV8 (cpdl)
(-)
△H
22
34
47
61
89
108
◀ I.C.
1
2
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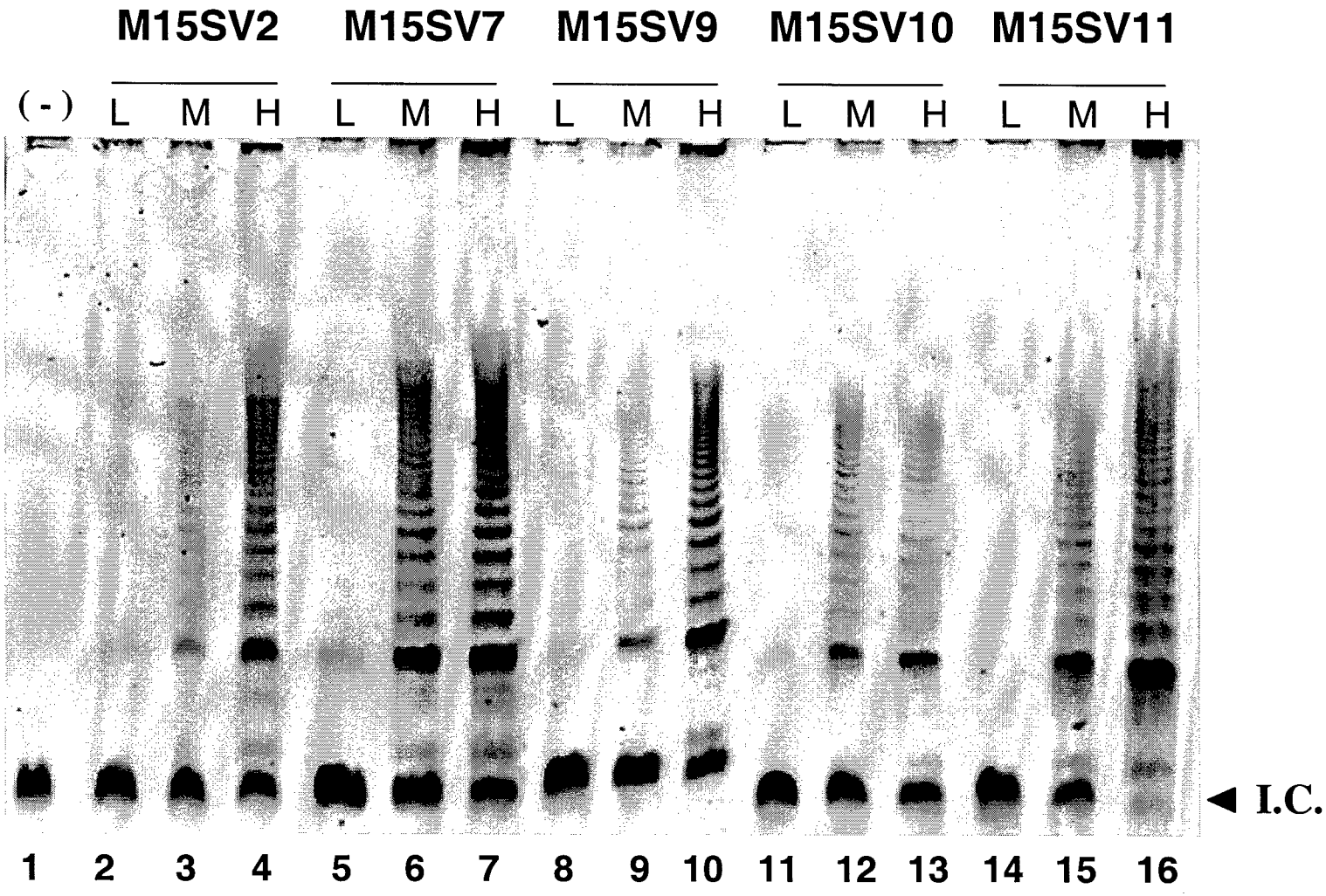
M15SV2
M15SV7
M15SV9
M15SV10
M15SV11
(-)
L M H L M H L M H L M H L M H
◄ I.C.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4B

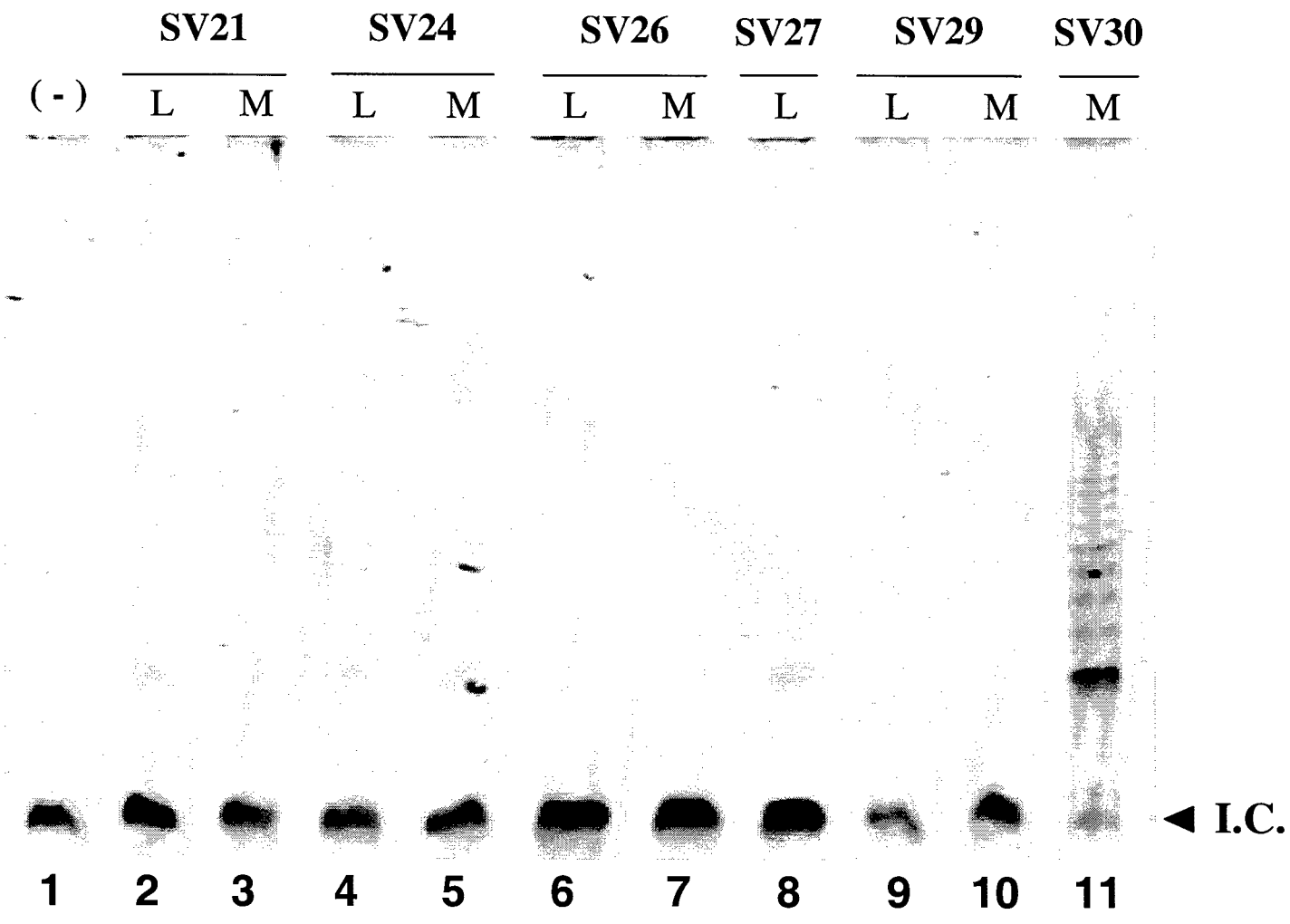

Table I

Cell Line	Parental Cell Type	Passage Levels			Immortalized
		Low	Middle	High	
M15SV1	Type I	ND	++++	++++	Yes
M15SV2	Type I	+	+	++++	Yes
M15SV3	Type I	+	+	++++	Yes
M15SV4	Type I	+	++++	++++	Yes
M15SV5	Type I	+	+++	+++	Yes
M15SV6	Type I	+	++++	++++	Yes
M15SV7	Type I	+	++++	++++	Yes
M15SV8	Type I	+	++++	++++	Yes
M15SV9	Type I	+	++++	++++	Yes
M15SV10	Type I	+	++++	++++	Yes
M15SV11	Type I	+	++++	++++	Yes
M15SV21	Type II	+	±	(Senescent)	No
M15SV24	Type II	+	±	(Senescent)	No
M15SV26	Type II	+	±	(Senescent)	No
M15SV27	Type II	+	ND	(Senescent)	No
M15SV29	Type II	+	±	(Senescent)	No
M15SV30	Type II	ND	++++	ND	Yes

*±, +, ++++ denote very weak, low and high telomerase activity, respectively; ND, not done.

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ROLES OF IONIZING RADIATION IN NEOPLASTIC TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

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Number of Figure: 1
Number of Table: 1

INTRODUCTION

Although advances in breast cancer research have been made in recent years, the etiology of breast cancer is not fully understood. However, a major role of lifetime exposure to estrogen in breast cancer seems to be well established. This estrogen-breast cancer theory is supported by the age-incidence curve which shows a lower rate of linear increase of breast cancer incidence after menopause (1) as well as by chemoprevention studies with the anti-estrogen tamoxifen and estrogen substitute, raloxifene, which reduced the risk of breast cancer. Many environmental agents might cause breast cancer by functioning as xenoestrogens or as estrogen potentiating factors (2). While the evidence for these compounds is not yet substantial, ionizing radiation is considered as the most well-established breast cancer carcinogen based on the excess risk of breast cancer associated with Japanese women exposed to atomic bomb radiation in Hiroshima and Nagasaki (3), as well as from radiation therapy of Hodgkin's disease (4) and other non-malignant conditions (5).

Although ionizing radiation as a carcinogen for breast cancer is convincing, questions concerning its mechanisms of action remain to be elucidated. These include: (a) **At which stage of carcinogenesis does the agent exert its effect?**; (b) **Is breast cancer caused by genetic and/or epigenetic effects of ionizing radiation?**; (c) **Which genes are affected by the agent and responsible for neoplastic transformation?**; and (d) **Are there target cells in mammary glands that specifically respond to the agent to induce breast cancer?** The latter is related to **the role of stem cells and differentiation in carcinogenesis.** This paper addresses these questions and suggests an *in vitro* cell culture system for these studies.

IONIZING RADIATION AFFECTS DIFFERENT STAGES OF CARCINOGENESIS

From *in vitro* studies, ionizing radiation has been shown to immortalize or neoplastically transform normal human fibroblasts (6-7) and keratinocytes (8) and to induce neoplastic conversion of immortalized human epidermal keratinocytes (9). For human breast epithelial cells, repeated γ-irradiation (2 Gy x 15) has been reported to produce an immortal cell line (10). These cells, although unable to grow in soft agar, were able to form tumors in nude mice.

An immortal non-tumorigenic cell line, derived from a normal human breast epithelial cell (HBEC) type with luminal and stem cell characteristics after transfection with SV40 large T-antigen, can be converted by x-rays (2 Gy x 2) into weakly tumorigenic cell lines which were selected as large fast-growing colonies in soft agar (11). These weakly tumorigenic cells, but not the parental immortal cells, responded to the ectopic expression of a mutated neu oncogene to enhance their tumorigenicity in nude mice. Although $p185^{C\text{-}erbB2/neu}$ proteins are highly expressed in both immortal and x-ray transformed tumorigenic cell lines after the infection with the neu oncogene, only in the latter were the $p185^{C\text{-}erbB2/neu}$ proteins tyrosine phosphorylated (11). The mechanism for the differential response is not known. One possible mechanism is that a protein tyrosine phosphatase is present in the immortal cell line but not in the weakly tumorigenic cell lines transformed by x-rays. The possible deletion of a protein tyrosine phosphatase gene such as the PTEN tumor suppressor (12) is to be investigated. In a different series of experiments, we found that normal human breast epithelial cells can be converted to cell lines with extended lifespan (i.e., bypassing senescence). It is clear that ionizing radiation is capable of extending the lifespan, immortalizing and developing tumorigenicity in HBECs.

GENETIC AND EPIGENETIC EFFECTS OF IONIZING RADIATION ON BREAST CARCINOGENESIS

Besides being a well-known physical mutagen, ionizing radiation has been shown to induce the expression of a variety of genes including DNA-binding transcription factors, growth factor and growth-related genes, proto-oncogenes, signal transduction and DNA repair genes (13-14). The importance of these effects on radiation-induced breast cancer remains to be determined.

Ataxia-telangiectasia (AT) heterozygotes who make up about 1 percent of the general population, have a 5.1-fold excess risk of breast cancer in women (15). The product of ATM gene, which is mutated in patients with AT, has been shown to phosphorylate IKβ-α (16). This suggests a role of ATM gene in NF-kβ activation and cell proliferation (16). Furthermore, AT cells were found to be more susceptible to the transcriptional activation of C-myc and XRCC1 than other human cells after x-ray irradiation (17).

GENES AFFECTED BY IONIZING RADIATION AND RELATED TO NEOPLASTIC TRANSFORMATION

Many genes with diverse functions have been shown to be inducible by ionizing radiation (13-14). These genes may be involved in cellular recovery and adaptation or could trigger signal transduction and transcriptional activation related to tumor initiation, promotion or metastasis.

There are few genes that are known to be mutated by ionizing radiation and shown to be important for neoplastic progression. The p53 tumor suppressor gene appears to be the best known. Skin fibroblast cultures, derived from Li-Fraumeni Syndrome (LFS), did not become immortal in culture but have been immortalized by x-ray irradiation (one of six cultures) (18). These results suggest that loss of the wild type p53 gene was necessary but not sufficient for immortalization. It is interesting that the only human breast epithelial cell line immortalized by γ-irradiation also lacked the p53 tumor suppressor protein (10). However, the p53 mutation may not be the earliest tumor initiation event, since p53 mutations have been found in mammary ductal carcinoma *in situ* but not in epithelial hyperplasia (19).

ROLE OF STEM CELL/DIFFERENTIATION IN BREAST CARCINOGENESIS

Epidemiological studies indicate that nulliparous or late parous increased the risk for breast cancer (20-21); lifetime lactation reduced the risk (21). This could be due to the induced mammary gland differentiation (22). Furthermore, from the atomic bomb studies in Japan, younger women with undifferentiated mammary glands were found to be at higher risk for radiation-induced breast cancer (23).

Recently, it has been shown that normal HBEC cultures lacking the $p16^{INK4a}$ expression can be immortalized by ectopic expression of the human telomerase gene (hTERT) whereas HBEC cultures expressing the $p16^{INK4a}$ failed to do so (24). Using a different culture system, we also found that HBEC cultures (Type II cells) derived from reduction mammoplasty vary in the expression of $p16^{INK4a}$. In our attempts to extend the lifespan by x-ray irradiation, we found that one culture deficient in $p16^{INK4a}$ expression was more susceptible to x-ray irradiation to acquire extended lifespan (average cumulative population doubling of 24 clones = 32) than 3 other cultures derived from different patients that expressed $p16^{INK4a}$ (Table I and Figure 1). These extended life (EL) clones eventually stopped proliferating. The p53 and p21 were frequently and concomitantly elevated in these EL clones. However, they appear to contain the wild-type p53

since the cells showed radiation-induced G1 arrest (C.J. Albert Liu, MS Thesis, Michigan State University, 1997).

Table I. Induction of extended lifespan (EL) clones from different HBEC cultures by x-ray irradiation

HBEC Culture	p16INK4a	No. of Experiments	X-ray Treatment[a] (dose x times)	Independent EL Clones Obtained
HME-5	+[b]	2	2 Gy x 4	None
HME12	+	2	2 Gy x 4	None
HME14	+	3	2 Gy x 3	None
HME14	+	2	4 Gy + 2 Gy x 3	None
HME15	–	1	2 Gy x 4	11
HME15	–	1	2 Gy x 5	5
HME15	–	1	2 Gy x 6	6
HME15	–	1	4 Gy x 5	2

[a]Approximately 5 x 10^6 Type II HBECs were used in the initial treatment with x-rays.
[b]$p16^{INK4a}$ expression determined by Western blot analysis shown in Fig. 1.

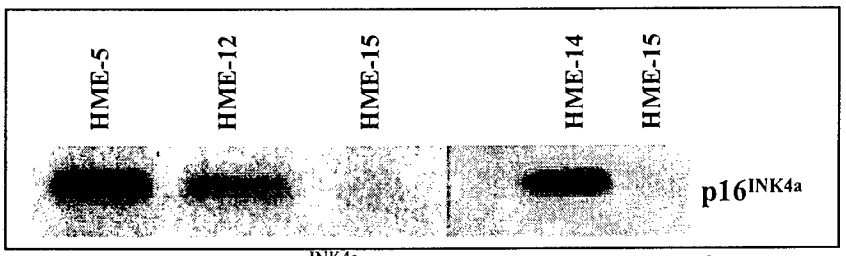

Fig. 1. Expression of $p16^{INK4a}$ in different normal HBEC cultures

We have previously developed two types of normal HBEC from reduction mammoplasty (25). Type I HBECs, in contrast to Type II cells that expressed basal epithelial phenotype, were deficient in gap junctional intercellular communication, expressed estrogen receptors and luminal epithelial cell markers, and showed stem cell characteristics (i.e., the ability to differentiate into other cell type and to form budding/ductal structures in Matrigel) (25-26). Although both cell types can be equally transformed by SV40 large T-antigen to acquire extended lifespan, they differ in the ability to become immortal spontaneously (11/11 Type I EL clones became immortal compared to 1/10 for Type II EL clones). Both parental Type I and Type II cells, as well as their transformed EL clones at early passages (~30 cpd), showed a low level of telomerase activity measured by the TRAP assay. For all the 11 Type I and 1 Type II EL clones that became immortal, telomerase activities were invariably activated at middle (~60 cpd) or late passage (~100 cpd). For the senescent EL clones, the telomerase activities were found to be diminished at mid-passage before the end of lifespan. Thus, Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalization, a basis that they may be major target cells for breast carcinogenesis (W. Sun, K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang, Submitted for publication). We would like to use this cell culture system to test if Type I HBECs with stem cell features are also more susceptible to neoplastic transformation by ionizing radiation.

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Stem Cell Differentiation and Reduction as a Potential Mechanism for Chemoprevention of Breast Cancer+

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ABSTRACT

From laboratory animal studies of genistein and human chorionic gonadotropin, induction of mammary gland differentiation could be a mechanism for chemoprevention. Besides incorporating this strategy into a blueprint for cancer prevention proposed by Weinstein (1991), we have reviewed recently reported mechanisms of action of various chemopreventive agents and potential chemopreventive agents for breast cancer. Furthermore, we described a recently characterized normal human breast epithelial cell type with stem cell characteristics (Type I HBEC). These cells have been shown to be more susceptible to neoplastic transformation than the conventional cell type (Type II HBEC) and could be differentiated into other cell types. This *in vitro* differentiation model could be useful to identify chemopreventive agents that induce mammary gland differentiation. Our study with two putative breast cancer chemopreventive agents, genistein and vitamin D_3, indeed, shows that these compounds could preferentially inhibit the growth and/or differentiate Type I HBEC. These results support the hypothesis that chemopreventive agents might differentiate and/or inhibit the growth of stem cells, thereby reducing the target cells for neoplastic transformation.

Key words: Stem cell differentiation and inhibition; Chemoprevention mechanisms; Breast cancer.

INTRODUCTION

In his review on recent progress and future opportunities for cancer prevention,[1] We-

+ This paper is dedicated to Professor Kuang-Chao Wang on the occasion of his honorary retirement.

instein believed that knowledge gathered from studies of cancer epidemiology, carcinogenesis, biochemistry and molecular biology is sufficient to provide new strategies and to draft a blueprint for a comprehensive approach to cancer prevention. The plan he proposed includes: (a) expanded epidemiological and laboratory studies to identify the specific causes of human cancers; (b) intervention studies, such as risk reduction by the cessation of cigarette smoking, dietary modifications, chemoprevention, and the development of vaccines for viral agents implicated in human cancer and; (c) early detection and screening. He further modified strategies for cancer chemoprevention from one developed by Wattenberg.[2] In this scheme, various chemopreventive agents may intervene at four steps:(a) carcinogen formation, absorption; (b) carcinogen activation, DNA damage, mutagenesis; (c) detoxification of carcinogens and; (d) cell proliferation, promotion and tumor formation. The last step refers to initiated or premalignant cells. Conspicuously lacking in the scheme, however, is the reduction of normal target cells for neoplastic transformation although stem cells have been hypothesized to be major target cells for neoplastic transformation[3-5] and chemopreventive agents (e. g. genistein, human chorionic gonadotropin, hCG) have been shown to affect differentiation of stem or target cells.[6-7] Apparently, the reason that this strategy was not considered in chemoprevention is due to the lack of evidence about the involvement of stem cells in carcinogenesis and chemoprevention. In the past few years, we have characterized a normal human breast epithelial cell type with stem cell characteristics. Furthermore, we have shown that this cell type is more susceptible to neoplastic transformation. This provides an opportunity to test the hypothesis that some chemopreventive agents might function by inducing the differentiation and inhibiting the growth of stem cells. Indeed, the results from studies of two putative breast cancer chemopreventive agents, genistein and vitamin D_3, have provided the evidence to support this hypothesis. The incorporation of this strategy into a modified blueprint for cancer prevention is shown in Table 1.

MECHANISMS OF CHEMOPREVENTION

The blueprint for cancer prevention listed in Table 1 provides a useful system to classify chemopreventive agents according to their mechanisms of function. Many new chemopreventive agents and mechanisms have been reported in the past few years. These will be briefly described as follows.

Reducing Target Cells for Neoplastic Transformation

Epidemiological studies indicate that the life time risk of breast cancer developing in child-bearing women seems to be linearly related to the age at which a women has her first

Table 1. Blueprint for Cancer Prevention*

1. Identify causes of cancer: epidemiology/laboratory studies.
2. Intervention
   A. Risk factor reduction: prevention or reduction of exposure to cancer causing agents; vaccination
   B. Chemoprevention
      a) reducing target cells for neoplastic transformation
      b) prevention of carcinogen formation, absorption
      c) detoxification of carcinogens / prevention of carcinogen activation, DNA dam age, mutagenesis
      d) prevention of proliferation, promotion of premalignant cells
3. Early detection /screening and removal of precancerous cells

*Modified from Weinstein, I. B. *Cancer Research (suppl.)* **1991**, *51*, 5080S-5085S

full-term pregnancy[8] and that breast cancer develops more frequently in those who are nulliparous or late parous.[9-10] This has been explained by John Cairns[3] as related to stem cell multiplication that occurs commencing at the time of puberty and during each ovarian cycle until, but not after, the first pregnancy. Alternatively, pregnancy may induce full differentiation of the gland, with elimination of terminal end buds, resulting in refractoriness of the gland to carcinogenesis.[7] Human chorionic gonadotropin (hCG), a placental hormone, administered exogenously produces in the rat mammary gland a degree of differentiation similar to that induced by pregnancy.[11] In rat, hCG treatment has been shown to significantly reduce the 7,12-dimethyl-benz[a]anthracene (DMBA)-induced mammary adenocarcinomas.[7] Genistein, an isoflavonoid derived from soy, has been reported to suppress DMBA-induced mammary tumor in rats when treated at prepubertal stage and to enhance mammary gland differentiation.[6-12] Although the specific cells differentiated by these compounds were not identified, the treatment apparently reduced or altered a specific population of cells that are targets for carcinogen-induced tumorigenesis.

Prevention of Carcinogen Formation

The mechanisms of many of the chemopreventive agents are not fully understood, making it difficult to organize them in precise category. Wattenburg classifies them in three categories in sequence of time in carcinogenesis when they are effective.[13] The first category of compounds that prevent the formation of carcinogens from precursor substances includes vitamin C or E which prevents the formation of carcinogenic nitrosamines or phenolic diazonium compounds.

Detoxification of Carcinogens/Prevention of Carcinogen Activation, DNA Damage and Mutation

This second category of agents, which Wattenburg refers to as "blocking agents", prevents carcinogenic agents from reaching or reacting with critical target tissues. Examples of blocking agents that act by detoxification of carcinogens are organosulfur compounds found in allium species that induce glutathione S-transferase activity and vitamins (A, C, E), β-carotene, and selenium which are antioxidants and remove the reactive oxygen species (ROS). Although DNA could be a target for ROS generated by oxidative stress, more evidence now have shown that ROS may alter signal transduction[14,15] which may be important for tumor promotion and progression. In human cancer prevention experiments, β-carotene increased risk of lung cancer in current smokers. The results do not reconcile with epidemiological data which indicate increased consumption of β-carotene-rich food and higher blood level of β-carotene are associated with reduced risk of lung cancer. This leads to the hypothesis that β-carotene can be an antioxidant or a prooxidant depending on dose.[16] As listed by Wattenberg,[13] blocking agents that act by inhibiting carcinogen activation[17-19] include aromatic isothiocyanates (in cruciferous vegetables), organosulfur compounds (diallyl disulfide in Allium sp), monoterpenes (D-limonene in citrus fruit oils) and glucosinolates (glucobrassicin, glucotropaeolin in cruciferous vegetables). Other compounds such as sulforaphane, an aliphatic isothiocyanate, and brassinin, an indole-based dithiocarbamate, both found in cruciferous vegetables and induce phase 2 drug metabolizing enzymes[20] and S-allylcysteine, a sulfur compound in garlic powder that inhibits DNA adduct formation induced by DMBA[21] also belong to this list.

Prevention of Proliferation of Precancerous Cells

This class of chemicals was termed by Wattenberg[22] as "suppressing agents" which prevent the evolution of neoplastic process in carcinogen-initiated cells. The mechanisms of function of these chemicals are very diversified and include the following.

A. Hormone Receptor-mediated Gene Expression

The progesterone has been shown to reduce endometrial mitotic activity[23] and to reduce estrogen-enhanced risk of endometrial cancer[24] in estrogen replacement therapy. In a recent large scale Breast Cancer Prevention Trial, tamoxifen, an antiestrogen, has been found to drastically reduce the breast cancer while increasing the uterine cancer.[25]

B. Expression of Genes that Regulate Cell Cycle Progression

Vitamin D_3 has been found to induce the expression of cyclin-dependent kinase (cdk) inhibitor p27 and $p21^{WAF1/CIP1}$. The latter contains vitamin D_3 receptor (VDR)-responsive element in the promoter of the gene.[26] The expression of $p21^{WAF1/CIP1}$ and other genes

might induce cellular differentiation.[26] Similarly, retinoic acid directly signals a decline in cyclin E and cyclin D_1 protein expression through induced proteolysis in human bronchial epithelial cells.[27-28]

C. Induction of Apoptosis

Phenethyl isothiocyancte and other structurally related isothiocyanates have been reported to induce apoptosis and caspase-3-like protease activity.[29] Several other chemopreventive agents such as Sulindac, curcumin and phenylethyl-3-methylcaffeate have also been shown to induce apoptosis in rat colon tumors.[30] The non-steroidal anti-inflammatory drug such as aspirin and Sulindac have been found to reduce colon cancer.[31-32] The chemopreventive action of these compounds and curcumin may be mediated through cyclooxygenase (COX) inhibition which could mediate the induction of apoptosis.[33-34]

D. DNA Methylation

There is evidence that carcinogenesis in rodents is significantly influenced by dietary supplies of lipotropes, a group of nutrients that includes choline, methionine, folic acid and vitamin B12.[35] These compounds regulate the supply and metabolism of methyl groups and affect gene expression through DNA methylation which may play a role in tumor evolution.

E. Prevention of the Blockage of Intercellular Communication

Although the tumor-prevention activities of green and black tea have been demonstrated convincingly in animal studies, such effect has been shown in some, but not other, epidemiological studies.[36] The anti-tumor activity is believed to be mainly due to the antioxidative and antiproliferative effects of polyphenolic compounds in green and black tea. One of the effect of green tea extract is the inhibition of tumor promotion *in vivo* and the prevention of the blockage of gap-junctional intercellular communication induced by tumor promoters.[37-38]

POTENTIAL CHEMOPREVENTIVE AGENTS FOR BREAST CANCER

Before identifying potential agents for chemoprevention trials, it is necessary to know what causes cancer? For breast cancer, besides estrogen and ionizing radiations, other potential carcinogens are not certain. The most convincing evidence that lifetime estrogen exposure causes breast cancer came from age-incidence curve which shows a higher rate of linear rise of incidence with age until menopause, thereafter the rate of rise is considerably diminished.[39] The shift in focus of breast cancer chemoprevention to hormone occurred when epidemiological study failed to find an association between high fat diet and breast cancer.[40] The impressive reduction in risk of contralateral breast cancer by tamoxifen therapy provided the most compelling argument for extending the use of tamoxifen to healthy

women at high risk of breast cancer.[39] The results from such a highly heralded trial (The Breast Cancer Prevention Trial funded by US NCI and publicized in April, 1998) involving 13,388 women, indeed, showed that women who took tamoxifen had a 45 % lower incidence of breast cancer than those who took placebos. However, until further improvement, tamoxifen comes with so many caveats (i.e. increasing uterine cancer and blood clotting) that it won't help most women.

The estrogen-breast cancer connection may be found in comparative studies of African-American and US white women. African-American women have earlier menarche than white US women, and an early menarche may be associated with higher estrogen levels in adulthood.[41-42] The higher baseline estrogen level in African-American women have been confirmed[43] and could partly explain why African-American under 40 year of age have a higher risk of breast cancer than other women in that age group.[44] On the other hand, the late menarche and low estrogen level in Chinese women may account for their lower breast cancer risk relative to US white women.[45] Based on this estrogen theory, dietary modification to reduce estrogen level may be an important way to reduce breast cancer. Several reports have shown that low fat and/or high fiber could significantly reduce the estrogen levels.[46-51]

The effects of fibers could be mediated by diverse mechanisms including the reduction of enterohepatic circulation of estrogen by (1) trapping unconjugated estrogens and (2) changing the bowel flora in favor of bacteria with low β-glucuronidase activity which increases fecal excretion and lowers plasma levels of estrogens.[52] In animal experiment, an equal combination of psyllium (a soluble fiber that suppresses β-glucuronidase) and wheat bran (an insoluble fiber that traps estrogen) produced the strongest protection against N-methylnitrosourea-induced mammary tumors.[53] The fiber-estrogen theory, however, is not consistent with the lack of variation in serum and urinary estrogen levels among treatment groups,[53] and the tumor prevention effect observed in both ovariectomized and nonovariectomized rats.[54] In human epidemiological studies, the chemopreventive effects were not consistently observed.[55-58]

The 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$ D_3], the hormonally active form of vitamin D, is a major regulator of calcium and phosphorus levels in the blood and bone formation/remodeling.[59] 1,25-$(OH)_2$ D_3 can also elicit growth inhibitory and differentiation effects on a variety of cell types.[60] These effects of 1,25-$(OH)_2$ D_3 may be mediated by vitamin D_3 receptor (VDR) on genes containing the vitamin D response element (VDRE) or nuclear proteins binding to a c-myc intron element. C-myc and the cell-cycle regulatory gene, $p21^{WAF1/CIP1}$ [61] appear to be major target genes. A recent review of epidemiological and laboratory studies found evidence that sunlight deprivation and the associated reduction in blood levels of vitamin D metabolites may lead to an increase in breast cancer. In a case-

control study of breast cancer risks and blood 1,25-$(OH)_2$ D_3 levels, the odd ratio for the lowest relative to the highest quartile was found to be 4.8.[62] These data support the hypothesis that 1,25-$(OH)_2$ D_3 could be an important chemopreventive agent for breast cancer.

Among Western countries, the breast cancer rates are relatively low in Mediterranean countries.[63] The traditional Mediterranean diet is relatively rich in carbohydrates, vegetables and fruits, but not the total fat intake due to the high consumption of olive oil.[64] A case-control study of more than 5000 women in Italy to define low-risk diet for breast cancer has identified the consumption of vegetables, olive, and seed oil and fruit as being protective for breast cancer.[65]

Poland is another Western country with low incidence of breast cancer (about one-third of the incidence rate of U.S. women). The polish immigrants to U.S., however, had an incidence rate approaching that of U.S. women.[66] Dietary factors such as phytochemicals, micronutrients and fiber present in Cruciferous vegetables are suspected to be responsible.[67] High intake of fruits, vegetables, carotenoid vitamin A has been associated with a lower risk of breast cancer in epidemiological studies,[68-71] although other studies do not support this conclusion.[58,72,73]

DIFFERENTIATION OF MAMMARY EPITHELIAL STEM CELLS AS A POTENTIAL MECHANISM FOR CHEMOPREVENTION OF BREAST CANCER

As mentioned previously, the study of genistein and human chorionic gonadotropin in rats indicates that induction of mammary gland differentiation could be a mechanism of chemoprevention. In our laboratory, we have developed a culture method to grow two types of normal human breast epithelial cells (HBEC) from reduction mammoplasty.[74] Type II HBEC show basal epithelial cell phenotypes. Type I HBEC are deficient in gap-junctional intercellular communication, and express luminal epithelial cell markers and estrogen receptors.[74,75] Significantly, Type I HBEC also show stem cell characteristics (i.e. the ability to differentiate into Type II HBEC by cyclic AMP-inducing agents and to form budding/ductal structures on Matrigel) and are more susceptible to neoplastic transformation.[74,76] This cell culture system provides an *in vitro* model to study whether chemopreventive agents could induce differentiation of mammary epithelial stem cells, thereby reducing target cells for neoplastic transformation. Two potential chemopreventive agents were chosen for this study: genistein and Vitamin D_3. The former, a component of soy products which are present in traditional diet of oriental women. Epidemiological studies have demonstrated a relationship between a diet high in soy and a low incidence of breast cancer.[77,78] There is also evidence correlating low blood level of vitamin D_3 and a high risk

for breast cancer for American women as mentioned previously.[62] We have studied the effects of these two compounds on cell differentiation, cell growth and cell cycle progression in Type I and Type II HBEC.

Genistein

Genistein has many biological and biochemical effects (see references in 6) including the promoting effect at physiological concentrations on cell growth *in vitro* and tumor growth *in vivo*[79] which is mediated by the estrogen receptor. At high concentrations, genistein may inhibit cell growth[79] and tumor growth[80] by different mechanisms. In our

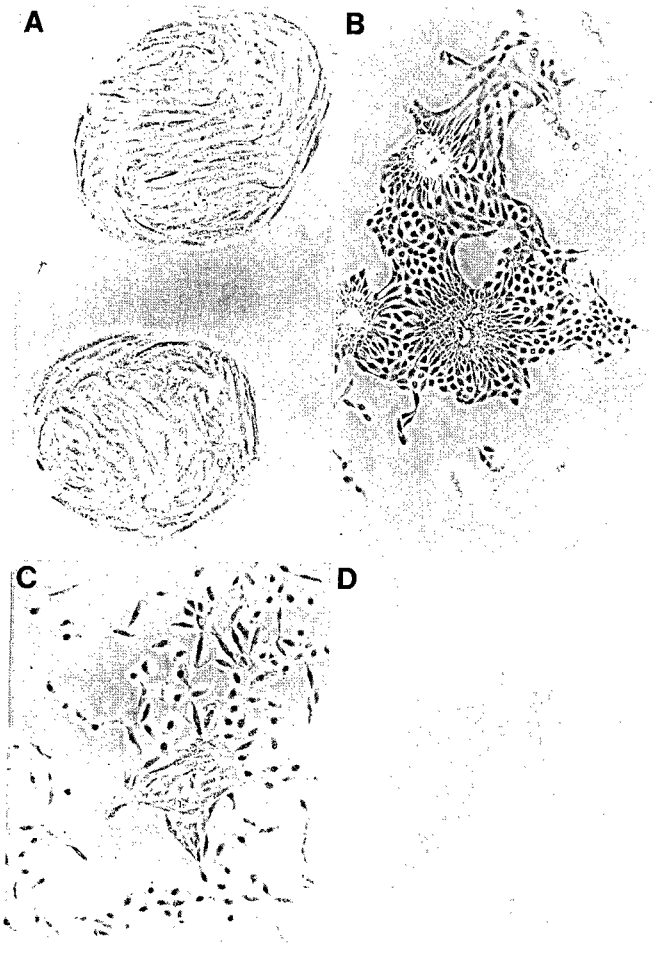

Fig. 1. Induction of differentiation of Type I to Type II human breast epithelial cells (HBEC) by genistein and vitamin D_3. The cells were treated with A. Ethanol (0.3 %, v/v), solvent control; B. Cholera toxin (1 ng/mL), positive control; C. Genistein (10^{-7} M) and D. vitamin D_3 (10^{-9} M). Photos were taken 12 days after treatment.

study, genistein, at concentrations lower than 1 μM, significantly increased the differentiation of Type I HBEC to Type II cells in two of three primary cultures derived from different human subjects (Fig. 1). Genistein completely arrested cell growth of Type I HBEC at concentrations higher than 5 μM and Type II HBEC at concentrations higher than 50 μM after 72 h treatment in all the 6 independent primary cultures examined[81] (Fig. 2). Flow cytometric analysis revealed that genistein was able to arrest cell cycle progression of both Type I and Type II HBEC at both G1/S and G2/M checkpoints.[81] Western blot analysis showed that the level of $p21^{WAF1/CIP1}$, which negatively regulates the G1/S transition, and cdc2 protein, which positively regulates the G2/M transition, are significantly enhanced and decreased respectively after 72 h genistein treatment (50 μM) in both Type I and Type II HBEC.[81]

1α,25-(OH)$_2$ Vitamin D$_3$

We have studied the effect of 1α,25-(OH)$_2$ vitamin D$_3$ on cell growth and differentiation in both Type I and Type II HBEC. The results show that 1α,25-(OH)$_2$ vitamin D$_3$, at concentration of 10^{-9} M, is able to induce the differentiation of Type I cells into Type II cells (Fig. 1) in 31 % of Type I colonies compared to 1.2 % for the solvent control. At higher concentration (10^{-7} M), 1α,25-(OH)$_2$ vitamin D$_3$, inhibited the growth of Type II HBEC but not Type I HBEC (Fig. 3). Furthermore, at this concentration, the Vitamin D$_3$ was found to increase cytokeratin 19 positive cells (a luminal epithelial cell type) in the Type I cell popula-

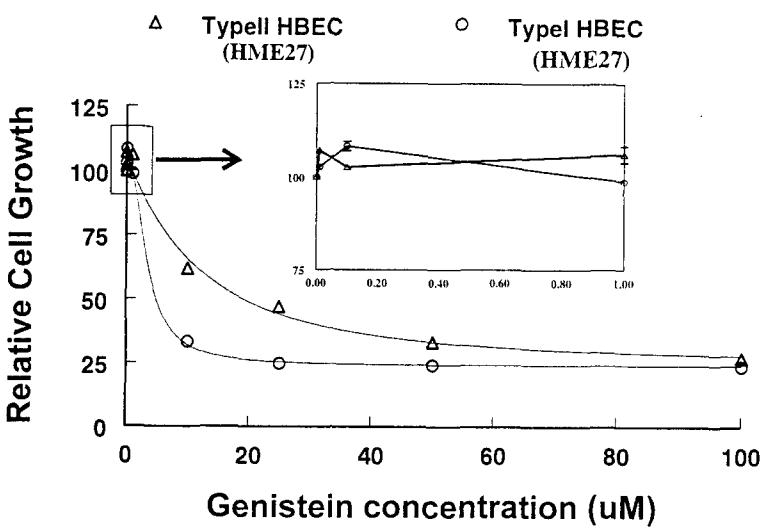

Fig. 2. The effects of various concentrations of genistein on growth of Type I and Type II HBEC. Cell growth was determined by quantitative measure of DNA contents in cell cultures 7 days after treatment.

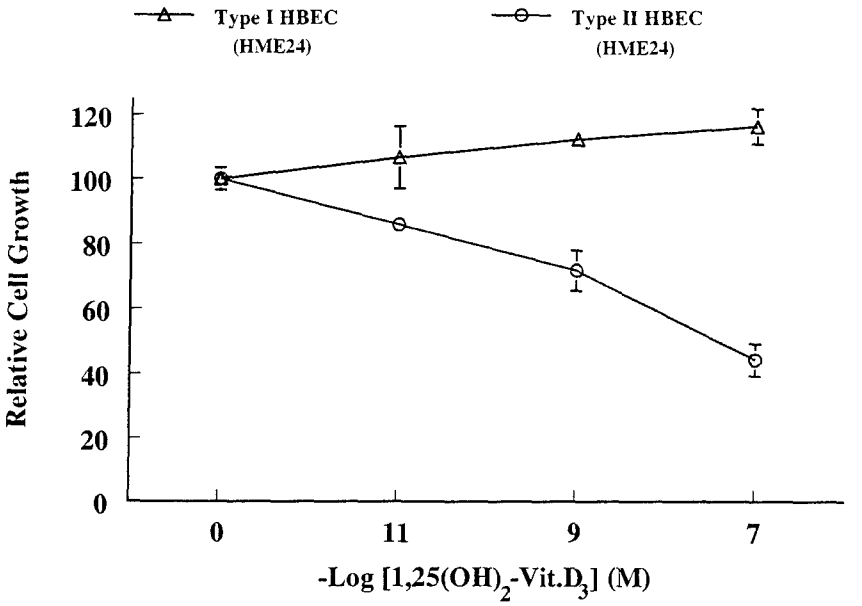

Fig. 3. The effects of different concentrations of vitamin D3 on growth of Type I and Type II HBEC. Cell growth was measured by DNA quantitation in cell cultures 6 days after treatment.

tion, although it had no effect on differentiation of Type I to Type II cells (a basal epithelial cell type), indicating that different concentrations of 1α,25-$(OH)_2$ vitamin D_3 may induce different cell differentiation.

Together, these results suggest that both genistein and 1α,25-$(OH)_2$ vitamin D_3 might function as chemopreventive agents by preferentially inhibiting the growth and/or inducing the differentiation of breast epithelial stem cells, thereby reducing the target cells for neoplastic transformation.

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Deregulation of *cdc2* gene expression correlates with overexpression of a 110 kDa CCAAT box binding factor in transformed cells

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Eukaryotic cell cycle progression is regulated by an orderly and sequential activation of several cyclin-dependent kinases, which phosphorylate key substrates during this process. $p34^{cdc2}$, the catalytic subunit of cdc2 kinase, is expressed at the late G_1/S boundary and is required for the $G_2 \rightarrow M$ phase transition. Transactivation of the human *cdc2* promoter by the DNA tumor virus-encoded oncogenic protein SV40 large T antigen is mediated by induction of a novel 110 kDa CCAAT box binding factor (CBF/cdc2). To investigate whether induction of CBF/cdc2 is an intrinsic property of the viral oncoprotein or is a common event during transformation of normal cells, expression of CBF/cdc2 was analyzed in many human tumor cell lines and in rodent cells spontaneously transformed or stably expressing various oncogenes. Our results showed that CBF/cdc2 was overexpressed in all transformed cells examined, including human 293, MCF-7, HeLa and HepG2 cells. Moreover, expression of CBF/cdc2 was elevated in spontaneously transformed rat liver epithelial cells (C4T), but not detectable in the non-tumorigenic parental (RLE) cells. The elevated levels of CBF/cdc2 expression in C4T cells correlated well with increased *cdc2* mRNA and $p34^{cdc2}$ levels. CBF/cdc2 was also overexpressed in a rat liver epithelial cell line (WB) stably transfected with various oncogenes, v-*myc*, v-Ha-*ras* and mutated rat *neu* and v-*src*. Using an electrophoretic mobility shift assay, specific binding of CBF/cdc2 to the CCAAT box motifs of the human *cdc2*, *cycA* and *cdc25C* promoters was detected, suggesting that transcription of these cell cycle regulatory genes are coordinately activated by CBF/cdc2.

Introduction

Cell cycle progression in eukaryotic cells is tightly regulated by orderly expression and function of the regulatory proteins cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (1–9; for reviews see refs 10–15). Deregulation of expression and/or loss of function of any of the cell cycle regulatory proteins will result in disruption of cellular growth control, which is one of the hallmarks of cancer (for reviews see refs 14,16). Moreover, mutations in the cell cycle regulatory genes commonly found in human tumors are often involved in G_1 checkpoint control (reviewed in refs 17–20).

$p34^{cdc2}$, the product of the *cdc2* gene, is expressed in $G_1 \rightarrow S$ phase and is required for the $G2 \rightarrow M$ phase transition (7–9,21). The activity of cdc2 kinase is regulated by post-translational modifications and is linked to the growth state of the eukaryotic cell (4,21; for reviews see refs 13,22,23). Moreover, human *cdc2* mRNA and $p34^{cdc2}$ protein levels are very low when cells are in quiescence or undergo differentiation, but become significantly elevated as the cells pass through the G_1 restriction point and enter S phase (21,24–27). These studies indicate that $p34^{cdc2}$ is required for cell proliferation and plays an important role in phosphorylation of key substrates involved in the $G_1 \rightarrow S$ transition (for reviews see refs 11,13,22,23 and references therein).

The oncogenic proteins encoded by DNA tumor viruses such as SV40, adenovirus, polyoma and papilloma viruses have contributed immensely to our understanding of cell cycle events in normal cells. Expression of these viral oncoproteins in cells, which are normally in quiescence or a terminally differentiated state, induces many activities involved in cell cycle progression and host DNA synthesis, including endogenous *cdc2* mRNA and $p34^{cdc2}$ kinase (28,29; for reviews see refs 30–32).

The growth-promoting activities of these oncoproteins are at least partially attributable to their abilities to associate with a number of cellular proteins which negatively regulate cell cycle. The best-studied cellular targets of these viral oncoproteins are the tumor suppressor proteins, the retinoblastoma susceptibility gene product (pRB) and p53. A number of cell cycle genes are activated by the E2F-like transcription factors during cell proliferation, but they exist as transcriptionally inactive complexes with pRB in growth-arrested cells. These inactive complexes are disrupted by viral oncoproteins when cells are driven to enter S phase and the E2F-like transcription factors are activated (33–37; for reviews see refs 31,32 and references therein).

The human *cdc2* promoter has multiple regulatory elements for binding of several transcription factors, including ATF, c-Myb, Sp1, E2F and CCAAT box motifs (27,38). pRB was shown to negatively regulate the *cdc2* promoter through two E2F binding sites (27), suggesting that E2F-like transcription factors may play a role in transactivation of *cdc2* gene expression by viral oncoproteins in the $G1 \rightarrow S$ phase transition. Moreover, a previous study also revealed that activation of the human *cdc2* promoter by SV40 large T antigen (SV40-LT) in cycling cells, when E2F is already active, is mediated by induction of a 110 kDa CCAAT box binding factor (CBF/cdc2) which specifically interacted with the two inverted CCAAT box motifs in the promoter (39).

Abbreviations: CDKs, cyclin-dependent kinases; SV40-LT, SV40 large T antigen; CBF/cdc2, CCAAT box binding factor; DMEM, Dulbecco's modified Eagle's medium; EMEM, Earl's minimal essential medium; FBS, fetal bovine serum; HME, human breast epithelial; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RLE, rat liver epithelial.

This study was initiated to examine the relationship between expression of CBF/cdc2 and oncogenic transformation or carcinogenesis. Our results show that CBF/cdc2 is overexpressed in diverse human tumor cell lines and in a spontaneously transformed tumorigenic rodent cell line, as well as in rodent cells stably transfected with different oncogenes known to induce neoplastic transformation. However, expression of CBF/cdc2 is barely detectable in cells of non-tumorigenic counterparts. Expression of *cdc*2 mRNA and $p34^{cdc2}$ correlate well with increased expression of CBF/cdc2. CBF/cdc2 also specifically binds the CCAAT box motifs of two other key cell cycle regulatory genes, cyclin A and cdc25C. These results, taken together, indicate that the CBF/cdc2 transcription factor is overexpressed not only in cells stably transformed by viral oncoproteins, but also in transformed cells of diverse etiologies, such as in established human tumor cell lines and in rodent cells transformed spontaneously or by stable expression of oncogenes. Our results also suggest that CBF/cdc2 is likely to be involved in transactivation of other cell cycle genes, such as *cycA* and *cdc*25C.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Earl's minimal essential medium (EMEM), F12 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from DuPont-NEN (Wilmington, DE). Nitrocellulose membranes for northern blot analyses were from Dupont-NEN and PVDF membranes for southwestern and western blot analyses were from Bio-Rad (Bio-Rad, Hercules, CA). The reagent for RNA extraction, RNA STAT-60, was from TEL-TEST Inc. (Friendwood, TX). Ready-To-Go DNA labeling beads for preparation of probes for northern blot analyses were from Pharmacia (Piscataway, NJ). Quick-Hyb hybridization solution was from Stratagene (La Jolla, CA). The pCMV-cdc2 plasmid was a kind gift from Dr J.Campisi.

Cell culture

Human embryonic kidney cells transformed by early region 1 of adenovirus (human 293 cells) (40) were cultured in EMEM supplemented with 10% FBS. Human cancer cell lines MCF7, HeLa and HepG2 and normal monkey kidney (CV-1) cells were cultured in DMEM with 10% FBS. A primary culture of normal human breast epithelial (HME) cells, which was derived from reduction mammoplasty, were cultured in MSU-1 medium as described previously (41). Rat liver epithelial (RLE) cells, spontaneously transformed RLE (C4T) (42) and Fischer-344 RLE (WB) cells transfected with a variety of oncogenes (WB-*myc*, WB-*ras*, WB-*src* and WB-*neu*) or with the neomycin resistance marker alone as control (WB-*neo*) (43) were cultured in a 1:1 mixture of DMEM and F-12 medium supplemented with 10% FBS. All media contained 50 μg/ml each of streptomycin and penicillin and all cell cultures were incubated at 37°C in humidified air and 5% CO_2.

The origins of v-*myc*, v-Ha-*ras*, v-*src* and the mutated rat *neu* oncogenes used to transfect WB cells have been described (43). The parental WB and WB-*myc* cells are immortal and non-tumorigenic. WB-*ras*, WB-*neu* and WB-*src* were tumorigenic in F-344 rat livers when hepatically injected through portal veins.

Nuclear extracts

Cells grown to 70–80% confluence in 75 cm^2 cell culture flasks were washed with ice-cold phosphate-buffered saline (PBS) three times and collected in 3 ml PBS. Cell suspensions were centrifuged at 1200 *g* at 4°C for 10 min and the pellet resuspended in hypotonic buffer [20 mM HEPES–KOH, pH 7.4, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After incubation at 0°C (ice) for 30 min, cells were lysed by passing through a 1 ml syringe fitted with a 27G needle 20 times (44,45). The nuclear pellet was recovered by centrifugation at 5000 *g* at 4°C for 10 min and was resuspended in a high salt buffer (50 mM HEPES–KOH, pH 7.4, 5 mM KCl, 0.5 mM $MgCl_2$, 0.2 M NaCl, 10% sucrose, 1 mM PMSF). After incubation at 0°C for 45 min, the nuclear extract was obtained by centrifugation at 12 000 *g* for 10 min. Protein concentration was measured as described by Bradford (46) and the samples were kept at –70°C until use.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously (39). Briefly, the nuclear extracts (5 μg/assay) were mixed with a labeled probe (5×10^5 c.p.m./assay) in 20 μl reaction buffer [25 mM HEPES–KOH, pH 7.9, 5 mM KCl, 0.5 mM EDTA, 0.15 mg/ml BSA, 0.25 mM dithiothreitol, 10% glycerol, 0.1 mg/ml poly(dI·dC)]. The probe was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction mixture was incubated at 37°C for 30 min and stopped by adding 2 μl stop solution containing 50 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and 5% glycerol. The DNA–protein complexes were fractionated by electrophoresis on 4% non-denatured polyacrylamide gels. The gels were dried and subjected to autoradiography using X-ray film (Reflection; Du Pont-NEN). The probes used for EMSA were synthetic oligonucleotides containing the CCAAT box motifs in the promoters of the human *cdc*2 gene (5′-CATGGGCTCTGATTGGCTGCTTTG, GenBank/EMBL accession no. L06298) (38), human *cycA* gene (5′-CGAGCGCTTTCA-TTGGTCCATTTC, GenBank/EMBL accession no. X68303) (47) and the *cdc*25C gene (5′-GCGCGCGGAGATT-GGCTGACGCAG, GenBank/EMBL accession no. Z29077) (48).

Southwestern blot analyses

Southwestern blot analyses was carried out as described (39,49). Briefly, 10 μg nuclear proteins from each sample were fractionated by SDS–PAGE (7.5%) and electrotransferred to a PVDF membrane. After denaturation and renaturation steps, the proteins immobilized on the membrane were probed with the 5′-labeled *cdc*2/CCAAT probe (10^6 c.p.m./ml). The membranes were washed and subjected to autoradiography at –70°C.

Northern blot analyses

The cells were cultured to 70–80% confluency in 100 mm Petri dishes. Total RNA was extracted using RNA-STAT-60 according to the protocol supplied by the manufacturer and used for northern blot analyses. Briefly, 20 μg total RNA were separated on 1% agarose/6% formaldehyde gels and transferred onto a nitrocellulose membrane. After heating the membrane at 80°C for 2 h, the membrane was hybridized with a ^{32}P-labeled probe using Quick-Hyb hybridization solution. The probe was a 0.4 kb fragment generated by digesting the pCMV1-cdc2 plasmid with *KpnI* and *BglII* and labeled with [α-^{32}P]dCTP using Ready-To-Go DNA labeling beads. After extensive washing, the membrane was subjected to autoradiography at –70°C.

Western blot analyses

Nuclear extracts (10 μg) were fractionated by SDS–PAGE (10%) and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk and incubated with a monoclonal anti-cdc2 antibody (sc-54) at a 1:5000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed using a chemiluminescence detection method (ECL kit; Amersham Life Science, Cleveland, OH).

Results

Expression of 110 kDa CBF/cdc2 in human 293, RLE and C4T cells

SV40-LT and adenovirus E1A are known to induce DNA synthesis and $p34^{cdc2}$ kinase in human fibroblasts that are quiescent and/or senescent (50–56). Previous work from this laboratory showed that transactivation of the human *cdc*2 promoter by SV40-LT was mediated through the two inverted CCAAT box motifs and by induction of a 110 kDa transcription factor, CBF/cdc2, in cycling cells, when E2F is active. CBF/cdc2 was overexpressed in monkey kidney (COS-7) cells transformed by SV40-LT and in human diploid fibroblasts conditionally expressing SV40-LT (39).

In this study, we sought to determine whether overexpression of CBF/cdc2 is unique to cells transformed by the viral oncoprotein SV40-LT or is a common feature in other tumorigenic cells of human and rodent origin having viral and non-viral etiologies. Especially, our aim was to ascertain whether there is any relationship between overexpression of CBF/cdc2 and continuous cell proliferation or oncogenic cell transformation.

Normal RLE cells and their spontaneously transformed counterpart, C4T cells, and adenovirus early region 1-transformed human 293 cells (40) were grown under continuously

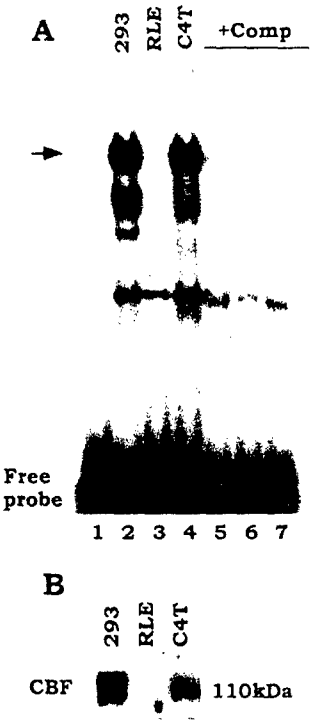

Fig. 1. Expression of the110 kDa CBF/cdc2 in human 293, RLE and C4T cells. (A) EMSA using nuclear extracts from human 293, RLE and C4T cells were carried out as described under Materials and methods. The arrow shows the position of the CBF/cdc2–DNA complexes. The competition experiments were carried out by adding a 100-fold excess of unlabeled cold oligonucleotide containing the CCAAT box motif of the human *cdc*2 promoter (+ Comp.). (B) Southwestern blot analyses using nuclear extracts from 293, RLE and C4T cells were carried out as described under Materials and methods. The 110 kDa CBF/cdc2 (CBF), detected in 293 and C4T cells, is indicated.

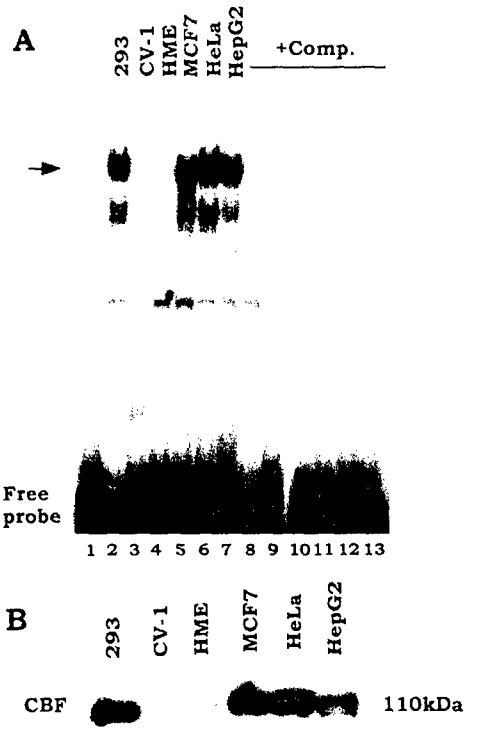

Fig. 2. Expression of the 110 kDa CBF/cdc2 in different human cancer cells. (A) For EMSA, nuclear extracts of human 293, CV-1, normal HME, MCF7, HeLa and HepG2 cells were used as indicated in lanes 2–7. The 110 kDa CBF/cdc2-specific DNA–protein complexes are shown by an arrow. In lanes 8–13, a 100× excess of cold oligonucleotide containing the CCAAT box motif of the human *cdc*2 promoter was included in the EMSA reactions in the order shown in lanes 2–7 (+ Comp.). (B) Southwestern blot analyses of the nuclear extracts used in (A) are shown.

cycling conditions in the presence of 10% FBS. The extracts prepared from these cells were first analyzed for DNA binding activity of CBF/cdc2 by EMSA. For EMSA, the same amounts of nuclear proteins, as determined by the Bradford (46) method, were loaded. The results shown in Figure 1A indicate that both human 293 and rodent C4T nuclear extracts formed a predominant DNA–protein complex (complex I, as shown by the arrow) with the cdc2 probe (lanes 2 and 4). However, the extract from the non-tumorigenic RLE cells formed no detectable DNA–protein complex (lane 3). The specificity of the DNA–protein complexes formed with the cdc2 probe was established using the unlabeled cdc2/CCAAT box motif as competitor (lanes 5–7).

Expression of CBF/cdc2 was further analyzed by southwestern blot analyses (Figure 1B). The nuclear extracts were fractionated by SDS–PAGE, transferred to a membrane and probed with the labeled CCAAT/cdc2 oligonucleotide. Distinct bands corresponding to a 110 kDa size were detected in nuclear extracts from the human 293 and the C4T cells, but not in nuclear extracts from RLE cells (Figure 1B). These results indicate that induction of the 110 kDa CBF occurred not only in SV40-transformed monkey kidney (COS) cells and human diploid fibroblasts conditionally expressing SV40-LT (39), but also in human 293 cells transformed by the adenovirus E1 region and in spontaneously transformed rat liver epithelial (C4T) cells (this study). However, CBF/cdc2 was not detectable in the normal counterparts, CV-1 (Figure 2) and RLE cells. These experiments were repeated three times and the results were reproducible.

Expression of cdc2 mRNA and p34^{cdc2} protein in RLE and C4T cells

Since the results shown in Figure 1 indicate that the 110 kDa CBF/cdc2 was overexpressed in spontaneously transformed C4T cells but was not detectable in normal RLE cells, we sought to determine the correlation between CBF/cdc2 expression and *cdc*2 mRNA and p34^{cdc2} protein levels. Transcription of the human *cdc*2 gene gives rise to two transcripts, 1.6 and 2.0 kb in length, whereas in rat cells only the 1.6 kb transcript is reportedly expressed (57). The results of northern blot analyses using a human *cdc*2 cDNA probe showed that tumorigenic C4T cells expressed a 3-fold higher level of the 1.6 kb transcript than non-tumorigenic RLE cells (Figure 3A). The p34^{cdc2} protein levels were also analyzed by western blot analyses using a monoclonal anti-p34^{cdc2} antibody (Figure 3B). The results showed that the level of p34^{cdc2} protein was also elevated in the C4T cells, whereas it was not detectable in RLE cells under these conditions (Figure 3B).

Overexpression of the 110 kDa CBF/cdc2 in human tumor cell lines

Oncogenic transformation of normal cells is often the result of aberrant cell cycle control leading to continuous cell proliferation. Next, we sought to determine whether CBF/cdc2 is also overexpressed in several established human tumor cell lines. For this study, HeLa (cervical cancer cells with human papilloma virus etiology) (58), MCF-7 (human breast cancer cells) and HepG2 (hepatocellular carcinoma cells) were chosen. As controls, HME (41) and monkey kidney (CV-1) cells were used. The results of EMSA showed that nuclear extracts from

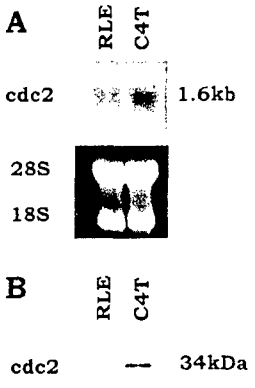

Fig. 3. *cdc*2 mRNA and $p34^{cdc2}$ protein levels in RLE and C4T cells. (**A**) Northern blot analyses were carried out using RNA isolated from RLE and C4T cells as described under Materials and methods. Equal amounts of RNA were loaded for electrophoresis, as shown by the intensities of 28S and 18S rRNAs stained with ethidium bromide. The 1.6 kb rat *cdc*2 transcript is shown. (**B**) Western blot analyses for the detection of $p34^{cdc2}$ in RLE and C4T were carried as described under Materials and methods using a mouse monoclonal anti-$p34^{cdc2}$ antibody.

all three tumor cell lines exhibited much higher CCAAT/cdc2 binding activity (indicated by an arrow in Figure 2A, lanes 5–7) compared with those from the primary culture of HME cells (lane 4) when equal amounts of proteins were loaded. The results of EMSA were confirmed by southwestern blot analysis (Figure 2B) using the CCAAT/cdc2 probe. CBF/cdc2 was expressed at significantly higher levels in human cancer cell lines as well as in human 293 cells compared with the levels found in normal cells, which were either barely detectable (as in HME cells) or undetectable (as in CV-1 cells; Figure 2B). These results indicate that induction of CBF/cdc2 and concomitant transactivation of the *cdc*2 promoter are not unique attributes of the viral oncoproteins encoded by SV40 and adenovirus. These are common events in a variety of tumor cell lines of both human and rodent origin

Expression of the 110 kDa CBF/cdc2, cdc2 mRNA and $p34^{cdc2}$ in various oncogene-transfected cell lines

Overexpression of oncogenes in mammalian cells often leads to immortalization and loss of anchorage dependence for growth. To examine whether stable expression of oncogenes could lead to induction of CBF/cdc2, we analyzed expression of CBF/cdc2 in a rat liver epithelial cell line (WB cells) stably transfected by various oncogenes. The nuclear extracts from all of the oncogene-transfected cell lines analyzed formed specific DNA–protein complexes with the CCAAT/cdc2 probe similar to that from human 293 cells (indicated by an arrow in Figure 4A, lanes 1 and 4–7). However, nuclear extracts from the parental WB cells as well as from WB-*neo* cells transfected with the vector alone did not form any specific DNA–protein complexes (Figure 4A, lanes 2 and 3). The results of EMSA were confirmed by southwestern blot analysis (Figure 4B), which showed that CBF/cdc2 was expressed at different levels in the oncogene-transfected cell lines but was barely detectable in the control cell extracts (Figure 4B, lanes WB and WB-*neo*). Among the WB cell lines transformed by different oncogenes, WB-*src* expressed the lowest amount of CBF/cdc2, which was still higher than the barely detectable level observed in the parental WB cell line. This lower level of CBF/cdc2 in *src*-transfected cells correlated well with reduced *cdc*2 mRNA and $p34^{cdc2}$ protein levels (Figure

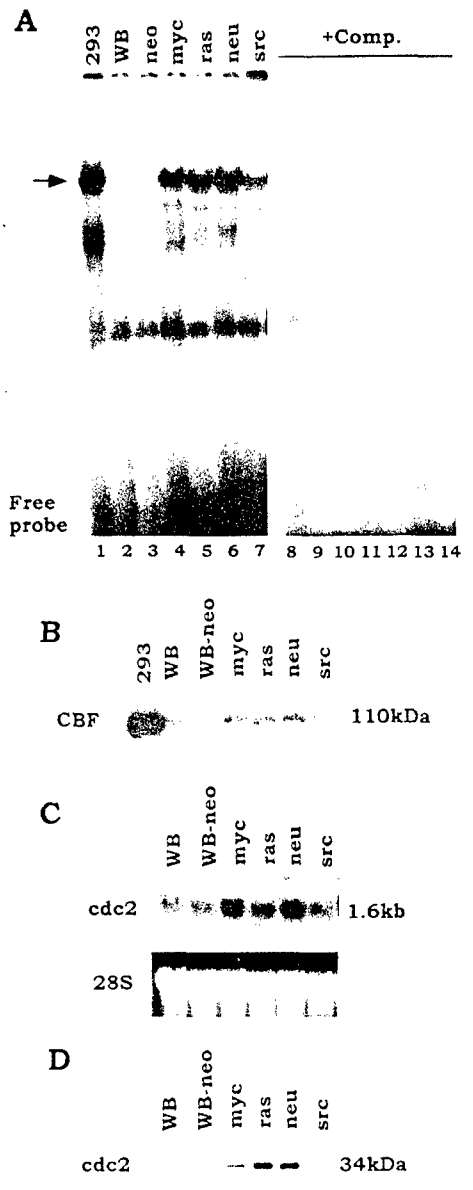

Fig. 4. Analyses of expression of CBF/cdc2, *cdc*2 mRNA and $p34^{cdc2}$ in WB cells stably transfected with various oncogenes. (**A**) For EMSA, nuclear extracts from WB, WB-*neo* and various oncogene-transfected WB cell lines were used as indicated. The arrow indicates the position of the CBF/cdc2–CCAAT box complexes. Lanes 1–7, no competitor; lanes 8–14, a 100× excess of the same unlabeled competitor, as in Figure 3 (+Comp.). (**B**) Southwestern blot analyses were carried out using the same nuclear extracts as in (A). (**C**) Northern blot analyses were carried out for quantitation of *cdc*2 mRNA levels. Equal amounts of RNA were loaded in each lane, as indicated by ethidium bromide staining of 28S rRNA. (**D**) Western blot analyses were carried out for quantitation of $p34^{cdc2}$ levels.

4B–D). This may reflect the fact that, unlike other cell lines, the WB-*src* cell population contained a significant proportion of terminally differentiated cells as well as proliferating cells (C.C.Chang, unpublished results). In contrast, the levels of CBF/cdc2 were significantly higher in c-*myc*-, *ras*- and mutated *neu* oncogene-transfected cells, which again correlated well with correspondingly increased expression of *cdc*2 mRNA and $p34^{cdc2}$ protein.

Increased expression of CBF/cdc2 also correlated well with higher levels of *cdc*2 mRNA in the oncogene-transfected cells relative to those in the control WB and WB-*neo* cells, as

shown by northern blot analysis (Figure 4C). Furthermore, $p34^{cdc2}$ protein levels were also much higher in cells expressing the oncogenes, as shown by western blot analyses (Figure 4D).

Specificity of DNA binding activity of CBF/cdc2 to CCAAT box motifs of cdc2, cycA and cdc25C promoters

The oncoproteins of DNA tumor viruses induce $p34^{cdc2}$ kinase, DNA synthesis and cell proliferation in quiescent cells (27,29,50–56). Interaction of cyclin A or cyclin B with the $p34^{cdc2}$ catalytic subunit is required for its enzymatic activity as a protein kinase (for a review see ref. 22). The $p34^{cdc2}$ catalytic subunit is activated by a dual specificity tyrosine/serine-threonine phosphatase, cdc25C. This phosphatase removes inhibitory phosphate residues from the $p34^{cdc2}$ catalytic subunit which then becomes activated to allow cell cycle progression from G_2 to M phase (59,60; for a review see ref. 61). Since the promoters of *cycA* and the *cdc*25C contain CCAAT box motifs (47,48), we sought to examine the possibility that CBF/cdc2 also recognizes the CCAAT box motifs in the *cycA* and *cdc*25C promoters.

Nuclear extracts from WB cell lines were analyzed for binding to the CCAAT box motifs of the *cycA* and *cdc*25C promoters. The data presented in Figure 5 clearly indicate that the nuclear extracts from human 293 cells as well as the oncogene-transfected cells formed specific DNA–protein complexes with the CCAAT box motifs of the *cdc*25C and *cycA* promoters (shown by arrows in Figure 5A and B, respectively) as labeled probes. The specificities of these DNA–protein complexes were analyzed by competition experiments using a 100-fold excess of the respective unlabeled homologous competitor (Figure 5A and B, right, + Comp.). Moreover, binding of CBF/cdc2 to a labeled *cdc*2 CCAAT box motif was effectively competed out by an unlabled *cdc*25C or *cycA* CCAAT box motif. Likewise, binding of CBF/cdc2 to a labeled *cdc*25C or *cycA* motif as probe was also competed out by an unlabeled *cdc*2 CCAAT motif (data not shown). These results, taken together, indicate that CBF/cdc2, overexpressed in a variety of oncogene-transfected cell lines, binds to the CCAAT box motifs of the *cdc*2, *cyc*A and *cdc*25C promoters.

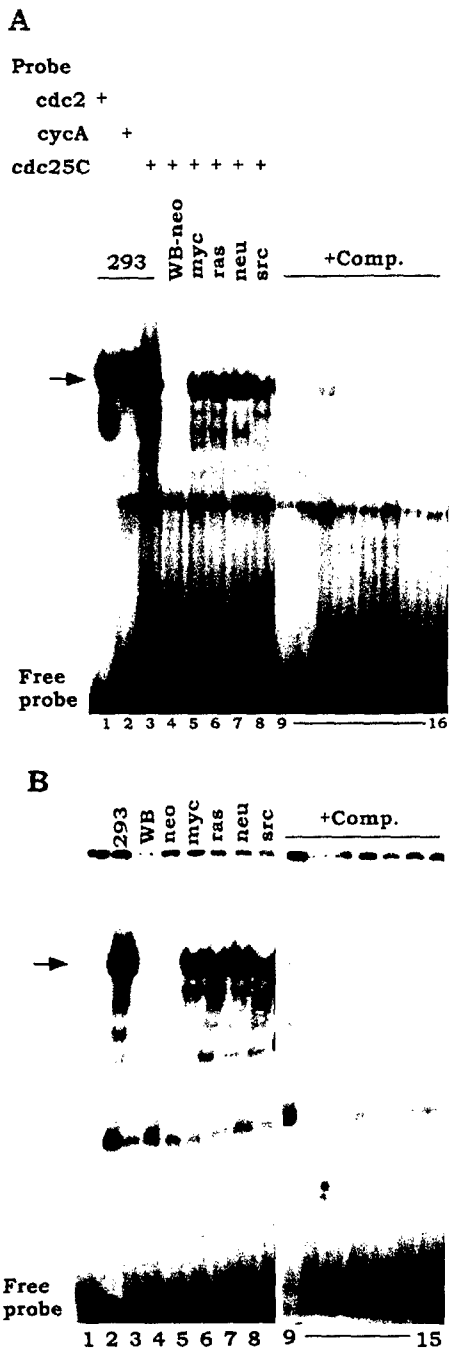

Fig. 5. Specificity of DNA binding activity of CBF/cdc2 to CCAAT box motifs of *cdc*25C and *cyc*A promoters. For EMSA, the labeled *cdc*25C (**A**) and *cycA* (**B**) CCAAT motifs as probes and nuclear extracts from WB cells stably transfected with the various oncogenes (or WB-*neo* as the negative control) were used as indicated. For comparison of the DNA binding activities, human 293 cell extracts and labeled CCAAT motifs of the *cdc*2, *cycA* and *cdc*25C promoters as probes (A) or the labeled *cycA* promoter motif alone (B) were used. The arrow shows the position of the CBF–DNA complexes. Competition experiments were carried out using a 100-fold excess of unlabeled *cdc*25C (A) and *cyclA* (B) CCAAT motif as homologous competitors (right panels, + Comp.).

Discussion

Our previous study showed that transactivation of the human *cdc*2 promoter by SV40-LT is mediated through the two inverted CCAAT box binding motifs and a novel 110 kDa protein which specifically interacted with these motifs in EMSA and southwestern blot analyses. Furthermore, SV40-LT activated endogenous *cdc*2 gene expression and, concomitantly, expression of CBF/cdc2 was induced in monkey kidney cells either transiently expressing SV40-LT or stably transformed by SV40 (COS cells), as well as in human diploid fibroblasts conditionally expressing SV40-LT (39). In this study, we show that this CBF/cdc2 is also overexpressed in other virally transformed cell lines, such as human 293 and HeLa cells, as well as in established tumor cell lines and in oncogene-transformed rodent cells, whereas it is expressed at very low to undetectable levels in normal cells. In human 293 cells, the two E1A proteins of 243 and 289 amino acid residues and the E1B 55 kDa protein are expressed (for a review see ref. 62) and in HeLa cells, the oncoproteins E6 and E7 are expressed (58).

Spontaneously transformed and tumorigenic C4T rat liver epithelial cells were derived by continuous passage of its non-tumorigenic parental RLE cells in culture (42). This spontaneous transformation model *in vitro* suggested that a genetic alteration(s) might occur during the multiple steps of the carcinogenesis process. This spontaneous transformation process is considered as an *in vitro* model for human carcinogenesis. Therefore, delineation of the differences between RLE and C4T cells is likely to contribute to our understanding of the transformation process. In this study, we observed that the

110 kDa CBF/cdc2 was overexpressed in C4T but not in normal RLE cells. These results suggest that overexpression of CBF/cdc2 in C4T cells may be involved in the transformation of RLE to C4T cells.

This study also provides evidence for the first time of overexpression of this transcription factor in a variety of human tumor cells and cells which are stably transfected with oncogenes, such as c-*myc*, Ha-*ras*, *neu* and *src*. Activation of oncogenes, such as *src*, *myc*, *ras* and *neu*, has been reported in many human clinical samples and human cancer cell lines (63,64). Moreover, these oncogene products are known to be transducers of intracellular signaling pathways which are multidirectional and show extensive cross-talk among them (18,65; for a review see ref. 20). The human *cdc*2 promoter is also cooperatively transactivated by c-Myc and activated H-ras (66). Our results suggest the possibility that CBF/cdc2 is one of the targets of the oncogene signaling cascades. Since WB-*myc* cells express a high level of CBF/cdc2 but are non-tumorigenic, this result suggests that overexpression of CBF/cdc2 alone is not sufficient for attainment of the tumorigenic state. Our results also indicate that there is a good correlation between overexpression of CBF/cdc2 and elevated levels of *cdc*2 mRNA and $p34^{cdc2}$ protein in a variety of transformed cells of both human and rodent origin, suggesting that CBF/cdc2 plays an important role in transactivation of *cdc*2 gene expression.

There is increasing evidence that aberrations in expression of cell cycle regulatory proteins such as cyclins, CDKs and CDK inhibitors are closely linked to the development and progression of carcinogenesis (16,17,19). Many abnormalities in human cancers affect the $G_1 \rightarrow S$ phase transition, but some in the $G_2 \rightarrow M$ transition are also known. At late S to G_2 phase of the cell cycle the cdc25C phosphatase is predominantly expressed (59). A positive feedback activation mechanism exists between cdc2 and cdc25C, whereby cdc25C dephosphorylates and activates cdc2 and activated cdc2 then phosphorylates and activates cdc25C (60; reviewed in ref. 61). Thus we suggest that induction of CBF/cdc2 may be an early event in carcinogenesis which triggers overexpression or unscheduled expression of a number of cell cycle genes which contain the CCAAT box motif in their promoters. Indeed, overexpression of the *cdc*2 gene has been reported in human tumors (67–70). Our findings of overexpression of CBF/cdc2 in tumor cells and its specific binding to the CCAAT motif of the *cdc*2, *cycA* and *cdc*25C genes suggest that these genes are coordinately regulated by CBF/cdc2.

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