AD_____

GRANT NUMBER DAMD17-96-1-6099

TITLE: Characterization of an In Vitro Human Breast Epithelial Organoid System

PRINCIPAL INVESTIGATOR: Chia-Cheng Chang

CONTRACTING ORGANIZATION: Michigan State University
East Lansing, MI 48824-1046

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE Characterization of an In Vitro Human Breast Epithelial Organoid System

5. FUNDING NUMBERS
DAMD17-96-1-6099

6. AUTHOR(S)
Chia-Cheng Chang

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Michigan State University
East Lansing, MI 48824-1046

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19990127 079

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We have characterized organoids formed by two types of normal human breast epithelial cells (HBEC) on Matrigel. The results show that mammary gland-like structures can be formed in one day after inoculation of right number and ratio of Type I and Type II HBEC. These organoids later may form ductal and actively proliferating terminal end bud-like structures. The two cell types that formed organoids in Matrigel for months can be recovered as actively growing monolayer culture, on plastic, which then can be used to form organoids again. Thus, a monolayer-organoid-monolayer-organoid cycle is able to preserve HBEC for a long time, reaffirming stem cell nature of Type I HBEC. Our study also reveals that a low level of telomerase activity was present in both Type I and Type II HBEC and their SV40 transformed clones at early passages. However, this innate telomerase activity appears not sufficient for immortalization and can be easily activated in transformed Type I cells at late passages which became immortal at high frequency (10/11). In contrast, Type II transformed clones with extended lifespan seem to lack the ability of telomerase activation and rarely became immortal (1/10). These results provide a basis for high susceptibility of Type I cells to neoplastic transformation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Stem Cells, Breast Epithelial Organoid, Telomerase, Neoplastic Transformation, Estrogen Receptor	67
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A <.<.<. In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

<.<.<. ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A <.<.<. In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A <.<.<. In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A <.<.<. In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Chia-Cheng Chang Aug. 28'98

PI - Signature Date

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

Table of Contents

Page No.

I. Front Cover 1

II. Standard Form 298 Report Documentation Page 2

III. Foreword 3

IV. Introduction 4-5

V. Body 6-50

VI. Conclusions 51-52

VII. References 53-54

VIII. Appendices 55

IV. 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). 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 (4). 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 (5). 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 is sufficient for neoplastic transformation. This became an additional task for this project.

V. BODY

A. 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)

Introduction

Telomerase activity has been found in most cancer cells and in many immortal cell lines, as well as in germ line cells (4). In contrast, it is generally undetectable in most normal human somatic cells (4, 6). Progressive telomere shortening and reactivation of telomerase activity have been considered as the key mechanisms respectively for cellular senescence and immortalization. However, some tissues with cell renewal ability, such as basal cells of the epidermis in human skin and a subpopulation of cells of normal hematopoietic lineage which may contain stem cells, have been reported to express telomerase activity (7-9). Furthermore, the role of telomerase in malignant transformation has been questioned by a recent study that observed the presence of telomerase activity in both normal and tumorigenic human cells including breast epithelial cells (5). This study, however, did not quantitatively measure the telomerase activity during the course of clonal progression in neoplastic transformation. Since we have the putative human breast epithelial stem cells and since we have a series of SV40 transformed cell lines, derived from single cells, preserved at different cumulative population doubling levels during the course of neoplastic transformation,

we have the opportunity to determine (a) whether telomerase activity is present in human breast epithelial stem cells, and (b) whether the high and low susceptibility of SV40 large T-antigen transformed Type I and Type II HBEC respectively to spontaneous immortalization is correlated with their potential for telomerase activation.

Material and Methods

Cell Culture and Development of Normal and SV40 Large T-antigen Transformed Type I and Type II HBEC. The cell culture and method to develop the two types of normal HBEC culture from reduction mammoplasty are as described previously (1). The transformation of normal HBEC was achieved by lipofectin-mediated transfection (1) with SV40 DNA (GIBCO-BRL) (M13SV1 derived from HME 13) or a plasmid carrying the G418-resistance gene and an origin-defective SV40 genome expressing a wild type large T-antigen (PRNS-1, obtained from Johng S. Rhim of the National Cancer Institute) (M15SV 1-11, 21-30 derived from HME 15). The derivation of weakly and highly tumorigenic HBEC lines from the SV40 immortalized HBEC (M13SV1) following sequential treatment with x-rays and neu oncogene was recently reported (10).

During the course of determining the potential cumulative population doubling level (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 lysate.

Measurement of Telomerase Activity by TRAP (Telomeric Repeat Amplification Protocol). Cells grown to about 70% confluency were harvested by trypsinization. After cell count, 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 tube. After cells were centrifuged and PBS carefully removed, the cell pellets were stored at –85°C. For 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 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 from individual cell line. Telomerase activity was examined by utilizing TRAPeze™ Telomerase Detection Kit (Oncor, Gaithersburg, MD) which 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 sample), heat-

inactivated control (sample incubated at –85°C for 10 minutes prior to the assay) and positive control (breast carcinoma cell line MCF-7). The product of TRAP assay were resolved by electrophoresis in a nondenaturing 12% polyacrylamide gel electrophoresis (PAGE) in a buffer containing 54 mM Tris-HCl (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. Image was captured and analyzed by AlphaImager™ (Alpha Innotech Corporation, SanLeandro, CA).

Results

Telomerase Activity in Type I and Type II HBEC. As shown in Fig. 1, telomerase activity was present in both Type I and Type II HBEC. The level of activity, however, was weak compared to the breast carcinoma cell line, MCF-7. At 10 times less in cell number (250 vs. 2,500), the telomerase activity in MCF-7 was significantly higher than Type I and Type II HBEC. In contrast, telomerase activity was almost undetectable in human breast stromal fibroblasts at early passage. The weak telomerase activity in normal Type I and Type II HBEC is not due to the presence of telomerase inhibitor in these cells, since the cell lysate from these cells did not affect the telomerase activity of MCF-7 cells in lysate mixture (Fig. 2).

Telomerase Activity in SV40 Transformed Type I HBEC at Different CPDL. We have isolated 11 independent SV40 transformed clones with extended lifespan (cpdl > 22). Each of these clones was derived from a single cell since they were isolated from individual colonies. Ten of the 11 clones eventually became immortal (cpdl > 100). Four of these clones were randomly selected for telomerase assay (Fig. 3). Similar to the parental normal HBEC, all these clones at early passage (low cpdl) showed low levels of telomerase activity. Invariably, the telomerase activities in these clones were greatly activated at mid or late passage (high cpdl). The one exception that did not become immortal showed undetectable telomerase activity at early or mid-passage.

Telomerase Activity in SV40 Transformed Type II HBEC at Different CPDL. In contrast to Type I cells, SV40 transformed Type II HBEC rarely became immortal (1/10). These cells usually senesced before mid-passage (60 cpdl). Three of the 9 senescing clones were randomly selected for telomerase assay. As shown in Fig. 4, the telomerase activities in these clones were low at early passage and diminished at mid-passage when cells became senescent. The one exception that became immortal (M15SV30) showed a greatly activated telomerase activity at mid-passage.

Telomerase Activity During Malignant Transformation. We have transformed SV40 immortalized Type I HBEC into weakly and highly tumorigenic cells by stepwise treatment with x-ray irradiation and neu

oncogene transfection (10). An examination of the telomerase activity in this series of clones revealed that telomerase activity may be further enhanced when the cells became weakly and highly tumorigenic compared to immortal non-tumorigenic cells. But there is no significant difference between weakly and highly tumorigenic cells (Fig. 5).

Discussion

Our results clearly indicate that telomerase activity was present in both Type I and Type II HBEC. This innate level of telomerase activity, however, appears not sufficient for immortalization since in all the 5 immortalized cell lines assayed, the telomerase activity was invariably activated either at mid or late passage. In contrast, for the 4 senescent clones assayed, the telomerase activity was never activated but diminished at the time when they became senescent. It is significant to note the correlation between the high susceptibility of SV40 transformed Type I HBEC clones to immortalization (10/11) and their high potential for telomerase activation and vice versa for Type II HBEC which infrequently became immortal (1/10).

Unlike a previous report concluding that telomerase is not a biomarker for malignant transformation (5), our results show that telomerase activation is a major event during the transition from extended lifespan to immortalization. This high telomerase activity was maintained when the immortal HBEC was malignantly transformed. We believe our results are more convincing since we measured telomerase

activity during the clonal evolution of a transformed cell instead of comparing tumor cells and unrelated normal cells. Furthermore, the mere presence of telomerase activity in both normal and tumor cells is not sufficient to conclude that telomerase activity is not a biomarker for malignant transformation since our results show that there is a striking quantitative difference in telomerase activity between normal and immortal or malignant HBEC.

Our study of this task is essentially completed. After assaying for a few more clones for both types of cells, we will prepare a manuscript for publication.

B. Characterization of Budding and Ductal Structures formed by Normal HBEC on Matrigel (Task 1)

Introduction

We have previously shown that Type II HBEC formed hollow spheres while Type I HBEC formed acini and, in conjunction with Type II cells, budding/ductal structures on Matrigel. The latter provides strong evidence that Type I HBEC contain both stem cell and luminal epithelial cells. The organoid system, when characterized, may be used 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 mammary carcinogenesis. The major objectives of this study are to determine the roles of the two types of cells in organoid

formation and to characterize (a) the organoid structures and (b) the conditions required for the formation of human breast epithelial organoids.

Materials and Methods

Cell Culture. The derivation of the two types of HBEC from reduction mammoplasty tissues and cell culture methods has been described previously (1).

Inoculation of HBEC on Matrigel for Organoid Formation and Growth. Growth factor-reduced Matrigel Matrix (Becton Dickinson Labware, Bedford, MA) was stored frozen in a -20°C freezer and thawed to liquefy in a 4°C refrigerator overnight prior to use. Either single-chamber Supercell culture slides (Fisher Scientific, Pittsburgh, PA) to be used for histological sectioning or 35-mm tissue culture dishes and 2-chamber Lab-Tek culture slide (Nunc, Inc., Naperville, IL) to be used for all other purposes such as organoid growth and immunofluorescent staining, were prepared in the sterile tissue culture hood. Once Matrigel is taken out of the refrigerator, Matrigel was kept on ice all the time to prevent premature gelling while it was being poured onto the dishes, after which it was put back right away in the refrigerator. 1 ml of Matrigel was poured onto the culture dishes or the slides to completely cover their surface. The dishes or the slides with the newly-poured Matrigel that is still liquid were rotated immediately to ensure that the Matrigel was

evenly distributed on the dish surface before the Matrigel solidifies. Matrigel sets at room temperature in a few minutes, after which ~1 ml of the cell suspension may then be plated on top of it. Matrigel remains solid in the 37°C humidified incubator where the cells are allowed to aggregate for a period of one day according to their specific affinities with each other. The next day, the cells will be attached to the Matrigel and the medium can be pipetted out. 1 ml of Matrigel is then poured on the freshly organized structures to prevent the structures from balling up because of excessive intercellular attraction and to keep the organoids viable for a long time. The overlaid Matrigel is then allowed to gel at room temperature for ~30 min, after which 1 ml of MSU-1 medium (1) is given on top of the overlay. The medium can diffuse through the gelled Matrigel to serve as the cells' source of nutrients during incubation and may be replaced every 3 days.

Replating of Organoids on Plastic. After supporting the organoids on Matrigel for a month, organoids and their parts may be harvested under the microscope in the hood using a micropipette and placed back on 35-mm plastic dishes. They are pressed down on the plastic using sterile needles in order to initiate their attachment on plastic as well as because the wounding process may stimulate epithelial growth. 1 ml of MSU-1 medium is then fed to the harvested organoids for incubation. Refeeding may require microcentrifugation if the organoids are not yet attached to

the plastic and is done every 3 days. Outgrowth of cells from attached organoids may be observed in a few days' time.

Alternate Growth of HBEC on Plastic and in Matrigel. Thus it is possible to transfer HBEC from growth on plastic into Matrigel and vice versa. The plastic (polystyrene) dishes can support growth up to 2 weeks, while Matrigel can support it much longer, but usually the transfers are done on or after 1 month of growth on Matrigel.

Immunofluorescent Staining of Organoids on Matrigel. The existing medium in the 35-mm Matrigel-covered dish containing adherent organoids was replaced with 4% formaldehyde (Mallinckrodt Specialty Chemicals Co., Paris, KY) + 0.5% Triton X-100 (X-100; Sigma) in PBS and incubated for 10 min at room temperature to fix and permeabilize the cells' plasma and nuclear membranes. Then it was replaced sequentially with absolute methanol for 30 sec to stabilize membrane proteins and .05% saponin (S-7900; Sigma) in PBS for 30 min. The cells were then rinsed with PBS 3x before 10% NGS (normal goat serum) in PBS was added and incubated for 1 hr to block nonspecific binding of the antibodies. Then the blocking solution was replaced with primary antibodies diluted at 1:50-200 in 1% NGS + 0.1% bovine serum albumin (A-3294; Sigma) in PBS and incubated overnight in a shaker in the cold room. Next day, the primary antibody solution is rinsed off with 3 changes of PBS to remove any remaining primary antibody that can compete with the binding of the secondary antibody to be added. Then a

compatible secondary antibody conjugated with a fluorescent dye (those from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA are specific and have minimal cross-reactivity with other epitopes) diluted at 1:200 with the same antibody diluent that was used with the primary antibody replaces the last PBS wash and incubated for 1 hr in a shaker at room temperature. The dish is covered from light during the entire incubation time to prevent weakening of fluorescence. The last step is rinsing with 3 changes of PBS without discarding the last wash to prevent drying while viewing and taking photographs of the immunofluorescent-stained cells under the fluorescent microscope using the appropriate filter. Positive and negative control cells, if known, were used, and controls for nonspecific staining were performed by deleting the primary antibodies from the protocol.

Results

Organoid Structures Formed by Two Types of HBEC. When Type I and Type II cells were plated separately on Matrigel, the Type I cells characteristically formed acinar structures (Fig. 6) and cell aggregate with some capability for limited budding (Fig. 7), while Type II cells form hollow spheres (Fig. 8) and elongated structures. Hollow spheres have a bigger central lumen with the surrounding layers of cells lying flat against each other, while the cells of an acinus are arranged in a radial column like the sections of an orange, having almost no lumen in the center. When the two types of cells are plated together on Matrigel, they

form buds and ducts as well (Figs. 9 and 10). Type I and Type II cells mixed in 1:2 ratio formed bud/duct-like structures in one day (Fig. 11). Almost all the cells plated were involved in the formation of the structures. These bud/duct-like structures may form continuous lumen-like structures after extended culture (Fig. 12). Trypan Blue solution injected from the broken end of a duct was able to fill the lumen-like area, indicating the existence of a cavity in that area (13). The forming of budding/ductal structures is reproducible using different HBEC cultures from different women (e.g., HME 15, Fig. 9; HME 27, Fig. 10). Aggregates of Type I and Type II cells can also form extensive budding structures which are cauliflower-like (Fig. 14).

Conditions for Budding/Ductal Structure Formation. We have identified two conditions as being critical for the cells to form budding/ductal structures on Matrigel: The two types of cells have to be in right numbers (~5 x 10^5 per 4 cm^2 chamber) and ratio (Type II/Type I HBEC = 2 or more) on initial plating and they only grow in our formulated MSU-1 medium, which contains some growth factors and hormones but is serum-free. Fetal bovine serum (FBS) inhibits budding/ductal growth on Matrigel, and deleting any of the major growth factor/hormone supplements (EGF, hydrocortisone, insulin) drastically reduces their growth as was found in monolayer culture on plastic (2). Under the culture conditions using serum-free MSU-1 medium, the cells

are able to form organoid structures and sustain long-term culture (over 1 month).

The organoids with budding/ductal structures can be formed on top of Matrigel (Fig. 9) or between 2 layers of Matrigel (embedded) (Fig. 10). The latter seems to allow the organoids to grow longer in healthy state.

Alternate Growth of HBEC as Monolayer on Plastic and Organoid in Matrigel. Growing the cells on plastic may support growth of the cells for only about 2 weeks before *in vitro* cellular senescence becomes evident. This might be because the growth of cells on plastic changes the stem cell phenotype too quickly. In contrast, Matrigel might allow organoids to preserve its stem cell character longer. This reality may be rooted on the different signalling pathways that the various cellular integrins discharge. For instance, the polystyrene dishes were initially developed for the purpose of culturing fibroblasts and exhibit a greater affinity for the fibronectin receptors of the cell. In contrast, Matrigel consists mostly of laminin (~60%) and collagen IV (~30%), which makes it more suitable for the epithelial cells' laminin receptors.

The budding/ductal structures can be maintained on Matrigel for more than one month with initial active growth of terminal end-bud-like structures (cauliflower-like buds) followed by the formation of lumen-like structures in the center of a duct and final stage of quiescence in growth.

The transfer of these late stage quiescent organoids to a new Matrigel may initiate some new ductal and budding growth.

Using the technique described in Materials and Methods, the two types of HBEC found in the monolayer culture on plastic can be derived from organoids (Figs. 15 and 16). These cells can be used to develop organoids again on Matrigel similar to the original organoids with ductal and budding structures (Fig. 17).

Discussion

Since Type II HBEC did not form terminal end-bud-like structures, they do not exhibit stem cell characteristics. However, Type II cells appear to be essential for ductal framework formation since Type I cells alone lack the ability to form the ductal structure. Type I cells alone formed acinar structures similar to those acini described by Petersen and Bissel using luminal epithelial cells (11) and few bud-like structures with limited growth. Strikingly, Type I and Type II cells together are able to form ductal structures in one day on Matrigel. Many actively-proliferating buds were developed from the organoids (cauliflower-like). Therefore, we can conclude that Type I population contains both stem cells and luminal epithelial cells.

The stem cell nature of Type I cells is reinforced by the observation that organoids formed by Type I and Type II cells can be preserved for more than one month and healthy undifferentiated Type I and Type II cells can be derived from them as if they were freshly derived from

reduction mammoplasty tissues. The alternate cycling of these HBEC as organoid in Matrigel and as monolayer culture on plastic allow them to grow for at least 3 months.

The budding/ductal organoids formed by the two types of HBEC will be a useful *in vitro* model to analyze factors that regulate normal and abnormal growth and differentiation of human mammary gland. The model also will be useful to study the mechanism of human breast carcinogenesis since large numbers of target cells (believed to be in terminal end-bud) are available for long-term study in a structure under an environment resembling the *in vivo* condition.

This project is largely completed except for the immunofluorescent staining of the organoids to reveal gene expression. The technique for this part of the study has been developed as described in Materials and Methods. It has not been finished by this time mainly because my graduate research assistant who worked on this project had to leave the country because she was not successful in obtaining a waiver for her exchange visa.

C. 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 preliminary results from this study indicate that these three chemicals are essential for budding/ductal growth. A systematic study of these and other chemicals such as estrogen and prolactin will be carried out in the coming year.

D. 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).

This study will be a priority to complete in the coming year. 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).

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

This task was completed before the contract started. A detailed report was presented in last year's 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).

F. 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 in Appendix). 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.

We are in the process of immortalizing HBEC by x-rays. We will characterize the ER expression when these immortal cell lines are available.

G. 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. 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 will test this hypothesis in the coming year.

H. 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.

I. 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, 10). 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). Our preliminary results show that the wild type ER expressed *in vivo* and in Matrigel was responsive to estrogen as measured by cell growth and progesterone receptor expression which is inducible by estrogen. A more detailed study will be carried out in the coming year.

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

Cell Line	Parental Cell Type	Telomerase activity at different cpdl*			Immortalized
		Low	Middle	High	
M15SV3	Type I	+	+	++++	Yes
M15SV4	Type I	+	++++	++++	Yes
M15SV6	Type I	+	++++	++++	Yes
M15SV8	Type I	+	++++	++++	Yes
M15SV5	Type I	±	±	(Senescent)	No
M15SV21	Type II	+	±	(Senescent)	No
M15SV24	Type II	+	±	(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.

Figure Legends

Figure 1. Telomerase activity in Type I and Type II HBEC. Telomerase activity was detected by PCR-based telomeric repeat amplification protocol (TRAP) as described in Materials and Methods. Lane 1 and lanes 5, 9, 13, 15 represent negative control and heat inactivated controls, respectively. Type I, Type II HBEC and fibroblast 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) HBEC; the activity in fibroblasts was undetectable (lanes 10, 11, 12).

Figure 2. No telomerase inhibitor present in Type I and Type II HBEC. 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 HBEC was not due to the presence of telomerase inhibitors in these cells. Lane 3 and lane 6 show the telomerase activity in cell lysate 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, indicating no telomerase inhibitor was present in Type I and Type II HBEC.

Figure 3. Telomerase activity in SV40 transformed Type I HBEC. Four SV40 transformed cell lines (M15SV3, M15SV4, M15SV6, and M15SV8) which became immortal were examined for telomerase activity at low passage (L, ~25 cpdl), middle passage (M, ~55 cpdl), and high passage (H, >100 cpdl). Among eleven SV40 transformed clones, which acquired

extended lifespan, the only one that failed to become immortal (M15SV5) showed little or no detectable telomerase activity at low passage (lane 14). Cell lysate derived from 500 cells was used for telomerase activity assay for each sample.

Figure 4. Telomerase activity in SV40 transformed Type II HBEC. The majority of SV40 transfected Type II clones (nine out of ten) could not become immortal after extended lifespan. Three (M15SV21, M15SV24 and M15SV29) among these nine clones were analyzed for telomerase activity at low passage (L, ~25 cpdl) and middle 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 8). Cell lysate derived from 500 cells was used for telomerase assay for each sample.

Figure 5. Telomerase activity in HBEC during neoplastic transformation from immortal to malignant stage. In order to analyze the quantitative change of telomerase activity during the course of neoplastic transformation, DNA positive control (lane 2) was used as a standard to compare the level of telomerase activity in a series of cell lines transformed at different stages of neoplastic transformation. Lanes 4 to 6 show telomerase activity of normal Type I HBEC. M13SV1

(lanes 8 to 10) is an immortal cell line from Type I HBEC by SV40 transformation. M13SV1R2 (lanes 12 to 14) is a weakly tumorigenic cell line and derived from M13SV1 after x-ray irradiation. M13SV1R2N1 (lanes 16 to 18) is a highly tumorigenic cell line and derived from M13SV1R2 after neu oncogene transfection.

Figure 6. Acini structure formed by Type I HBEC. The structure is typically formed by normal luminal epithelial cells.

Figure 7. A bud-like structure formed by Type I HBEC (HME 15) on Matrigel in 19 days after inoculation.

Figure 8. Hollow balls formed in Matrigel by Type II HBEC (HME 23) in 16 days.

Figure 9. Terminal end-bud-like structures formed by Type I and Type II HBEC (HME 15) on Matrigel in 19 days.

Figure 10. Budding/ductal structures formed by Type I and Type II HBEC (HME 27) in Matrigel in 20 days.

Figure 11. Ductal structures formed by Type I and Type II HBEC (HME 27) in 1:2 ratio in one day after inoculation of cells on Matrigel.

Figure 12. Lumen-like structures formed in the center of duct-like structures by Type I and Type II HBEC (Top HME 15, bottom HME 27) in 30 days (HME 15) and 20 days (HME 27).

Figure 13. Trypan blue injected from the broken end of a duct was able to fill the lumen-like area, indicating the existence of a cavity in that area. The ducts were formed by Type I and Type II HBEC (HME 15).

Figure 14. Highly proliferative buds (cauliflower-like) formed by Type I and Type II HBEC (HME 15).

Figure 15. Type II HBEC (HME 15) derived from organoid formed by Type I and Type II HBEC on Matrigel, are proliferating on plastic dish.

Figure 16. Type I HBEC (HME 15), derived from organoid formed by Type I and Type II HBEC on Matrigel, are proliferating on plastic dish.

Figure 17. Budding/ductal structures formed by Type I and Type II HBEC (HME 15) derived from organoid in 28 days.

Figure 18. Detection of the 51 kd ER in Western blot either by C-terminal ER antibody or by N-terminal ER antibody. The tumorigenic cell line derived from HBEC cells, M13SV1R2-N1, was harvested under different conditions: **CP**, cell-culture on plastic; **TT**, tumor tissues developed in nude mice; **TTM**, tumor tissues removed from nude mice, then maintained on Matrigel for two weeks; **TTC**, tumor tissues removed from nude mice, then maintained in cell culture medium in a plate for two weeks. Tumors were developed either in male nude

mice (lanes A-2, 4, 6 and B-2, 4, 6) or in female nude mice (lanes A-3, 5, 7 and B-3, 5, 7). The result shows that the 51 kd ER expressed either in tumor or in Matrigel is different from the 48 kd ER not only in molecular weight but also in its detectability by the N-terminal ER antibody, which suggests a difference between their splicing patterns in post-transcriptional modulation.

Figure 19. The effects of estradial on growth of two tumorigenic Type I HBEC lines transformed *in vitro* by SV40, x-rays and neu oncogene. Cells were harvested 3 days after estradial treatment by a dose which promoted the growth of estrogen-responsive MCF-7 cells.

Figure 1. Telomerase Activity in Type I and Type II HBEC

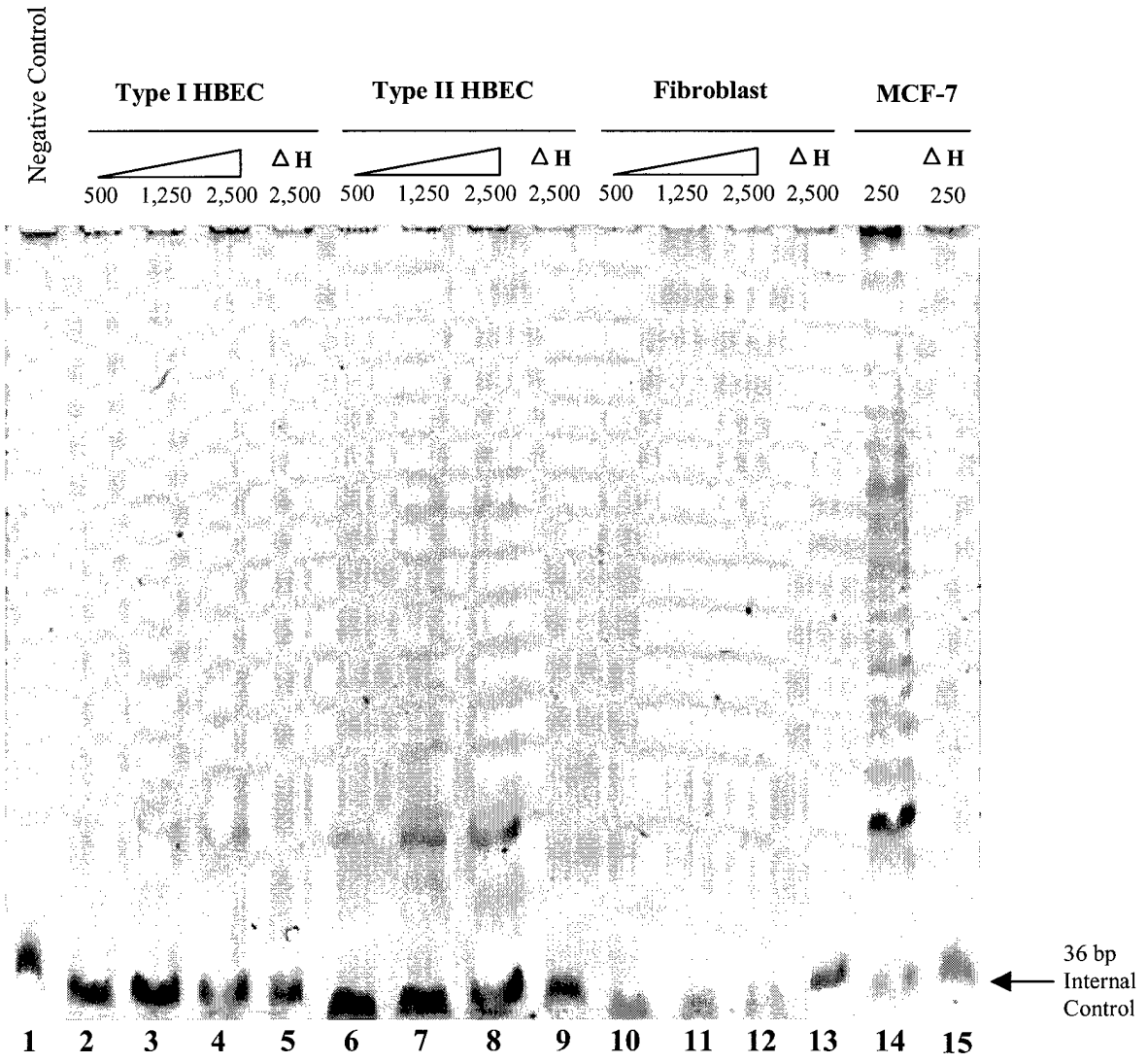


Figure 2. No Telomerase Inhibitor Present in Type I and Type II HBEC

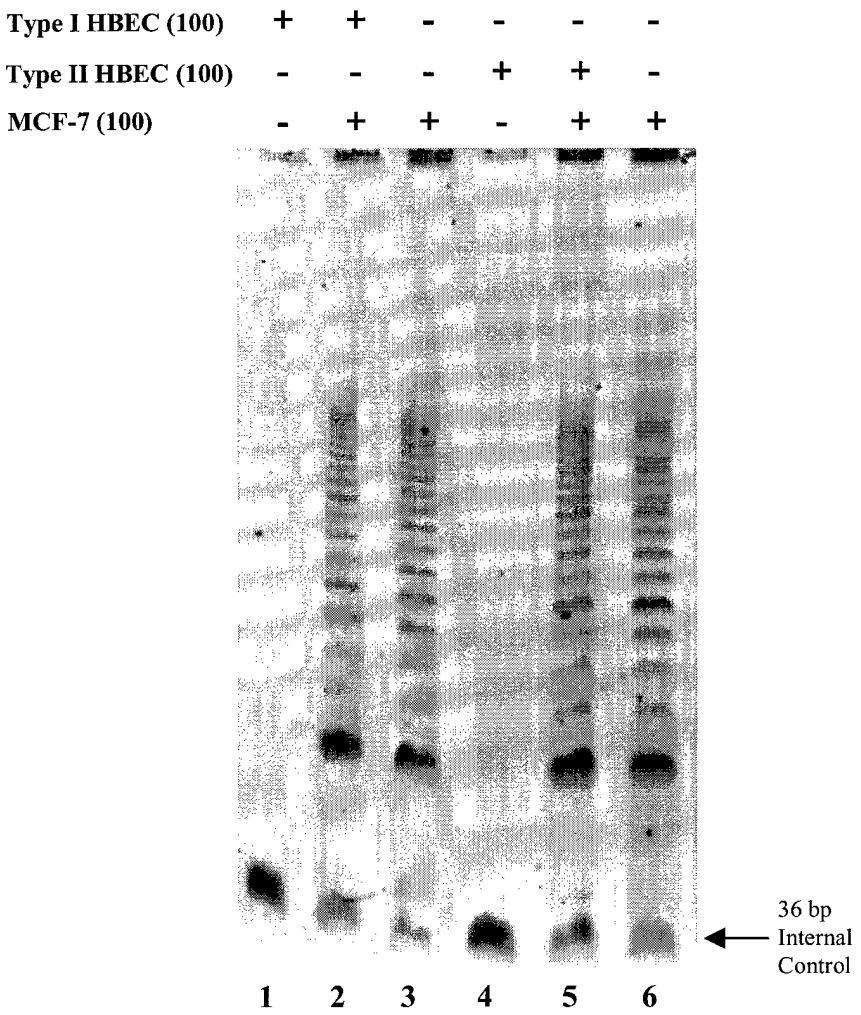


Figure 3. Telomerase Activity in SV40 Transformed Type I HBEC

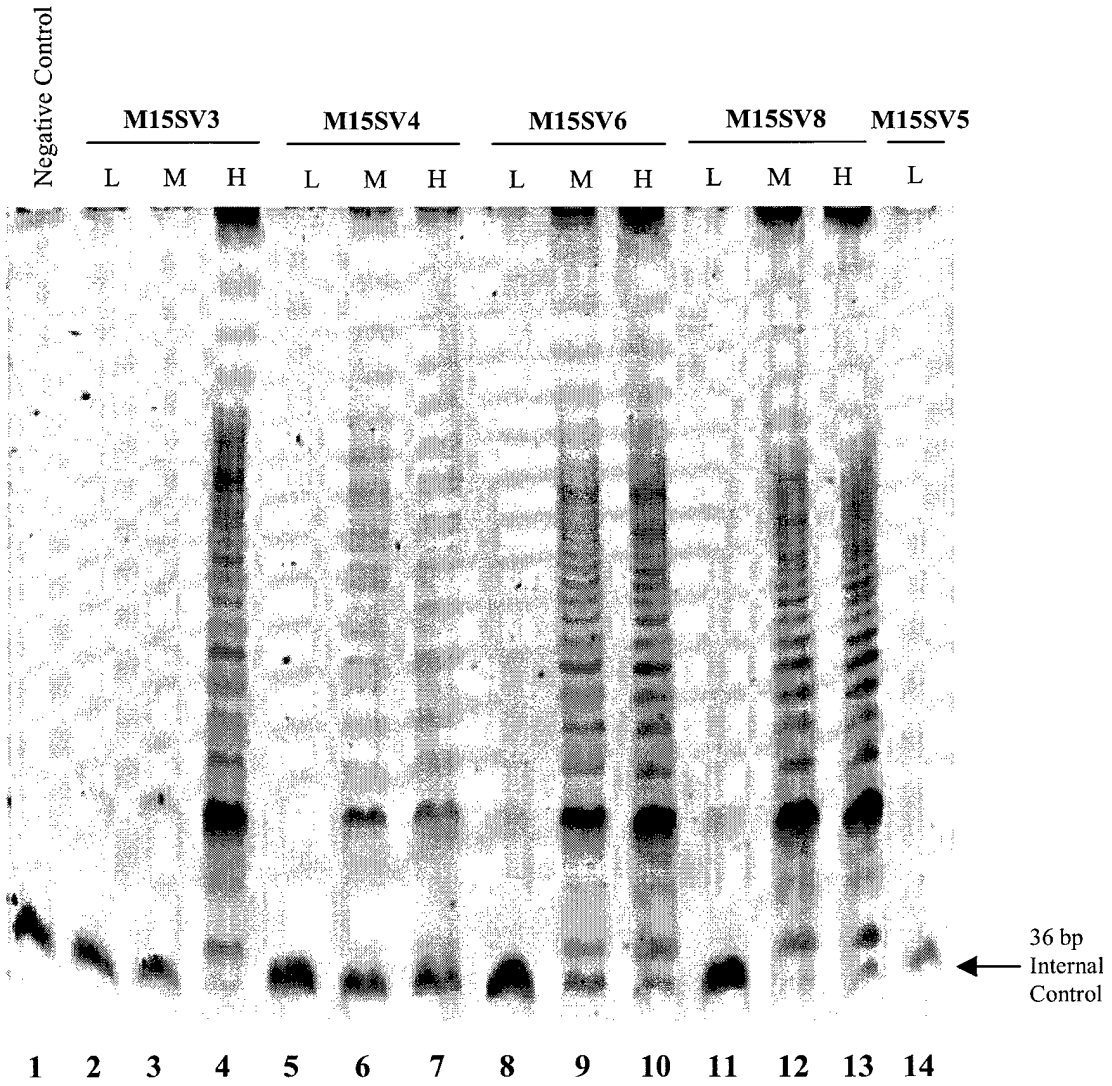


Figure 4. Telomerase Activity in SV40 Transformed Type II HBEC

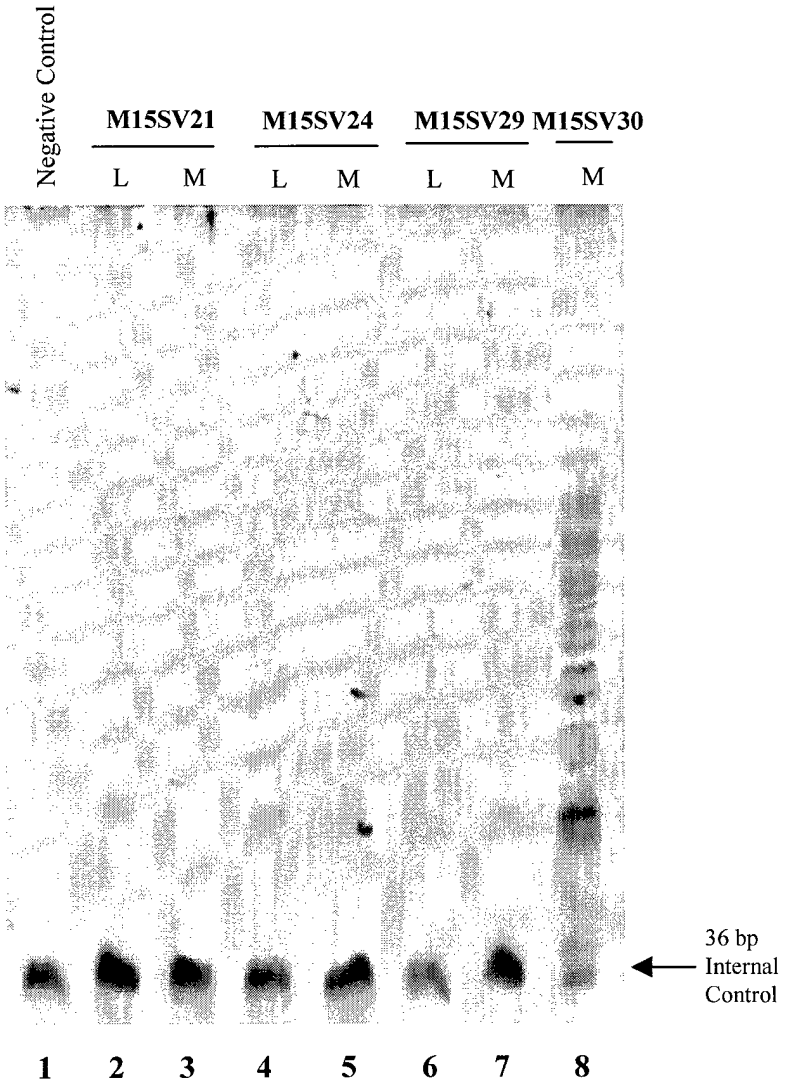


Figure 5. Telomerase Activity in HBEC Neoplastic Transformation Series

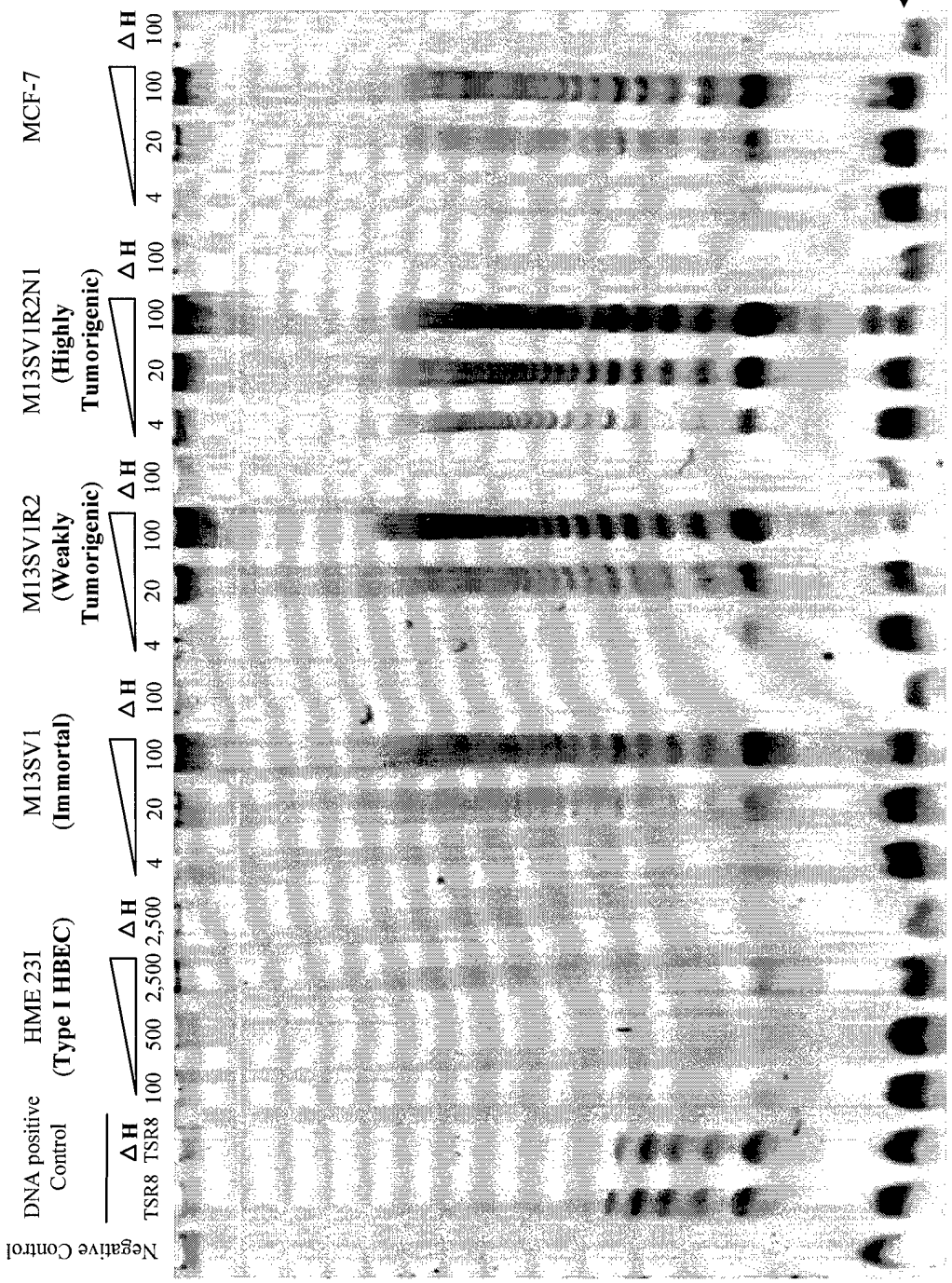


Figure 6

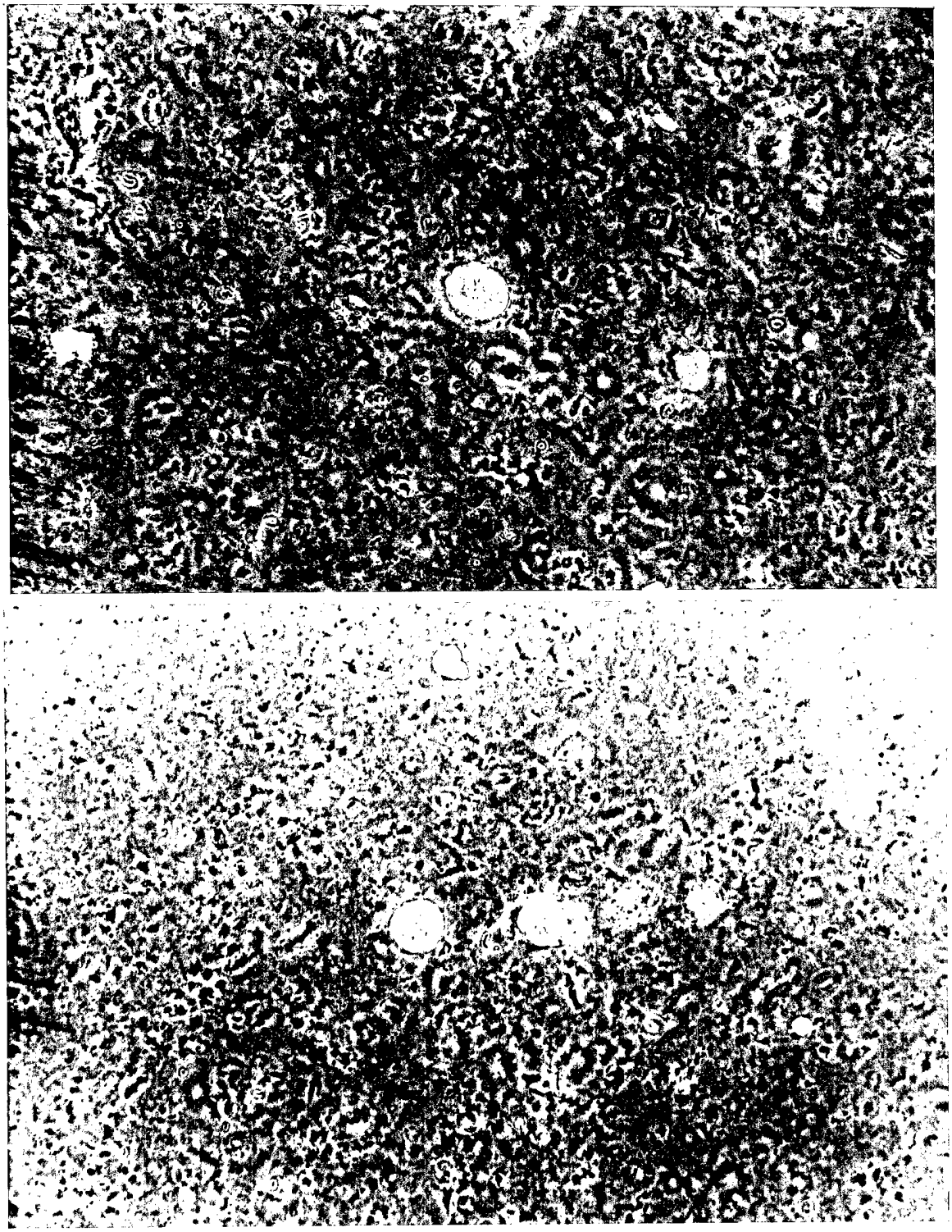


Figure 7

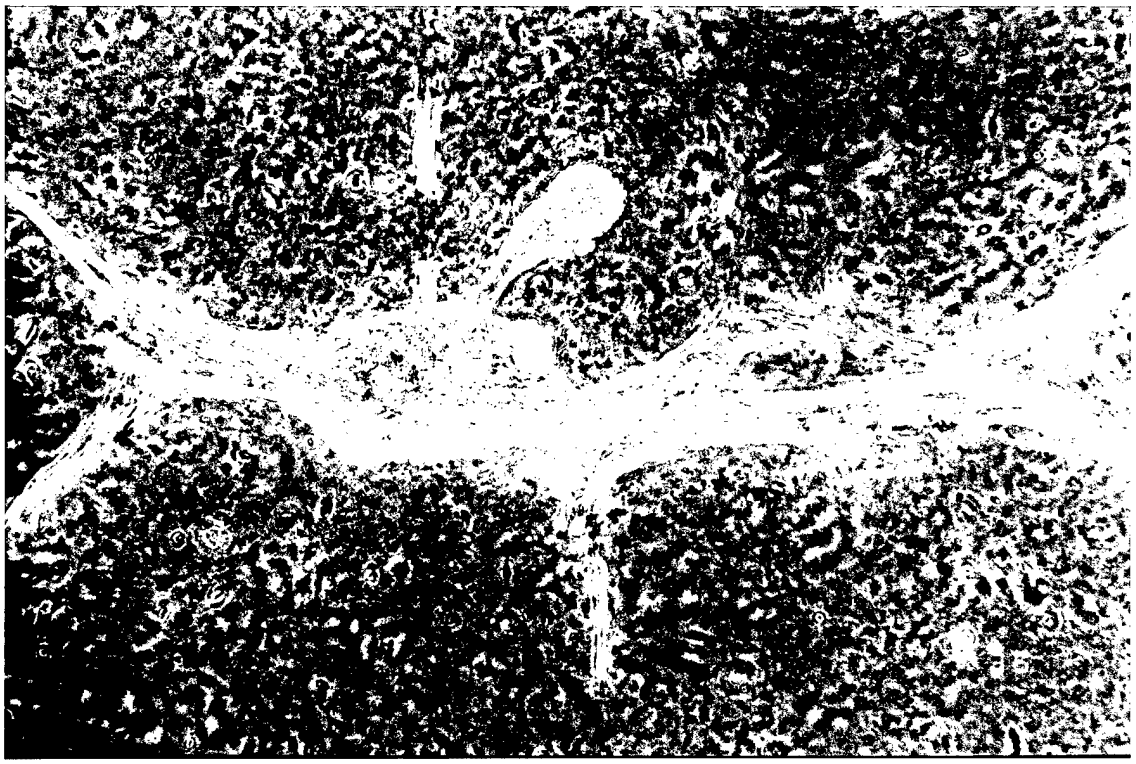


Figure 8

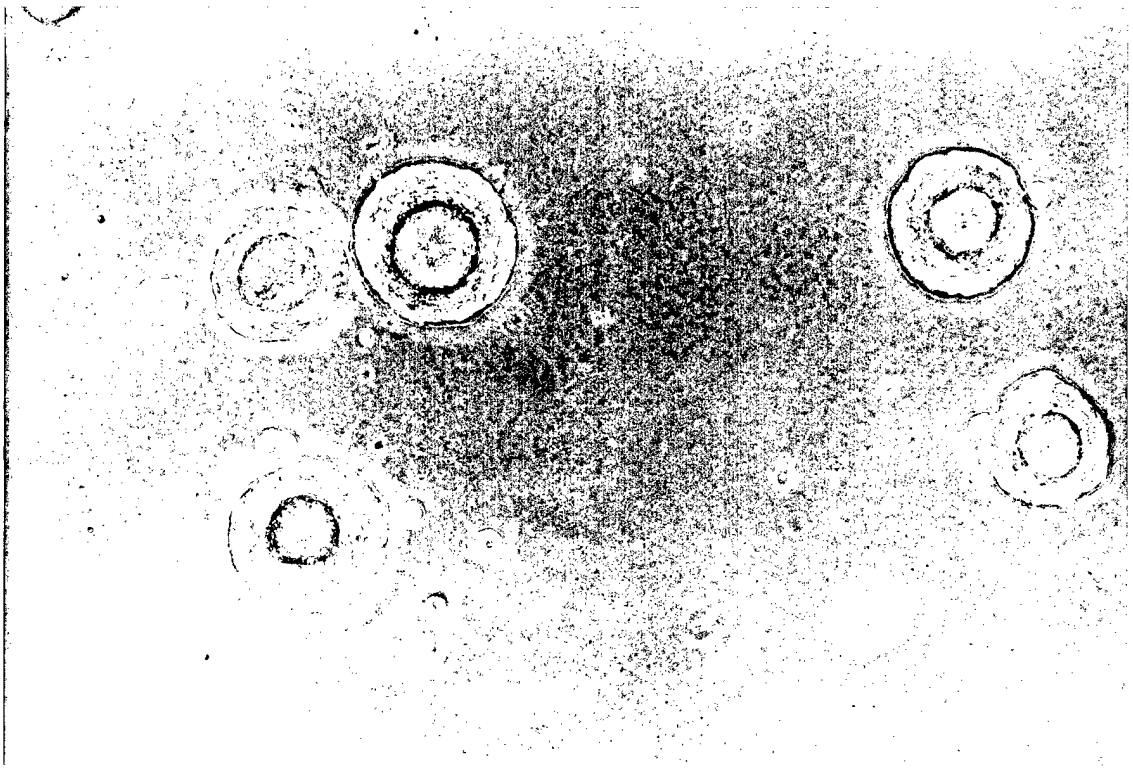


Figure 9

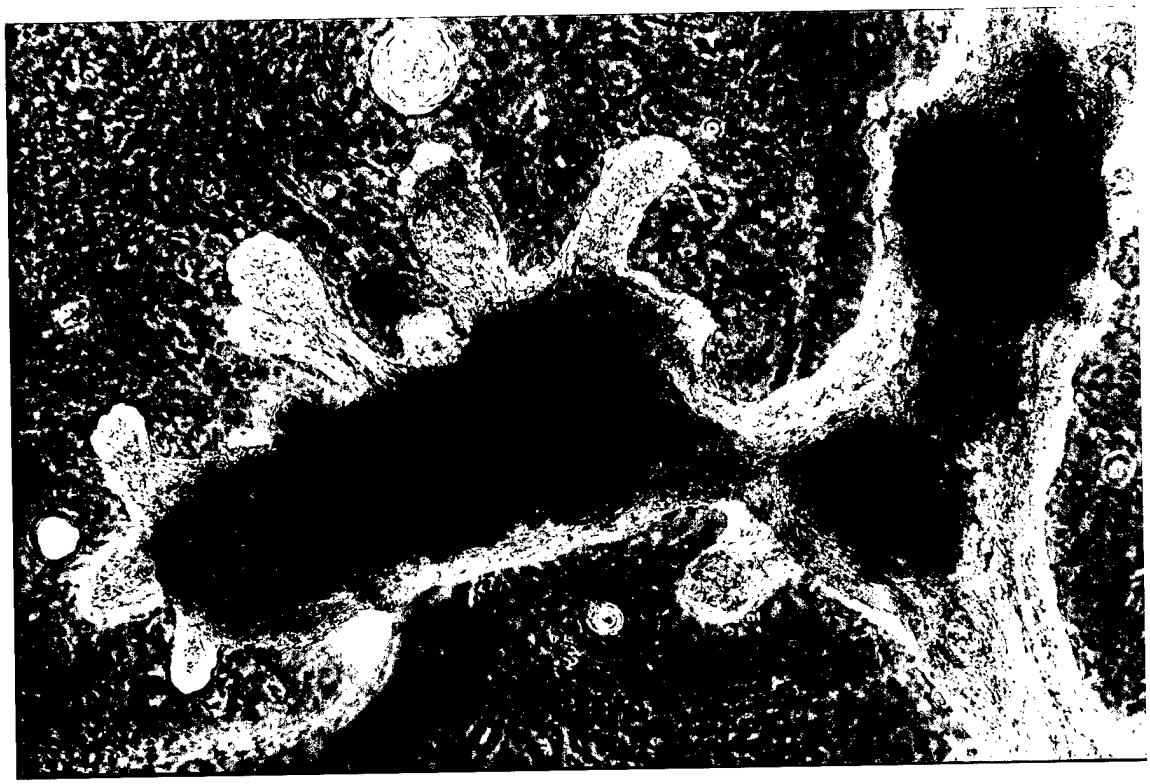


Figure 10

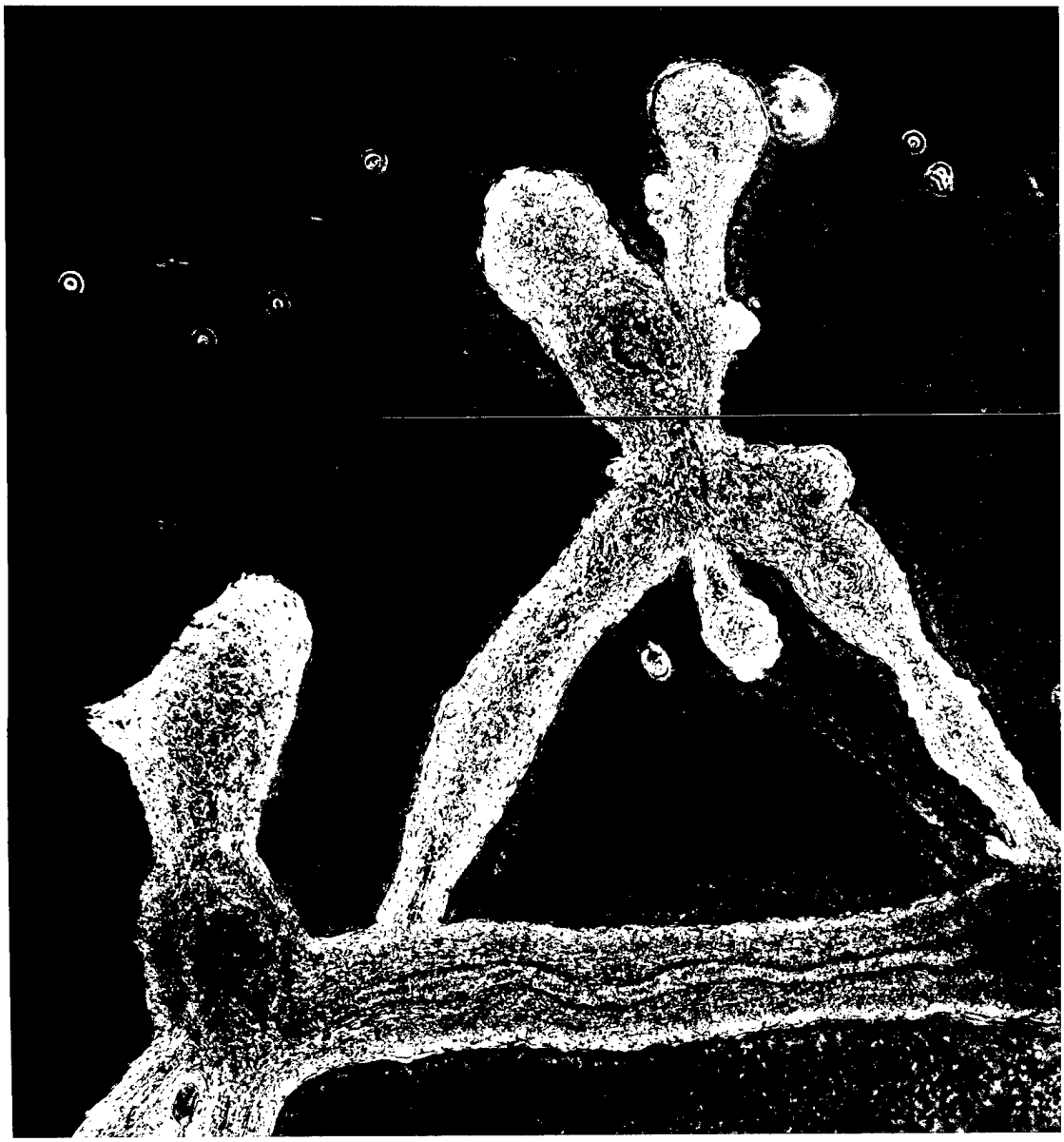


Figure 11

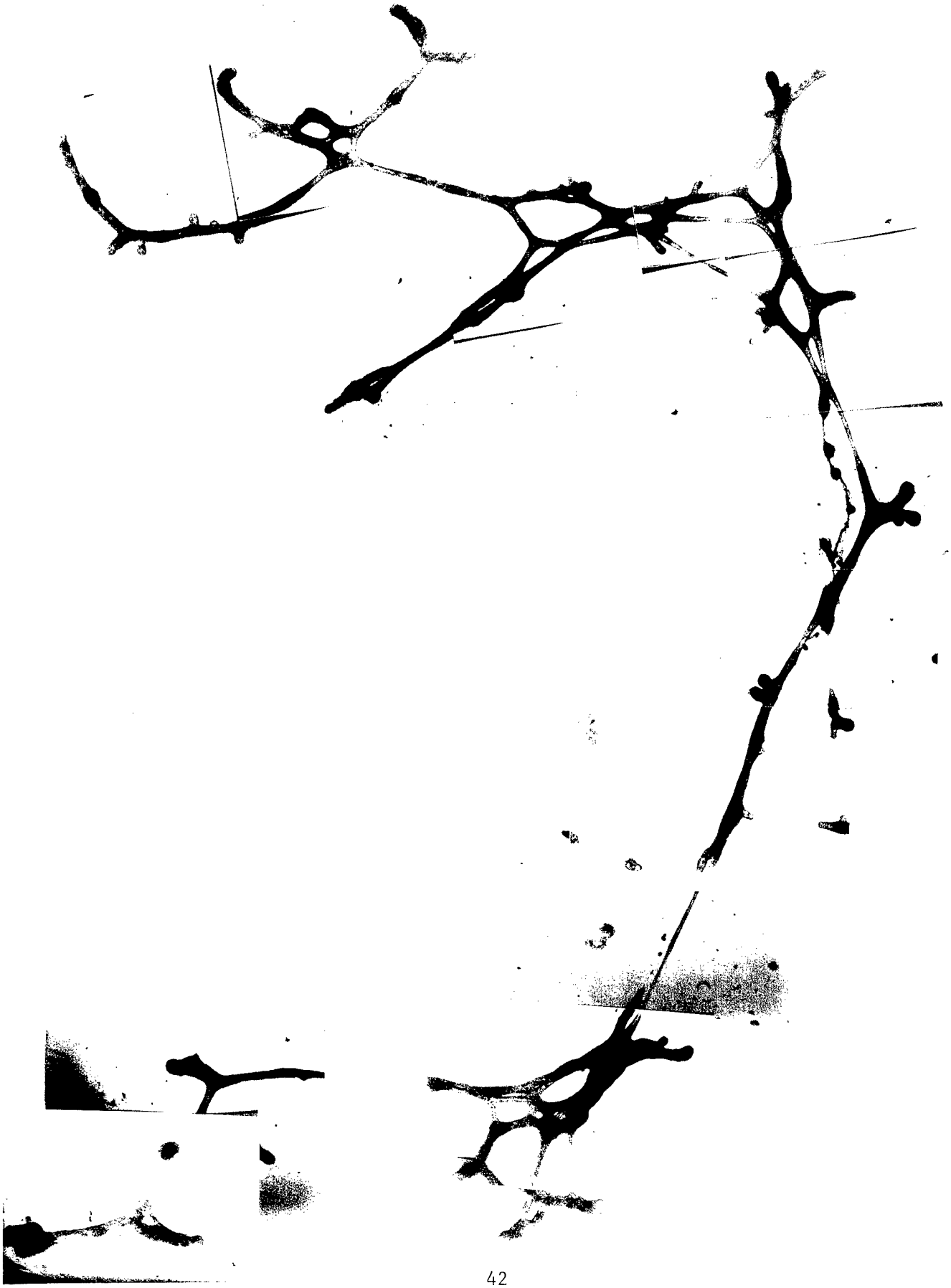


Figure 12

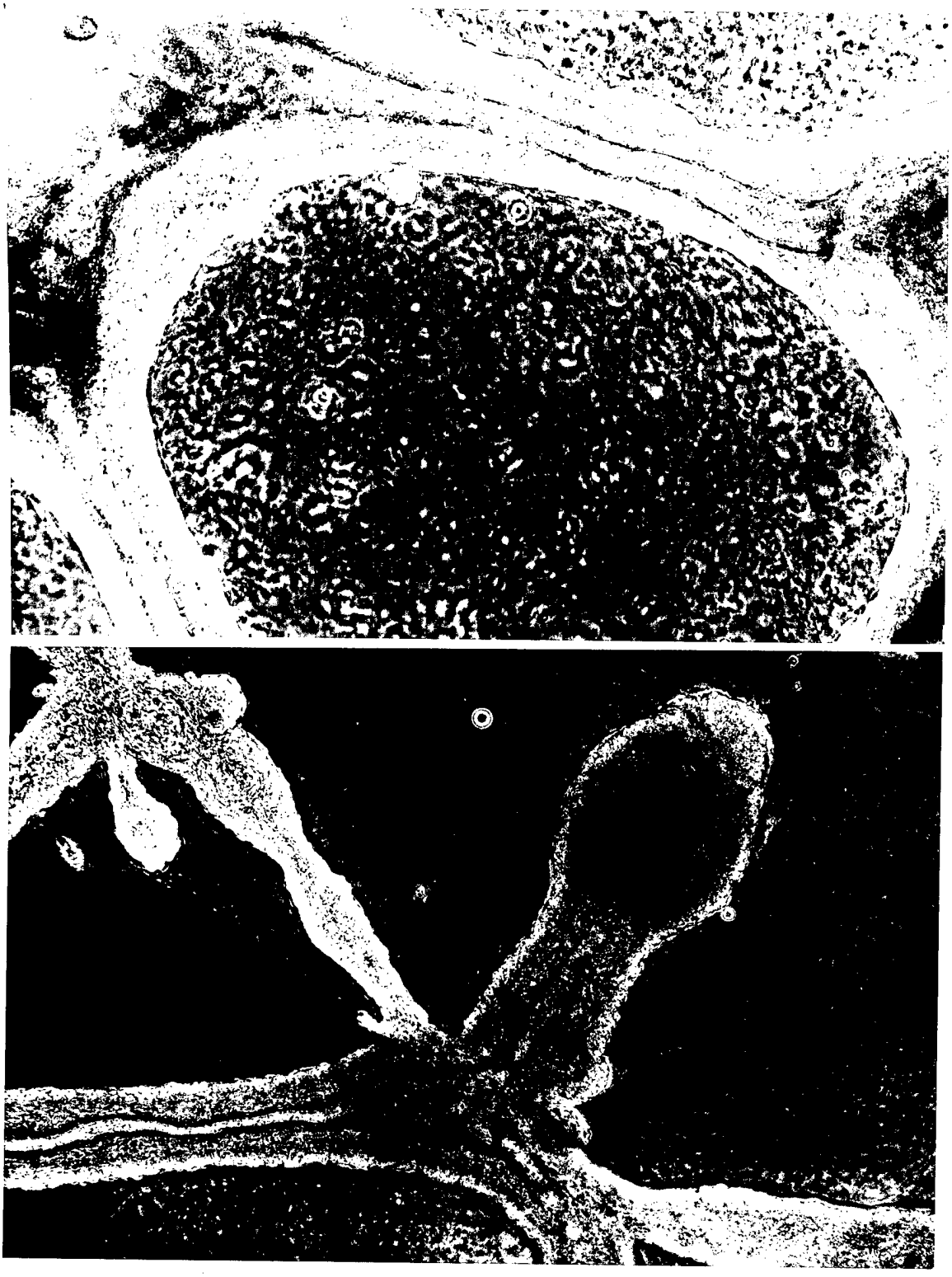
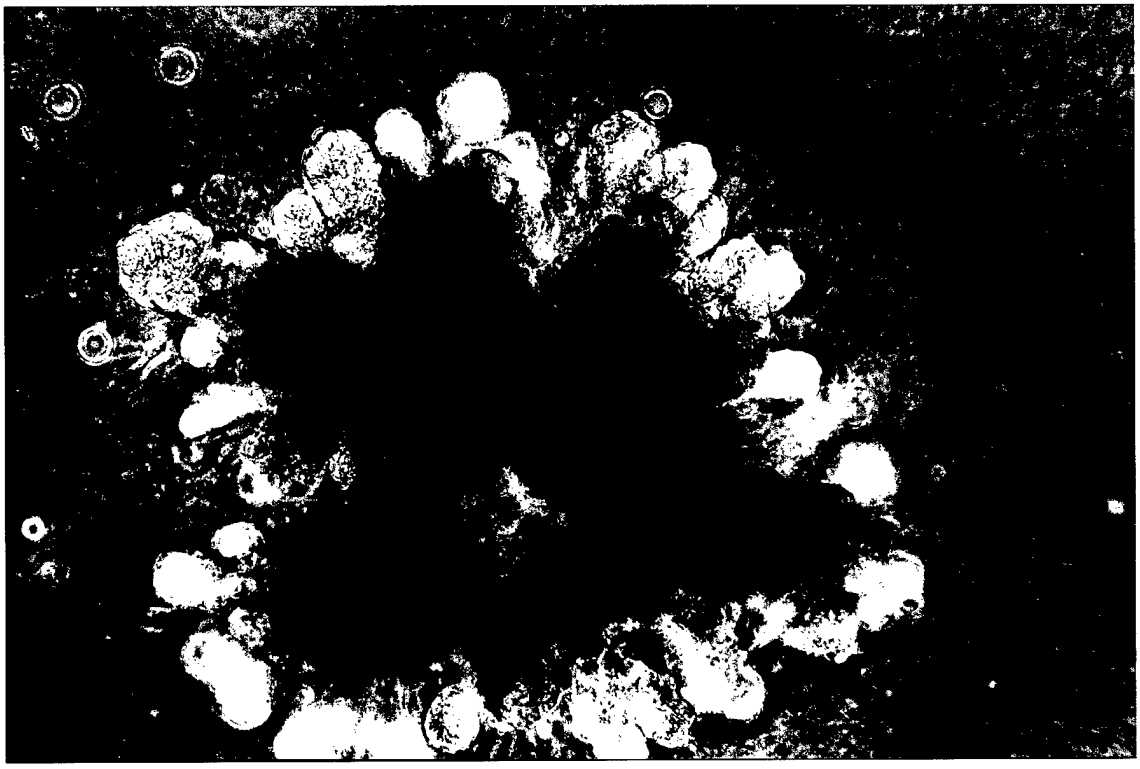


Figure 13

Figure 14



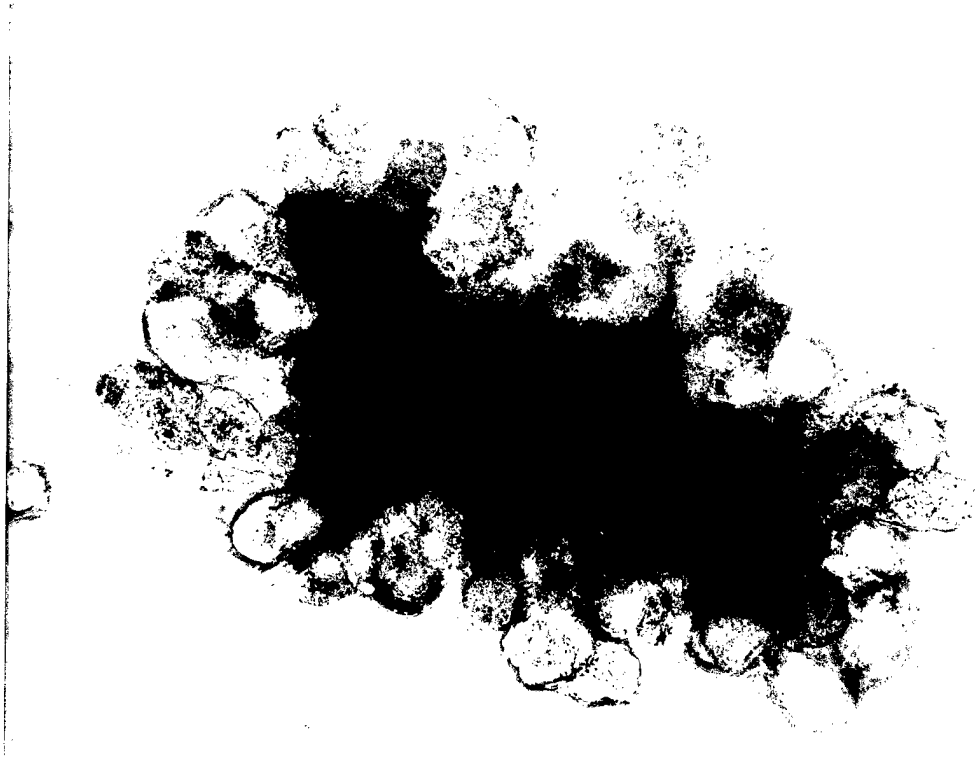


Figure 15

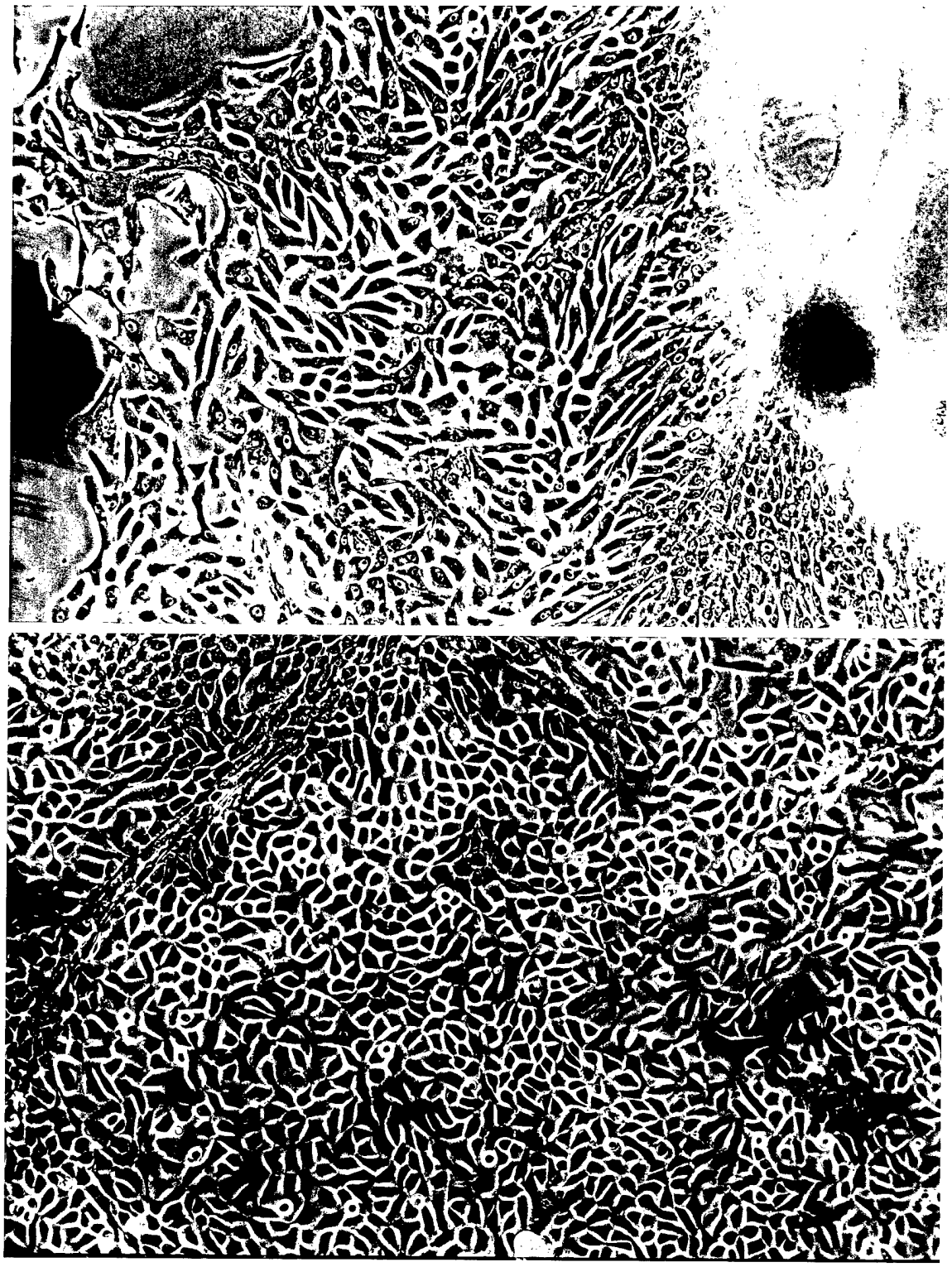


Figure 16

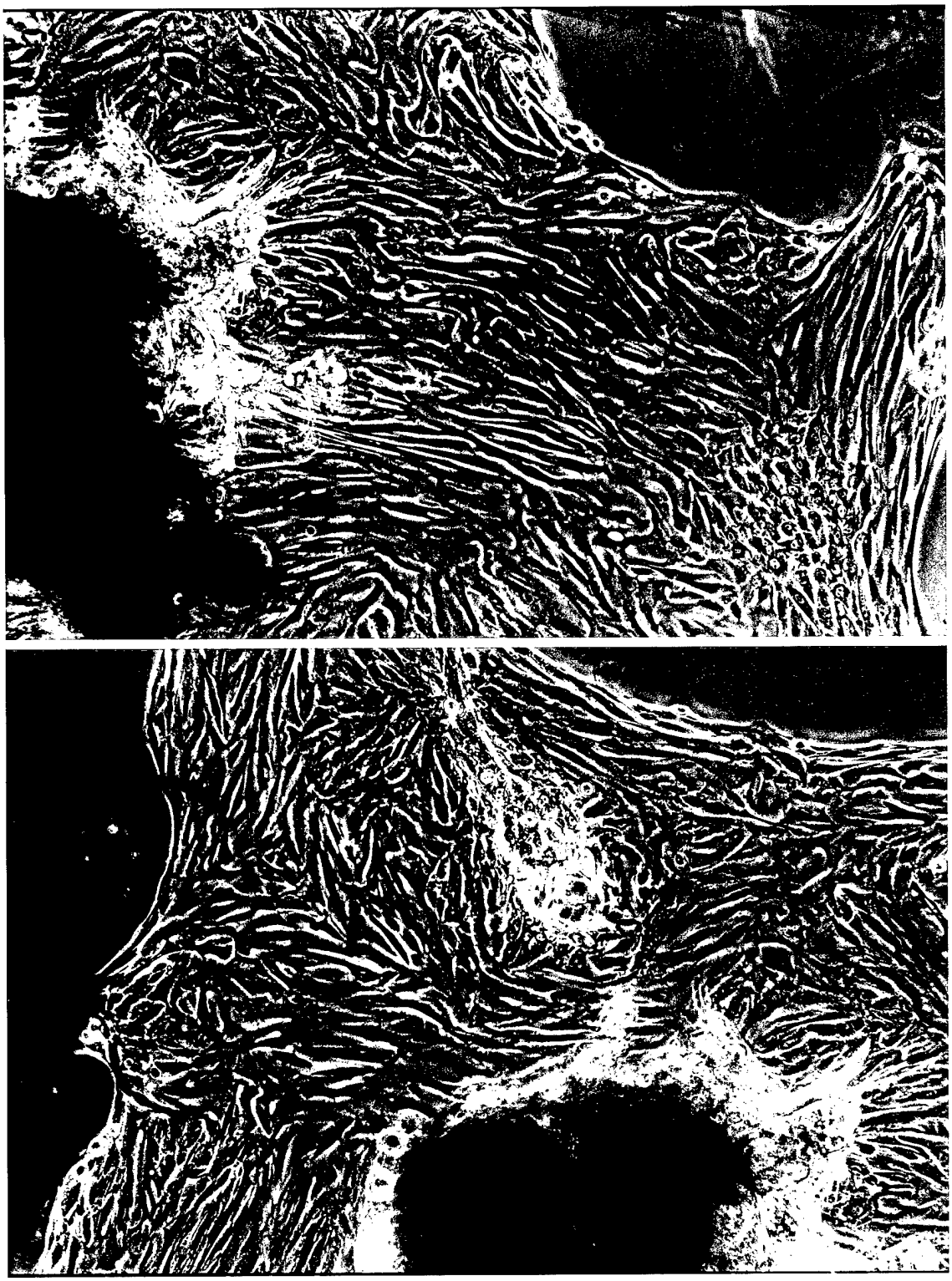


Figure 17

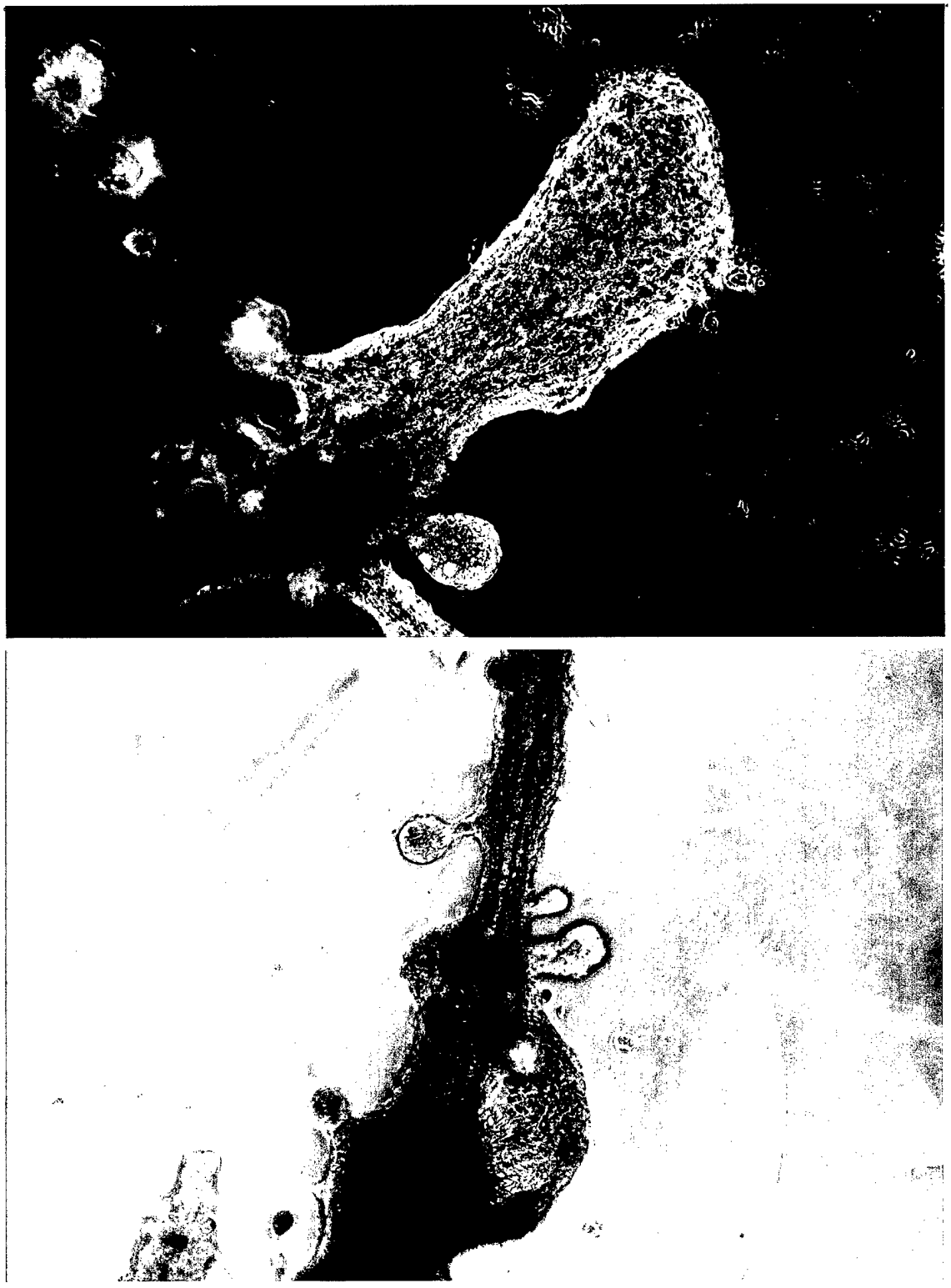


Figure 18. Detection of the 51 kDa ER in western blot either by C-terminal antibody (A) or by N-terminal antibody (B).

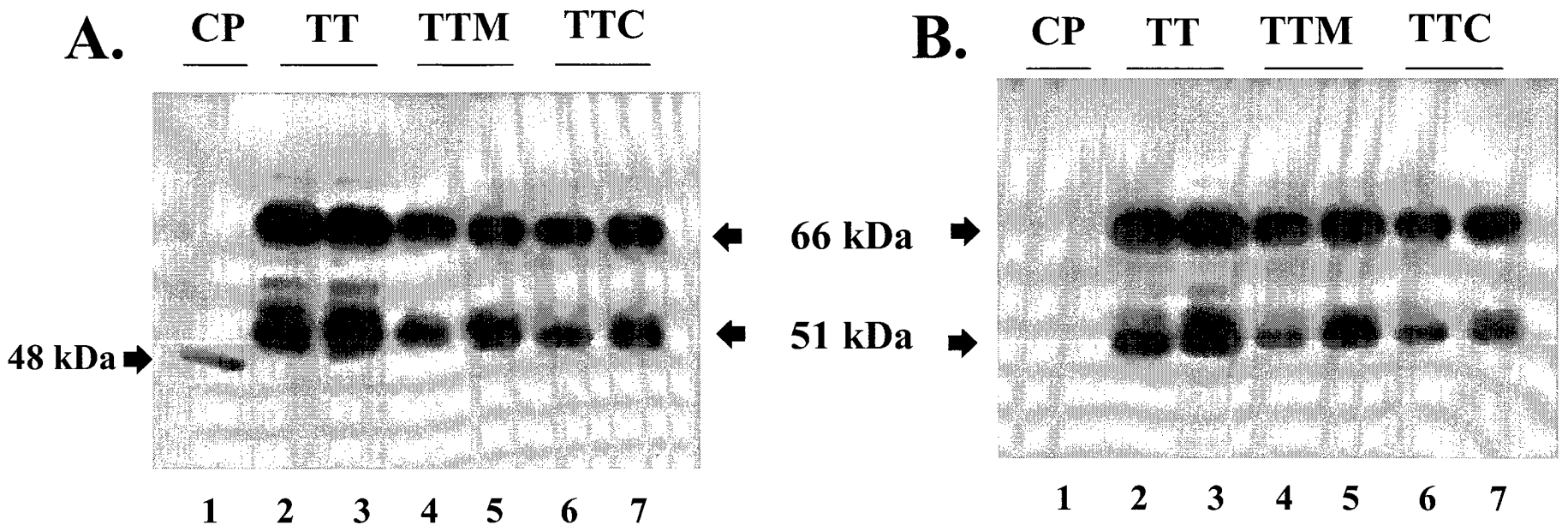
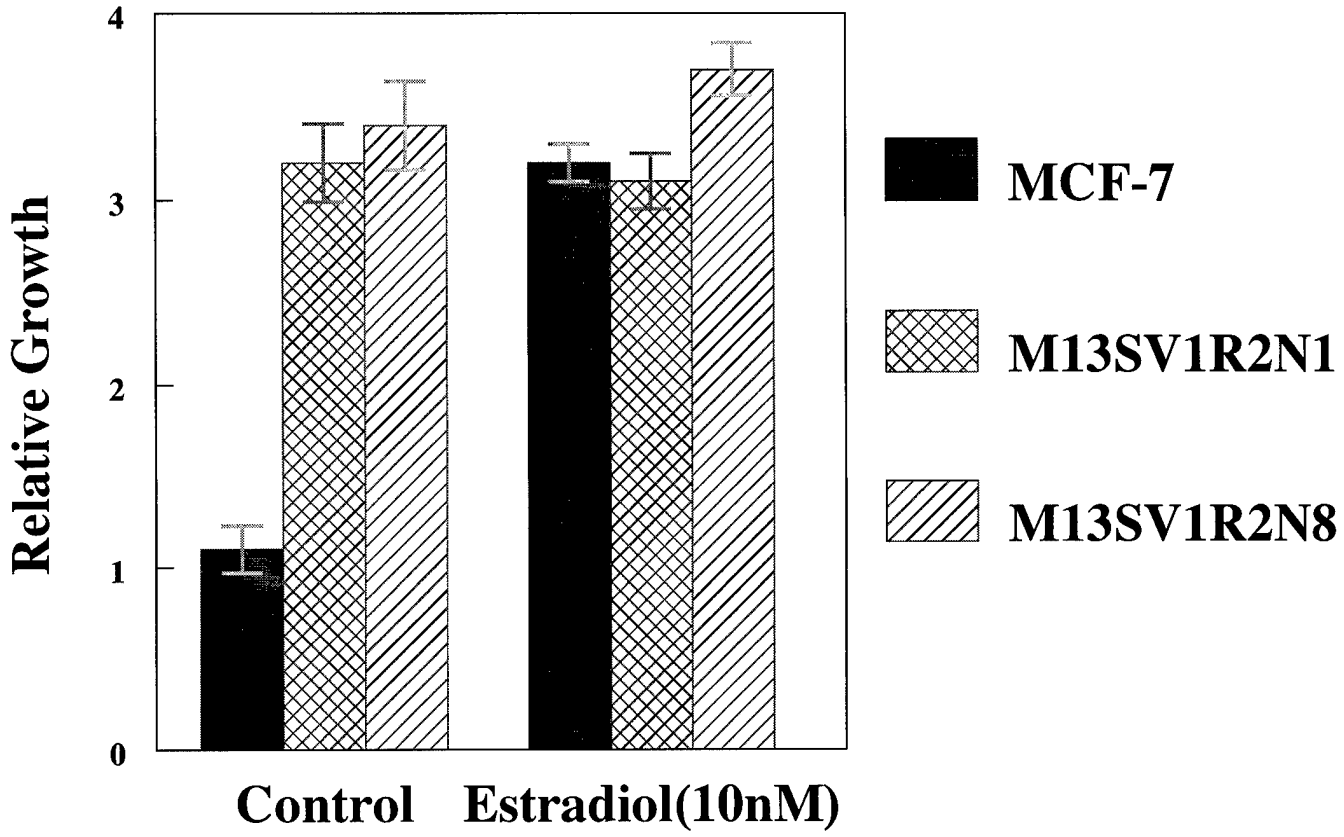


Figure 19


Relative Growth
4
3
2
1
0
Control
Estradiol(10nM)
MCF-7
M13SV1R2N1
M13SV1R2N8

VI. CONCLUSIONS

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 (10/11) appears to be correlated with their high potential for telomerase activation, and vice versa for Type II HBEC which became immortal at low frequency (1/10). Therefore, telomerase activation could be a major biomarker and mechanism for the transition from extended lifespan to immortalization, contrary to a previous report (5). 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 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.

VII. REFERENCES

1. C.Y. Kao, K. Nomata, C.S. Oakley, C.W. Welsch, C.C. Chang, *Carcinogenesis* **16**, 531-538 (1995).
2. C.Y. Kao, C.S. Oakley, C.W. Welsch, C.C. Chang, *In Vitro Cell. Develop. Biol.* **33**, 282-288 (1997).
3. K.S. Kang, I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko, C.C. Chang, *Carcinogenesis* **18**, 251-257 (1997).
4. N.W. Kim, M.a. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, J.W. Shay, *Science* **266**, 2011-2015 (1994).
5. C.D. Belair, T.R. Yeager, P.M. Lopez, C.A. Reznikoff, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13677-13682 (1997).
6. C. Chadeneau, K. Hay, H.W. Hirte, S. Gallinger, S. Bacchetti, *Cancer Res.* **55**, 2533-2536 (1995).
7. C. Harle-Bachor and P. Boukamp, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6476-6481 (1996).
8. S. Yasumoto, C. Kunimura, K. Kikuchi, H. Tahara, H. Ohji, H. Yamamoto, T. Ide, T. Utakoji, *Oncogene* **13**, 433-439 (1996).
9. C.M. Counter, J. Gupta, C.B. Harley, B. Leber, S. Bacchetti, *Blood* **85**, 2315-2320 (1995).
10. K.S. Kang, W. Sun, K. Nomata, I. Morita, A. Cruz, C.J. Liu, J.E. Trosko, C.C. Chang, *Mol. Carcinogenesis* **21**, 225-233 (1998).

11. O.W. Petersen, L. Ronnov-Jesen, A.R. Howlett, M. Bissell, *Proc. Natl. Acad. Sci. USA* **89**, 9064-9068 (1992).

VIII. APPENDICES

1. A revised Statement of Work.
2. Reprint of a published paper:

   K.S. Kang, W. Sun, K. Nomata, I. Morita, A. Cruz, C.J. Liu, J.E. Trosko, and C.C. Chang. Involvement of tyrosine phosphorylation of $p^{185C\text{-}erbB2/neu}$ in tumorigenicity induced by x-rays and the neu oncogene in human breast epithelial cells. *Molecular Carcinogenesis* 21: 225-233, 1998.

MICHIGAN STATE UNIVERSITY

DEPARTMENT OF PEDIATRICS/HUMAN DEVELOPMENT
B240 LIFE SCIENCES

EAST LANSING • MICHIGAN • 48824-1317
(517) 353-5042 FAX: (517) 353-8464

August 20, 1998

Nrusingha C. Mishra, Ph.D.
Science Program Manager
Congressionally Directed Medical Research Programs
U.S. army Medical Research and Materiel Command
MCMR-PLF, Building 524
Palacky Street, Ft. Detrick
Frederick, MD 21702-5024

Dear Dr. Mishra:

RE: Revised Statement of Work for Grant Number DAMD 17-96-1-6099

In response to my last annual report dated August 1997, you have indicated that some tasks seem to have been done before the work on the contract was initiated and that I need to submit a new Statement of Work (SOW) complete with time line and justification. The new SOW is now enclosed.

It is true that we have completed Task 4 ahead of schedule and published a paper on this work. To compensate for the extra time, we have embarked on a new project, i.e., the correlation of the susceptibility of a human breast epithelial cell type with stem cell characteristics to neoplastic transformation with its high potential for telomerase activation. The initial work was presented in the last annual report and reviewers considered that the work is justified but require a new SOW. Since the expression of telomerase in breast epithelial stem cell is not known and there is question about the role of telomerase activation in malignant transformation in breast carcinogenesis (see *Proc. Natl. Acad. Sci. USA* 94:13677-13682, 1997), we are in a good position to resolve this problem because we have these stem cells.

Besides this additional new Task (Task 9), the other original tasks remain the same, although the time lines for some tasks need to be changed for a realistic schedule of completion as is clear at this stage. I realize I should have sent this revised SOW earlier but I was not sure how long this new project would take especially when we were involved in the movement of our entire laboratory to a new building this year. As it turned out, I am not only pleased with the results of the study of this new project, but also confident that we will accomplish all the tasks in time.

Sincerely,

Chia-Cheng Chang

Chia-Cheng Chang, Ph.D.
Professor
Dept. of Pediatrics and Human Development

CC/rd

Enclosure

STATEMENT OF WORK

TECHNICAL OBJECTIVE 1: To identify factors that regulate the growth and differentiation of "ductal" and budding structures formed by two types of normal HBEC on Matrigel.

TASK 1: Months 1-30: Characterization of "ductal" and budding structures formed by normal HBEC on Matrigel .

TASK 2: Months 25-36: To study the effects of hormones and growth factors on growth and differentiation of "ductal" and budding structures formed by normal HBEC on Matrigel.

TECHNICAL OBJECTIVE 2: To characterize the expression of estrogen receptor in normal and in vitro neoplastically transformed human breast epithelial cells.

TASK 3: Months 7-30: Comparison of ER expression in two types of normal HBEC grown on plastic surface and on Matrigel.

TASK 4: Months 1-12: ER expression in SV40 large T-antigen transformed HBEC. (Completed before initiation of contract).

TASK 5: Months 37-48: ER expression in ionizing radiation transformed HBEC.

TASK 6: Months 25-36: Mechanism of the expression of ER in Type II HBEC by SV40 large T-antigen.

TECHNICAL OBJECTIVE 3: To determine the biological functions of estrogen receptors expressed in normal and in vitro neoplastically transformed HBEC.

TASK 7: Months 30-42: To determine the biological functions of estrogen receptors expressed in normal HBEC.

TASK 8: Months 37-48: To determine the biological functions of estrogen receptors expressed in SV40 large T-antigen and x-ray transformed HBEC.

TECHNICAL OBJECTIVE 4: To determine the role of telomerase in neoplastic transformation of HBEC.

TASK 9: Months 1-24: To determine if there is a correlation of the susceptibility of a HBEC type with stem cell characteristics to neoplastic transformation with its high potential for telomerase activation (a new task).

MOLECULAR CARCINOGENESIS 21:225–233 (1998)

ARTICLES

Involvement of Tyrosine Phosphorylation of p185$^{c\text{-}erbB2/neu}$ in Tumorigenicity Induced by X-Rays and the *neu* Oncogene in Human Breast Epithelial Cells

Kyung-Sun Kang, Wei Sun, Koichiro Nomata, Ikue Morita, Angela Cruz, Chia-Jen Liu, James E. Trosko, and Chia-Cheng Chang*

Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing, Michigan

Ionizing radiation is the exogenous agent best proven to induce breast cancer. c-*erbB2/neu* amplification and overexpression are known to occur in breast cancer and are correlated with aggressive tumor growth and poor prognosis. We have developed simian virus 40–immortalized cell lines from normal human breast epithelial cells (HBECs) with luminal and stem-cell characteristics. In this study, we examined whether x-rays and a mutated *neu* oncogene are capable of inducing tumorigenicity in these cells. The results indicated that x-rays were effective in converting immortal non-tumorigenic HBECs to weakly tumorigenic cells that then could be transformed to highly tumorigenic cells by the *neu* oncogene. The in vitro growth of these tumorigenic cells was significantly faster than that of the parental non-tumorigenic cells in growth factor– and hormone-supplemented or -depleted media. The *neu* oncogene, however, had no tumorigenic effect on immortal non-tumorigenic cells. The expression of p185$^{c\text{-}erbB2/neu}$ was elevated in *neu*-transduced immortal or weakly tumorigenic cell lines. However, only in the latter was p185$^{c\text{-}erbB2/neu}$ found to be phosphorylated at tyrosine residues. Thus, x-rays appear to induce a genetic alteration that confers weak tumorigenicity on immortal HBECs and interacts with p185$^{c\text{-}erbB2/neu}$ directly or indirectly to give rise to fast-growing tumors. *Mol. Carcinog. 21:225–233, 1998.*

Key words: human breast cells; tumorigenicity; x-ray; *neu* oncogene; tyrosine phosphorylation

INTRODUCTION

Although significant advances in cancer research have been made in recent years, the etiology and detailed mechanisms of human breast cancer are not well known. Among the known endogenous factors that cause breast cancers are breast cancer genes (e.g., *BRCA1* [1], *BRCA2* [2], the Li-Fraumeni-syndrome gene [3], and the ataxia telangectasia gene [4]) and developmental factors (e.g., early menarche [5], late menopause [6], obesity [7], old age at first birth, and nulliparity [8]). Stem-cell multiplication or differentiation [9,10] and cumulative exposure to estrogens [11] appear to be major determinants of these developmental factors. Many environmental agents (e.g., organochlorine compounds, such as DDE, PCBs, and chlordane, and polycyclic aromatic hydrocarbons) are also suspected to cause breast cancer by functioning as xenoestrogens [12], by inducing enzymes that metabolize estrogens or carcinogens, or by inhibiting gap-junctional intercellular communication [13]. While dietary factors are believed to play some role, there is no conclusive evidence supporting that hypothesis [14,15]. Ionizing radiation is the environmental agent best proven to cause breast cancers. The clearest demonstration of excess risk associated with exposure to ionizing radiation comes from studies of Japanese women exposed to atomic-bomb radiation in Hiroshima and Nagasaki [16] and of radiation therapy for Hodgkin's disease [17] and other nonmalignant conditions [18,19].

The human c-*erbB2/neu* gene encodes a 185-kDa transmembrane protein that is a member of the epidermal growth factor (EGF) receptor family [20,21]. The gene is amplified in approximately 30% of primary human breast carcinomas [22,23], and another 10% overexpress c-erbB2 without amplification of the gene [24–26]. The amplification of the gene indicates poor prognosis and predicts shorter overall survival and shorter time to relapse [22,27].

We have developed a culture method to grow a new human breast epithelial cell (HBEC) type from reduction mammoplasty tissues [28]. The new HBEC type, which expresses the estrogen receptor [29], and has luminal and stem cell characteristics, is more susceptible to neoplastic transformation by simian

*Correspondence to: Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, National Food Safety and Toxicology Bldg., East Lansing, MI 48824-1317.

Received 6 May 1997; Revised 2 October 1997; Accepted 29 October 1997

Abbreviations: EGF, epidermal growth factor; HBEC, human breast epithelial cell; SV40, simian virus 40; FBS, fetal bovine serum.

virus 40 (SV40) than the conventional HBECs are [28,30]. In this study, we investigated whether x-rays and a mutated *neu* oncogene are capable of inducing neoplastic transformation of these cells.

MATERIALS AND METHODS

Cell Culture

The SV40-immortalized cell line M13SV1 was derived from type I normal HBECs [28]. From this cell line, the weakly tumorigenic cell line M13SV1R2 and the *neu* oncogene–transduced cell lines M13SV1-N5 and -N6 from M13SV1 and M13SVR2-N1, -N4, and -N8 from M13SV1R2 were obtained as described in the following section. All of these cell lines were cultured in MSU-1 medium [28] supplemented with 5% fetal bovine serum (FBS) at 37°C in incubators supplied with 5% CO_2 and humidified air.

Neoplastic Transformation of Immortal HBEC by X-Rays

The immortal M13SV1 cells were non-tumorigenic in athymic nude mice. These cells formed small anchorage-independent colonies at a low frequency. After x-ray irradiation (two doses of 2 Gy 8 d apart), large colonies appeared among the anchorage-independent colonies. When replated in soft agar, these large colonies formed large colonies at higher frequencies. Two of these retested clones (M13SV1R2 and M13SV1R6) were weakly tumorigenic when inoculated into athymic nude mice. One of these two clones (M13SV1R2) was chosen for further studies.

Transduction of a Mutated Rat *neu* Oncogene into M13SV1 and M13SV1R2

The GLu664-neu virus–producing cell line was a gift from Dr. G. P. Dotto (The Cutaneous Biology Research Center, Boston, MA) [31]. The virus carries a complete cDNA copy of the rat *neu* oncogene with a point mutation at amino-acid 664. The point mutation at position 664 leads to a full oncogenic activation of the *neu* oncogene. The cells were plated in 10-cm plates and exposed to an undiluted viral stock in culture medium with 8 μg/mL polybrene (Sigma Chemical Co., St. Louis, MO). After 2–3 h of virus exposure, the virus-containing medium was replaced with normal culture medium. These infected cells were continuously cultured for 3 d before they were exposed to medium with G418 (400 μg/mL; GIBCO BRL, Gaithersburg, MD) for the selection of G418-resistant clones. The control cells were infected with a virus carrying the G418-resistance gene (PC6M$^-$neo) [31]. The control virus–producing cells were also kindly provided by Dr. Dotto.

Colony Formation in Soft Agar

Agarose (0.5% Type I, low EEO; Sigma Chemical Co.) prepared in MSU-1 medium at 39°C was added to 60-mm culture dishes and allowed to solidify in the incubator. HBEC (1×10^5) suspended in medium with 0.33% agarose were overlaid on top of the hard layer 0.5% agar. The plates were incubated at 37°C, and liquid medium was added 3 d after HBEC inoculation and renewed every 3 d. After 4 wk of inoculation, anchorage-independent colonies were observed between the two agar layers.

In Vivo Tumorigenicity Test

The immortal cell line (M13SV1), x-ray transformed cell lines (M13SV1R2 and M13SV1R6), and *neu* oncogene–transduced cell lines (M13SV1-N5 and -N6 and M13SV1R2-N1, -N4, and -N8), were inoculated subcutaneously into 6-wk-old female athymic nude mice (Harlan Sprague-Dawley Inc., Indianapolis, IN) for the tumorigenicity assay. The tumors formed from 6×10^6 cells inoculated in each of two sites per mouse were dissected and measured 4 wk after inoculation. Some of the tumors were excised and reestablished in cell culture for further characterization.

In Vitro Growth of Cells in Growth Factor– and Hormone-Supplemented and Depleted Media

To compare growth factor– and hormone-dependent and -independent growth among cell lines transformed at various stages, the cells were grown in various *MSU-1* media with or without specific growth factors and hormones. The growth of HBECs in various media was measured by quantitation of total nucleic acid extracted from the cell cultures. Briefly, HBECs (1×10^5 cells) were plated in 6-cm plates in triplicate in the various media. All cells were incubated for 3 or 6 d at 37°C, and the media were changed twice for the 6 d culture. The cells were then washed twice with phosphate-buffered saline and lysed with 1 mL of 0.1 N NaOH. The lysates were transferred into a 2.2-mL microcentrifuge tube and centrifuged for 2–3 min. The absorbance of the clear lysate at 260 nm was measured with a spectrophotometer.

Western Blot Analysis of $p185^{c\text{-}erbB2/neu}$ and Tyrosine Phosphorylation

Proteins were extracted from SV40-transformed HBECs in 10-cm dishes by treatment with 20% sodium dodecyl sulfate lysis solution containing several protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM antipain, 0.1 μM aprotinin, 0.1 μM sodium orthovanadate, and 5 mM sodium fluoride). After sonication with three 10-s pulses from a probe sonicator [29], the cell lysates were stored at –20°C until used. The amounts of protein were determined with the DC protein assay kit (Bio-Rad Co., Richmond, CA). The proteins were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 h. The expression of the *neu* oncogene and its phosphorylation

at tyrosine residues were detected with anti-erbB2/neu monoclonal antibodies Ab-1 and Ab-3 (Oncogene Science Inc., Uniondale, NY), which detect both rat and human $p185^{c\text{-}erbB2/neu}$; the monoclonal antibody Ab-6 (Oncogene Science, Inc.), which is specific for human $p185^{c\text{-}erbB2/neu}$; and the anti-phosphotyrosine monoclonal antibody Ab-2 (Oncogene Science, Inc.) after blocking with 5% dried skim milk in phosphate-buffered saline containing 0.1% Tween 20. This was then followed by incubation with horseradish peroxidase–conjugated secondary antibody and visualization with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL). The x-ray films were exposed to the membranes for 15 s to 1 min.

RESULTS

Isolation of Tumorigenic Cell Lines from an SV40-Immortalized HBEC Line after X-Ray Irradiation

The near-diploid HBEC line M13SV1, immortalized by SV40 transfection [28], was non-tumorigenic in the eight athymic nude mice tested (data not shown). The cells formed slow-growing colonies in soft agar (anchorage-independent growth) at a low frequency (about 0.03%). X-ray irradiation (two doses of 2 Gy 8 d apart), enhanced the frequency of anchorage-independent colonies approximately sevenfold. Some fast-growing large colonies also emerged in these x-ray–irradiated cell populations. Eight large anchorage-independent colonies were isolated from one experiment for further characterization. When replated, these clones showed a range of frequencies of anchorage-independent colonies (0.07–1.4%). Two of these clones with the highest frequencies of anchorage-independent growth (M13SV1R2 (1.4%) and M13SV1R6 (1.3%)) were propagated and inoculated into athymic nude mice for a tumorigenicity test. The results show that these two cell lines were weakly tumorigenic (in six of six and three of six mice innoculated with M13SV1R2 and M13SV1R6, respectively). Tumors less than 0.5 cm in diameter formed in 1 mo and did not grow larger in the subsequent month (data not shown).

Effect of a Mutated *neu* Oncogene on Tumorigenesis

To test this effect, the immortal cell line (M13SV1) and a weakly tumorigenic cell line derived from it by x-ray irradiation (M13SV1R2) were infected with a virus (GLu664-neu) carrying a mutated rat *neu* oncogene [31] and the G418-resistance selective marker. A vector control (PC6M$^-$neo) carrying the G418-resistance gene [31] was also included in the experiment. Randomly selected G418-resistant colonies were isolated from M13SV1 and M13SV1R2 cell lines after infection. Two and three putative *neu* oncogene–transformed cell lines derived from M13SV1 (M13SV1-N5 and -N6) and M13SV1R2 (M13SV1R2-N1, -N4, and -N8) were further characterized for the expression of the *neu* oncogene and for tumorigenicity in athymic nude mice. Western blot analysis showed that all these putative *neu* oncogene–transformed cell lines had higher levels of $p185^{c\text{-}erbB2/neu}$ expression than did the parental cells (M13SV1 and M13SV1R2) and vector controls (M13SV1-M1, -M2, and M13SV1R2-M1) (Figure 1A). In tumorigenicity experiments, the immortal cell lines with elevated *neu* expression (i.e., M13SV1-N5 and -N6) were non-tumorigenic. In contrast, the weakly tumorigenic cell lines with elevated expression of $p185^{c\text{-}erbB2/neu}$ (i.e., M13SV1R2-N1, -N4, and -N8) had significantly enhanced tumor growth (tumor weight about 16 times greater than that of tumors formed by parental cells) (Table 1). The derivation of these cell lines and their tumorigenicity are shown in Figure 2.

In Vitro Cell Growth of Various Cell Lines Transformed by X-Rays and the *neu* Oncogene

The growth requirement of normal HBECs [32] for the major growth factor and hormones (EGF, insulin, and hydrocortisone) was examined for the various cell lines transformed by x-rays and the *neu* oncogene. The results indicated that both *neu* and x-ray irradiation were capable of enhancing cell growth in vitro in a defined medium (MSU-1) with or without the supplement of one or two of the key growth factor and hormones (Figure 3). For the immortal cell line, M13SV1, the effect of x-ray irradiation (as in M13SV1R2) was better than that of elevated expression of $p185^{c\text{-}erbB2/neu}$ (as in M13SV1-N5 and -N6). Although the expression of the *neu* oncogene in M13SV1R2 was able to enhance tumor growth in athymic nude mice (as shown for M13SV1R2-N1, -N4, and -N8 in Table 1), the in vitro growth of these *neu*-transformed cell lines was not always better than that of the parental cells (M13SV1R2).

Expression and Tyrosine Phosphorylation of $p185^{c\text{-}erbB2/neu}$

In western blot analysis using the anti-$p185^{c\text{-}erbB2/neu}$ antibody Ab-1, the level of $p185^{c\text{-}erbB2/neu}$ protein was found to be significantly higher in *neu* oncogene–transduced cell lines derived either from the M13SV1 immortal cell lines (i.e., M13SV1-N5 and -N6) or from the M13SV1R2 weakly tumorigenic cell lines (i.e., M13SV1R2-N1, -N4, and -N8) than in the parental cell lines (M13SV1 and M13SV1R2) or the vector control cell lines (M13SV1-M1 and -M2 and M13SV1R2-M1) (Figure 1A). Although all the *neu* oncogene–transduced cell lines examined in this experiment expressed elevated levels of $p185^{c\text{-}erbB2/neu}$, only those cell lines, derived from the M13SV1R2 weakly tumorigenic cell lines (i.e., M13SV1R2-N1, -N4, and -N8), were found to contain a high level of tyrosine-phosphorylated $p185^{c\text{-}erbB2/neu}$ (Figure 1B).

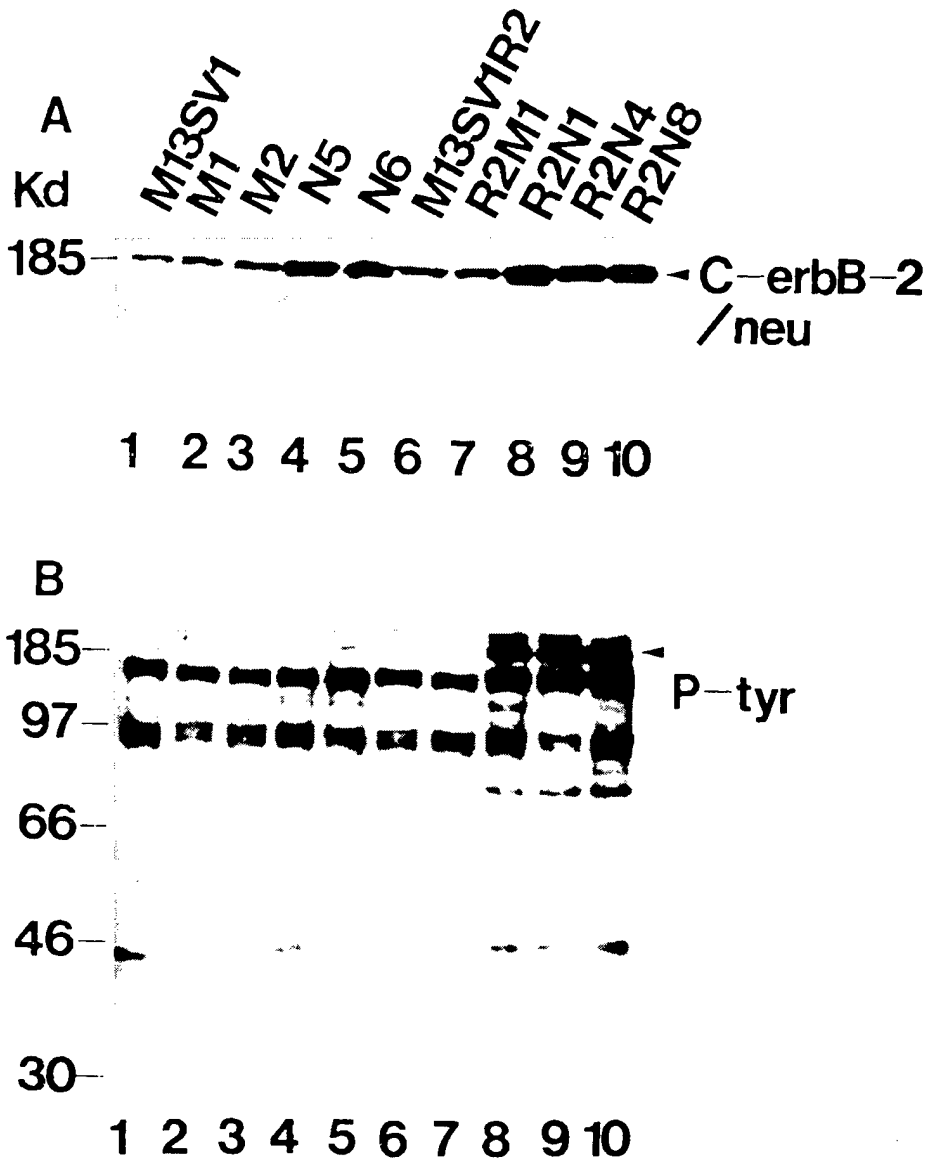


Figure 1. Expression and tyrosine phosphorylation of $p185^{c\text{-}erbB2/neu}$ studied by western blot analysis. (A) Expression of c-erbB2/neu detected with anti-neu antibody Ab-1 (Oncogene Science Inc.). (B) The blot in panel A reprobed with the anti-phosphotyrosine antibody Ab-2 (Oncogene Science Inc.). Lane 1, the SV40-immortalized HBEC M13SV1; lanes 2 and 3, M13SV1-M1 and -M2, respectively (vector controls for lanes 4 and 5); lanes 4 and 5, M13SV1-N5, and -N6, respectively (M13SV1 clones transduced by the mutated *neu* oncogene); lane 6, x-ray–transformed cell line M13SV1R2; lane 7, M13SV1R2-M1, the vector control for lanes 8–10; lanes 8–10, M13SV1R2-N1, -N4, and -N8, respectively (M13SV1R2 clones transduced by the mutated *neu* oncogene).

The parental immortal cell line (M13SV1) and the weakly tumorigenic cell line (M13SV1R2), derived from type I normal HBECs, did not express a significant level of tyrosine-phosphorylated $p185^{c\text{-}erbB2/neu}$ (Figure 1B). In a separate experiment using cell lysates harvested independently, we confirmed that the *neu*-transduced cell lines had elevated levels of $p185^{c\text{-}erbB2/neu}$ and that the parental cells and vector control cell lines had weak but detectable $p185^{c\text{-}erbB2/neu}$ (Figure 4, upper panel). By using an antibody specific for human $p185^{c\text{-}erbB2/neu}$, we also determined that the elevated $p185^{c\text{-}erbB2/neu}$ in *neu*-transduced cell lines was from the exogenous rat *neu*, not from the host human *c-erbB2/neu* gene (Figure 4, middle panel). Although overexpressed human $p185^{c\text{-}erbB2/neu}$ was readily detectable with Ab-6, the human-specific antibody is not as sensitive in detecting normal levels of human $p185^{c\text{-}erbB2/neu}$.

The medium used to grow these cells was MSU-1 medium supplemented with EGF, insulin, hydrocortisone, and fetal bovine serum. To test whether these growth factors and hormones affect the tyrosine

Table 1. Tumorigenicity of Immortal or Weakly Tumorigenic HBEC Lines Transduced by the *neu* Oncogene in Athymic Nude Mice

Cell line	No. of mice with tumors/total no. of mice	Tumor weight (mg)*	Tumor diameter (cm)*
M13SV1	0/5	—	—
M13SV1 M1	0/4	—	—
M13SV1 M2	0/4	—	—
M13SV1-N5	0/5	—	—
M13SV1-N6	0/5	—	—
M13SV1R2	4/4	14±10	0.24±0.07
M13SV1R2-M1	4/5	11±12.4	0.21±0.15
M13SV1R2-N1	4/4	295±12	1.12±0.16
M13SV1R2-N4	5/5	140±6	0.83±0.12
M13SV1R2-N8	5/5	230±100	1.03±0.17

*The values shown are means ± standard deviations.

phosphorylation of $p185^{c\text{-}erbB2/neu}$, the cells were grown in the defined MSU-1 medium without these supplements. The results revealed that tyrosine phosphorylation of $p185^{c\text{-}erbB2/neu}$ in the four different transformed cell lines (immortal and weakly tumorigenic cells with and without transduced *neu* oncogene) was not affected by these growth factor and hormones and confirmed that the highly tumorigenic cell line (M13SV1R2N1) expressed a tyrosine-phosphorylated $p185^{c\text{-}erbB2/neu}$ (Figure 5).

DISCUSSION

As reviewed in the Introduction, ionizing radiation is a known breast carcinogen, and c-*erbB2/neu* is known to be amplified or overexpressed in breast cancer. However, the specific genes that are altered by x-rays during carcinogenesis and the genes that interact with c-*erbB2/neu* are not known. We therefore attempted to shed light on the mechanism of action of this important breast carcinogen and oncogene by using an in vitro model involving HBEC lines at different stages of neoplastic transformation.

Our results revealed that x-rays were capable of inducing tumorigenicity in immortal non-tumorigenic HBECs. Weakly tumorigenic clones of these cells can be selected as large fast-growing anchorage-independent colonies. Although the gene induced by x-rays has not been identified, the mutated gene appears to be capable of conferring a growth advantage to these cells, as shown in in vitro cultures in soft agar or on plastic in medium with or without growth factors and hormones. That x-rays are capable of malignant transformation of immortal human cells has been demonstrated in human epidermal keratinocytes [33]. However, the carcinogenic alteration induced by ionizing radiation may not be limited to the stage subsequent to immortalization. Our preliminary study showed that HBEC clones with extended lifespans could be obtained after few repeated treatments with low dose x-rays (unpublished data). Furthermore, γ-irradiation has been reported to immortalize primary human mammary epithelial cells with the loss of p53 protein [34].

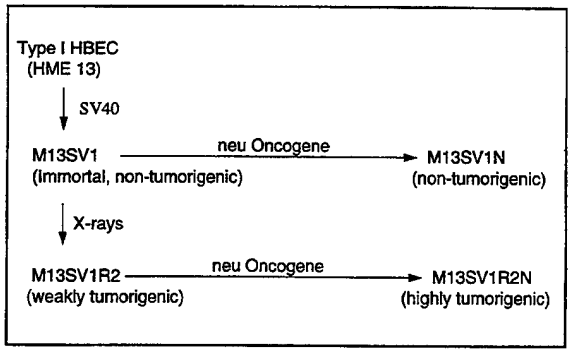


Figure 2. Derivation and tumorigenicity of various HBEC lines from HME 13, a normal human mammary epithelial culture from reduction mammoplasty.

The transduction of the mutated *neu* oncogene in an immortal cell line (M13SV1) and a weakly tumorigenic cell line (M13SV1R2) resulted in elevated expression of $p185^{c\text{-}erbB2/neu}$ in both cell lines but caused significantly faster growth only in the immortal cell line in medium with or without growth factors and hormones (i.e., M13SV1-N5 and -N6 grew better than M13SV1, but M13SV1R2-N1, -N4, and -8 did not grow consistently better than M13SV1R2). However, a significant level of tyrosine-phosphorylated $p185^{c\text{-}erbB2/neu}$ was found in the x-ray-transformed tumorigenic cell lines (i.e., M13SV1R2-N1, -N4, and -N8) but not in the immortal non-tumorigenic cell lines (i.e., M13SV1-N4 and -N5). The expression of a high level of tyrosine-phosphorylated $p185^{c\text{-}erbB2/neu}$ appears to be correlated with enhanced tumor growth in athymic nude mice. Human c-*erbB2* may be a better choice than the mutated rat *neu* oncogene for this study. However, without gene amplification, the expression of a transduced c-*erbB2* gene may not be sufficient to have a measurable effect. In fact, we have performed parallel experiments and found that c-*erbB2* transfection did not change the tumorigenicity in either the immortal M13SV1 cell line or the weakly tumorigenic cell line M13SV1R2 (data not shown).

The role of c-erbB-2/neu in neoplastic transformation has been shown to be different in rodent and human mammary epithelial cells. High levels of neu expression after *neu* oncogene transfer into mammary epithelial cells in situ frequently induce mammary carcinomas in rats [35]. MCF-10A, a spontaneously immortalized human breast epithelial cell line, however, remains non-tumorigenic in nude mice after infection and expression of c-*erbB2* in conjunction with Ha-*ras* or transforming growth factor-α, although these transformed clones form a high frequency of anchorage-independent colonies [36]. The overexpression of c-erbB2 in 184B5 cells, a chemically immortalized human breast epithelial cell line,

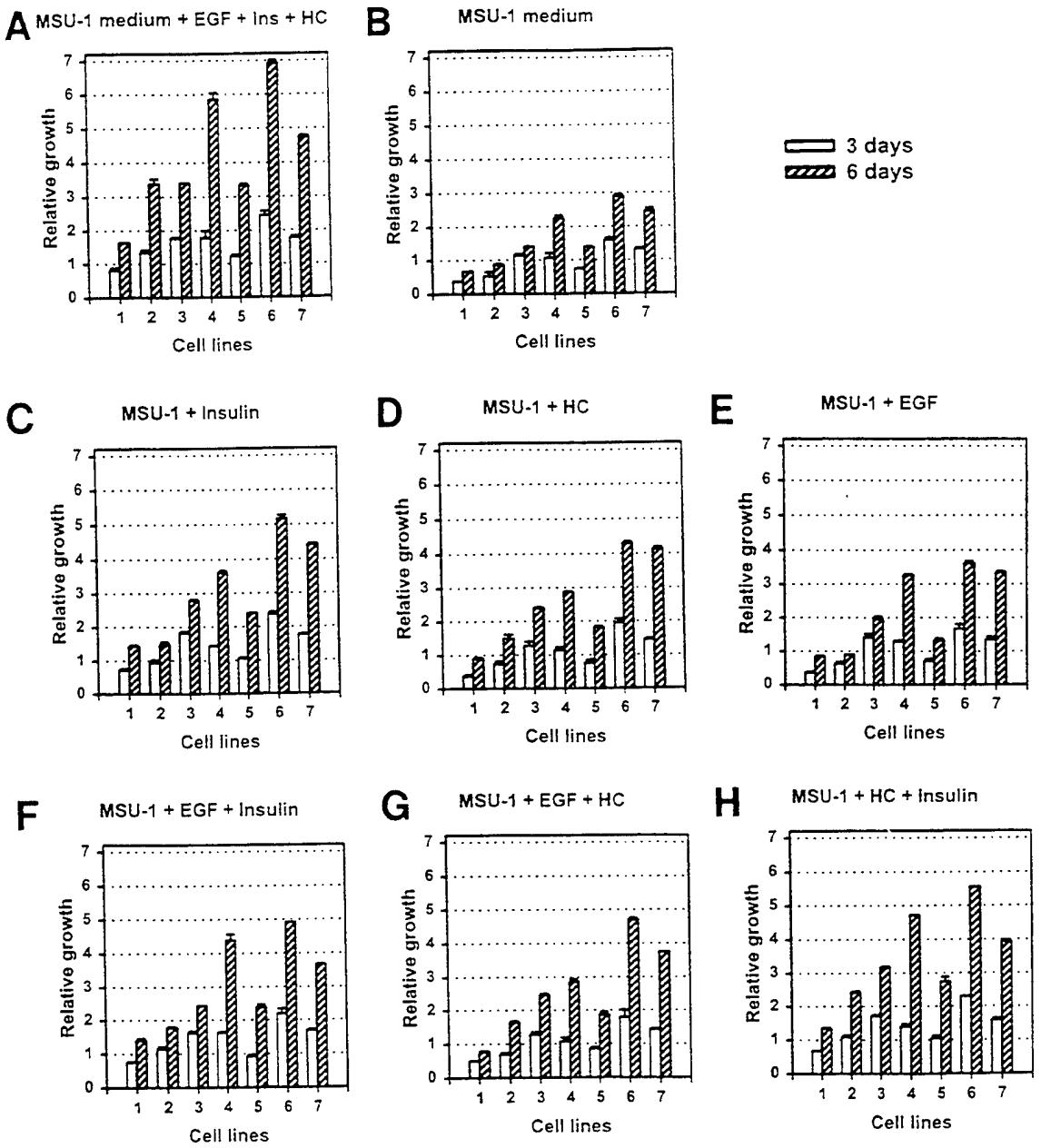


Figure 3. Growth factor and hormone requirements of immortal, weakly tumorigenic, and highly tumorigenic HBECs. Relative growth is the average total nucleic acid content of cells grown in triplicate dishes (6 cm) as measured by absorbance (OD) at 260 nm wavelength. 1, M13SV1; 2, M13SV1-N5; 3, M13SV1-N6; 4, M13SV1R2; 5, M13SV1R2-N1; 6, M13SV1R2-N4; 6, M13SV1R2-N8. (A) MSU-1 medium supplemented with hydrocortisone, EGF, and insulin; (B) MSU-1 medium without hydrocortisone, EGF, and insulin; (C) MSU-1 medium supplemented with insulin only; (D) MSU-1 medium supplemented with hydrocortisone only; (E) MSU-1 medium supplemented with EGF only; (F) MSU-1 medium supplemented with EGF and insulin; (G) MSU-1 medium supplemented with hydrocortisone and EGF; (H) MSU-1 medium supplemented with hydrocortisone and insulin.

leads to formation of colonies in soft agar, frequently induces transient nodules in athymic mice, and produces progressive tumors at a low frequency [37]. These results suggest that the expression of erbB2/neu is necessary but not sufficient to cause malignant transformation of human mammary epithelial cells. In several ways, our results are similar to those of a previous study comparing the expression of c-erbB2 in untransformed HBECs and breast cancer cell lines [38]. First, the normal non-tumorigenic cells expressed a low but detectable level of $p185^{c\text{-}erbB2/neu}$ that was not tyrosine phosphorylated. Second, the highly tumorigenic cell line neu-transfected and the metastatic breast cancer cell lines expressed a high

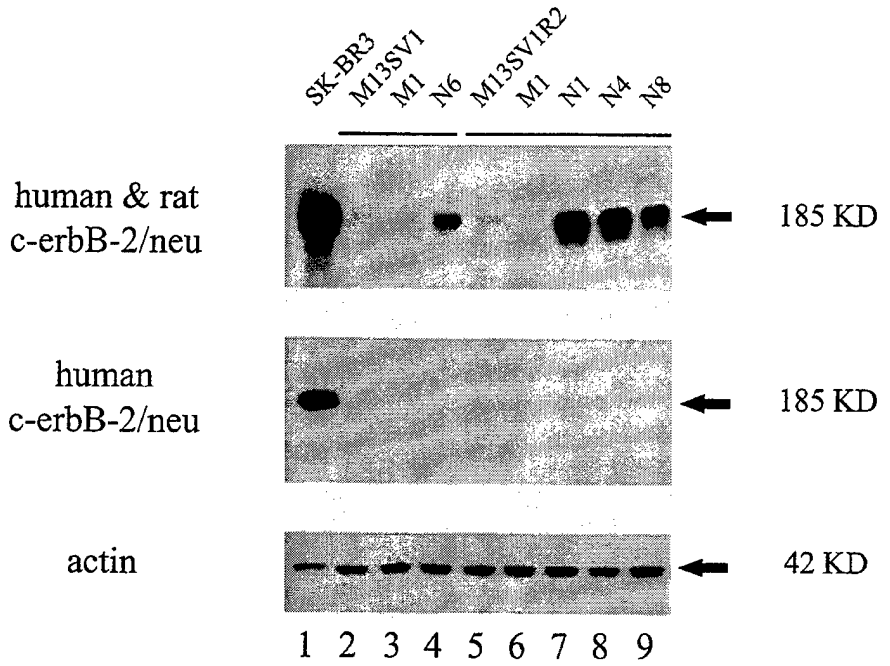
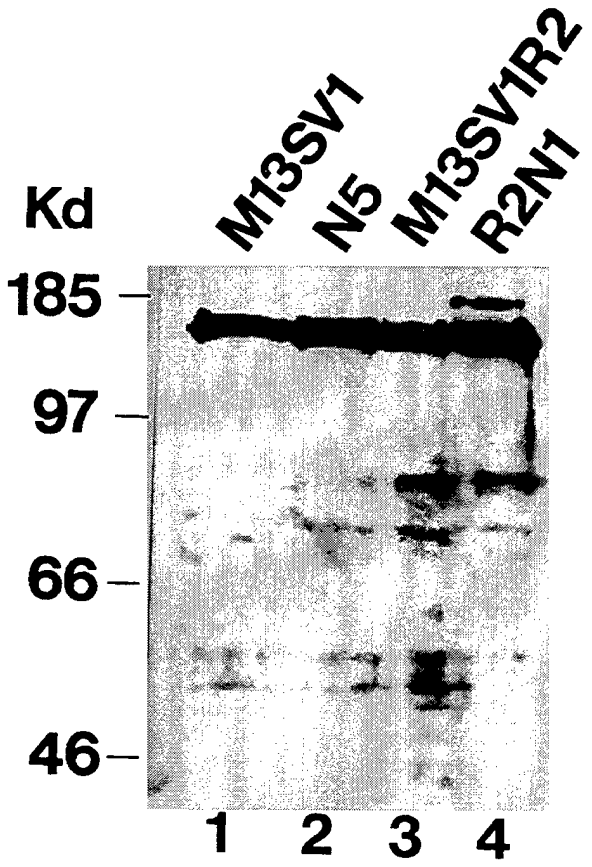


Figure 4. Western blot analysis of expression of p185$^{c\text{-}erbB2/neu}$ in various HBEC lines and in the breast cancer cell line SK-BR3. The proteins were detected by using an anti-p185$^{c\text{-}erbB2/neu}$ antibody recognizing both human and rat p185$^{c\text{-}erbB2/neu}$ (Ab-3) (upper panel), a human c-erbB2-specific anti-p185$^{c\text{-}erbB2/neu}$ antibody (Ab-6) (middle panel), and an anti-actin antibody (A-2066, Sigma Chemical Co.) (lower panel), used to monitor protein loading levels among samples. Lane 1, cell extract from SK-BR3, which constitutively expresses high levels of human p185$^{c\text{-}erbB2/neu}$ and was used as a positive control in this study; lane 2, M13SV1, an SV40-immortalized HBEC line; lane 3, M13SV1-M1, a vector control for lane 4; lane 4, M13SV1-N6, an M13SV1 clone tranduced by mutated rat *neu*; lane 5, M13SV1R2, an x-ray–transformed M13SV1 cell line; lane 6, M13SV1R2-M1, a vector control for lanes 7–9; lanes 7–9; M13SV1R2-N1, -N4, and -N8, respectively, M13SV1R2 clones transduced by a mutated rat *neu*.



level of p185$^{c\text{-}erbB2/neu}$ that was tyrosine phosphorylated. Third, the tumorigenic cell lines had a growth advantage or growth-factor independence in culture as compared with normal untransformed cells. Our results further indicated that the elevated expression of a non-phosphorylated p185$^{c\text{-}erbB2/neu}$ was not sufficient for tumor development.

This study showed that x-ray irradiation may induce a genetic change that gives rise to tumorigenicity and confers a growth advantage (i.e., the growth of x-ray–transformed cell line, M13SV1R2, in growth factor– and hormone-depleted medium was better than that of the parental cell line, M13SV1, in growth factor– and hormone-depleted or -supplemented medium (Figure 3, top panel)). At the molecular level, the mutation may be directly or indirectly responsible for the phosphorylation of the p185$^{c\text{-}erbB2/neu}$ in c-erbB-2/neu-overexpressing cells. Because tyrosine phosphorylation is an important regulatory mechanism in response to growth factors and the expression of oncogenes, the upstream receptor or non-receptor tyrosine kinase genes might be the genes activated by x-rays. These genes include *pp60*$^{c\text{-}src}$, which has been shown to be involved in two major signaling pathways (EGF and p185$^{c\text{-}erbB2/neu}$) in human breast

Figure 5. Tyrosine phosphorylation of p185$^{c\text{-}erbB2/neu}$ for cells grown in MSU-1 medium without growth factors, hormone, or FBS. Lane 1, M13SV1; lane 2, M13SV1-N5; lane 3, M13SV1R2; lane 4, M13SV1R2-N1.

cancer [39,40], and neu differentiation factor/heregulin, which has been reported to be produced in cancer cells and to be capable of activating $p185^{c\text{-}erbB2/neu}$ by direct binding to erbB3 or erbB4 [41]. Alternatively, a phosphatase might be inactivated by x-rays. In addition, phosphatidylinositol 3-kinase has been shown to be recruited by $p185^{c\text{-}erbB2/neu}$ and to be constitutively overexpressed in growth factor–independent breast carcinoma cells with c-*erbB2* gene amplification [38]. The recruitment of Grb2 and SOS proteins by $p185^{c\text{-}erbB2/neu}$ was also recently shown to occur constitutively in mammary carcinoma cells with c-*erbB2* gene amplification, and the level of ras-mediated mitogen-activated protein kinase pathway activation was directly related to the level of c-*erbB2* overexpression and constitutive $p185^{c\text{-}erbB2/neu}$ tyrosine kinase activity [42]. These events probably occur in our tumorigenic cells and will be explored in future studies.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. G. P. Dotto for providing the *neu* oncogene and control vector virus–producing cell lines. We thank Robbyn Davenport for assistance with word processing. This work was supported by grants from the National Institute of Environmental Health Sciences (ES07256), the U.S. Army Medical Research and Materiel Command Breast Cancer Program (DAM017-96-1-6099), and the National Cancer Institute (CA 21104).

REFERENCES

1. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266:66–71, 1994.
2. Wooster R, Bignell G, Lancaster J, et al. Identification of the breast susceptibility gene BRCA2. Nature 378:789–792, 1995.
3. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in familial syndrome of breast cancer sarcomas, and other neoplasms. Science 250:1233–1238, 1990.
4. Swift M, Morrell D, Massey RB, Chase CL. Incidence of cancer in 161 families affected by ataxia-telangectasia. N Engl J Med 325:1831–1836, 1991.
5. Bouchardy C, Le MG, Hill C. Risk factors for breast cancer according to age at diagnosis in a French case-control study. J Clin Epidemiol 43:267–275, 1990.
6. Ewertz M, Duffy SW. Risk of breast cancer in relation to reproductive factors in Denmark. Br J Cancer 58:99–104, 1988.
7. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81:1879–1886, 1989.
8. MacMahon B, Cole P, Brown J. Etiology of human breast cancer: A review. J Natl Cancer Inst 50:21–42, 1973.
9. Cairns J. Mutation selection and the natural history of cancer. Nature 255:197–200, 1975.
10. Russo LH, Kiszalka M, Russo J. Human chorionic gonadotropin and rat mammary cancer prevention. J Natl Cancer Inst 82:1286–1289, 1990.
11. Henderson BE, Ross RK, Pike MC. Hormonal chemoprevention of cancer in women. Science 259:633–638, 1993.
12. Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environ Health Perspect 101:372–377, 1993.
13. Kang K-S, Wilson MR, Hayashi T, Chang CC, Trosko JE. Inhibition of gap junctional intercellular communication in normal human breast epithelial cells after treatment with pesticides, PCBs, and PBBs, alone or in mixtures. Environ Health Perspect 104:192–200, 1996.
14. Kushi LH, Sellers TA, Potter JD, Folsom AR. Dietary fat and postmenopausal breast cancer-response. J Natl Cancer Inst 84:1667–1669, 1992.
15. Howe GR, Friedenreich CM, Jain M. A cohort study of fat intake and risk of breast cancer. J Natl Cancer Inst 83:336–340, 1991.
16. Land CE, Tokunaga M, Tukuoka S. Studies of breast cancer risks among atomic bomb survivors. *In:* Gerber GB, Taylor DM, Candis E, Thiessen JW (eds) British Institute of Radiology Report 22, London, 1991, pp. 49–54.
17. Pollner F. A holistic approach to breast cancer research. Environ Health Perspect 101:116–120, 1993.
18. Hildreth NG, Shore RE, Dvoretsky PM. The risk of breast cancer after irradiation of the thymus in infancy. N Engl J Med 321:1281–1284, 1989.
19. Shore RE, Hempelmann LH, Kowaluk E. Breast neoplasms in women treated with x-rays for acute postpartum mastitis. J Natl Cancer Inst 59:813–822, 1977.
20. Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature 319:226–230, 1986.
21. Yamamoto T, Ikawa S, Akiyama T, et al. Similarity of protein encoded by the human C-erb-B-2 gene to epidermal growth factor receptor. Nature 319:230–234, 1986.
22. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182, 1987.
23. Iglehart JD, Kraus MH, Langton BC, Huper G, Kerns BJ, Marks JR. Increased erbB-2 gene copies and expression in multiple stages of breast cancer. Cancer Res 50:6701–6707, 1990.
24. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6:605–610, 1987.
25. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707–712, 1989.
26. Hollywood DP, Hurst HC. A novel transcription factor, OB2-1, is required for overexpression of proto-oncogene C-erbB-2 in mammary tumor lines. EMBO J 12:2369–2375, 1993.
27. Borg A, Tandon AK, Sigurdsson H, et al. HER-2/neu amplification predicts poor survival in node-positive breast cancer. Cancer Res 50:4332–4337, 1990.
28. Kao CY, Nomata K, Oakley CS, Welsch CW, Chang CC. Two types of normal human breast epithelial cells derived from reduction mammoplasty: Phenotypic characterization and response to SV40 transfection. Carcinogenesis 16:531–538, 1995.
29. Kang K-S, Morita I, Cruz A, Jeon YJ, Trosko JE, Chang CC. 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, 1997.
30. Chang CC, Kang KS, Kao CY, Liu CJ, Trosko JE. A normal human breast epithelial cell type with stem cell characteristics and estrogen receptor expression is more susceptible to neoplastic transformation. In Vitro Cell Dev Biol Anim 32:14A–15A, 1996.
31. Dotto GP, Moellmann G, Ghosh S, Edwards M, Halaban R. Transformation of murine melanocytes by basic fibroblast growth factor cDNA and oncogenes and selective suppression of the transformed phenotype in a reconstituted cutaneous environment. J Cell Biol 109:3115–3128, 1989.
32. Kao GY, Oakley CS, Welsch CW, Chang CC. Growth requirements and neoplastic transformation of two types of normal human breast epithelial cells derived from reduction mammoplasty. In Vitro Cell Dev Biol Anim 33:282–288, 1997.
33. Thraves P, Salehi Z, Dritschilo A, Rhim JS. Neoplastic transformation of immortalized human epidermal keratinocytes by ionizing radiation. Proc Natl Acad Sci USA 87:1174–1177, 1990.
34. Wazer DE, Chu Q, Liu XL, Gao Q, Safall H, Band V. Loss of p53 protein during radiation transformation of primary human mammary epithelial cells. Mol Cell Biol 14:2468–2478, 1994.
35. Wang B, Kennan WS, Wasukawa-Barnes J, Lundstrom MJ, Gould MN. Frequent induction of mammary carcinomas following neu oncogene transfer into *in situ* mammary epithelial cells of susceptible and resistant rat strains. Cancer Res 51:5649–5654, 1991.

36. Ciardiello F, Gottardis M, Basolo F, et al. Additive effects of c-erbB-2, c-Ha-ras, and transforming growth factor-α genes on *in vitro* transformation of human mammary epithelial cells. Mol Carcinog 6:43–52, 1992.
37. Pierce JH, Arnstein P, DiMarco E, et al. Oncogenic potential of erbB-2 in human mammary epithelial cells. Oncogene 6:1189–1194, 1991.
38. Ram TG, Ethier SP. Phosphatidyl-inositol 3-kinase recruitment by $p185^{erbB-2}$ and erbB-3 is potentially induced by neu differentiation factor/heregulin during mitogenesis and is constitutively elevated in growth factor-independent breast carcinoma cells with c-erbB-2 gene amplification. Cell Growth Differ 7:551–561, 1996.
39. Ottenhoff-Kalff AE, Rijksen G, van Beurden EACM, Hennipman A, Michels AA, Staal GEJ. Characterization of protein tyrosine kinases from human breast cancer: Involvement of the c-src oncogene product. Cancer Res 52:4773–4778, 1992.
40. Luttrell DK, Lee A, Lansing TJ, et al. Involvement of pp60c-src with two major signaling pathways in human breast cancer. Proc Natl Acad Sci USA 91:83–87, 1994.
41. Stephen PE, Beatrice CL, Cheryl AD. Growth factor-independent proliferation of rat mammary carcinoma cells by autocrine secretion of neu-differentiation factor/heregulin and transforming growth factor-α. Mol Carcinog 15:134–143, 1996.
42. Jane PW, Daly RJ, deFazio A, Sutherland RL. Activation of the ras signaling pathway in human breast cancer cells overexpressing erbB-2. Oncogene 9:3601–3608, 1994.