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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The objectives of this study are 1) to identify factors that regulate the growth and differentiation of organoids formed by two types of normal human breast epithelial cells (HBEC) in Matrigel; 2) to characterize the expression and function of estrogen receptors (ER) in normal and *in vitro* neoplastically transformed HBEC; and 3) to determine if a HBEC type with stem cell characteristics (Type I) is more susceptible to telomerase activation and immortalization. The major results are 1) Type I HBEC in conjunction with Type II cells are capable of forming ductal and end bud or lobule 1-like structures in Matrigel which preserve the undifferentiated state of HBEC for a long time, evidence that Type I HBEC are stem cells; 2) Type I normal HBEC and their neoplastically transformed clones express a variant ER-Ã *in vitro* on plastic while expressing a wild type ER-Ã in tumors developed in nude mice or grown *in vitro* in Matrigel 3) The ~46kd variant ER-Ã is the result of splicing deletion of exon 1 in ER-Ã transcript; Type 1 HBEC use promoter A while immortal and tumorigenic Type 1 cells use both promoter A and C for transcription; 4) high susceptibility of Type 1 HBEC to telomerase activation and immortalization,, a basis on which Type 1 HBEC (stem cells) are major targets for carcinogenesis.

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V. INTRODUCTION

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

The mechanism that Type I HBEC were more susceptible to neoplastic transformation is not known. In general, tumor cells are known to express telomerase that maintains telomere length for continuous growth whereas normal cells lack the telomerase activity (5). One study, however, concludes

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

VI. BODY

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

The results of this study was reported in great detail in the second year Annual Report (1998) in regard to 1) conditions to form budding/ductal structures; 2) the types of organoids formed by the two types of HBEC; and 3) the alternate growth of HBEC as monolayer on plastic and organoid in Matrigel to preserve the undifferentiated state of HBEC for extended time. We also developed the techniques to section the organoid developed in Matrigel and to reveal gene expression by immunostaining have been developed. Using this technique, we found that the centers of the spherical balls formed by Type II HBEC in Matrigel are not hollow as they appear. The structure (Fig. 1 2000, Annual Report) is actually similar to the squamous metaplasia developed by rat mammary organoid in Matrigel (7). The Type II HBEC expressed cytokeratin 14 when grown on plastic as monolayer (1). In spherical structure, only the outermost cell layer that contacted with Matrigel showed the expression of cytokeratin 14 (Fig. 2, 2000, Annual Report) similar to basal epithelial cells in human mammary gland. The technique can be applied to budding/ductal structures formed by Type I and Type II HBEC to reveal their structure and gene expression (i.e., ER and cytokeratins) by immunostaining in future studies. Some major results of this study have been included in papers published in *Cancer Research* (4)

and *Radiation Research* (8). In Fig. 1 of the *Cancer Research* (4) paper, we show that Type 1 HBEC formed a limited number of bud-like structures on Matrigel whereas Type II HBEC typically formed spherical organoids. The combination of Type I and Type II HBEC in 1:2 or 1:3 ratios can generate many budding and ductal structures in 2-3 weeks. In Fig. 1 of the *Radiation Research* (8) paper, we show that Type 1 HBEC in conjunction with Type II HBEC formed budding and ductal organoids on Matrigel one day after inoculating the mixed population of cells. The structures are very similar to the human lobule Type I mammary gland of young nulliparous woman shown by Russo and Russo (9). A manuscript to report the remaining data is in preparation. These data together with the extended life of Type I HBEC, that remain undifferentiated in continuous alternate culture on plastic monolayer and on Matrigel as organoids, provide a strong evidence that Type I HBEC are mammary epithelial stem cells. Other evidence are the ability of Type I cells to differentiate into other cell types (1) and the high susceptibility of these cells to telomerase activation and immortalization to be reported later (Task 9).

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

The budding/ductal organoids can be formed in Matrigel in our defined MSU-1 Medium supplemented with EGF, insulin, hydrocortisone,

estradiol and human transferrin. The former three are essential for the growth of the two types of HBEC as monolayer on plastic (2) As described in the 1998 Annual Report, we also found that EGF, insulin and hydrocortisone are essential for the development of budding/ductal structure formed by the two types of HBEC on Matrigel. Fetal bovine serum which inhibits the growth of Type II cells but not Type I cells was found to inhibit the growth of budding/ductal organoids. In the past years, we found that the formation and development of organoids can be influenced by different cell cultures derived from different women and by the use of different lots of commercial Matrigel. Due to the early departure of a foreign graduate student (Angela Cruz) who was initially responsible for this study, a more systematic study of this task was not carried out.

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

A major difference between Type I and Type II HBEC is the expression of ER. In contrast to Type II cells, Type I HBEC were ER-positive as shown by immunostaining, RT-PCR and Western blotting (3). The latter showed that the ER expressed is a 48 kd variant ER. The *in vitro* neoplastically transformed Type I cells also expressed the 48 kd ER when grown on plastic *in vitro*. The same cells, however, expressed the 66 kd wild type ER and a 51 kd variant ER *in vivo* as tumors developed in immune-deficient mice. These results were included in the first year

annual report (1997). In addition, we reported that Matrigel was able to mimic the *in vivo* condition in inducing the expression of the wild type ER (66 kd), indicating a role of extracellular matrix in the regulation of ER expression. We also demonstrated that the structure of the 51 kd variant ER expressed *in vivo* (tumor in nude mice) and in Matrigel culture is clearly different from the 48 kd variant ER expressed in cells grown on plastic, i.e., an anti-ER antibody recognizing the N-terminal portion of ER can detect both the wild type ER and the 51 kd ER but not the 48 kd ER (Fig. 18 in 1998 Annual Report).

The major results of this study have been published (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) and 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). As reported in last year's Annual Report, we further defined the molecular structure and promoter usage of the variant ER by RT-PCR analysis as described in the following.

<u>RT-PCR Analysis of the Structure of a Variant ER, and Promoter Usage in Type I HBEC</u>.

In our previous study using a primer pair encompassing exon 2 in RT-PCR analysis, we found no ER-α transcript in Type I HBEC and concluded that the variant ER-α expressed in Type I HBEC has deleted the exon 2 (3). The primer pair is located near the translation-starting site in exon 1 and in exon 3. Using a different primer pair (E1U new near the 3′ end of exon 1 and E5L in exon 5, we found the 850 bp transcript was expressed in a transformed Type I cell line (M13SV1R2-2) grown on plastic and the MCF-7 breast cancer cell line which expressed the wild type ER-α (Fig. 3, 2000 Annual Report). Therefore, exons 2, 3 and 4 are present in the 46 kd variant ER and the deletion in the variant ER is upstream of the primer E1U new site in exon 1. Indeed, from the molecular weight calculation, the deletion of exon 2 alone will produce a 59 kd ER whereas the deletion of exon 1 will result in a 49 kd protein, which is close to the observed 46-48 kd ER-α in Type I HBEC. The translation of the variant ER-α at the 3′ end of exon 1 is possible since there is a translation starting codon (ATG) at 557-559 bp (the upstream ATG at 233-235 bp).

Three promoters have been identified for the transcription of human estrogene-α gene (10). Using the same primer pairs for RT-PCR analysis of the usage of promoter A, B and C, we found that Type I normal HBEC used promoter A but not promoter C. However, the immortal (M13SV1) or tumorigenic cell line (M13SV1R2N1) derived from Type I HBEC used both

promoter A and C similar to MCF-7 (Fig. 4, 2000 Annual Report). A low level of promoter B usage was found for both Type I and Type II HBEC as well as the immortal (M13SV1) and tumorigenic (M13SV1R2N1) Type I HBEC, similar to the ER-positive breast cancer cell lines MCF-7 and T47D. The ER-negative cancer cell line MDA-MB-231 did not use any of the 3 promoters.

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

This task has been completed. A detailed report was presented in the 1997 Annual Report. A paper was published on this work (3).(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). The results show that although only Type I normal HBEC expressed the variant ER-α, both SV40 large T-antigen transformed Type I and Type II HBEC expressed the ER-α. Furthermore, tumors formed in a thymic nude mice by in vitro transformed tumorigenic Type I cell lines expressed a high level of wild type ER which was undetectable in these cells grown in vitro before and after tumor formation.

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

We have characterized a series of immortal, weakly tumorigenic and highly tumorigenic cell lines derived from Type I HBEC following sequential treatment with SV40 large T-antigen, x-rays and neu oncogene (Kang, K.S. et al. 1998. Involvement of tyrosine phosphorylation of $p185^{C\text{-}erbB2/neu}$ in tumorigenicity induced by x-rays and neu oncogene in human breast epithelial cells. *Molecular Carcinogenesis* 21:225-233. Reprint submitted in 1998 Annual Report). This paper (11) is one of the few reported successes in the creation of human tumour cells *in vitro* as cited by a *Nature* paper (12) by Dr. Robert A. Weinberg. These immortal or tumorigenic cells, when grown on plastic, expressed the 48 kd variant ER (3). The tumorigenic cell lines expressed the wild type ER *in vivo* or in Matrigel as described in the previous section.

The original proposal plans to characterize the expression of ER in HBEC immortalized by x-rays. These immortal cells are to be produced by a different project supported by a different grant from NIEHS. Since that project only produced extended life clones (13) but not immortalized HBEC, we do not have x-ray immortalized HBEC line to test the expression of ER-α. Ectopic expression of other genes (i.e. co-transfection with c-myc and dominant negative mutant p53, transfection with the catalytic component of the telomerase gene, pBABE-hygro-hTERT, and cyclin D1, pcDNA3-cyclin D1) also failed to immortalize type I HBEC to be used to study the expression of ER-α.

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

The normal Type II HBEC did not express the ER. The SV40 transformed Type II cells, similar to Type I cells, expressed the 48 kd variant ER when grown on plastic (3). The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen may induce the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast carcinoma cells with SV40. These cells expressing the large T-antigen resulted from SV40 transfection, however, remain ER-negative (3). Alternatively, in the Type II HBEC population, there might exist a small population of ER-positive transitional cells, newly differentiated from Type I cells, which are the target cells for SV40 transformation. Indeed, we have observed a morphologically distinguishable atypical HBEC type, which attached on plastic early similar to Type II cells after trypsinization and subculture. These cells are also very likely to be derived from Type I cells as they may be found in some Type I cell colony. Preliminary study shows that these cells have mixed phenotypes of Type I and Type II cells (i.e., not growth-inhibited by FBS, proficient in gap junctional intercellular communication and ER-positive). It is possible that a small minority of these cells was present in Type II cell population and they are the target cells transformable by SV40. We have done one experiment to transfect 2 plates of atypical HBEC colonies, after removing other types of colonies by

scraping, with a plasmid carrying the wild type SV40 large T-antigen (PRNS-1) from Dr. Johng S. Rhim). The experiment, however, failed to produce the transformed colony.

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

Only Type I HBEC expressed the ER. When grown on plastic, these cells expressed the 48 kd variant ER which appears to be non-functional in DNA-binding (3) and not responsive to estrogen for cell growth (2). What is not known is whether the wild type ER is expressed in budding/ductal structures formed in Matrigel and responsive to estrogen for growth. Although we have developed the techniques for sectioning of organoids in Matrigel, we did not finish this part of the study before the end of the grant. I thought I could complete the work in the 5th year with no cost extension with little funding left. Then I realized that no funds were left to support a research associate to do the work.

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

We have developed and characterized SV40 and x-ray transformed Type I HBEC lines (3, 11). These cells expressed a 48 kd ER-α *in vitro* on plastic and the wild type ER-α 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-α expressed in cells on plastic appears not to respond to estrogen to stimulate cell growth (Fig. 19 in 1998 Annual

Report). Additional experiments show that these neoplastically transformed Type I HBEC (M13SV1R2N1) (11) also did not respond to estrogen for growth on plastic plates coated with different extracellular matrix components (collagen I, IV, fibronectin and laminin) (Biocoat Cellware, Collaborative Biomedical Products) (Fig. 8 in 1999 Annual Report). The Matrigel also seems not to modify the estrogen response for growth when these cells were inoculated on top, in between of two layers (overlay) or mixed (embedded) with Matrigel (Fig. 9 in 1999 Annual Report). This grant did not seek approval for animal experiments. Previous experiments carried out under a different grant (NIEHS ES07256) show that M13SV1R2N1 formed tumors in both male and female mice (11) and in ovariectomized nude mice with or without estrogen pellet (Table 3 in 1999 Annual Report).

All these experiments indicate that the *in vitro* transformed cells are non-responsive to estrogen for growth *in vitro* and do not require estrogen for tumor growth despite the expression of the wild type ER. Therefore, we suspect the non-responsiveness of these cells to estrogen could be the result of their transformation by SV40 large T-antigen. To test this hypothesis, we have transfected the ER-positive, estrogen-dependent breast cancer cells MCF-7. The results, indeed, show that the response of SV40 large T-antigen expressing MCF-7 cells have partially reduced their response to estrogen for cell growth *in vitro* (Fig. 10 in 1999 Annual Report).

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

This Task has been completed. A detailed report was presented in the 1998 Annual Report. A paper was published in *Cancer Research* (4) (W. Sun, K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Research* **59**: 6118-6123, 1999).

In this study, we have examined whether Type I HBECs with stem cell characteristics are more susceptible to telomerase activation and immortalization after transfection with SV40 large T-antigen as compared to Type II HBECs, which show basal epithelial phenotypes. The results show that both types of cells acquire extended life span [(EL); i.e., bypassing senescent] at a comparable frequency. However, they differ significantly in the ability to become immortal in continuous culture, i.e., 11 of 11 Type I EL clones became immortal compared with 1 of 10 Type II EL clones. Both parental Type I and Type II cells as well as their transformed EL clones at early passages [~30 cumulative population doubling level (cdpl)] showed a low level of telomerase activity as measured by the telomeric repeat amplification protocol assay. For all 11 of the Type I EL clones and the single Type II EL clone that became immortal, telomerase activities were invariably activated at middle

passages (~60 cpdl) or late passages (~100 cpdl). For the four Type II EL clones randomly selected from the nine Type II clones that did not become immortal, the telomerase activities were found to be further diminished at mid-passage, before the end of the life span. Thus, normal HBECs do have a low level of telomerase activity, and Type I HBECs with stem cell characteristics are more susceptible to telomerase activation and immortalization, a basis on which they may be major target cells for breast carcinogenesis.

I have presented these results as a plenary session speaker at the 22nd International Breast Cancer Research Congress of the International Association for Breast Cancer Research, Athens, Greece on September 26, 1998 (Human breast epithelial stem cells as targets for carcinogenesis and chemoprevention). The results were also presented at the 11th International Congress of Radiation Research, Dublin, Ireland, on July 22, 1999 (Roles of ionizing radiation in neoplastic transformation of human breast epithelial cells) (13) and its satellite meeting in Cork, Ireland on In Vitro Transformation which resulted in our publication of a paper in Radiation Research (8) (A human breast epithelial cell type with stem cell characteristics as target cells for carcinogenesis.)

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	Spherical and elongated structures
Anchorage independent growth	+ (small colony)	–
Response to SV40 large T-antigen:		
Anchorage independent growth	+ (large colony)	–
Immortalization	High frequency	Low frequency

VII. KEY RESEARCH ACCOMPLISHMENTS

- The discovery that normal Type I human breast epithelial cells (HBEC) and their neoplastically transformed derivatives express a variant ER (~48 kd) when grown *in vitro* on plastic and that tumors formed by these cells in nude mice expressed a wild type ER (~66 kd) and a different variant ER (~51 kd).
- Demonstrated that Matrigel is able to mimic the *in vivo* condition to activate the expression of the wild type ER.
- Obtained further evidence that Type I HBEC have stem cell characteristics, i.e., the ability to form budding/ductal structures similar to human lobul 1 mammary gland and to maintain the

undifferentiated state in Matrigel for a long time, in addition to previous observations that Type I cells are capable of differentiating into other types of cells.

- The demonstration that Type I cells are more susceptible to telomerase activation and immortalization following transfection by SV40 large T-antigen (i.e., Type I cells are target cells for breast carcinogenesis).
- Demonstrated that Type I HBEC can be neoplastically transformed by few steps of sequential treatments: transfection with SV40 large T-antigen (immortalization), x-ray irradiation (weakly tumorigenic), C-erbB2/neu (highly tumorigenic). This neoplastic transformation is consistent with a general view of the major events in carcinogenesis. i.e. (1) Altered cell cycle regulation - bypassing cellular senescence (extended lifespan)→ (2) Telomerase activation – immortilization → (3) Activation of a growth –promoting pathway – tumorigenic.
- The ER-α expressed by Type I HBEC in vitro on plastics is a splicing variant deleting exon 1.
- Type I HBEC use promoter A exclusively while immortalized Type I HBEC, similar to breast carcinoma cell lines, use both promoter A and C for ER-α transcription.

VIII. REPORTABLE OUTCOMES

A. Published Papers

1. Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang, 1997. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* 18: 251-257.
2. Kang, K.S., W. Sun, K. Nomata, I Morita, A. Cruz, C.J. Liu, J.E. Trosko and C.C. Chang, 1998. 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. *Molecular Carcinogenesis* 21: 225-233.
3. Tanimoto, A., C.Y. Kao, C.C. Chang, Y. Sasaguri and R. Padmanabhan, 1998. Deregulation of cdc2 gene expression correlates with overexpression of a 110 kDa CCAAT box binding factor in transformed cells. *Carcinogenesis*, 19: 1735-1741.
4. Hsieh, C.Y. and C.C. Chang, 1999. Stem cell differentiation and reduction as a potential mechanism for chemoprevention of breast cancer. *Chinese Pharm. J.* 51: 15-30.
5. Sun, W., K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang, 1999. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Res.* 59: 6118-6123.

6. Chang, C.C., C.J. Liu, W. Sun, M. Saitoh and J.E. Trosko, 2000. Roles of ionizing radiation in neoplastic transformation of human breast epithelial cells. In Radiation Res., Vol. 2 (M. Moriety *et al.*, Eds) pp. 576-579 Allen Press, Lawrence, KS.

7. Trosko, J.E., C.C. Chang, M.R. Wilson, B.L. Upham, T. Hayashi and M. Wade, 2000. Gap junction and the regulation of cellular functions of stem cells during development and differentiation. *Methods* 20: 245-264, 2000.

8. Chang, C.C., W. Sun, A. Cruz, M. Saitoh, M.H. Tai and J.E. Trosko, 2001. A human breast epithelial cell type with stem cell characteristics as target cells for carcinogenesis. *Radiation Res.* 155: 201-207

9. Trosko, J.E. and C. C. Chang, 2001. Role of stem cells and gap junctional intercellular communication in human carcinogenesis. *Radiation Res.* 155: 175-180.

B. Manuscripts in Preparation

1. Hsieh, C.Y., W. Sun, and C.C. Chang. The structure and function of a variant ER-α expressed in a human breast epithelial cell type with stem cells characteristics.

2. Hsieh, C.Y., M.H. Tai and C.C. Chang. Genistein and 1, 25$(OH)_2$ Vitamin D_3 could be chemopreventive agents by inducing differentiation of breast epithelial stem cells.

C. Abstracts, Presentations

1. Sun, W. and C.C. Chang, 1998. Matrigel mimics the *in vivo* condition in activating the expression of the wild type estrogen receptor in a human breast epithelial cell type. *Proc. Am. Assoc. Cancer Res.* 39: 407. New Orleans, LA. March 28-April 1.
2. Chang, C.C., C.-Y. Hsieh, A. Cruz, W. Sun and J.E. Trosko, 1998. Human breast epithelial stem cells as targets for carcinogenesis and chemoprevention. *Anticancer Res.* 18: 3832. Plenary session speaker. 22nd International Breast Cancer Research Congress of the International Association for Breast Cancer Research, Athens, Greece, September 24-27.
3. Sun, W., K.S. Kang, J.E. Trosko and C.C. Chang, 1999. A normal human breast epithelial cell (HBEC) type with stem cell characteristics is more susceptible to telomerase activation and immortalization. *Proc. Am. Assoc. Cancer Res.* 40: 635. AACR Annual Meeting, Philadelphia, PA, April 10-14.
4. Chang, C.C., C.Y. Hsieh, W. Sun, C.J. Liu, M. Saitoh and J.E. Trosko, 1999. Role of ionizing radiation in neoplastic transformation of human breast epithelial cells. 11th International Congress of Radiation Research, Dublin, Ireland, July 18-23. *Radiation Res.* Vol. 1, Congress Abstracts p. 74.
5. Chang, C.C., W. Sun, K.S. Kang and J.E. Trosko, 1999. A normal human breast epithelial cell (HBEC) type with stem cell

characteristics is more susceptible to telomerase activation and immortalization. 11th International Congress of Radiation Research Satellite meeting: In Vitro Transformation, Cork City, Ireland, July 24-25.

6. Saitoh, M., W. Sun, J.E. Trosko and C.C. Chang, 2000. Co-transfection of a mutant p53 and the human c-myc is an effective method to extend the lifespan of normal human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 41: 540. AACR Annual Meeting, San Francisco, CA, April 1-5.

7. Chang, C.C., W. Sun, C.-Y. Hsieh, M. Saitoh, M.-H. Tai, and J.E. Trosko, 2000. A human breast epithelial cell type with stem cell characteristics and estrogen receptor expression as a target for carcinogenesis. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Atlanta, Georgia, June 8-12. Proceedings, p. 408.

D. Personnel Receiving Pay by This Grant for Research Effort

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Wei Sun
Ching-Yi Hsieh
Mei-hui Tai

IX. CONCLUSIONS

Our characterization of organoids formed by the two types of HBEC on Matrigel provides strong evidence that Type I HBEC are breast epithelial stem cells. The Type I cells not only are capable of differentiating into other cell type (1) and forming budding/ductal structures on Matrigel (4, 13), 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 method 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 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 (11/11 Type I cells and 1/10 Type II cells with extended lifespan) assayed, the telomerase activity was invariably greatly activated at mid- or late passage, in contrast to senescent clones of Type II cells which failed to activate the telomerase. Furthermore, the ability of Type I HBEC to become immortal at high frequency (11/11) appears to be correlated with their high potential for telomerase activation. Therefore, telomerase activation could be a major biomarker and mechanism for the transition from extended lifespan to immortalization, contrary to the conclusion of a previous report (6) that telomerase activity is a biomarker of cell proliferation but not malignant transformation. The high potential of Type I HBEC to activate telomerase activity (11/11 extended lifespan clones) also provides a mechanism why Type I cells might be target cells for neoplastic transformation. If Type I stem cells are target cells for carcinogenesis, they should be also target cells for chemoprevention. Therefore, we hypothesize that agents capable of inducing differentiation and/or preferentially arrest the self-renewal of breast epithelial stem cells, thereby reducing the number of target cells for carcinogenesis, are potential chemopreventive agents for breast cancer. The hypothesis is supported by 1) our Type I stem cell experiments mentioned above; 2) animal experiments with genistein that induced mammary gland differentiation and reduced mammary tumorigenesis (14); 3) epidemiological evidence that early full-term pregnancy reduces, while nulliparous or late parous increases, breast cancer (15-17); and 4) our preliminary results

indicating that potential chemopreventive agents, genistein and 1,25-dihydroxy-vitamin D_3, induced differentiation of breast epithelial stem cells (18).

Our Type I HBEC can be converted to immortal, weakly tumorigenic and highly tumorigenic clones following sequential treatment with SV40 large T-antigen, x-rays and C-erbB2/neu (8). This paper is one of the few reported successes in the creation of human tumor cells in vitro as cited by a Nature paper by Dr. Robert A. Weinberg (11). These results demonstrate a general sequence of the major events in neoplastic transformation, i.e.,

(1) Altered cell cycle regulation – bypassing cellular senescenc (extended lifespan). → (2) Telomerase activation – immortalization →(3) Activation of a growth-promoting pathway – tumorigenic → (4) Altered cell adhesion, mobility and protease/collagenase activity – invasion and metastasis.

The fact that our neoplastically transformed Type I HBEC and breast carcinomas such as MCF-7 show many similar phenotypes as Type I HBEC (i.e. expression of luminal epithelial cell markers, EMA, cytokeratin 18, ER-α and deficiency in gap junctional intercellular communication) strongly supports the "oncogeny as blocked ontogeny" concept (19) or stem cell theory of carcinogenesis.

The Type I cells transformed by sequential treatment with SV40, x-rays and neu oncogene did not require estrogen for cell growth or tumor development. This could be due to the expression of SV40 large T-antigen as shown by the reduced sensitivity to estrogen for growth in T-antigen

expressing MCF-7 cells. This can be confirmed by testing whether Type I HBEC transformed by other means such as hTERT or mutant p53 could be estrogen-dependent similar to MCF-7 cells in future studies.

Our finding of the differential expression of ER-α variant or wild type forms *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.

Undoubtedly, we have developed a highly relevant *in vitro* model to study the mechanism of carcinogenesis and chemoprevention of breast cancer. Unfortunately, in the past year, we have failed to obtain funding to continue this study. Instead, our efforts have been diverted to the development of a novel culture system to grow multi-potential pancreatic stem cells from human pancreatic islets. This new development with its important implication in treatment of diabetes and pancreatic cancer research is partly due to our experience working with human breast epithelial stem cells.

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XI. APPENDICES

Reprints of published papers and manuscripts in press:

1. Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang. 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.
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Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines

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Although approximately two-thirds of breast cancers are estrogen receptor (ER)-positive, only a small proportion of epithelial cells in the mammary gland express the ER. The origin of the ER-positive breast cancers is unknown. Recently, we have developed a culture method to grow two morphologically and antigenically distinguishable types of normal human breast epithelial cells (HBEC) derived from reduction mammoplasty. In this report, we studied the expression of ER in these two types of cells and their transformed cell lines. The results indicate that Type I HBEC with luminal and stem cell characteristics expressed a variant ER (~48 kd) by Western blot analysis. This variant ER contains a deletion in the DNA binding domain (exon 2) as revealed by RT-PCR analysis. The lack of the DNA-binding domain of the variant ER was also confirmed by the ER-estrogen responsive element binding assay, as well as by the immunofluorescence staining of the ER using anti-ER antibodies which recognize either the C-terminal or N-terminal region. In contrast, Type II HBEC with basal epithelial phenotype are ER-negative. Simian virus 40 (SV40) transformed Type I and Type II HBEC lines also expressed the variant ER. Tumors formed in athymic nude mice by *in vitro* transformed tumorigenic Type I cell lines, however, expressed a high level of wild type ER which was undetectable in these cells grown *in vitro* before and after tumor formation. Thus, there appears to be a differential ER mRNA splicing between the *in vitro* and *in vivo* mileu.

Introduction

The estrogen receptor (ER*) is a ligand-inducible transcription factor which regulates the expression of a variety of genes including some growth factors, hormones and oncogenes important for the growth of breast cancer (1,2). Expression of the ER plays an important role in the pathogenesis and maintenance of breast cancer. In breast cancer patients, about two-thirds of tumors are ER-positive (3); 50% of these ER-positive tumors are estrogen-dependent and respond to endocrine therapy (4). Breast carcinomas occurring in post-menopausal women are often ER-positive (5), and many of these tumors express significantly more ER than does the normal mammary epithelium (6).

On the other hand, it has been proposed that the cumulative exposure of breast tissue to hormones associated with ovarian activity is a major determinant of breast cancer risk (7,8). Furthermore, some environmental agents and dietary components might influence breast cancer development by functioning as xenoestrogens or estrogenic potentiating factors (9).

The ER gene spans 140 kb and is comprised of 8 exons which are spliced to yield a 6.3 kb mRNA, encoding a 595-amino acid protein with a molecular weight of 66 kd (10,11). The ER protein is comprised of several discrete functional domains (12). Two transcriptional activation functions (TAF-1 and TAF-2) reside in exons 1 and 8, respectively (13,14). The DNA binding domain of ~70 amino acids is located at exons 2 and 3. Exons 4 through 8 translating into 250 amino acids in size are necessary and sufficient for ligand binding (12,15). A 22 amino acid sequence necessary for subunit dimerization has been located in exon 7 (16).

Recently, it has been reported that both the wild type and variant estrogen receptors were coexpressed in some human breast carcinoma cell lines (17–23). Variant human breast tumor ER with constitutive transcriptional activity has been identified in tumor specimens (24,25). Therefore, estrogen receptor variants might have a role in human breast cancer. However, to date, the role of the estrogen receptor variant in carcinogenesis and the regulation of its expression are poorly understood.

The human mammary gland contains a small but distinct population of ER-positive cells, comprising ~7% of the total epithelial cell population from all biopsies (26). The ER-positive cells were distributed as scattered single cells, most of them (87%) were luminal epithelial cells or occupied an intermediate position in the duct wall. The highest frequency of ER-positive cells has been found in the lobules as compared to the interlobular ducts (26). Normal HBEC grown in the commonly used media, MCDB 170 (27) and DFCI-1 (28), including the commercially available normal HBEC (Clonetics), exhibited basal epithelial, but not luminal epithelial cell characteristics. These cells have not been shown to express the estrogen receptor.

Recently, we have developed a culture method to grow two morphologically and antigenically distinguishable normal human breast epithelial cell (HBEC) types from reduction mammoplasty (29). Type I HBEC is deficient in gap junctional intercellular communication and has luminal and stem cell characteristics, i.e. the differentiation of Type I into Type II HBEC by a cyclic AMP-inducing agent (29) and the unique ability of Type I HBEC to form budding and ductal structures on Matrigel matrix (30), whereas Type II HBEC is capable of gap junctional intercellular communication (29,31,32) and expresses basal epithelial cell phenotypes (29).

In this study, we characterized the expression of estrogen receptors in these two types of normal HBEC and the neoplastically transformed cell lines derived from these cells.

***Abbreviations:** ER, estrogen receptor; ERE, estrogen responsive element; HBEC, human breast epithelial cell; kd, kilodalton; PBS, phosphate buffered saline; NGS, normal goat serum; PgR, progesterone receptor; RT-PCR, reverse transcription-polymerase chain reaction; SV40, simian virus 40.

Materials and methods

Cells and cell culture

Normal human breast epithelial cells were isolated from reduction mammoplasty of four different women (21–29 years of age, designated HME-5, -12, -14, -15). The media and the procedure used to develop the two types of normal HBEC have been described previously (29). The primary cultures developed *in vitro* for one week were stored in liquid nitrogen. Early passage cells, after recovery from liquid nitrogen storage, were used in these experiments. All cell cultures were grown at 37°C in incubators supplied with 5% CO_2 and humidified air.

Transformation of normal HBEC was achieved by lipofectin-mediated transfection of HBEC (29) with SV40 DNA (GIBCO-BRL) (M11SV2, M13SV1, M13SV22 derived from normal HBEC cultures HME-11 and HME-13, respectively) 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) (M15SV1-11, M15SV21-29 derived from HME-15). In addition to the ER-positive MCF-7, T47D and the ER-negative MDA-MB-231 breast cancer cell lines, a HBEC line (M12B4 derived from HME-12), with extended lifespan after 5-bromodeoxyuridine treatment, was also included in the study of ER expression.

Immunofluorescence staining of estrogen receptor

Cells were fixed by 4% paraformaldehyde for 20 min, postfixed with absolute methanol for 30 s and then rehydrated with phosphate buffered saline (PBS). Subsequently, nonspecific binding sites were blocked with 10% normal goat serum (NGS) in PBS for 30 min at room temperature. The cells were incubated overnight at 4°C with a primary antibody against ER (Ab-1, Oncogene Science, NY; diluted 1:200 in PBS containing 0.1% bovine serum albumin and 1% NGS), washed three times with PBS, and then incubated with a second anti-mouse antibody conjugated with rhodamine (Jackson Immunoresearch Lab, West Grove, PA; diluted 1:100 in PBS) for 30 min at room temperature. The cells were washed extensively with PBS containing 0.1% bovine serum albumin and 1% NGS and mounted with coverslips on Polyaquamount (Polysciences, Inc., Warrington, PA). The cells were examined and photographed using the Ultima laser confocal scanning microscope (Meridian Instruments, Okemos, MI).

SDS-PAGE and Western blot analysis

Proteins were extracted from normal and SV40-transformed HBEC, and from MCF-7, T47D, MDA-MB-231 cells in 100 mm dishes by treatment with 20% SDS 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, 5 mM sodium fluoride). After sonication at three 10-s pulses from a probe sonicator, the cell lysates were stored at −20°C until use (31). The protein amounts were determined by the DC protein assay kit (Bio-Rad Co., Richmond, CA). Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 h. ER was detected by the anti-ER monoclonal antibody (NCL-ER-LH2, Vector Lab., Burlingame, CA) which recognizes the amino (N)-terminal portion of ER and by the anti-ER monoclonal antibody (Ab-1, oncogene science, NY) which recognizes the carboxyl (C)-terminal portion of ER after blocking with 5% dried skim milk in PBS containing 0.1% Tween 20. This was then followed by incubation with horseradish peroxidase-conjugated secondary antibody and detected with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL). The membranes were exposed to X-ray film for 15 s to 1 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by using Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The extracted RNA was dissolved in RNase-free water, and its concentration and purity was determined by a spectrophotometer.

cDNA was synthesized from the isolated RNA by reverse transcription in 20 μl reaction solution containing 2.5 μM of random hexamers (Perkin Elmer, Madison, WI), 50 units of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Madison, WI), 1 μg of total RNA, 2 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris−HCl, pH 8.3), 5 mM $MgCl_2$, 1 mM of each dNTP, 20 units of RNase inhibitor (Perkin Elmer, Madison, WI), and 2 μl of RNase free water (Promega, Madison, WI). The reaction mixture was incubated at room temperature for 10 min and then at 42°C for 15 min, heated to 99°C for 5 min, and then quick-chilled on ice. The 20 μl of solution which contains the reverse transcribed cDNA were added to 30 μl of PCR reaction mixture containing 2 mM $MgCl_2$, 3 μl of 10× PCR buffer, 25 pmol of each primer and 1.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Madison, WI). The primers chosen for PCR are sequences surrounding the border between exons 7 and 8 of the ER (5′-GCACCCTGAAGTCTCTGGAA-3′, 5′-TGGCTAAAGTGGTGCATGAT-3′) (33) and sequences encompassing exon 2 of the ER (5′-TACTGCATCAGATCCAAGGG-3′, 5′-ATCAATGGTGCACTGGTTGG-3′) (34). The primers used to generate the 306-base pairs of GAPDH products are 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. Thermal cycling was performed in a GeneAMP 9600 PCR system (Perkin Elmer, Madison, WI) by using the following two steps amplification profile: an initial denaturation at 95°C for 105 s, 35 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s; and a final elongation step at 72°C for 7 min. The PCR products were then electrophoresed in a 2% agarose gel and stained with 0.5 μg/ml ethidium bromide. The size of cDNA product was determined by comparison to DNA size markers (the HaeIII digested X174 DNA, GIBCO-BRL, Gaithersburg, MD).

ER-ERE binding assay

Nuclear extracts were prepared as previously described (35). Cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM, $MgCl_2$, pH 7.5) and nuclei were pelleted by centrifugation at 3000 *g* for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, and 10% glycerol with 1 mM DTT, 1 mM PMSF, and 1 μg/ml of aprotinin and leupeptin. Following lysis, the samples were centrifuged at 12 000 *g* for 30 min, and the supernatant was collected for use in the DNA binding assay. Double-stranded oligonucleotides, containing a wild-type estrogen responsive element (ERE) consensus sequence (5′-GTCCAAAGTCAGGTCAGTGACCTGATCAAAGTT-3′), that corresponds to −308/−342 of the promoter/upstream element of the Xenopus vitellogenin A2 gene (35), was synthesized at the Biotechnology Facility of Michigan State University. The ERE was annealed, and end-labeled with [γ-^{32}P]. The nuclear extract (3 μg) was incubated with the reaction buffer [70 mM KCl, 30 mM HEPES (pH 8.0), 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, and 2 μg poly (dI-dC)] in the presence or absence of unlabelled oligonucleotide competitor, or antibody for 10 min, followed by a 20 min incubation at room temperature with the ^{32}P-labelled ERE DNA probe. The products of ER and ERE DNA binding activity was separated from free probe in a 4.8% polyacrylamide gel by electrophoresis using TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried and subjected to autoradiography.

Tumor development in athymic nude mice

Cells suspended in 0.25 ml of MSU-1 medium (29) with 5% fetal bovine serum were injected subcutaneously into each of two sites on a female athymic nude mouse (Harlan−Sprague−Dawley). The tumors formed from 6×10^6 cells innoculated in each site were dissected and measured four weeks after innoculation. The tumor cells were removed for protein extraction for Western blot analysis and for re-establishment of cell cultures which were also harvested for Western blot analysis.

Results

Expression of estrogen receptor in normal HBEC

Type I and Type II normal HBEC derived from reduction mammoplasty of four different women (HME-5, -12, -14, -15) were examined for their ER expression by Western blot analysis using an anti-ER antibody which recognizes the C-terminal region of ER (Ab-1, Oncogene Science). The results show that all the four Type I HBEC expressed an ER, whereas all the four Type II HBEC did not express any ER (Figure 1A and B). The ER expressed in Type I HBEC, however, is not the wild type ER since its molecular size (~48 kd) is smaller than the wild type ER (~66 kd). This variant ER appears to contain a deletion in N-terminal region, based on the observation that, unlike the ER-positive MCF-7 and T47D cells which express both wild type and variant ER, the ER was not detectable in Type I or Type II HBEC when anti-ER antibodies which recognize the N-terminal region (NCL-ER-LH2, Vector Laboratory; C314, Santa Cruz) were used (Figure 1C).

The expression of ER in Type I, but not Type II, normal HBEC was confirmed by immunofluorescence staining using Ab-1 anti-ER antibody (Figure 2C and E). In this study, the ER was found to be expressed in all Type I HBEC colonies examined (>100) and in every cell in a colony (data not shown). Similar to Western blot analysis, the ER was detectable

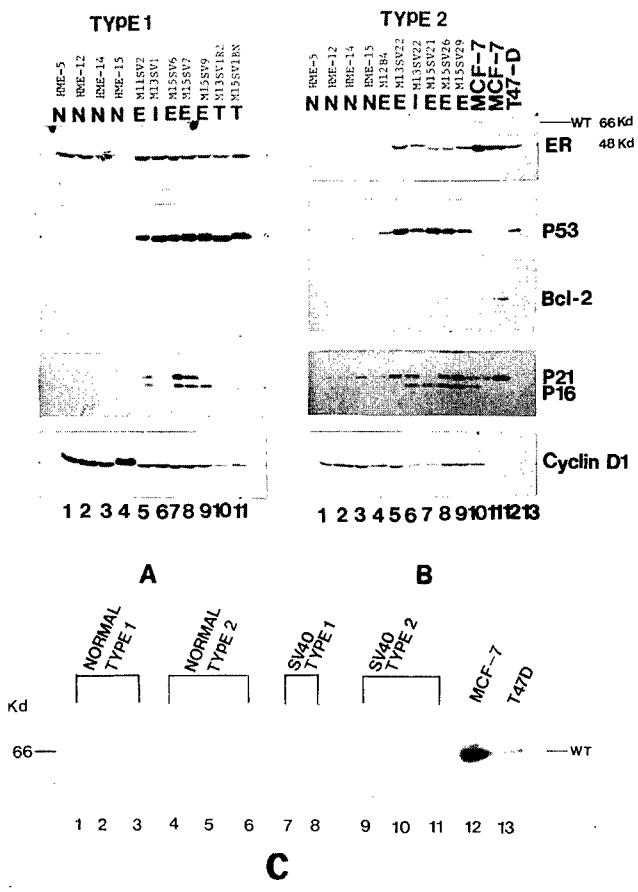

Fig. 1. Western blot analysis for the expression of estrogen receptor, p53, bcl-2, p21, p16, and cyclin D1 expression. (**A**) Same amount of protein from normal Type I (N) and SV40-transformed Type I HBEC at different stages of transformation (extended lifespan, E, immortal, I and tumorigenic, T) blotted on the same membrane were probed for the expression of cell cycle-related proteins and ER; (**B**) same as panel A, except normal Type II and SV40-transformed Type II HBEC and the control MCF-7 and T47D cells were used. In both experiments, the Ab-1 (Oncogene Science) anti-ER antibody which recognizes the C-terminal domain of ER was used; (**C**) the expression of ER in normal and SV40-transformed Type I and Type II HBEC was studied using the NCL-ER-LH2 (Vector lab.) anti-ER antibody which recognizes the N-terminal domain of ER. The cells used are HME-12, -14, -15 (lanes 1–3), HME-12, -14, -15 (lanes 4–6), M13SV1 (lane 7), M15SV1 (lane 8), M13SV22 (lane 9), M15SV26 (lane 10), M15SV29 (lane 11).

using the C-terminal anti-ER antibody (i.e. Ab-1) and not detectable using the N-terminal anti-ER antibodies (i.e. NCL-ER-LH2 and C314).

ER expression in SV40-transformed Type I and Type II HBEC

Similar to the Type I normal HBEC, SV40 transformed Type I HBEC lines (derived from three different primary cultures) at different stage of neoplastic transformation (extended lifespan, immortal, and tumorigenic) expressed the variant ER (~48 kd) (Figure 1A) at similar expression levels as their parental cells, detected by Western blotting. Interestingly, unlike Type II normal HBEC, SV40 tranformed Type II HBEC lines also expressed this variant ER (Figure 1B). In contrast, a cell line with extended lifespan after 5-bromodeoxyuridine treatment (BrdU) (M12B4) did not express the ER (Figure 1B). As expected, the ER of SV40-transformed Type I and Type II HBEC were found in the nuclei (Figure 2D and 2F) similar to MCF-7 cells (Figure 2A) using the Ab-1 anti-ER antibody. However, we could not detect any ER in the SV40-transformed

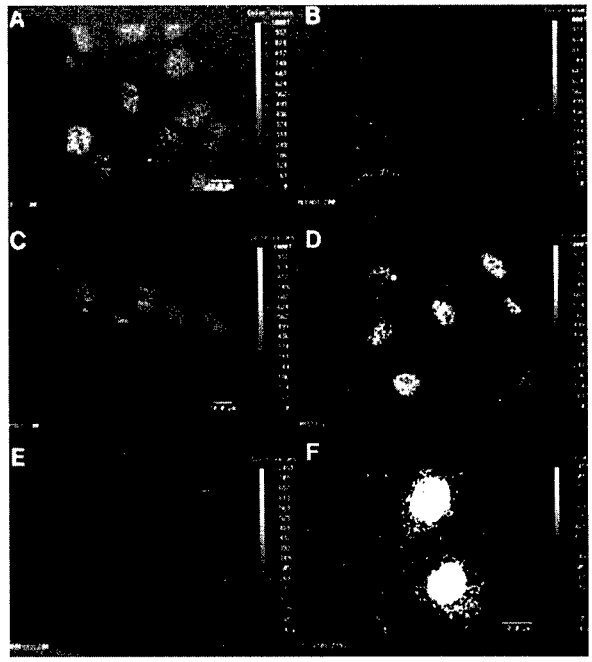

Fig. 2. Immunofluorescence staining for ER using the Ab-1 (Oncogene Science) anti-ER antibody. (**A**) and (**B**) MCF-7 with and without primary antibody, respectively as controls; (**C**) and (**D**) normal and SV40-transformed Type I HBEC (HME15 and M15SV1), respectively; (**E**) and (**F**) normal and SV40-transformed Type II HBEC (HME15 and M15SV29), respectively.

Type I and Type II HBEC, using antibodies which recognize the N-terminal region of the ER (NCL-ER-LH2, Vector lab; C314, Santa Cruz), by Western blot analysis (Figure 1C) or by immunofluorescence staining (data not shown). In order to understand the function of this variant ER, we also examined progesterone receptor (PgR) expression which might be induced by estrogen in cell strains and cell lines studied in Figure 1A and 1B. None of these cells were found to express the PgR (data not shown).

ER expression by RT-PCR analysis

To verify the above results and to gain more insight into the nature of the expressed variant ER, RT-PCR was performed using primer pairs encompassing exon 2 or a contiguous region bordering exons 7 and 8. As controls, RNA from the ER-positive MCF-7, T47D and the ER-negative MDA-MB-231 breast cancer cell lines were reverse transcribed, and then the cDNAs were amplified by PCR in conjunction with studies using Type I and Type II normal HBEC, as well as SV40-transformed Type I and Type II HBEC. As shown in Figure 3, when primer pairs surrounding a region bordering exon 7 and exon 8 of the ER were used for PCR, a ~470 base pair product was detected in Type I but not in Type II normal HBEC or in the ER negative MDA-MB-231 cells. The amplified DNA was also found in SV40 transformed Type I and Type II HBEC, as well as in the ER-positive MCF-7 and T47D cells. The RT-PCR condition appears similar for all the cell strains or cell lines tested as judged from the uniform control amplification for the GAPDH gene. When primer pairs encompassing the exon 2 of ER were used for the PCR, none of the normal or SV40 transformed Type I or Type II HBEC yielded the ~650 base pair product as did the ER-positive MCF-7 and T47D cells (Figure 4). These results confirm previous results that showed a variant ER was expressed in normal Type I HBEC and both Type I and Type II SV40 transformed HBEC.

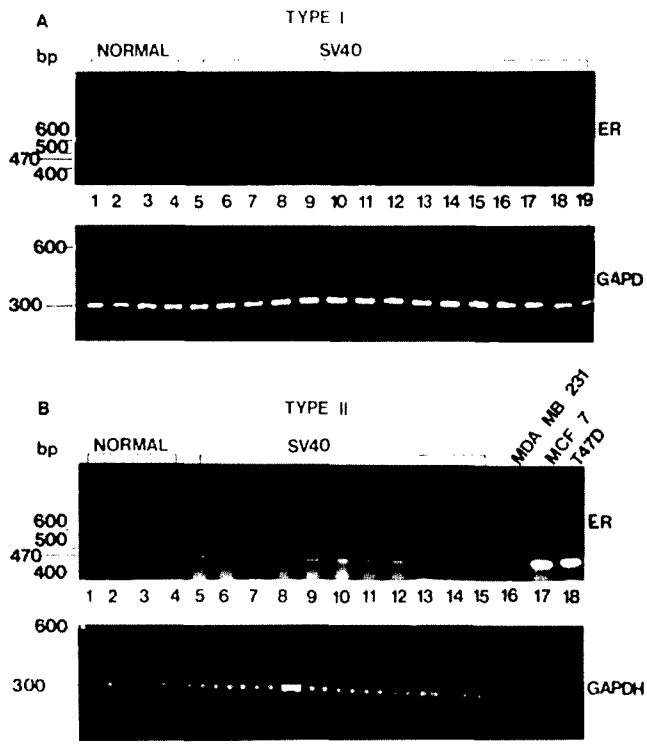

Fig. 3. RT-PCR analyses for detecting ER transcripts using primer pairs in the border between exon 7 and exon 8. (**A**) Type I and SV40-transformed HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–19 (M11SV1, M11SV2, M13SV1, M13SV2, M15SV1–11, respectively); (**B**) normal Type II and SV40-transformed Type II HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–15 (M13SV22, M15SV21–30, respectively). The control RT-PCR for GAPDH are also shown.

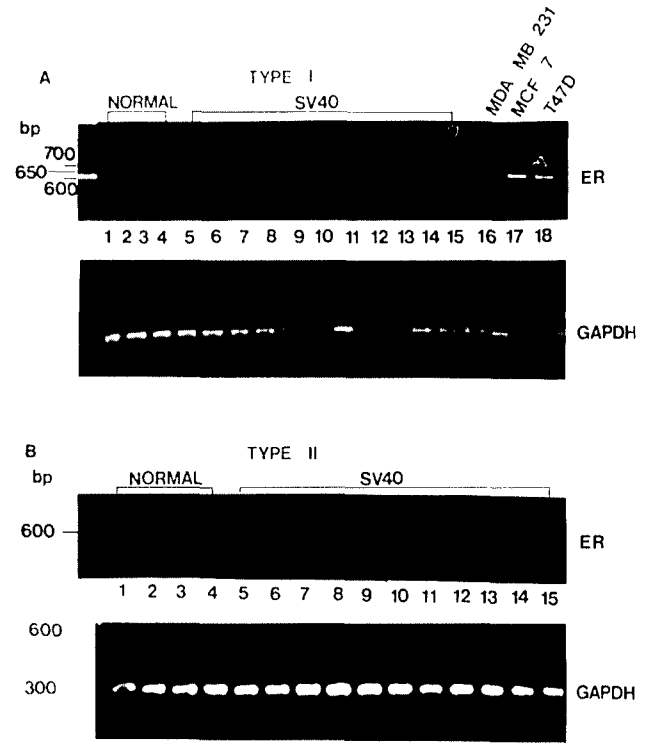

Fig. 4. RT-PCR analyses for detecting ER transcripts using primer pairs encompassing exon 2. (**A**) Type I and SV40-transformed HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–16 (M11SV1, M13SV1, M15SV1–10, respectively); (**B**) normal Type II and SV40-transformed Type II HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–15 (M13SV22, M15SV21–30, respectively). The control RT-PCR for GAPDH are also shown.

In addition, the study further indicates that the variant ER contains a deletion in the exon 2 region of the ER.

The ability of the variant ER to bind an estrogen responsive element

If the expressed variant ER contains a deletion in the DNA binding domain (exons 2 and 3), this ER is expected to lose its ERE DNA binding ability. To test if this is the case, nuclear extracts were prepared from cell lines expressing the variant ER and then subjected to a ER-ERE binding assay using a ERE motif, the cognate binding site for estrogen receptor. The results showed that the variant ER expressed in normal and SV40 transformed cells was deficient in ERE binding activity, while MCF-7 cells, as a positive control, produced a strong binding signal (Figure 5A). The specificity of the retarded ER-ERE band observed for MCF-7 cells was confirmed by the addition of an excess of unlabelled double-stranded oligonucleotide. As shown in Figure 5B, the retarded band was supershifted when the nuclear extract was preincubated with anti-ER antibody (Ab-1) before its binding to the ERE probe (Figure 5B). These results are consistent with previous RT-PCR and Western blot analyses and clearly indicate that this variant ER had a deleted DNA binding region.

Expression of wild type and variant ER in tumors formed in nude mice by neoplastically transformed Type I HBEC

The SV40 immortalized Type I HBEC line (M13SV1) reported previously (29) was non-tumorigenic. After X-ray irradiation (2 Gy, twice), large colonies formed in soft agar were found to be weakly tumorigenic in athymic nude mice (a cell line,

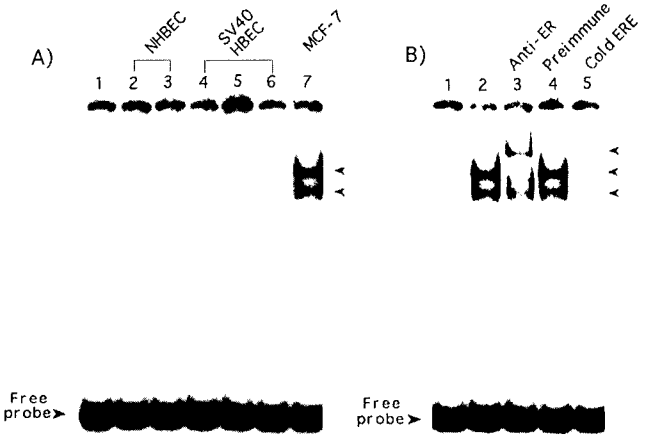

Fig. 5. Electrophoretic Mobility Shift Assay (EMSA). (**A**) ER-ERE binding: free probe only (lane 1), and nuclear extracts from normal Type I HBEC (lane 2, HME-15) and normal Type II HBEC (lane 3, HME-15), SV40-transformed Type I HBEC (lanes 4 and 5, M13SV1 and M15SV6, respectively), SV40 transformed Type II HBEC (lane 6, M13SV22) and MCF-7 (as a positive control, lane 7); (**B**) the specificity of the retarded bands was confirmed by the addition of an excess of unlabelled double-stranded oligonucleotide. The retarded band was supershifted dramatically when the nuclear extract was preincubated with anti-ER antibody (Ab-1, Oncogene Science) before binding to ERE probe (lane 3), lane 1, free probe; lane 2, MCF-7 without anti-ER antibody; lane 4, MCF-7 with preimmune serum from mouse; lane 5, unlabeled oligonucleotide of ERE.

M13SVIR2 was used in this study). The weakly tumorigenic cells became highly tumorigenic (tumor > 1 cm formed in 4 weeks) after infection with a viral vector carrying a mutated *neu* oncogene (i.e. M13SV1R2-N1, -N4, -N8) (manuscript in

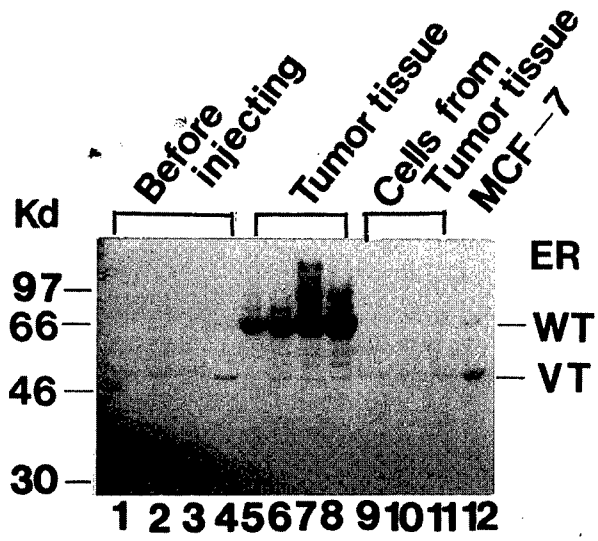

Fig. 6. Western blot analysis for the expression of ER. The expression of ER in weakly tumorigenic cell line, M13SV1R2 and highly tumorigenic cell lines, M13SV1R2-N1, -4, -8, before innoculation for tumor growth in nude mice (lanes 1–4) and after reestablishment of cell culture from tumors (lanes 9–11), were compared with that in tumors formed by these cells (lanes 5–8). The cell lysates loaded are; lanes 1, 5, 9, M13SV1R2; lanes 2, 6, 10, M13SV1R2-N1; lanes 3, 7, 11, M13SV1R2-N4; lanes 4, 8, M13SV1R2-N8. The positions of wild type ER (WT) and variant ER (VT) are indicated.

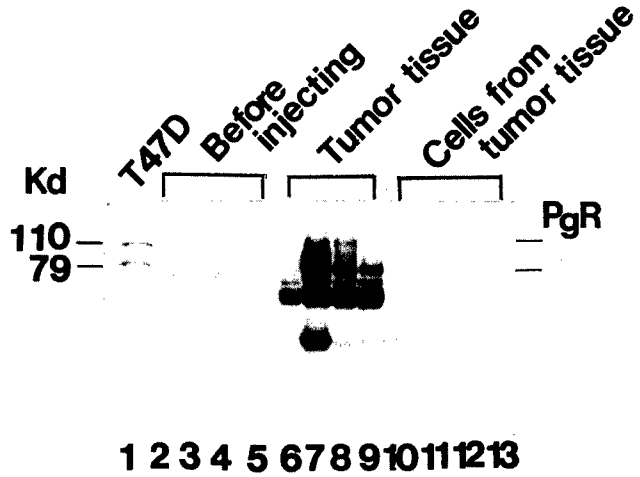

Fig. 7. The proteins from cell lines used in experiments presented in Fig. 6 also were used for Western blot analysis for the expression of PgR. The PgR expression in these cells before innoculation for tumor growth in nude mice (lanes 2–5) and after re-establishment of cell culture from tumors (lanes 10–12) were compared with that in tumors formed by these cells (lanes 6–9). The cell lysate loaded are: lanes 2, 6, 10, M13SV1R2; lanes 3, 7, 11, M13SV1R2-N1; lanes 4, 8, 12, M13SV1R2-N4; lanes 5, 9, 13, M13SV1R2-N8.

preparation). We have tested the expression of ER in these tumorigenic cell lines before innoculation in nude mice, in tumors formed by these cells in nude mice and in cell cultures reestablished from tumors. The results showed that cells, before injection into nude mice, and cells, reestablished from tumor tissue, *in vitro*, only expressed the variant ER (~48 kd) using an anti-ER antibody recognizing the C-terminal region of the ER (Ab-1, Oncogene Science). Significantly, the tumor tissues expressed a high level of the wild type ER (~66 kd) and less amount of the variant ER (~48 kd) by Western blotting using the Ab-1 (Oncogene Science) (Figure 6) or the human ER specific antibody (D75, kindly provided by Dr Geoffrey Greene of the University of Chicago) (data not shown). Western blot analysis for the expression of progesterone receptor using an anti-PgR antibody (Ab-1, Oncogene Science) was also carried out in these cells. The results are similar to that observed for the expression of ER (i.e. expression was found in tumor tissues but not in cells grown *in vitro*) (Figure 7), indicating that the wild type ER expressed *in vivo* might be functional.

Expression of cell cycle related proteins (p53, $p21^{waf}$, $p16^{INK4}$, cyclin D_1)

The same Western blots studying ER expression (Figure 1) were also probed for the expression of cell cycle related proteins. As expected, high levels of p53 were found in SV40 transformed cell lines, confirming the involvement of large T antigen in their transformation. The p21 and p16 proteins were frequently elevated in transformed Type I and Type II HBEC cells. The cyclin D1 was highly expressed in Type I normal HBEC compared to their Type II HBEC counterparts. The transformation by SV40 reduced the expression of cyclin D1 in Type I but not Type II HBEC.

Discussion

The major finding of this study is that an estrogen receptor was expressed in all the Type I HBEC with luminal and stem cell characteristics examined while their Type II HBEC counterparts with basal cell characteristics did not express any ER. Furthermore, all the SV40 transformed Type I and Type II HBEC lines examined (15 and 11 cell lines, respectively) were also found to express the ER. Although the human mammary gland is known to contain a small population of ER-positive cell (26), the previously reported normal HBEC in culture have not been shown to express significant level of ER. The expression of ER in our Type I HBEC or SV40 transformed cells is unambiguous since it has been observed by three different methods (i.e. Western blot analysis, immunofluorescence staining, and RT-PCR).

The ER expression, however, is not the wild type ER. It is a variant ER with smaller molecular weight (~48 kd) than the wild type ER (~66 kd). This variant ER was detectable by Western blot analysis and immunofluorescence staining using an anti-ER antibody (Ab-1, Oncogene Science) recognizing the C-terminal portion of the ER but was undetectable when anti-ER antibodies recognizing the N-terminal portion of the ER (NCL-ER-LH2, Vector laboratory, C314, Santa Cruz) were used. This observation suggests that the variant ER contains a deletion in the N-terminal region. By RT-PCR analysis using primer pairs in the C-terminal or N-terminal region, the deletion was found to be in the exon 2 region. Since exon 2 is a part of DNA binding domain, the ER with deletion in this region is expected to lose its DNA-binding activity. This was found to be true in the ER-ERE binding assay. That the variant ER deleting DNA binding domain would be non-functional is revealed by the non-expression of the PgR which is positively regulated by the ER (36). Recently, ER variants with deletion in exon 2, exon 3, or both were observed in normal human breast tissue (37). Further RT-PCR analysis will reveal whether our variant ER also includes deletion in exon 3.

Of the eight exons that constitute the ER mRNA transcript, most of them (exons 2–7) have been found to be involved in aberrant splicing events in breast tumor cell lines or tissues (38–42). Many of these variants disrupt critical regions such that they become non-functional (20). The variant ER expressed

in our cells appears to belong to this category. Some other variants may play an important role in tumor growth, e.g. variant ER lacking the hormonal binding domain is suspected to be a dominant positive transcriptional activator that accounts for hormone independent growth of tumors (24,25). Furthermore, variants ER lacking the DNA binding domain might function as negative or positive transcription factors through protein–protein interaction (43–45). Indeed, Yang *et al.* have recently shown that human transforming growth factor-β3 gene can be activated by the estrogen receptor in the presence of estrogen metabolites or estrogen antagonists. Activation was mediated by a polypurine sequence, termed the raloxifene response element, and did not require the DNA binding domain of the estrogen receptor (45).

Although the ER expressed in Type I and SV40 transformed HBEC appears to be non-functional, there is evidence that this is only an *in vitro* phenomenon. From studies presented in Figures 6 and 7, tumorigenic cell lines derived from Type I HBEC were found to express high level of wild type ER in tumors formed by these cells in athymic nude mice. The same cells did not express the wild type ER before innoculation for tumor growth or after the tumor cells were cultured in vitro. In this study, the wild type ER expressed in tumors have been shown to be human ER as they were observed by Western blot analysis using a human ER specific antibody. The expressed wild type ER in tumors appears to be functional since the PgR was simultaneously expressed. The differential *in vitro* and *in vivo* expression of different splicing variants of ER could be due to factors provided only by the *in vivo* condition such as 3-dimensional tissue structure or the presence of stromal cells and extracellular matrix. This will be examined in future studies.

The expression of the variant ER in SV40 transformed Type II HBEC is unexpected. The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen induced the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast adenoma cells with SV40. The MDA-MB-231 cells expressing the large T-antigen resulted from SV40 transfection, however, remained ER-negative (our unpublished results). 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 were the target cells for SV40 transformation. Except for ER-expression, the phenotypes of SV40 transformed Type II HBEC are substantially different from that of SV40 transformed Type I cells (29). Therefore, these hypothetical transitional cells are quite different from the Type I HBEC.

The implication of the results from this study are two-fold. First, the expression of ER in Type I HBEC provides additional similarity between breast cancer cells and Type I HBEC. As mentioned previously, the origin of the ER-positive tumors is not known. One possible origin is that they were derived from ER-positive normal HBEC stem cells similar to our Type I HBEC. During the neoplastic transformation, many of the parental target cell phenotypes are largely preserved. Those include deficiency in gap junctional intercellular communication, expression of luminal epithelial cell markers (29), and expression of telomerase (our unpublished results). Our study also indicates that Type I HBEC are more susceptible to neoplastic transformation by an oncogenic (SV40) stimulus (i.e. to become immortal and capable of anchorage independent growth) (29). The phenotypic similarity between Type I HBEC and breast cancer cells mentioned above are consistent with the notion of oncogeny as blocked or partially blocked ontogeny (46). Second, our results indicate that there is a differential splicing in ER mRNA under *in vitro* and *in vivo* conditions. The expression of a variant ER under our *in vitro* culture condition might be an indication that the same cells might express the wild type ER *in vivo*. Furthermore, the results suggest a potential strategy to control the growth of estrogen-dependent human breast tumors, i.e. the modulation of the tissue environment in order to facilitate the alternative splicing that results in the expression of non-functional ER. Lastly, this study raises an awareness concerning limitations in interpretation of experimental results. First, the use of immunostaining to classify tumors as ER-positive or ER-negative based on the use of one antibody may not be adequate. It may not detect all alternatively spliced ER nor provide the information concerning its function. Second, the phenotype of a normal cell type used for comparison with that of tumor cells may be irrelevant. For example, when we describe normal HBEC as proficient in gap junctional intercellular communication and ER-negative, they apply to Type II but not Type I HBEC. In other words, not all normal cells have the same phenotype in a given tissue. Thirdly, gene expression *in vitro* could be dramatically different from gene expression *in vivo*. This is exemplified by the wild type ER expression in Type I HBEC-derived tumorigenic cells as demonstrated in this study.

Acknowledgements

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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

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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.* © 1998 Wiley-Liss, Inc.

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

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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).

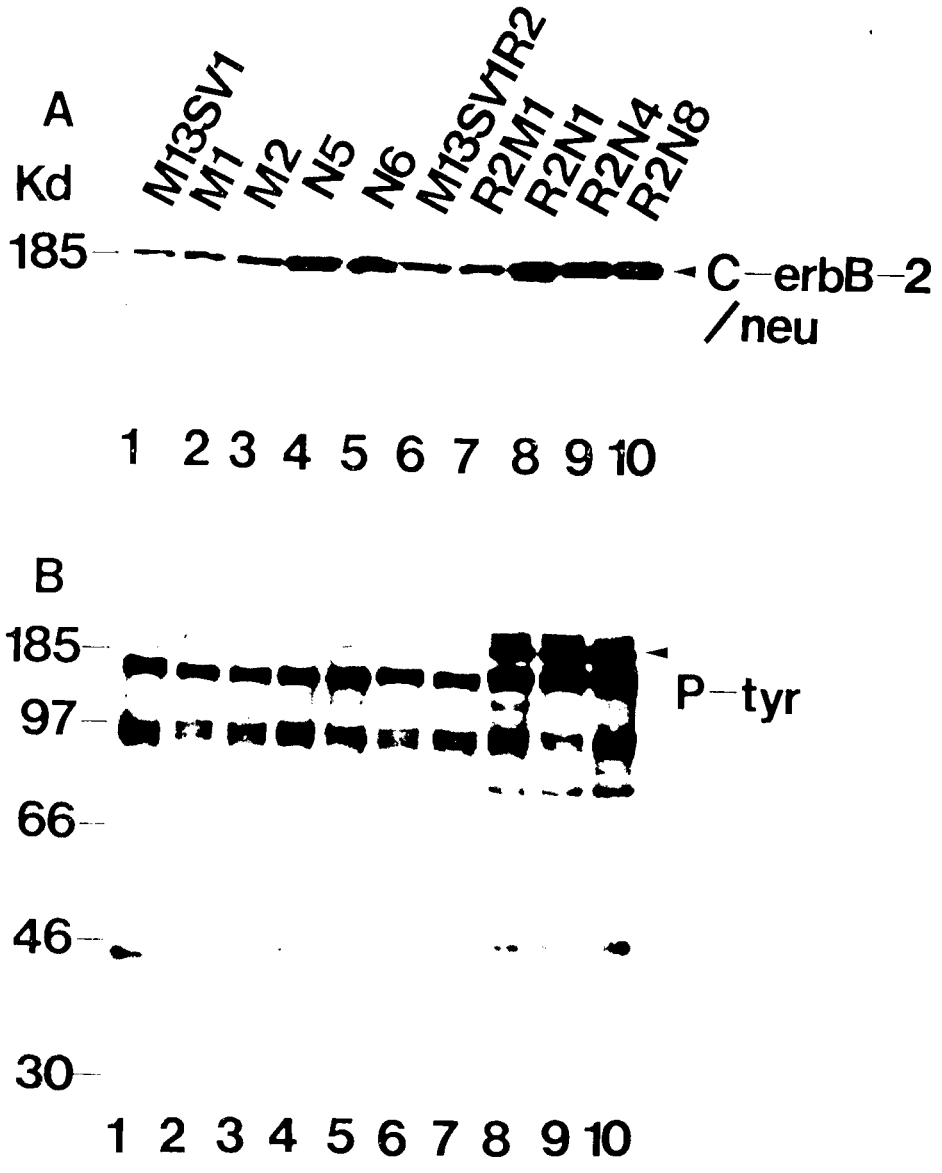


Figure 1. Expression and tyrosine phosphorylation of $p185^{c\text{-}erbB2/neu}$ studied by western blot analysis. (A) Expression of c-erbB2/neu detected with anti-neu antibody Ab-1 (Oncogene Science Inc.). (B) The blot in panel A reprobed with the anti-phosphotyrosine antibody Ab-2 (Oncogene Science 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].

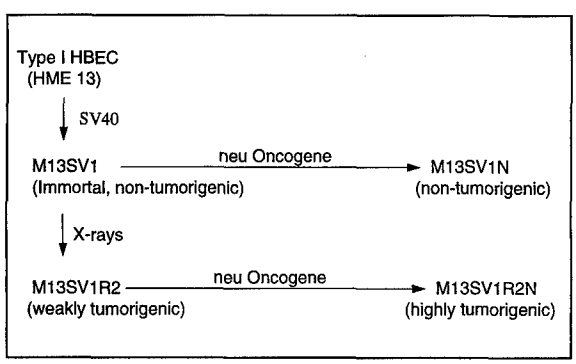


Figure 2. Derivation and tumorigenicity of various HBEC lines from HME 13, a normal human mammary epithelial culture from reduction mammoplasty.

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,

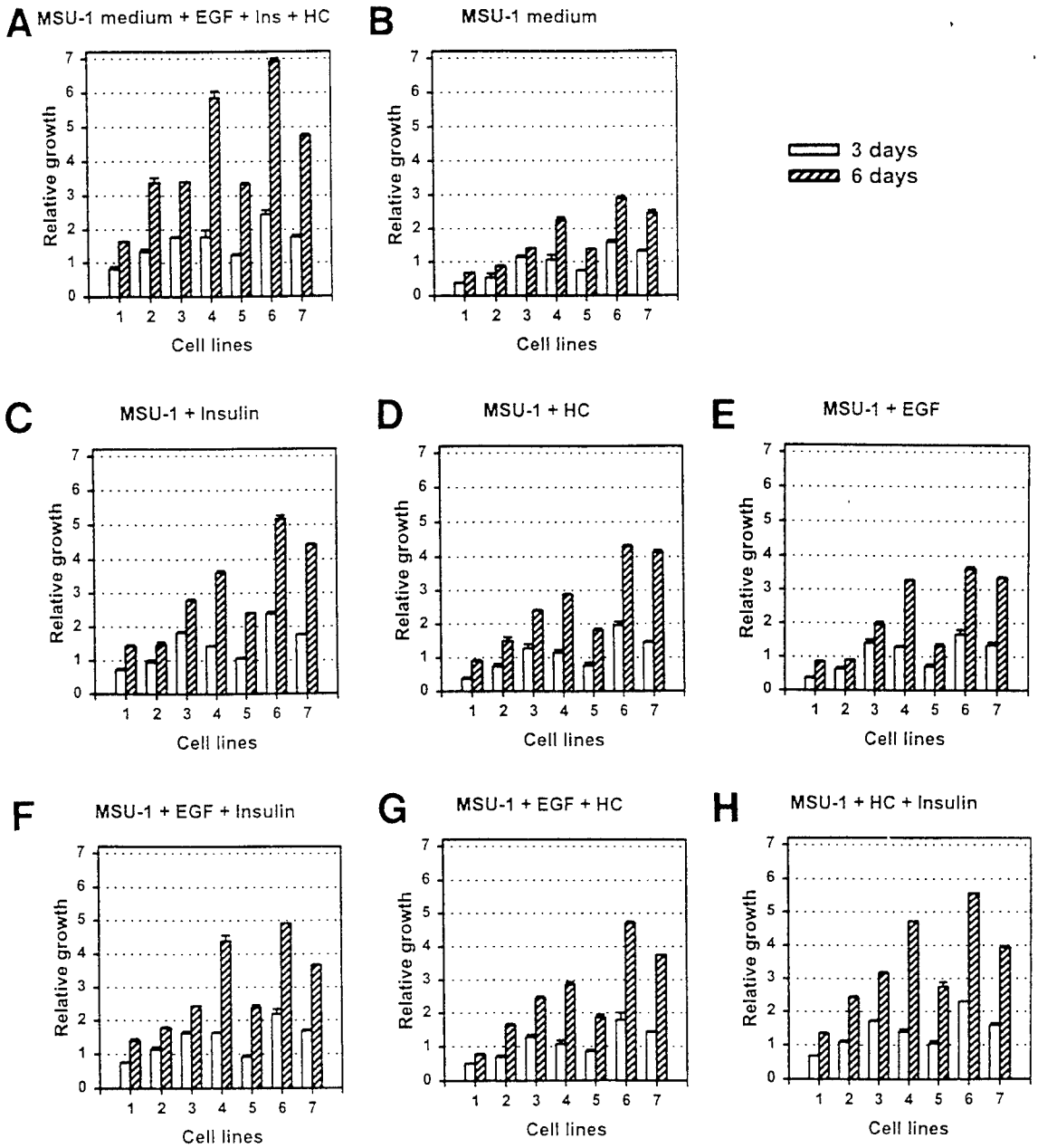


Figure 3. Growth factor and hormone requirements of immortal, weakly tumorigenic, and highly tumorigenic HBECs. Relative growth is the average total nucleic acid content of cells grown in triplicate dishes (6 cm) as measured by absorbance (OD) at 260 nm wavelength. 1, M13SV1; 2, M13SV1-N5; 3, M13SV1-N6; 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

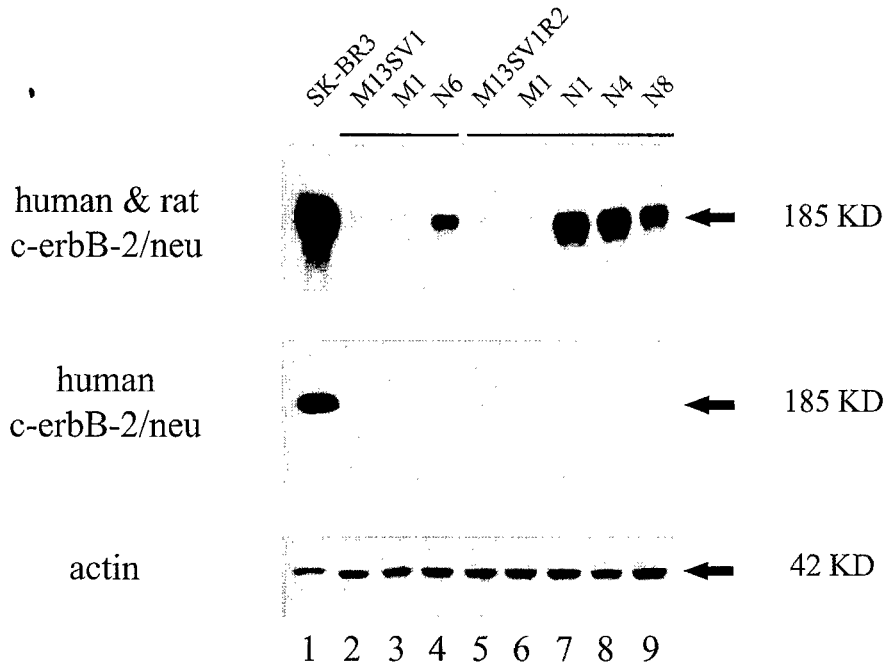
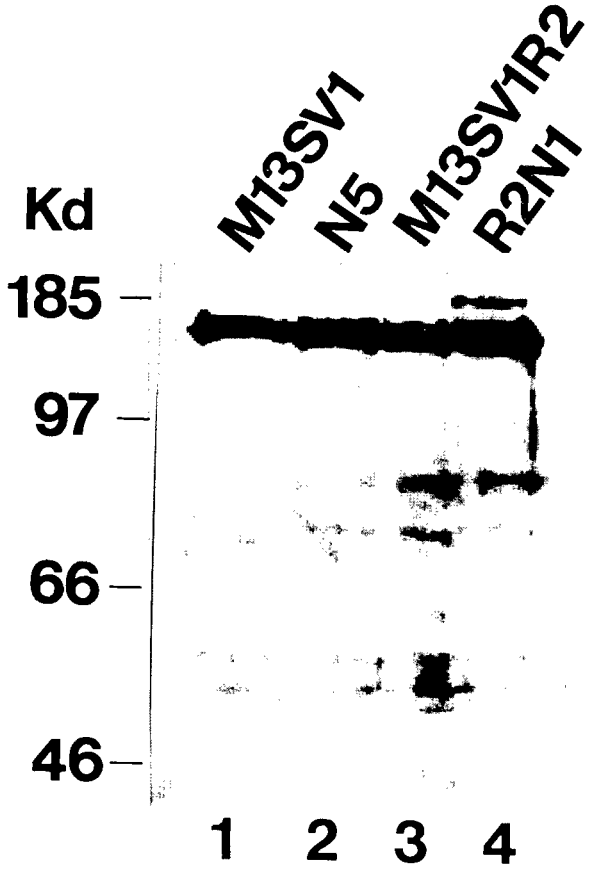


Figure 4. Western blot analysis of expression of p185$^{c\text{-}erbB2/neu}$ in various HBEC lines and in the breast cancer cell line SK-BR3. The proteins were detected by using an anti-p185$^{c\text{-}erbB2/neu}$ antibody recognizing both human and rat p185$^{c\text{-}erbB2/neu}$ (Ab-3) (upper panel), 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.

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

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Eukaryotic cell cycle progression is regulated by an orderly and sequential activation of several cyclin-dependent kinases, which phosphorylate key substrates during this process. $p34^{cdc2}$, the catalytic subunit of cdc2 kinase, is expressed at the late G_1/S boundary and is required for the $G_2 \rightarrow M$ phase transition. Transactivation of the human *cdc2* promoter by the DNA tumor virus-encoded oncogenic protein SV40 large T antigen is mediated by induction of a novel 110 kDa CCAAT box binding factor (CBF/cdc2). To investigate whether induction of CBF/cdc2 is an intrinsic property of the viral oncoprotein or is a common event during transformation of normal cells, expression of CBF/cdc2 was analyzed in many human tumor cell lines and in rodent cells spontaneously transformed or stably expressing various oncogenes. Our results showed that CBF/cdc2 was overexpressed in all transformed cells examined, including human 293, MCF-7, HeLa and HepG2 cells. Moreover, expression of CBF/cdc2 was elevated in spontaneously transformed rat liver epithelial cells (C4T), but not detectable in the non-tumorigenic parental (RLE) cells. The elevated levels of CBF/cdc2 expression in C4T cells correlated well with increased *cdc2* mRNA and $p34^{cdc2}$ levels. CBF/cdc2 was also overexpressed in a rat liver epithelial cell line (WB) stably transfected with various oncogenes, v-*myc*, v-Ha-*ras* and mutated rat *neu* and v-*src*. Using an electrophoretic mobility shift assay, specific binding of CBF/cdc2 to the CCAAT box motifs of the human *cdc2*, *cyc*A and *cdc*25C promoters was detected, suggesting that transcription of these cell cycle regulatory genes are coordinately activated by CBF/cdc2.

Introduction

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

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

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

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

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

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

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

Materials and methods

Chemicals and reagents

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

Cell culture

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

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

Nuclear extracts

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

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously (39). Briefly, the nuclear extracts (5 μg/assay) were mixed with a labeled probe (5×10^5 c.p.m./assay) in 20 μl reaction buffer [25 mM HEPES–KOH, pH 7.9, 5 mM KCl, 0.5 mM EDTA, 0.15 mg/ml BSA, 0.25 mM dithiothreitol, 10% glycerol, 0.1 mg/ml poly(dI·dC)]. The probe was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction mixture was incubated at 37°C for 30 min and stopped by adding 2 μl stop solution containing 50 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and 5% glycerol. The DNA–protein complexes were fractionated by electrophoresis on 4% non-denatured polyacrylamide gels. The gels were dried and subjected to autoradiography using X-ray film (Reflection; Du Pont-NEN). The probes used for EMSA were synthetic oligonucleotides containing the CCAAT box motifs in the promoters of the human *cdc2* gene (5′-CATGGGCTCTGATTGGCTGCTTTG, GenBank/EMBL accession no. L06298) (38), human *cycA* gene (5′-CGAGCGCTTTCATTGGTCCATTTC, GenBank/EMBL accession no. X68303) (47) and the *cdc*25C gene (5′-GCGCGCGGAGATT-GGCTGACGCAG, GenBank/EMBL accession no. Z29077) (48).

Southwestern blot analyses

Southwestern blot analyses was carried out as described (39,49). Briefly, 10 μg nuclear proteins from each sample were fractionated by SDS–PAGE (7.5%) and electrotransferred to a PVDF membrane. After denaturation and renaturation steps, the proteins immobilized on the membrane were probed with the 5′-labeled *cdc2*/CCAAT probe (10^6 c.p.m./ml). The membranes were washed and subjected to autoradiography at –70°C.

Northern blot analyses

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

Western blot analyses

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

Results

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

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

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

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

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

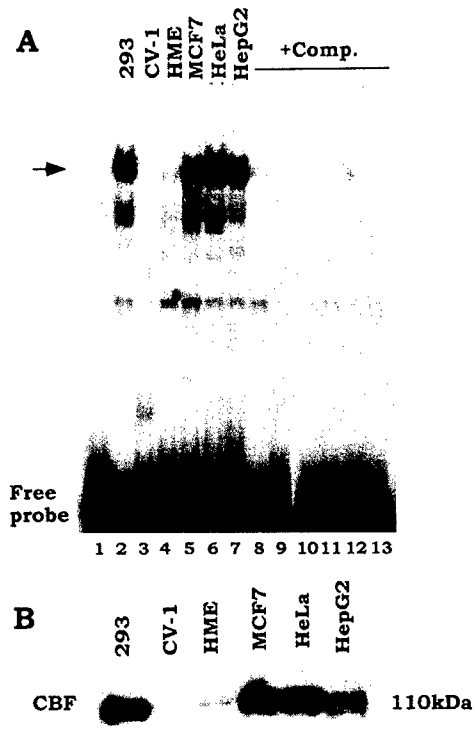

Fig. 2. Expression of the 110 kDa CBF/cdc2 in different human cancer cells. (**A**) For EMSA, nuclear extracts of human 293, CV-1, normal HME, MCF7, HeLa and HepG2 cells were used as indicated in lanes 2–7. The 110 kDa CBF/cdc2-specific DNA–protein complexes are shown by an arrow. In lanes 8–13, a 100×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

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

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

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

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

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

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

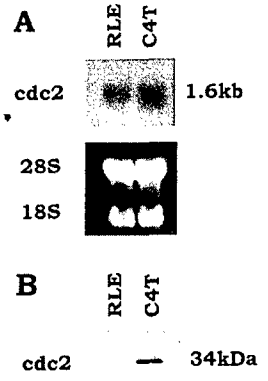

Fig. 3. *cdc*2 mRNA and p34^{cdc2} protein levels in RLE and C4T cells. (**A**) Northern blot analyses were carried out using RNA isolated from RLE and C4T cells as described under Materials and methods. Equal amounts of RNA were loaded for electrophoresis, as shown by the intensities of 28S and 18S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

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

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

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

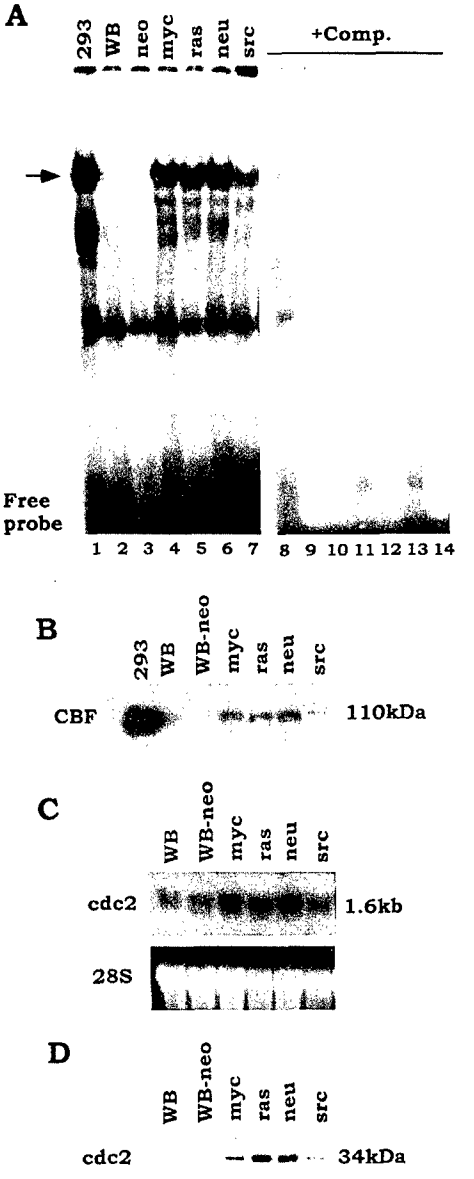

Fig. 4. Analyses of expression of CBF/cdc2, *cdc*2 mRNA and p34^{cdc2} in WB cells stably transfected with various oncogenes. (**A**) For EMSA, nuclear extracts from WB, WB-*neo* and various oncogene-transfected WB cell lines were used as indicated. The arrow indicates the position of the CBF/cdc2–CCAAT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

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

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

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

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

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

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

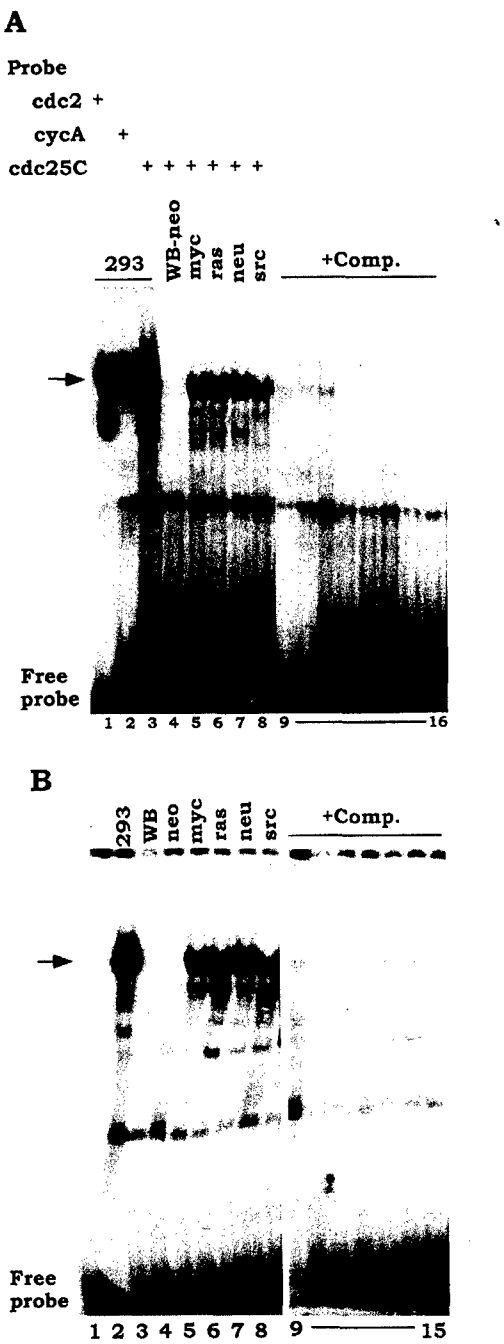

Fig. 5. Specificity of DNA binding activity of CBF/cdc2 to CCAAT box motifs of *cdc*25C and *cyc*A promoters. For EMSA, the labeled *cdc*25C (**A**) and *cycA* (**B**) CCAAT motifs as probes and nuclear extracts from WB cells stably transfected with the various oncogenes (or WB-*neo* as the negative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

Discussion

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

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

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

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

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

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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

MECHANISMS OF CHEMOPREVENTION

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

Reducing Target Cells for Neoplastic Transformation

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

Table 1. Blueprint for Cancer Prevention*

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

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

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

Prevention of Carcinogen Formation

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

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

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

Prevention of Proliferation of Precancerous Cells

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

A. Hormone Receptor-mediated Gene Expression

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

B. Expression of Genes that Regulate Cell Cycle Progression

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

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

C. Induction of Apoptosis

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

D. DNA Methylation

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

E. Prevention of the Blockage of Intercellular Communication

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

POTENTIAL CHEMOPREVENTIVE AGENTS FOR BREAST CANCER

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

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

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

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

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

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

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

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

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

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

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

Genistein

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

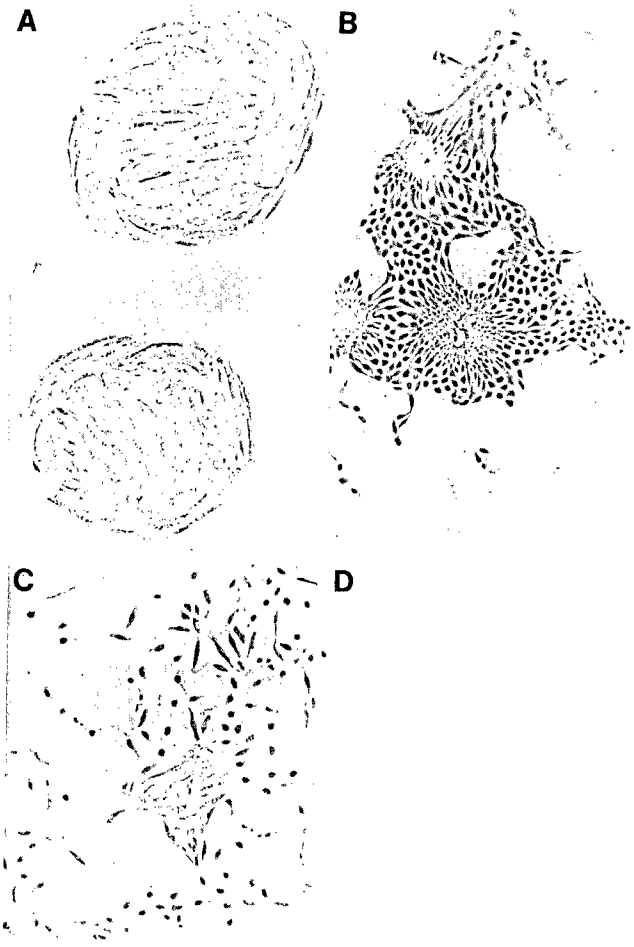

Fig. 1. Induction of differentiation of Type I to Type II human breast epithelial cells (HBEC) by genistein and vitamin D_3. The cells were treated with A. Ethanol (0.3 %, v/v), solvent control; B. Cholera toxin (1 ng/mL), positive control; C. Genistein (10^{-7} M) and D. vitamin D_3 (10^{-9} M). Photos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

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

1α,25-$(OH)_2$ Vitamin D_3

We have studied the effect of 1α,25-$(OH)_2$ vitamin D_3 on cell growth and differentiation in both Type I and Type II HBEC. The results show that 1α,25-$(OH)_2$ vitamin D_3, at concentration of 10^{-9} M, is able to induce the differentiation of Type I cells into Type II cells (Fig. 1) in 31 % of Type I colonies compared to 1.2 % for the solvent control. At higher concentration (10^{-7} M), 1α,25-$(OH)_2$ vitamin D_3, inhibited the growth of Type II HBEC but not Type I HBEC (Fig. 3). Furthermore, at this concentration, the Vitamin D_3 was found to increase cytokeratin 19 positive cells (a luminal epithelial cell type) in the Type I cell popula-

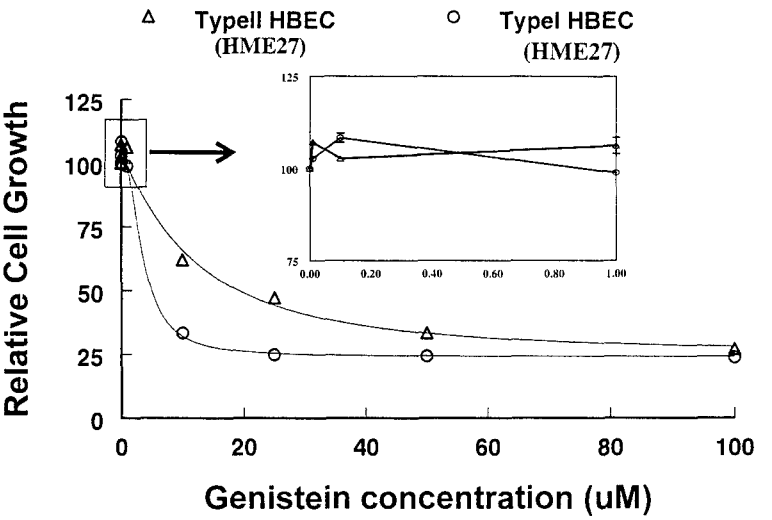

Fig. 2. The effects of various concentrations of genistein on growth of Type I and Type II HBEC. Cell growth was determined by quantitative measure of DNA contents in cell cultures 7 days after treatment.

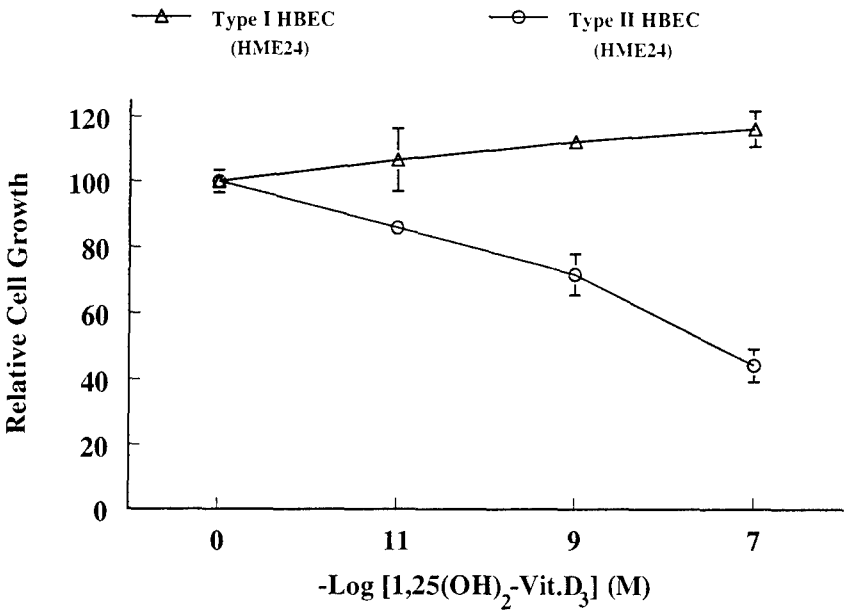

Fig. 3. The effects of different concentrations of vitamin D3 on growth of Type I and Type II HBEC. Cell growth was measured by DNA quantitation in cell cultures 6 days after treatment.

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

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

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High Susceptibility of a Human Breast Epithelial Cell Type with Stem Cell Characteristics to Telomerase Activation and Immortalization[1]

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ABSTRACT

We have recently characterized two types of normal human breast epithelial cells (HBECs) from reduction mammoplasty. Type I cells express estrogen receptor, luminal epithelial cell markers, and stem cell characteristics (*i.e.*, the ability to differentiate into other cell types and to form budding/ductal structures on Matrigel), whereas Type II cells show basal epithelial cell phenotypes. In this study, we have examined whether Type I HBECs are more susceptible to telomerase activation and immortalization after transfection with SV40 large T-antigen. The results show that both types of cells acquire extended life span [(EL); *i.e.*, bypassing senescence] at a comparable frequency. However, they differ significantly in the ability to become immortal in continuous culture, *i.e.*, 11 of 11 Type I EL clones became immortal compared with 1 of 10 Type II EL clones. Both parental Type I and Type II cells as well as their transformed EL clones at early passages [~30 cumulative population doubling level (cpdl)] showed a low level of telomerase activity as measured by the telomeric repeat amplification protocol assay. For all 11 of the Type I EL clones and the single Type II EL clone that became immortal, telomerase activities were invariably activated at middle passages (~60 cpdl) or late passages (~100 cpdl). For the four Type II EL clones randomly selected from the nine Type II clones that did not become immortal, the telomerase activities were found to be further diminished at mid-passage, before the end of the life span. Thus, normal HBECs do have a low level of telomerase activity, and Type I HBECs with stem cell characteristics are more susceptible to telomerase activation and immortalization, a basis on which they may be major target cells for breast carcinogenesis.

INTRODUCTION

Cancer cells are generally recognized as being in a relatively undifferentiated state and could arise from stem cells with blocked or partially blocked differentiation (1, 2). Epidemiological studies indicate that the lifetime risk of developing breast cancer in child-bearing women seems to be linearly related to the age at which a women has her first full-term pregnancy (3) and that breast cancer risk is higher in those who are nulliparous or late parous (4, 5). This has been hypothesized to be due to stem cell multiplication during each ovarian cycle before but not after the first pregnancy (6) or to the induction of mammary gland differentiation by pregnancy with the elimination of terminal end buds, resulting in refractoriness of the gland to carcinogenesis (7). Although the role of stem cells in cancer is implicated, whether stem cells are more susceptible to neoplastic transformation has not been examined experimentally.

Normal somatic cells have a finite life span. Telomere shortening as a consequence of the end-replication problem has been proposed as a mitotic clock for cellular senescence (8). Telomerase, a ribonucleoprotein complex with reverse transcriptase activity that uses a RNA template to add TTAGGG hexonucleotide repeat onto the end of chromosomes, is capable of maintaining the telomere length and replicative activity of cells (9). Whereas normal human cells or adult tissues, in general, lack telomerase activity, the great majority of human tumors from various tissues and immortal cell lines show telomerase activity (10, 11). A current predominant hypothesis proposes that the reexpression of telomerase occurs in most tumors and is probably a critical event responsible for continuous tumor cell growth (12), recognizing the existence of alternative mechanism for immortalization (13–15). Additional studies have revealed that telomerase activity is expressed in human germ-lines (testes and ovaries) (12), human embryonic stem cells (16), human lymphocytes and hematopoietic progenitor cells (17–20), candidate stem cells from the fetal liver (21), human epidermal cells expressing a basal cell marker (22), and from the basal layer (23) and in human endothelial (24) and uroepithelial cells (25). The latter two cell types showed proliferation-dependent expression of telomerase (24, 25). 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 (25). However, this study did not quantitatively measure telomerase activity during the course of neoplastic transformation.

We have developed a cell culture method to grow two types of normal HBECs[4] from reduction mammoplasty (26). One type of cell (Type II HBECs), similar to those commercially available or used by most other laboratories, shows basal epithelial cell markers. The other cell type (Type I HBECs) expresses luminal epithelial phenotypes and estrogen receptors (26, 27). Importantly, Type I HBECs also show stem cell characteristics (*i.e.*, the ability to differentiate into other cell types by cyclic AMP-inducing agents and to form budding/ductal structures in Matrigel). Because telomerase activity in human stem cells has not been well studied, our first objective is to determine whether telomerase is expressed in our two types of cells. Furthermore, it offers an opportunity to examine whether the HBECs with stem cell characteristics (Type I HBECs) are more susceptible to telomerase activation and immortalization.

MATERIALS AND METHODS

Cell Culture and Mammary Organoids Formation. The cell culture and the method to develop the two types of normal HBEC culture from reduction mammoplasty were as described previously (26). The two types of HBECs are morphologically distinguishable and substantially different in many phenotypes (26). In contrast to Type II cells, the major features of type I HBECs are the deficiency in gap junctional intercellular communication, the growth promotion by fetal bovine serum, and the expression of estrogen receptors and luminal epithelial cell markers (*i.e.*, epithelial membrane antigen and cytokeratin 18; Refs. 26–28).

Growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) was used to study mammary organoid structure formation with Type I and Type II HBECs. Approximately, 1–1.5 $\times$ 10^6 cells were plated on 35-mm culture dishes or two-chamber Lab-Tek culture slides with a layer of Matrigel. Matrigel remains solid in the 37°C humidifed incubator where cells were

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[4] The abbreviations used are: HBEC, human breast epithelial cell; EL, extended lifespan; cpdl, cumulative population doubling level; TRAP, telomeric repeat amplification protocol; AIG, anchorage-independent growth; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

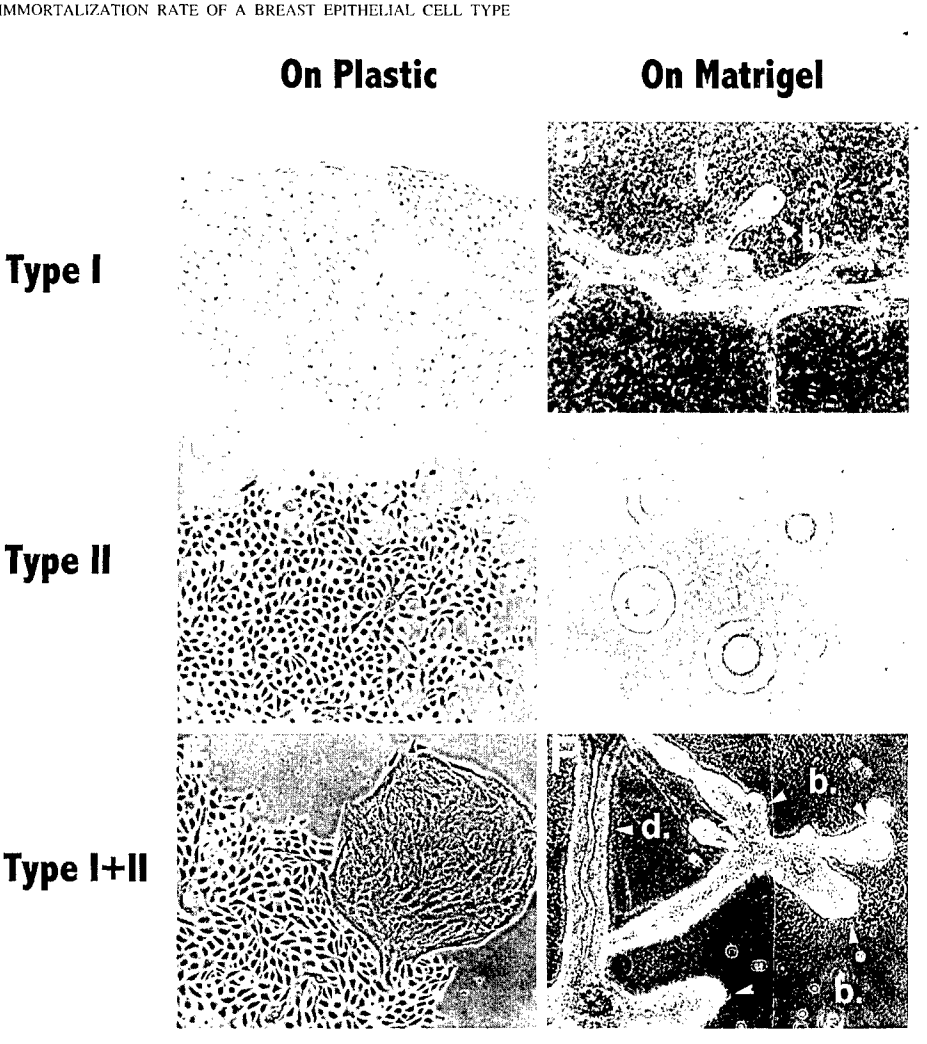

Fig. 1. HBEC colonies on plastic and organoids on Matrigel formed by two types of normal HBECs. Type I and Type II colonies developed on plastic (*A* and *C*, respectively) are morphologically distinguishable. On Matrigel, Type II cells typically formed spherical organoid (*s.o.*) (*D*), whereas Type I cells formed a limited number of bud-like (*B*) structures and acini (not shown). The combination of Type I and Type II cells (*E*; two types of cells on plastic) in 1:2 or 1:3 ratios can generate many budding (*B*)/ductal (*D*) structures in Matrigel (*F*) in 2–3 weeks.

allowed to aggregate for 1 day. After cells attached to Matrigel, the medium was changed or a second layer of Matrigel was placed on top of the first layer. Culture medium was changed once every 2 days.

Development of SV40 Large T-Antigen-transformed Type I and Type II HBECs. The normal HBECs were transfected with a plasmid carrying an origin-defective SV40 genome expressing a wild-type large T-antigen [PRNS-1; a gift from Johng S. Rhim, Uniformed Services University of the Health Sciences, Bethesda, (M15SV1–11 and M15SV21–30 were derived from Type I and Type II HME 15, respectively)] by Lipofectin (Life Technologies, Inc., Gaithersburg, MD). The actively proliferating colonies were selected by their resistance to G418 (0.4 mg/ml for M15SV1–11 from Type I HBECs and 0.15 mg/ml for M15SV 21–30 from Type II HBECs). The proliferation potential of transformed clones was determined by their total cpdls using the formula cpdl = ln(*Nf*/*Ni*)/ln 2, where *Ni* and *Nf* are initial and final cell numbers, respectively, and ln is the natural log. The initial cell number was 2×10^5 for each propagation.

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

PCR-based Telomerase Assay. Cells grown to about 50–70% confluence were harvested by trypsinization. After cell counts, the cells were centrifuged to remove trypsin solution. The cell pellet for each culture was washed with 10 ml of PBS and then centrifuged to remove PBS. Cells were then suspended at 1×10^6 cells/ml in PBS and aliquoted to Eppendorf tubes. After cells were centrifuged and PBS was carefully removed, the cell pellets were stored at −85°C. For the telomerase assay, the cell pellet was thawed and resuspended in 200 μl of 1× CHAPS lysis buffer/10^6 cells and left on ice for 30 min. The samples were spun in a microcentrifuge at 12,000 × *g* for 20 min at 4°C. The cell lysate for each sample was aliquoted to several new tubes and stored at −85°C. The original lysate represents a concentration of 5,000 cells/μl. Further dilution of the cell lysate was adjusted based on the level of telomerase activity for the individual cell line. Telomerase activity was examined by the TRAP assay (10) using the TRAPeze Telomerase Detection Kit (Oncor, Gaithersburg, MD). This protocol includes primers of a 36-bp internal control (I.C.) for quantitating the amplification efficiency, thus providing a positive control for accurate quantitation of telomerase activity within a linear range close to 2.5 logs. Each analysis included a negative control (CHAPS lysis buffer instead of cell lysate), a heat-inactivated control (the sample was incubated at 85°C for 10 min before the assay), and a positive cell line control (breast carcinoma cell line MCF-7). For RNase treatment, 10 μl of extract were incubated with 1 μg of RNase for 20 min at 37°C. The products of the TRAP assay were resolved by electrophoresis in a nondenaturing 12% PAGE in a buffer containing 54 mM Tris-HCl (pH 8.0), 54 mM boric acid, and 1.2 mM EDTA. The gel was stained with Syber Green (Molecular Probes, Inc., Eugene, OR) and visualized by a 302-nm or 254-nm UV transilluminator. Images were captured and analyzed by AlphaImager (Alpha Innotech Corp., San Leandro, CA) or NucleoVision760 (NucleoTech Corp., San Mateo, CA). Quantitation of the products generated from TRAP assay was calculated using the following formula: *TPG* (total product generated units) = $((x - x_o)/c)/((r - r_o)/c_R) \times 100$, where x and x_o represent signals corresponding to the TRAP product ladder bands of non-heat-treated and heat-treated sample lanes, respectively, r and r_o represent signals from 1× CHAPS lysis buffer control (*i.e.*, primer-dimer/PCR contamination control) and TSR8 (DNA quantitation control), respectively. c and c_R are the signal from the internal standard (TSK1) in non-heat-treated samples and the signal from TSR8 quantitation control, respectively.

Table *Telomerase activity in SV40 large T-antigen-transformed Type I and Type II HBECs at different cpdls*

Cell line	Parental cell type	Telomerase activity at different passage levels[a]			Immortalized (cpdl)[b]
		Low	Middle	High	
M15SV1	Type I	ND	++++	++++	Yes (>110)
M15SV2	Type I	+	+	++++	Yes (>106)
M15SV3	Type I	+	+	++++	Yes (>107)
M15SV4	Type I	+	++++	++++	Yes (>103)
M15SV5	Type I	+	+++	+++	Yes (>105)
M15SV6	Type I	+	++++	++++	Yes (>107)
M15SV7	Type I	+	++++	++++	Yes (>102)
M15SV8	Type I	+	++++	++++	Yes (>109)
M15SV9	Type I	+	++++	++++	Yes (>113)
M15SV10	Type I	+	++++	++++	Yes (>103)
M15SV11	Type I	+	++++	++++	Yes (>104)
M15SV21	Type II	+	±	(Senescent)	No (32)
M15SV24	Type II	+	±	(Senescent)	No (76)
M15SV26	Type II	+	±	(Senescent)	No (39)
M15SV27	Type II	+	ND	(Senescent)	No (31)
M15SV29	Type II	+	±	(Senescent)	No (62)
M15SV30	Type II	ND	++++	ND	Yes (>107)

[a] ±, +, and ++++ denote very weak, low, and high telomerase activity, respectively; ND, not done.
[b] The cpdl for other Type II clones are as follows: M15SV22, 32; M15SV23, 37; M15SV25, 39; and M15SV28, 43.

RESULTS

Stem Cell Characteristics of Type I HBECs as Indicated by Organoid Formation and Growth. Previously, we have shown that Type I HBECs have the ability to differentiate into Type II HBECs by cyclic AMP-inducing agents (cholera toxin and forskolin; Refs. 26 and 29). Additional evidence that Type I cells have stem cell characteristics came from the study of organoid formation and growth on Matrigel. When Type I and Type II cells were plated separately on top of Matrigel or in between two layers of Matrigel, Type I cells characteristically formed acinar structures that are formed by luminal epithelial cells as shown previously (30) and onganoid showing some limited budding structure formation (Fig. 1*B*), whereas Type II cells with basal epithelial phenotypes formed a spherical organoid (Fig. 1*D*). When the two types of cells were plated together on Matrigel, they formed a ductal and terminal end bud-like structure (Fig. 1*F*). Because mammary stem cells are known to be present in the end bud for ductal morphogenesis and elongation (31, 32), the ability of Type I HBECs to form these structures strongly indicates that the Type I HBEC population contains mammary epithelial stem cells that are capable of giving rise to luminal and basal epithelial cells.

High Susceptibility of Type I HBECs to Immortalization. Both Type I and Type II HBECs were transfected with an origin-defective SV40 genome expressing the wild-type large T-antigen (PRNS-1) and selected by G418. Eleven independent clones were isolated from $\sim 3 \times 10^6$ Type I HBECs, whereas 10 clones were derived from $\sim 4 \times 10^6$ Type II HBECs. All these Type I and Type II clones were able to bypass senescence and acquire an EL (*i.e.*, more than 30 cpdl; normal HBECs never grew more than 20 cpdl). Therefore, the ability of SV40 large T-antigen-transformed Type I and Type II HBEC clones to acquire EL appears to be comparable. However, the frequency at which EL clones became immortal differs significantly between Type I and Type II HBEC-derived clones. All of the 11

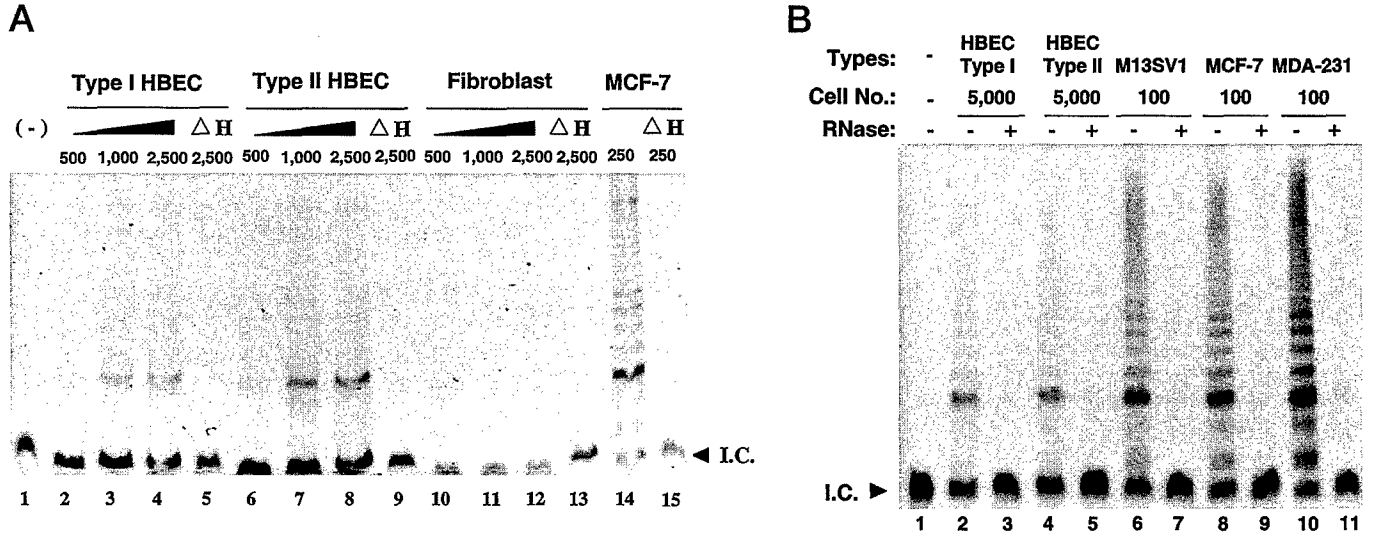

Fig. 2. Telomerase activity in Type I and Type II HBECs at passage two. *A*, telomerase activity was detected in cell lysates derived from different cell numbers as shown by a PCR-based TRAP assay, as described in "Materials and Methods." *Lane 1* and *Lanes 5, 9, 13*, and *15* represent negative controls without cell lysate (−) and heat-inactivated controls (Δ *H*), respectively. The Type I HBECs, Type II HBECs, and fibroblasts used in this assay were all derived from the 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 were detected in both normal Type I (*Lanes 2–4*) and Type II (*Lanes 6–8*) HBECs; the activity in fibroblasts was undetectable (*Lanes 10–12*). *B*, RNA dependency of the weak telomerase activity detected from primary Type I and Type II HBECs (lysate from 5000 cells), and the high activity from SV40-immortalized Type I HBECs (M13SV1) and two breast cancer cell lines (MCF-7 and MDA-MB-231). The extract equivalent to 100 cells of lysate was used in each assay. *I.C.*, the internal control for quantitating the amplification efficiency in the TRAP assay.

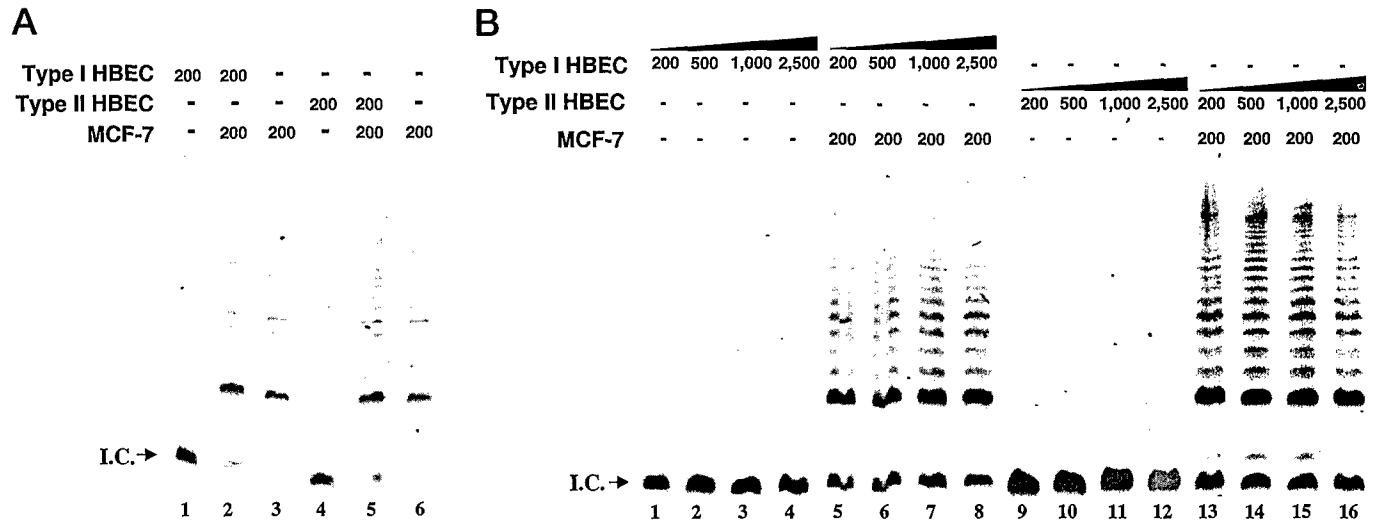

Fig. 3. Absence of telomerase inhibitor in Type I and Type II HBECs. Telomerase activity from a mixture of telomerase-positive MCF-7 cell lysate and HBEC lysate was examined to clarify that low levels of telomerase activity in both types of HBECs were not due to the presence of telomerase inhibitors in these cells. The numbers represent an equivalent amount of cells in the lysate. *A* and *B* are two independent experiments. No significant difference in telomerase activity was found between MCF-7 alone and the mixed lysates. *I.C.*, the internal control in the TRAP assay.

(100%) EL clones derived from Type I cells have become immortal (*i.e.*, actively proliferating after more than 100 cpdl). In comparison, only 1 of 10 (10%) EL clones derived from Type II cells has become immortal (Table 1). Excluding the immortal clone, the average life span of EL clones from Type II HBECs was 43 ± 5 cpdl. It is clear that Type I HBECs with stem cell characteristics were more susceptible to immortalization after SV40 large T-antigen transfection.

Telomerase Activity in Primary HBECs. To study the potential mechanisms underlying the high susceptibility of Type I HBECs to immortalization, we first studied the telomerase activities in primary Type I and Type II HBECs without SV40 transfection. As shown in Fig. 2*A*, telomerase activity was present in both Type I (*Lanes 2–4*) and Type II (*Lanes 6–8*) HBECs. However, the level of activity was weak compared with that of the breast carcinoma cell line MCF-7 (*Lane 14*). Using one-tenth of the cell number in the assay (250 *versus* 2500 cells), the telomerase activity in MCF-7 was significantly higher than that in Type I and Type II HBECs. In contrast, telomerase activity was undetectable in human breast stromal fibroblasts at early passages (*Lanes 10–12*). The telomerase activities in normal and in immortal or tumorigenic HBECs are dependent on enzyme activity from the catalytic subunit and RNA template, as shown by the elimination of the activity by heat inactivation or RNase treatment (Fig. 2, *A* and *B*). To exclude the possibility that the low level of telomerase activity from primary HBECs may be due to the existence of potential telomerase inhibitors, assays with mixed cell lysates from both HBECs and MCF-7 were carried out (Fig. 3, *A* and *B*). Because HBECs did not affect the telomerase activity of MCF-7 cells in the lysate mixtures, no telomerase inhibitor was detected in either Type I or Type II HBECs.

The Weak Telomerase Activity in Normal HBECs Is Insufficient for Immortalization. Because a low level of telomerase activity was found in primary HBECs, it is important to study whether further telomerase activation is required for these cells to become immortal. We have investigated the telomerase activity during the course of cell passage toward crisis or immortalization (Fig. 4, *A* and *B*). The same level of telomerase activity was found in both early-passage (before 50 cpdl) SV40-transformed Type I and Type II clones as in normal HBECs. There was a dramatic increase of the activity around 50–60 cpdl for clones that became immortal, and the activity remained high thereafter. In contrast, Type II EL clones without telomerase activation always stopped proliferating at crisis, as shown later (Fig. 6). The fact that the activated levels of telomerase activity

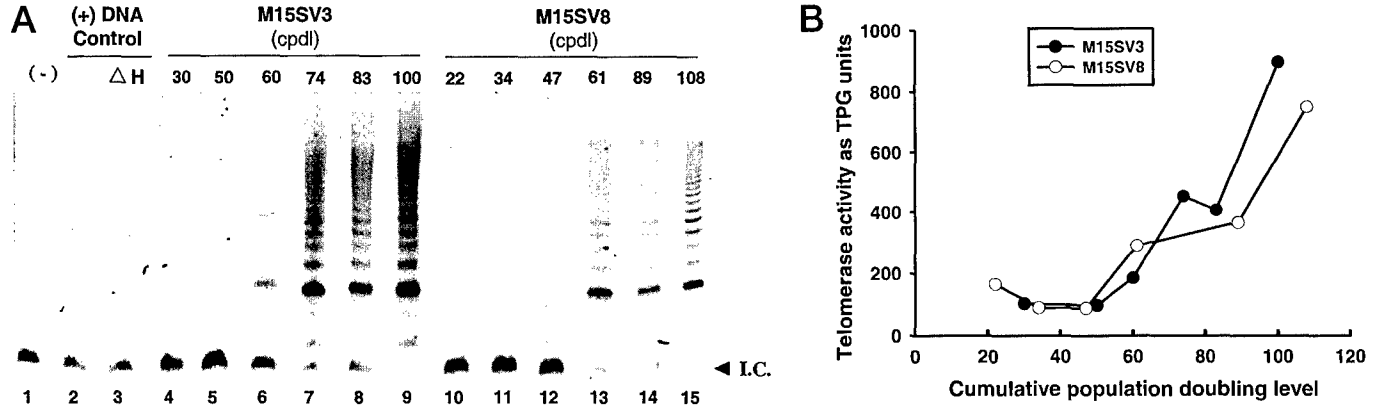

Fig. 4. Telomerase activation during the course of immortalization. A detailed analysis of telomerase activity at the expanded range of cpdl for two SV40-transformed Type I HBEC lines (M15SV3 and M15SV8) is shown. *A*, an elevation of telomerase activity was found at 50–60 cpdl for both cell lines. Cell lysate derived from 500 cells was used for the telomerase assay. *I.C.*, the internal control in the TRAP assay. *B*, quantitative measurement of telomerase activity in *A*. Telomerase activity was measured as total product generated (*TPG*), as described in "Materials and Methods."

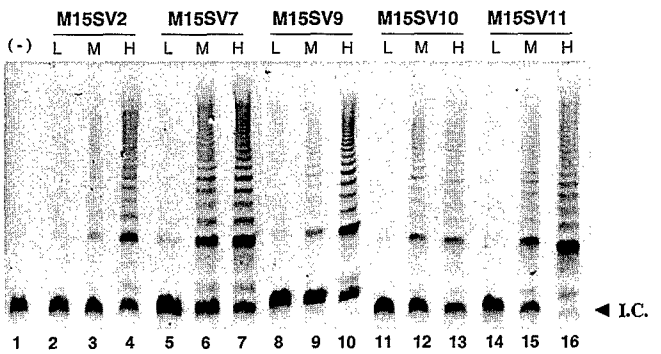

Fig. 5. Telomerase activity in SV40-transformed Type I HBEC-derived cell lines. Five SV40-transformed cell lines (M15SV2, M15SV7, M15SV9, M15SV10, and M15SV11), which became immortal, were examined for telomerase activity at low passage (*L*; ~22–30 cpdl), middle passage (*M*; ~50–60 cpdl), and high passage (*H*; >100 cpdl). In all of these cell lines, telomerase activities were elevated at middle or late passage. Cell lysate derived from 500 cells was used for the telomerase assay for each sample. *I.C.*, the internal control in the TRAP assay.

were found in all immortal cell lines and the fact that the majority of Type II EL clones did not become immortal indicate that the low level of telomerase activity detected in normal HBECs is insufficient for immortalization.

High Susceptibility of Type I HBECs to Telomerase Activation. Cell populations harvested at early passage (EL, ~30 cpdl), middle passage (around the potential crisis period, ~50–60 cpdl), and late passage (immortalization; >100 cpdl), respectively, were assayed. All 11 Type I HBEC-derived clones showed a significant increase in telomerase activity at middle or late passage (Table 1; only the results from seven clones are shown in Figs. 4 and 5). However, among Type II HBEC-derived clones, the only one that became immortal has shown activated telomerase activity (Fig. 6). Four of the nine Type II clones that did not become immortal were randomly selected for the telomerase activity assay. None of these clones showed telomerase activation at mid-passage before the advent of crisis. As summarized in Table 1, all Type I and Type II clones with activated telomerase activity eventually became immortal. These results suggest that Type I HBEC clones have a high potential for telomerase activation, which results in a high rate of immortalization.

DISCUSSION

The major findings of this study are as follows: (*a*) Type I HBECs were more susceptible to immortalization (11 of 11 clones) than Type II HBECs (1 of 10 clones) after SV40 large T-antigen transfection; (*b*) both normal Type I and Type II HBECs had a low level of telomerase activity that was insufficient to maintain continuous cell proliferation unless it was activated; and (*c*) the high potential of telomerase activation for Type I HBECs resulted in a more efficient immortalization compared to Type II HBECs. Because Type I HBECs have stem cell characteristics, these results suggest that the aforementioned mechanism is the reason why stem cells are more likely to be target cells for neoplastic transformation.

We have previously shown that Type I and Type II HBECs differ substantially in their response to an oncogenic (SV40) stimulus; *i.e.*, Type I cells were AIG^+, whereas SV40-transformed Type II cells totally lack the ability to grow in soft agar (AIG^-; Ref. 26). We were able to confirm that the SV40-transformed Type I HBEC clones obtained in this study are capable of AIG (eight of eight clones[5]). Therefore, this study provides additional and stronger evidence that Type I HBECs are more susceptible to tumorigenic initiation by acquiring two major and common tumor cell phenotypes, *i.e.*, AIG^+ and immortality.

Whereas it is reasonable to expect Type I cells with stem cell characteristics to have telomerase activity, the presence of telomerase activity in Type II HBECs seems difficult to reconcile with previous reports that normal breast tissues (10) and normal HBECs (33) do not have telomerase activity. It is possible that our Type II cells are newly derived from Type I cells. Therefore, they could be considered as progenitor cells for basal epithelial cells. Indeed, type II cells can be derived from Type I cells after treatment with cyclic AMP-inducing agent (26). The newly derived cells are similar to early-passage Type II cells from breast tissue in cell phenotype and proliferation potential. The low telomerase activity in normal HBECs and early-passage SV40-transformed HBECs is not due to the presence of telomerase inhibitors, as demonstrated in Fig. 3, or to the lower proportion of cycling cells because all cell cultures were harvested at near log phase of cell growth. However, this low level of telomerase activity in both Type I and Type II cells seems insufficient for unlimited growth for SV40-transfected cells, as shown by the requirement of telomerase activation in immortalized clones and the incapability of most Type II EL clones to become immortal.

The ability of Type I HBECs to form budding/ductal structures strongly suggests the stem cell characteristics of a proportion of these cells. The similarity of phenotypes between breast carcinoma cells and Type I cells (*i.e.*, deficiency in gap junctional intercellular communication, expression of estrogen receptors, and luminal epithelial cell markers) further indicates that breast cancers could be derived from Type I cells as a result of blocked differentiation, consistent with the oncogeny as blocked or partially blocked ontogeny theory of carcinogenesis (2).

The differential susceptibility of different HBEC types to immortalization by human papilloma virus (34) and by the catalytic component of telomerase, hTERT (33), has been reported previously. None of these cells are similar to the Type I HBECs.

The high susceptibility of SV40-transfected Type I cells to immortalization may provide a basis for the idea that stem cells are major targets for neoplastic transformation. In turn, the high potential of susceptibility to telomerase activation seems to be a mechanism that Type I cells become immortal at higher rate than Type II HBECs. However, the mechanism that telomerase is more easily activated in

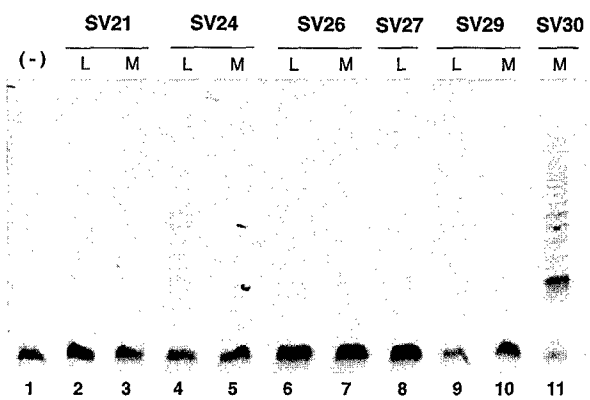

Fig. 6. Telomerase activity in SV40-transformed Type II HBECs. The majority of SV40-transfected Type II clones (9 of 10) did not become immortal beyond EL (Table 1). Five (M15SV21, M15SV24, M15SV26, M15SV27, and M15SV29) of these nine clones were analyzed for telomerase activity at low passage (*L*; ~25 cpdl) and mid-passage (*M*; ~40–50 cpdl). Telomerase activities in these clones diminished from low to middle passage when they were approaching crisis. Only the immortalized Type II HBECs (M15SV30) showed telomerase activation at middle passage (*Lane 11*). Cell lysate derived from 500 cells was used for each assay. *I.C.*, the internal control in the TRAP assay.

[5] Unpublished observations.

Type I cells remains unknown. Our results show that it is not due to a higher level of telomerase activity in Type I cells than Type II cells. In human tumor cells, the activity of telomerase has been shown to be cell cycle dependent, with the highest level of activity detected in S phase, and the lowest level found in cells arrested at G_2-M phase (35). Overexpression of cyclin D1 and/or cyclin E was a typical feature of breast cancers with high telomerase activity (36). It is likely that telomerase expression may be partially under the same control that regulates G_1 to S-phase transition. In this respect, our previous observation that the expression of cyclin D1 was higher in Type I HBECs than in Type II cells (27) may be relevant.

A previous report concludes that telomerase activity may not be a biomarker for malignant transformation because it is present in both normal and tumor cells (25). This conclusion may be misleading, because there is no quantitative comparison, and the comparison was made between tumor cells and unrelated normal cells. Using a well-characterized quantitative assay, we found that although both normal and immortal or tumorigenic cells have telomerase activity, the activities in immortal or tumorigenic cells are dramatically higher than that seen in normal cells. Indeed, the transition from low to high telomerase activity may be an indicator of when immortal cells are present in the population during the course of immortalization.

This study also has implications concerning the mechanism of carcinogenic initiation of breast epithelial cells. Because the function of SV40 large T-antigen is to inactivate p53 and pRb and to induce the CCAAT box binding factor that transactivates cell cycle-regulating genes such as cdc2 (37), alteration in cell cycle regulation seems to be the major event to acquire an EL for normal HBECs. The subsequent conversion of a cell with EL to an immortal cell clearly involves the activation of telomerase, as shown in this study. This is consistent with a recent report that both Rb/$P16^{INK4a}$ inactivation and telomerase activity are required to immortalize HBECs (33).

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Gap Junctions and the Regulation of Cellular Functions of Stem Cells during Development and Differentiation

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In multicellular organisms, the role of gap junction intercellular communication (GJIC) in the regulation of cell proliferation, cell differentiation, and apoptosis is becoming increasingly recognized as one of the major cellular functions from the start of the fertilized egg, through normal development of the embryo and fetus, to the sexual maturation of the adult and ultimately to the maintenance of health of the aging adult. Given that the function of this membrane-associated protein channel is to synchronize electrotonic or metabolic functions, differential regulation of function at the transcriptional, translational, and posttranslational levels of a family of highly evolutionarily conserved genes (connexins) needs to be considered. Both inherited mutations and environmental modulation of GJIC could, in principle, affect the function of gap junctions to control cell proliferation, cell differentiation, and apoptosis, thereby leading to a wide variety of pathologies. We review a few techniques used to characterize the ability of stem and progenitor cells to perform GJIC.

Nothing in biology makes sense except in the light of evolution.
T. Dobzhanski (1973, *Am. Biol.* **35**, 125–129)

This quotation by Dobzhanski (1) is being used to set the stage for the rationale to study a family of genes that code for the proteins called connexins. The cellular function of direct transfer of ions and small molecules between contiguous homologous or heterologous cells in a multicellular organism and the structural entity on the cell membrane, the gap junction, responsible for the means to connect the cytoplasms of these cells were only relatively recently discovered (2). With the transition of the single cell to the first multicellular organism came several important new phenotypic changes and the attendant genes responsible for these new functions.

Single-cell organisms survived by having all the intracellular signaling mechanisms to adapt to environmental changes and by unlimited proliferation (only physical factors such as temperature, pH, and external nutrients restricted cell proliferation). If the environment changed dramatically so as to cause death of most individual cells, the existence of a few spontaneous mutations in various genes might allow one of the population to survive to carry on the species. When the first multicellular organism appeared, the phenotypes of (a) the control of cell proliferation by "contact inhibition"; (b) the process of differentiation; (c) programmed cell death or apoptosis; and (d) the regulation of adaptive responses of terminal differentiated cells appeared (3). The process of "development" in a multicellular organism, therefore, must entail (a) the start from a single "totipotent" stem cell or fertilized egg (a cell that contains all the genetic material to form all the cell types and functions of the multicellular organism; a cell that has the ability to divide both symmetrically to expand its population and asymmetrically to have one daughter cell to terminally differentiate and the other to maintain "stemness"); and (b) the ability to selectively restrict the differentiation of this totipotent stem cell into a series of "pluripotent" stem cells (which can give rise to a restricted number of cell types for one

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or more tissues). These pluripotent stem cells can proliferate and differentiate into cells with even more restricted potentials to differentiate or proliferate (the progenitor cells of a given cell type or the terminally differentiated cells).

Of course, the transition from the single-cell organism to the first multicellular organism was accompanied by the appearance of many new genes. One new family, the connexin genes, which code for proteins needed for the formation of a hexameric hemichannel (the "connexon") to bond with the corresponding hemichannel in the contiguous cell membrane, appeared when the multicellular cellular organism appeared (4). Was it by chance, was it by coincidence, or was it causal that the connexin gene appeared when the control of cell proliferation, cell differentiation, programmed cell death, and adaptive responses of differentiated cells, processes needed for development, first appeared? Single cells could communicate with each other in a population by primitive molecules that could have been progenitors to hormones and growth factors (5). Single cells also had developed sophisticated intracellular signaling mechanisms that were the progenitors to many found in the multicellular organism. The delicate orchestration of cell growth, cell differentiation, and programmed cell death between the totipotent stem cells, pluripotent stem cells, their progenitors, and terminally differentiated daughters appears to have been mediated by the modulation of the genes that code for connexins that regulate gap junction function.

While at this stage, the aforementioned description of the transition from the single-cell organism to the multicellular organism is speculation, early attribution of the importance of gap junctions to the control of cell growth and differentiation (6), to adaptive functions of differentiated cells (7), and more recently to apoptosis (8, 9) has been made when one compares normal cells, which can have functional gap junction intercellular communication (GJIC), to that of cancer cells, which appear not to have either homologous or heterologous GJIC (10). It is interesting to note that cancer cells are characterized to be phenotypically unable to contact inhibit, to terminally differentiate, or to apoptose (11). Cancer has been described as a disease of differentiation (12), a stem cell disease (13), or as oncogeny as partially blocked ontogeny (14). In other words, if a stem cell is exposed to a carcinogenic agent that prevents it from completing its terminal differentiation program, it will be able to proliferate in an uncontrolled manner and is less sensitive to programmed cell death signals. In view of the demonstration that cancers appear to be monoclonally derived (15), can be induced to terminally differentiate or apoptose by certain chemotherapeutic agents (16, 17), and to have normal restored growth control and restricted tumor growth by agents that can induce GJIC or by transfection with connexin genes (11), the cancer cell, without functional GJIC, appears to be a throwback to evolution. The cancer cell behaves like a single-cell bacterium, having only intracellular signaling to respond to extracellular signals for proliferation, but none for growth control or differentiation.

Several important concepts appear to be emerging that demand explanations about the speculated roles that gap junctional intercellular communication plays during the transition of totipotent stem cells to the many pluripotent stem cells, progenitor and terminally differentiated cells as an organism goes through development. This process must allow stem cells to go from one cell to many (symmetrical cell division) and to differentiate while maintaining a stem cell pool for future growth and wound repair or tissue replacement (asymmetrical cell division). In addition, the mechanism allows a stem cell to maintain its stemness after its asymmetric cell division in the midst of its differentiated daughters. Consequently, isolation and characterization of human stem cells that are involved in development, differentiation, growth control, regulation of asymmetric/symmetric cell division, and apoptosis will be the objective of our recent work.

As a stimulus for our research into the role of stem cells in carcinogenesis, we needed to isolate human stem cells to test the hypothesis that stem cells are the target cells for carcinogenesis. Based on the observation that the totipotent stem cell or fertilized egg did not seem to express connexin genes or did not have the ability to perform GJIC (18), we assumed that all stem cells (toti- and pluripotent stem cells) maintained their primitive or undifferentiated state by being sequestered from their differentiated daughters. Two mechanisms seemed to provide this kind of sequestration: one being a physical barrier between the stem cell and its differentiated daughter; the other being the inability to couple via gap junctions with its differentiated daughter. While the lack of functional GJIC was observed in fertilized eggs and early embryos of *Xenopus, Drosophila,* and mouse (18), we assumed that in the tissues of most or all organs there existed many terminally differentiated cells and progenitor cells with functional GJIC. One other important observation suggested that the target cells for carcinogenesis did not have functional GJIC. In Syrian hamster embryonic cells, used to induce neoplastic transformants with chemical carcinogens, there exists a small population of contact-insensitive cells that were highly transformable by chemical carcinogens (19). Contact insensitivity appears to be the result of the lack of functional GJIC.

Lastly, in view of the potential of using human stem cells for tissue regeneration (20), the ability to isolate stem cells, based on their absence of GJIC, and to study the role of GJIC in the growth and differentiation of

stem cells is gaining importance. Fundamental questions that still must be answered are: (a) Is GJIC critical for growth control? (b) Is GJIC needed for cell differentiation? (c) Is GJIC necessary for apoptosis? (d) Do stem cells lack functional GJIC? Experiments and attendant techniques to answer these questions are described in this article.

DESCRIPTION OF METHODS

A. Techniques to Measure Gap Junction Function

A.1. The Scrape Loading/Dye Transfer Assay: Background

During development and differentiation, the alteration of GJIC occurs due to changes in the amount and type of connexin gene expression and posttranslational modifications of connexin proteins (21). Measurement of gap-junctional intercellular communication can be accomplished by many methods: metabolic cooperation, microinjection of single cells, fluorescence redistribution after photobleaching (FRAP), scrape loading dye transfer (SL/DT), and patch clamp to measure electrical conductance. Of all the techniques to measure GJIC, SL/DT is the fastest and simplest. This method was first described by El-Fouly *et al.* (22). Since then it has been used to assess the GJIC status of many cell types in various biological circumstances [for review, see (3)]. Since SL/DT can measure dye transfer in multiple cells almost simultaneously, it is the only visual method available to assess the overall GJIC of a population of cells (Fig. 1). We have used this assay to determine changes in GJIC after cell populations are transformed with oncogenes (Fig. 1B) or treated with a chemical tumor promoter (Figs. 1C, 1D) or a combination of chemical treatment of oncogene transformed cells (23).

A.2. Theory

Most gap junction channels exclude molecules greater than approximately 1000 Da. A dye such as Lucifer Yellow with a molecular mass of 457 Da can easily pass through gap junction channels. When a cell is loaded with such a dye, it will diffuse from one cell to another via gap junction channels if the cell is coupled with its neighboring cells as shown in Fig. 2A. Since Lucifer Yellow is brightly fluorescent (absorbance at 428 nm, emission at 536 nm), it is easily seen under a microscope using a UV light source. Other fluorescent dyes that are larger than the gap junction channel exclusion limit are employed as a control to label the initially loaded cell and show that dye transfer from cell to cell is not by other means such as cytoplasmic membrane fusions, cytoplasmic bridge formation, or leaky membranes that can occur under cytotoxic conditions. Rhodamine–dextran conjugates (10,000 MW, absorbance at 555 nm, emission at 580 nm) are most commonly used to label the primary (scraped) cells (Fig. 2B). The amount of dye transferred from one cell to its neighbor is dependent on the number of gap junction that are coupled and the gating properties of individual channels.

A.3. Materials

1. CaMg-PBS buffer: 137 mM NaCl (8g/liter), 2.68 mM KCl (0.2g/liter), 8.10 mM Na_2HPO_4 (1.15g/liter), 1.47 nM KH_2PO_4 (0.2g/liter), 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$. The PBS is sterilized by autoclaving. (Note: CaMg is added to the PBS buffer to prevent some monolayer cells from detaching from substrate. However, some cell types may be sensitive to this level of Ca^{2+}; therefore, by preparing the Lucifer yellow in CaMg-free buffer, one this problem might be alleviated).
2. Lucifer Yellow CH lithium salt (Molecular Probes, Eugene, OR), tetramethylrhodamine dextran (Molecular Probes).
3. 0.5 mg/ml Lucifer Yellow in CaMg PBS,
   0.5 mg/ml Lucifer Yellow and 0.5 mg/ml rhodamine–dextran in CaMg-PBS,
   0.5 mg/ml rhodamine–dextran in CaMg PBS.
   (Note: Up to 2 mg/ml rhodamine dextran may be used.)
   The solutions are filter-sterilized.
4. 4% (w/v) Formalin in CaMg-PBS.
5. Scalpel blades and Parafilm.

A.4. General Procedure

1. Cells are usually grown in 35- or 60-mm tissue culture dishes for 1 or 2 days.
2. The cells in question are rinsed multiple times with CaMg-PBS. The excess liquid may be removed with a pipet.
3. The Lucifer Yellow (with or without rhodamine–dextran) or rhodamine–dextran solution is added to the cell monolayer.
4. The end of a sterile scalpel blade is gently placed in contact with the cell monolayer and rolled in one direction to extend the contact with gentle pressure to wound cells in a straight line. Multiple scrapes should be performed on each monolayer. The plate is left undisturbed in dark or dim light for 3 min for complete dye transfer. The dye enters cells through wounds created by the scalpel blade and the wounded cell membrane heals in a few seconds. If cells contain functional gap junctions, the Lucifer Yellow dye travels through the gap junctions away from the cells along the scrape line into neighboring cells while rhodamine–dextran is retained in the initially loaded cells directly adjacent to the scrape line. The distance the Lucifer Yellow dye travels, within a designated period (usually a 3-min

period), away from the scrape line is indicative of the level of GJIC within a culture.

5. Cells are rinsed extensively with CaMg-PBS and fixed by adding 4% (v/v) formalin. The cells in PBS or medium can also be observed for GJIC without fixation.

6. Plates can be viewed immediately using an epifluorescence phase microscope with a UV light source and/or stored at 4°C for extended periods (months) sealed in Parafilm. Some workers have air-dried the fixed plates for extended storage and rehydrated them for viewing.

A.5. Quantitation of GJIC Using the SL/DT Assay

The distance the dye travels through a monolayer can be measured using a variety of methods: (1) two-dimensional determination of fluorescent area, (2) one-dimensional measurement with a ruler laid against a photograph, and (3) counting the number of fluorescently labeled cells. These methods are described below.

Fluorescent area. Typically we scan monolayers with the Ultima interactive laser cytometer (Meridian Instruments, Okemos, MI) to obtain digitized images. Any method used to obtain a digitized image of the scrape line and surrounding cells should be adequate. The fluorescence area, not intensity, is used to determine the extent of dye transfer within a monolayer. Many image analysis software programs have a subroutine for determining fluorescence area of a fluorescent image. The fluorescence areas of

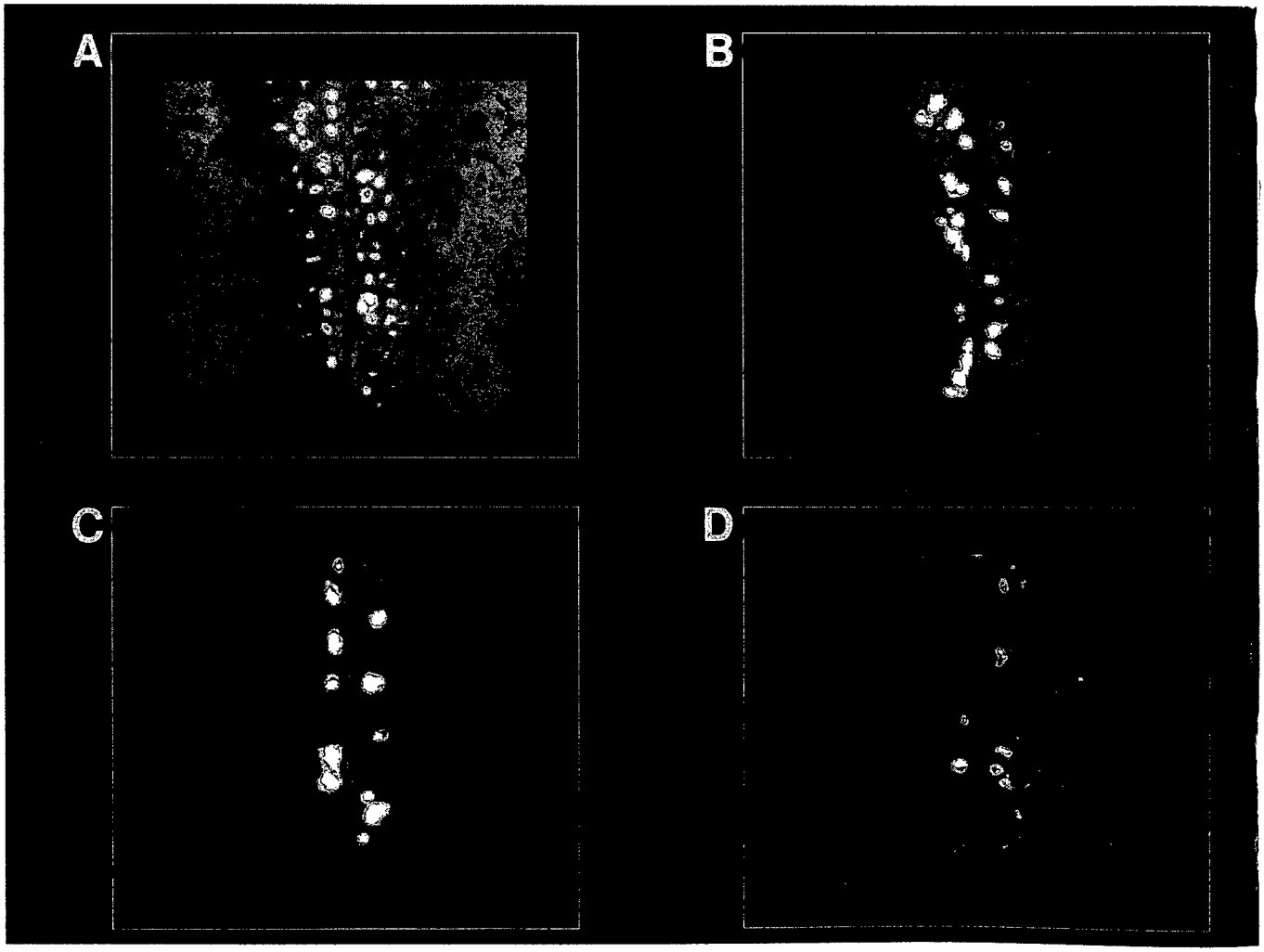


FIG. 1. Example of the scrape load/dye transfer (SL/DT) assay. Normal WB-F344 cultures (A), Ha-Ras transformed Wb-F344 cell line (B), and 30-min and 3-h TPA-treated WB-F344 (C and D, respectively) were subjected to the SL/DT assay as described in the text. Plates were scanned with a laser cytometer to obtain these digitized images. Spectral imaging was used to highlight the change in fluorescence intensity moving outward from the scrape line. White is the highest and pink is the lowest intenstity of fluorescence. Fluorescence area measurements were determined and percentage GJIC [as compared with normal WB-F344 (A)] was calculated as 17, 15, and 35% for (B), (C), and (D), respectively. Images are approximately equivalent to 200× magnification.

digitized images, which reflect extent of dye migration away from the scrape line, are subtracted from background fluorescence areas (areas of the monolayer well away from any scrape lines within the same test plate) to obtain a corrected fluorescence area value. These values can then be normalized to a control (test value divided by control) to obtain the fraction of control. Direct comparison of fluorescence areas between cultures is dependent on the consistency of the cells. All cells being compared must be relatively uniform with respect to growth state, cell type, and cell size. Generally, the average of 10 scrape line fluorescence areas is an adequate average to obtain a reasonable standard deviation for statistically significant data. A large standard deviation reflects a high degree of heterogeneity of growth,

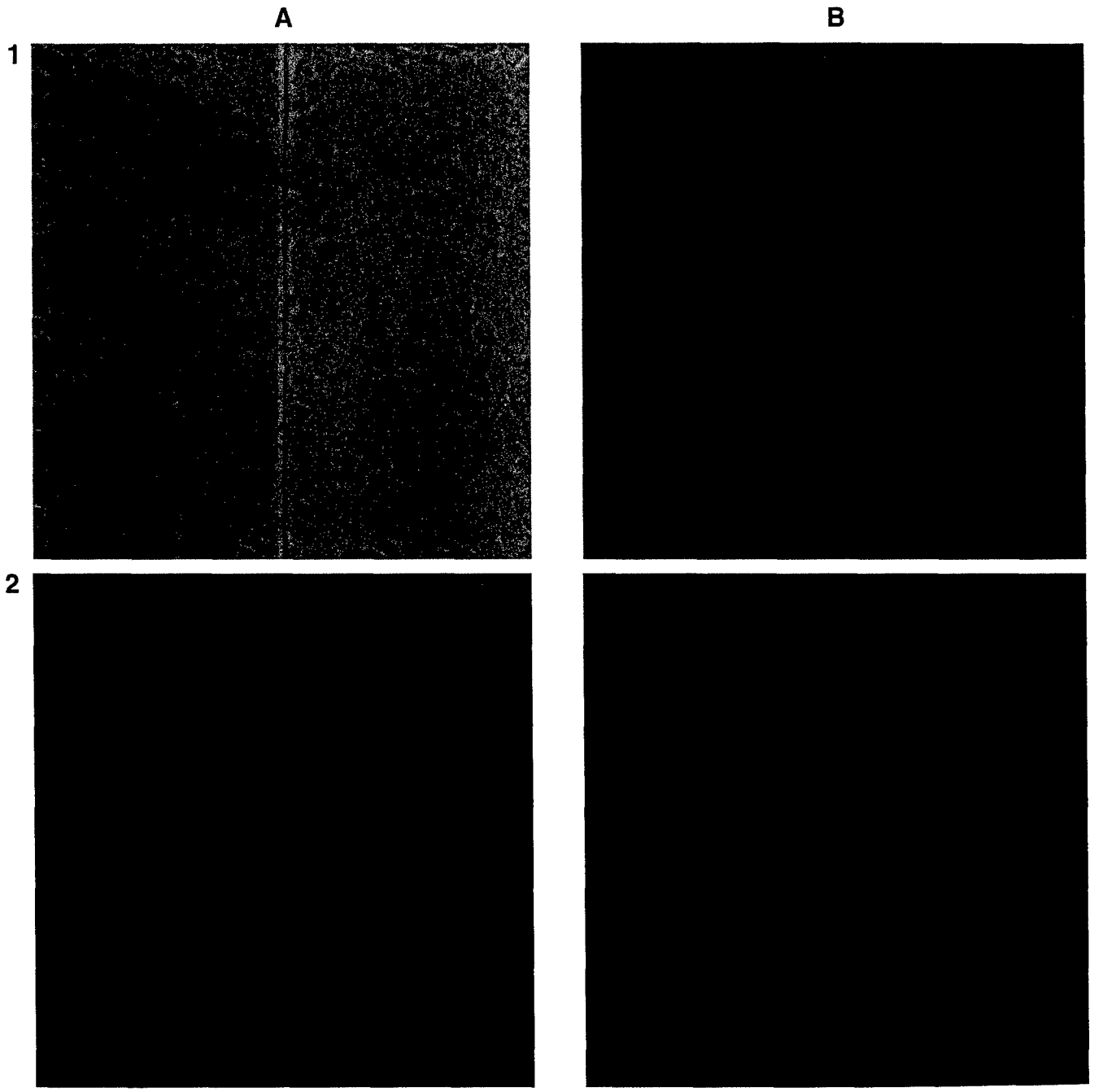


FIG. 2. SL/DT assay using a fluorescent dye excluded by gap junctions. WB-F344 cells were subjected to the SD/DT assay using Lucifer Yellow (A) and rhodamine conjugated to dextran (B). Phase-contrast (1) and UV transilluminated (2) photomicrographs were taken at 200× magnification. Lucifer Yellow was detected using a blue dichroic cube (410- to 485-nm exciter filter, 515- to 545-nm barrier filter) and rhodamine dextran was visualized using a green dichroic cube (535- to 550-nm exciter filter, 580-nm barrier filter).

size, and/or type of cells within a culture. If this is the case, a larger number of scrape line fluorescence areas should be included in the average.

One-dimensional measurement. If digitizing equipment is not readily available, or time consuming, measurement of distance traveled by the dye, using a standard ruler and photograph of the scrape lines, yields results similar to those obtained with fluorescence area measurements. However, this method can be highly subjective, since the experimenter decides extent of dye transfer. The distance the dye migrates is measured from the cell layer at the scrape to the edge of the dye front that is visually detectable. Due to the slight irregularity of the dye front edge, the distance along one scrape per dish is measured every 1 cm for a total of 8 cm (at 200×, 1 cm = 50 μm). The average cell length is 25 μm; therefore the 1-cm measurement on the photograph was equivalent to two cells. Each scrape chosen for photographic analysis is from a group of cells that are homogeneous in cell morphology and confluency. The nine measurements of distance are averaged and reported as the distance the dye traveled for the chosen group of cells representing the cell population of one monolayer. The photographs are taken and developed at the same time. Three plates per treatment are measured as described and the values are reported as an average ± 1SD.

Counting the number of fluorescently labeled cells. An alternative method of quantitating GJIC is by counting the number of cell rows adjacent to the scrape line that contain dye. There is a direct linear relationship between number of cell rows and level of fluorescence area if cell size, type, and growth state are similar (Fig. 3). Data calculations are similar to those described for the above two methods. This method may be useful when comparing populations of different cell size, type, and growth state.

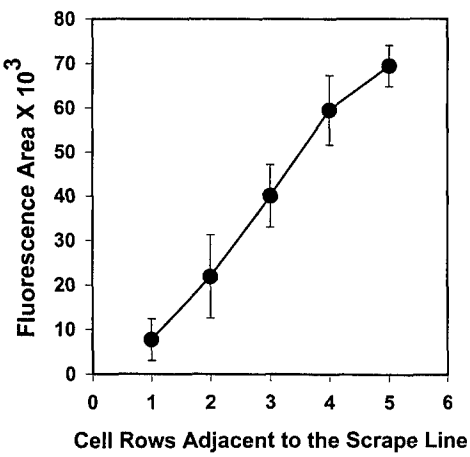


FIG. 3. Relationship between fluorescence area (ordinate) and number of cell rows (abscissa) Lucifer Yellow dye travels after cell monolayers are subjected to the SL/DT assay. Because the dye front can vary from one to two cells in a straight line, the data reflect n and $n \pm 1$ cells at each data point.

A.6. Data Analysis Considerations

When comparing different cell types or different treatment groups, it is absolutely essential that all cells compared are of uniform size. Generally we use analysis of variance for a test of significance for the results obtained by any of the methods listed above. Dunnett's multimean range t test is usually adequate for a test of significance between cell treatment groups and the control.

A.7. SL/DT: Drawbacks and Potential Misinterpretations of the Assay

The SL/DT is not useful for connexon channels that exclude molecules smaller than Lucifer Yellow. For example, connexin 45 channels cannot be assessed using Lucifer Yellow. If the connexin channels of a particular cell are unknown, then SL/DT using Lucifer Yellow as the transfer dye does not necessarily represent an accurate portrayal of the GJIC "competency" of that cell population. For channels that exclude Lucifer Yellow, smaller dyes such as biotin conjugates have been used (24).

The GJIC levels of some cell types of irregular shape are not easily quantitated using the SL/DT assay. For example, gap junction function of neuronal cells with extensive processes or long spindly fibroblasts cannot be easily quantified because the distance dye travels is not easily "trackable."

A.8. Fluorescence Recovery after Photobleaching (FRAP) in Cultured Cells: Background

The procedure described here was modified from one first reported (25) to measure GJIC between neighboring cells. In contrast to SL/DT, which measures GJIC in cell populations, FRAP is useful when studying individual cells within a population. This is extremely useful when assessing GJIC competency in cells displaying distinct morphologies such as associated with apoptosis and mitosis (9) or cells of neuronal origin (26) and fibroblasts in which GJIC is difficult to determine using SL/DT assay.

The general principle is to use a nontoxic, small-molecular-weight fluorescent dye that can pass through gap junctions in adjacent cells. Carboxyfluorescein diacetate (CFDA) was a suitable molecule. By selectively bleaching cells, the rate of transfer of fluorescent dye from the adjacent labeled cell back into the bleached cell could be calculated. In addition, it is possible to treat cells in a variety of ways that might affect their GJIC and document a corresponding effect on the fluorescent recovery rates.

A.8.a. Methods

Cell Labeling

Cultured cells are plated 1 to 2 days before the experiment is to be performed to allow for cell attachment, recovery from typsinization, and restoration of gap junctions. Cells should not be fully confluent since they need to be metabolically active to load with fluorescent dye and to communicate. However, there needs to be a significant number of touching cells for accurate measurements. For cultured human teratocarcinoma cells, if they were plated at a density of $1–2 \times 10^4$ cells per 35-mm dish, they would be suitable for experiments after 1 day of incubation.

Cells are incubated with CFDA (7 μg/ml; Molecular Probes, 1 mg/ml stock in ethanol) for 15–30 min at 37°C. In that form, CFDA is not fluorescent, but is permeable to the cells. An esterase enzyme function in the cells is necessary for the fluorescent molecule, carboxyfluorescein, to be released. There are some cell types with reduced esterase activity, and as mentioned above, senescent cells in confluence have extremely reduced levels and will not normally load sufficiently for accurate measurements. For maximum loading, a serum-free medium or isotonic buffer should be used (see recommended buffer described below). However, in experiments where serum was vital for parameters being measured, a reduced or normal serum concentration could be used. In these instances, increased CFDA concentration or incubation times may be necessary as the esterases in the serum often cleave a portion of the dye in solution, making it unavailable to the cells. After dye loading, the dishes were rinsed three times with buffer and kept in buffer or serum-free medium.

A.8.b. Photobleaching Measurements

The experiments described here were done using the Ultima (Meridian Instruments, Inc., now Genomic Solutions, Ann Arbor, MI). The computer-controlled instrument was equipped with an inverted microscope, argon laser tuned to 488 nm to excite the carboxyfluorescein, a microprocessor-controlled stage, and acoustoptic modulator to control beam intensity.

The dish of labeled cells was placed on the stage and a suitable field of cells was identified using a 40× objective. If desired, these experiments could be done with a stage system that maintains the culture dish at 37°C. For the optimum area, several touching cells should be located, along with one cell that is isolated from the rest which can serve as a control.

The field was scanned to generate a digital image of the fluorescence (Fig. 4, prebleach). Parameters of laser power, intensity used for scanning, photomultiplier setting, etc., were optimized to provide near-saturation levels in the image with minimal photobleaching. At this point, all the cells should uniformly be loaded with fluorescence. Often the nuclear region of the cell may appear brighter; this is typically a function of the cell being the thickest at that area and having the largest volume of fluorescent label.

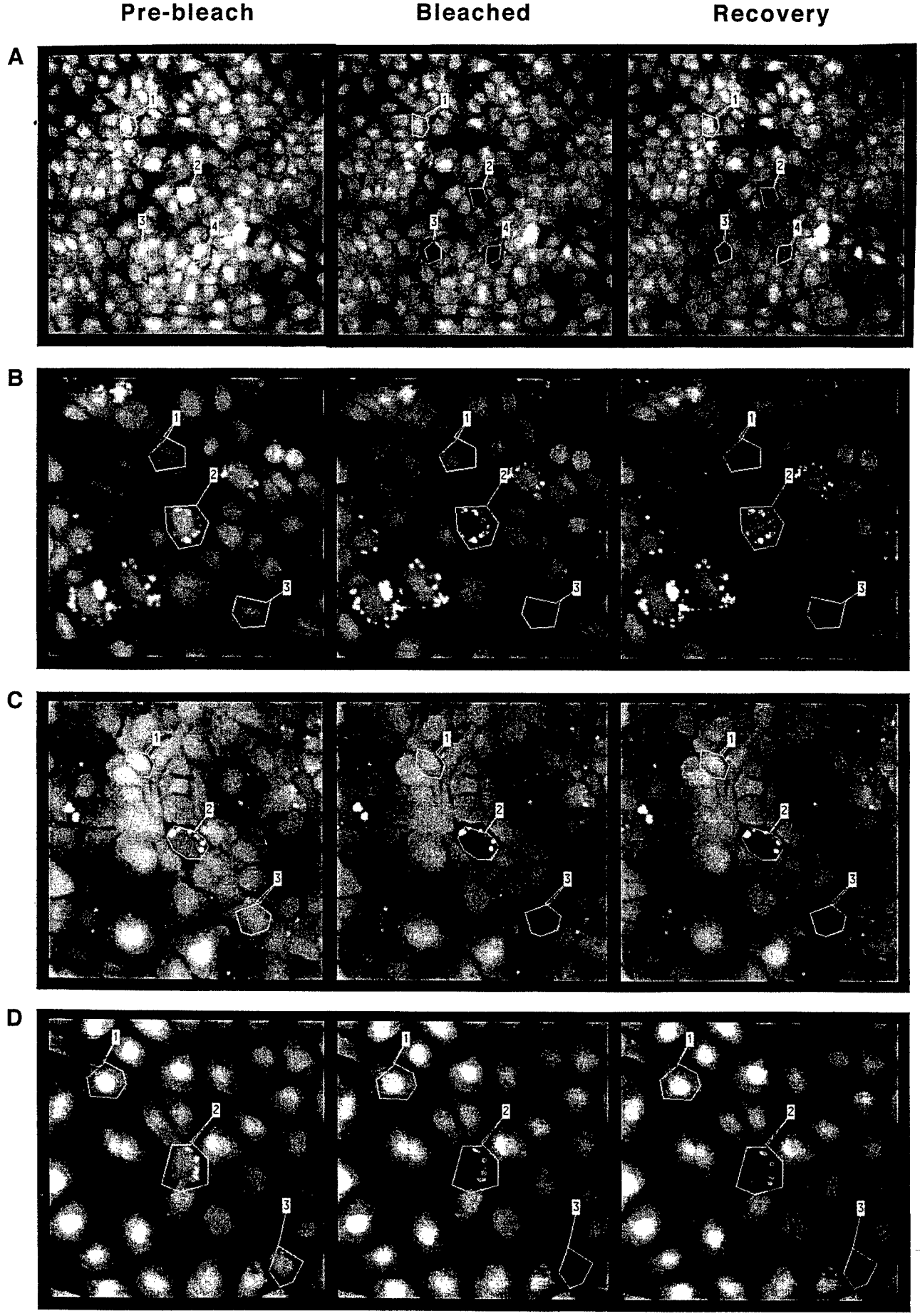
After the initial scan, selected cells were photobleached (Fig. 4A, bleached). With the Ultima, this is accomplished automatically with the software program by simply denoting which cells should be bleached with a more intense laser beam. Through use of an acoustooptic modulator, the intensity of the beam can be changed by orders of magnitude in microseconds, allowing scanning and bleaching to occur rapidly and sequentially.

After the bleaching has been accomplished, sequential scans to detect fluorescence recovery in bleached cells (Fig. 4A, recovery) are done at time intervals as determined by the experimenter. These images are digitally recorded for analyses. In addition to the adjuncent cells, several other controls should be done. These include an isolated cell that is bleached and should not recover because there are no adjacent cells from which dye can be transferred. Also several other nonbleached cells should be monitored to provide control data on the effect of several scans. Several fields can be rapidly scanned and bleached within one dish of cells to provide multiple data points.

A.8.c. FRAP Analysis

At the conclusion of the experimental portion of the experiment, the stored images can be recalled and the fluorescence quantified in the cells. This was accomplished by delineation of the cells to be monitored in the initial prebleached image that was stored. With the Ultima software, this same region was then monitored for fluorescence in all subsequent images and displayed as a percentage of the initial fluorescence recovery.

A typical plot of fluorescence recovery after photobleaching is shown in Fig. 5. The data have been normalized to the fluorescent signal present in each cell prior to photobleaching. In the cells that were bleached, the fluorescent signals drop to approximately 20% that of the prebleached levels, then gradually increase as fluorescent dye transfers into the cells through gap junctions. In an unbleached control cell, which was monitored for loss of fluorescent signal throughout the scanning process, the signal level remained constant at nearly 100% of the initial scan (Fig. 5, plot 1). In cells that were photobleached the positive slope of plots indicates recovery of fluorescence (Fig. 5, plots 2–4). In this example, cells 2, 3, and 4 showed rates of fluorescence recovery of 6.8, 12.7, and 13.0% per minute within the first 3 min of photobleaching. Additional detailed description of FRAP data analysis can be found in Stein *et al.* (27).


Pre-bleach
Bleached
Recovery
A
B
C
D

BUFFER FOR CELL LOADING AND EXPERIMENTATION

KCl	5.36 mM
NaCl	136.9 mM
KH_2PO_4	0.44 mM
$NaHCO_3$	4.16 mM
Na_2HPO_4 ($7H_20$)	0.336 mM
D-Glucose	11.101 mM
Hepes	5.0 mM
$CaCl_2 \cdot 2H_2O$	2.0 mM
BSA	0.1%

Adjust to pH 7.3; filter sterilize.

A.9. Heterologous GJIC: The FRAP Assay of Fluorescent Microbead-Labeled Cells: Background

Alteration of cell–cell communication between heterologous cell types is an important indicator of interactions that occur during various biological etiologies of differentiation, carcinogenesis, apoptosis, and mitosis (3). The difficulty in carrying out such experiments is in being able to distinguish between different cell types. For these experiments, one or both cell types are labeled with fluorescent microbeads; the cells are then mixed together in ratios such that one cell type is surrounded by another cell type (28). The FRAP assay is performed to determine the extent of GJIC of the cell type that is surrounded by the other cell type. This approach has been used successfully when assessing if GJIC has been altered after cells are transfected with chromosomes or plasmids or chemically/X-ray mutated (29–31).

A.9.a. Considerations

Although this is a relatively simple technique, many technical considerations must be addressed:

The bead concentration added to cells. If the concentration of beads in the medium is too high, then the cells tend to "gorge themselves" with beads and this affects the level of GJIC. If the bead concentration is too low, it is difficult to distinguish beaded from unbeaded cells.

The ratio of cells types mixed. Ideally the ratio is such that cells of one cell type completely surround a single bead-loaded cell of the other cell type and there are enough "heterologous clusters" to easily find fields to perform FRAP analysis. Typically a 1:25 ratio of beaded to unbeaded cells is adequate.

Cell confluency. The mixture of cells for each plate should be enough to form a monolayer of cells (90–95%) near confluency. Cells must be touching each other to form gap junctions.

Growth rate of cells. It is necessary to perform experiments within the first generation time after mixing labeled and unlabeled cell types. When a cell that contains microbeads divides, the beads do not necessarily segregate evenly to the daughter cells; thus a labeled cell can divide into an unlabeled cell and labeled cell, which leads to misidentification of the unlabeled daughter as the other cell type. A way to avoid this dilemma is to use beads labeled with two distinct dyes and label both cell types.

A.9.b. Materials

We typically use Polysciences, Inc., Fluoresbrite Carboxylate Microspheres, 1.16 μm in diameter. Molecular Probes is another good source of fluorescent beads of various sizes and fluorescent tags.

A.9.c. General Procedure

LABELING CELLS WITH FLUORESCENT BEADS

1. Grow cells in 35-mm cell culture dishes until they are 80% confluent.
2. Prepare beads: Dilute and wash the fluorescent bead stock by adding 1 drop of bead stock solution to 10 ml PBS. Microfuge 1 min at 2000 rpm. Discard PBS and resuspend the fluorescent beads in 10 ml of cell culture medium.
3. Remove medium from the cultures to be labeled. Transfer 2 ml of the bead suspension to each culture. Incubate overnight as per the cell growth conditions. The next day, rinse cultures extensively to remove any excess beads and check to see if the beads are evenly distributed within all cells in the culture. The concentration of beads in the medium and the confluency of cells used may have to be determined empirically for each particular cell type.

PREPARING COCULTURES

1. Trypsinize labeled and unlabeled cultures. Typically we add trypsin to a culture and remove it within

FIG. 4. Homologous and heterologous GJIC using the fluorescence redistribution after photobleaching (FRAP) assay. (A) Homologous GJIC WB-F344 cells were subjected to the FRAP assay as described in the text. Cell 1 remained unbleached throughout the assay as a control. Cells 2, 3, and 4 were bleached with 150-m sec pulses from a 488-nm argon laser. Fluorescence within these cells was monitored during the first 3 min after photobleaching and percentage fluorescence recovery was calculated to be 7, 13, and 13 %/min for cells 2, 3, and 4, respectively. Images depicted as 200× magnification equivalent. (B–D) Heterologous GJIC between Wb-F344 cells and WB.ab1, a mutant derivative of WB-F344 (60), was determined. Cells were labeled with fluorescent beads as described in the text, mixed (1:25 as WB*:WB (B), WB*:WB.ab1 (C) and WB.ab1*:Wb.ab1 (D) (where the asterisk denotes cells that were fluorescent bead labeled) and subjected to the FRAP assay. In all cases, cell 1 is the unbleached control, cell 2 is the fluorescent bead-labeled cell surrounded by unlabeled cell type, and cell 3 is usually an unbeaded cell. In (B), fluorescence recovery was 15%/ min in cells 2 and 3, whereas there was negligible fluorescence recovery in photobleached cells depicted in (C) and (D). Images depicted as 400× magnification equivalent.

30 s, then allow the cell to become detached from the plate.

2. Add 2.0 ml of cell culture medium to each culture; gently pipet up and down.

3. Transfer 80 μl of trypsinized fluorescent bead-labeled cells to a trypsinized culture of unlabeled cells containing 2.0 ml of trypsinized cells. Mix by gently pipetting up and down. If both cultures are 80–100% confluent, then the ratio of beaded to unbeaded cells in the coculture is approximately 1:25. Incubate overnight in medium containing reduced serum (1%) to minimize cell division.

FRAP Analysis

1. FRAP analysis can be performed essentially as described in Section A.8.

2. To obtain significant data we typically subject approximately 50 cells per coculture group to FRAP analysis.

3. Controls: For each coculture pair, homologous and heterologous bead controls must be included in all experiments. For example, if we want to determine if cell type A will form gap junctions with cell type B, the following coculture groups should be assessed by FRAP (an asterisk denotes fluorescent bead-labeled cells):

A*/A B*/B
A*/B A/B*

Data are expressed as the rate of fluorescence recovery per minute. Examples of typical data are described in Figs. 4B, 4C, and 4D.

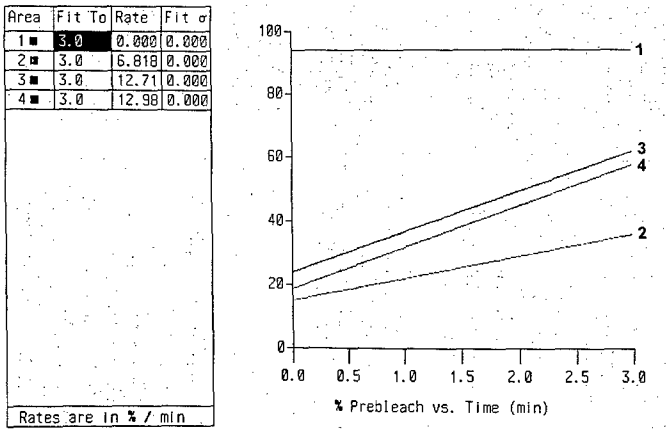


FIG. 5. Fluorescence recovery plot of a FRAP experiment. Data are for the experiment depicted in Fig. 4A. Cell 1 (unbleached control) is used to correct for entire field photobleaching due to multiple laser scanning of each field. Percentage prebleach fluorescence (ordinate) was plotted against time, in minutes, after photobleaching (abscissa).

A.10. Regulation of Human/Rat Connexin Transcription in Cells: Quantitation of mRNA

A.10.a. Expression of mRNA Transcripts Measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR): Background

Amplification of individual RNA molecules can be achieved by a method that combines reverse transcription (RT) and polymerase chain reaction (PCR). This method has been demonstrated to be extremely sensitive for mRNA analysis, and semiquantitative information can be obtained. Therefore, this technique is a powerful tool to study the regulation of transcription. In the PCR step, the mRNA of interest is specifically amplified by using two gene-specific oligonucleotide primers that anneal in two different exons to discriminate between genomic DNA. Relative RNA levels can be measured by relating the amplicons derived from the mRNA of interest to those of a housekeeping gene. The latter functions as control because its transcription is not affected by inducers.

A.10.b. Materials

RNA Preparation

Total RNA was extracted from cells by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions (32). Briefly, cells (2–5 × 10^6 cells) were washed twice with PBS, lysed by adding 1 ml of TRI Reagent, and transferred to microcentrifuge tubes. The samples were then incubated for 5 min at room temperature, 0.2 ml of chloroform was added with vigorous shaking for 15 s, and the mixtures were incubated at room temperature for another 10 min and centrifuged at 12,000 rpm for 15 min at 4°C. Each aqueous phase was transferred to a fresh tube, and the RNA was precipitated by mixing with 0.5 ml of 2-propanol. The samples were then incubated at room temperature for 5–10 min and centrifuged at 12,000 rpm for another 10 min at 4°C. The gel-like RNA pellet was washed with 75% ethanol by vortexing, centrifuged at 12,000 rpm for 10 min at 4°C, and air-dried for 10 min. The extracted RNA was dissolved in $T_{10}E_1$ with a RNase-free DNase cocktail containing (per sample) 10 μl of 100 mM $MgCl_2$/10 mM dithiothreitol (DTT) solution, 0.2 μl of 2.5 mg/ml RNase-free DNase, 0.1 μl placental ribonuclease inhibitor, and 39.7 μl TE buffer. The samples were mixed and incubated for 15 min at 37°C. The DNase reaction was stopped by adding 25 μl DNase stop mix containing 50 mM EDTA, 1.5 M sodium acetate, and 1% sodium dodecyl sulfate (SDS). The RNA was extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The extracted RNA was precipitated by adding the same volume of ethanol and centrifuged at 12,000 rpm for 10 min at 4°C. The RNA pellet was washed with 75% ethanol by vortexing,

centrifuged at 12,000 rpm for 10 min at 4°C, and air-dried for 10 min. Finally, the extracted RNA was dissolved in RNase-free water, and its RNA concentration was determined by spectrophotometry at 260 nm.

A.10.c. General Procedure

Reverse Transcription

It is advantageous to prepare total cDNA as PCR template, through the use of an oligo(dT) primer for the RT reaction. This alternative to using a gene-specific RT primer ensures an opportunity to reuse each sample multiple times and to reserve samples for future analysis for the expression of additional mRNAs. Reverse transcription of 1 μg of total RNA was performed in a final volume of 20 μl for 1 h at 37°C, using 50 U of M-MLV reverse transcriptase (Strata Script RNase H^- reverse transcriptase, Stratagene, La Jolla, CA) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 25 U of RNase inhibitor, 1 mM each of dATP, dGTP, dCTP, and dTTP, and 2.5 mM oligo(dT) (Pharmacia AB, Uppsala, Sweden). The samples were then heated to 90°C for 10 min to terminate the RT reaction, and quick-chilled on ice.

Oligonucleotides Used for PCR Amplification

Oligonucleotides were synthesized on an Applied Biosystems automated synthesizer. The primers for the PCR amplification of human or rat Cx26, Cx32, and Cx43 are RNA-specific primers. The 5′ primers are located on the first exon, and the 3′ primers are located on the second exon. The sequences are listed in Table 1.

PCR Amplification

The reverse-transcribed cDNA obtained from total RNA was added to a reaction mixture that contained a final concentration of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 mM each primer of Cx26, Cx32, or Cx43, 0.5 mM each primer of GAPDH, as a control for integrity of RNA, and 2.5 U of *Taq* polymerase (Perkin-Elmer–Cetus, Norwalk, CT) in a final reaction volume of 20 μl. The mixture was heated at 95°C for 30 s in a Perkin-Elmer DNA thermocycler (Perkin-Elmer–Cetus). Amplification was performed in 15 to 35 sequential cycles at 95°C for 20 s, 58°C for 40 s, and 72°C for 40 s, followed by incubation for 7 min at 72°C. The PCR products were analyzed on a 8% polyacrylamide gel in 1× Tris–borate/EDTA buffer. Gels were stained with ethidium bromide and photographed using Polaroid Type 667 film.

Optimal Conditions of RT-PCR

Each PCR assay needs to be optimized in several respects. First, specific amplification of the sequence of interest must be ensured. This step involves initially determining that PCR amplification yields discrete products of the expected size on ethidium bromide-stained polyacrylamide gels. Next, PCRs are saturable, and distinction between inputs is lost at cycle numbers that over- or underamplify. Analysis of products from reactions that have not been optimized yields large errors in estimation of target mRNA concentration. Such errors usually bias toward underestimation of differences between samples. Cycle number titrations are conveniently performed by scaling PCRs to 100 μl and removing 10-μl aliquots at each step. Preparation of parallel reactions that are subjected to varying cycle numbers is an adequate alternative.

As an example, two kinds of cell lines, G401.2/6TG.1 epithelial cell line (G401.2/6TG.1) and WB-F344 cell line (WB), were used to determine optimal PCR cycle number. The G401.2/6TG.1 epithelial cell line, derived from the kidney of a Wilms' tumor patient, exhibited extensive GJIC (33). The Fisher 344 rat liver-derived epithelial cell line, WB-F344, was obtained from J. W. Grisham (University of North Carolina) (34). The relationship between cycle number and PCR product was determined with these cell lines. The kinetics of RT-PCR amplification of human and rat GAPDH and Cx43

TABLE 1

Oligonucleotides of 5′ Primers and 3′ Primers of Connexin Genes and GAPDH Genes

mRNA species	5′ Primer	3′ Primer	PCR product (bp)
Human			
Cx26	GATTTAATCCATGACAAACT	CCACACCTCCTTTGCAGC	558
Cx32	GGGAAAGGGCAGCAGGAG	CCCATGGCCCTCAAGCCG	627
Cx43	GCGTGAGGAAAGTACCAAAC	GGGCAACCTTGAGTTCTTCC	506
GAPDH	GTTCGACAGTCAGCCGCATC	GTGGGTGTCGCTGTTGAAGTC	933
Rat			
Cx26	CGCGGCCGTCCGCTCTCCCAA	GAAGTAGTGGTCGTAGCACAC	481
Cx32	AGGTGTCGCAGTGCCAGGGAG	CCCGTGCCCCTCAAGCCGTAG	373
Cx43	GCGTGAGGAAAGTACCAAAC	GTGAAGCCGCCGCCAAAGTTG	527
GAPDH	TGAAGGTCGGTGTCAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	983

genes in the G401.2/6TG.1 and WB-F344 cell lines are shown in Figs. 6A and 6B and Fig. 7A and 7B, respectively. The A panels are densitometric quantitations of each band in the gel shown in the B panels. RT products are subjected to cycle number titration, typically in two stages. Initially, cycles are increased in increments of 5 from 15 to 35 cycles, producing a broad cycle number titration for this primer pair. The relationship between cycle number and PCR product is then determined. An optimal cycle number is determined at a titration across the exponential portion of the curve, using increments of a cycle at each step; the cycle number that represents the midexponential portion of the curve is selected. The cycle number that is defined in this fashion is then validated by cDNA input titration, using serial dilution of RT reaction product to program PCR amplifications, at the established optimal cycle number. Cycle number optima that are practically useful will yield outputs that are linear with respect to input across 2 logs of serial cDNA input dilutions. The successful application of a predetermined optimal PCR cycle number relies on the assumption that varying levels of mRNA starting material will produce RT reaction products that directly correspond to input. This assumption is directly tested for each assay by RNA input titrations into the optimized assay. The output should remain linear across at least 1 log of input.

Assessment of Cx mRNA Expression

As an example, the Cx mRNA levels of two cell lines, G401.2/6TG.1 and WB, were examined. As shown in Fig. 8, these cell lines were shown to express both Cx26 and Cx43 gap junction proteins. The mRNA of human/rat Cx26 and Cx43 was amplified and detected by RT-PCR. On the other hand, rat hepatocytes were shown to express both Cx26 and Cx32 gap junction proteins and the mRNA of rat Cx26 and Cx32 was amplified. These results confirm the previous results obtained from Western blot analysis and immunostaining analysis with specific antibodies against Cx26, Cx32, and Cx43 proteins.

A.10.d. Comment on RT-PCR Analysis

Quantitative PCR methods for comparing the relative amounts of mRNA in different cells have been reported (35, 36) and the quantitation has since been improved by competitive PCR, the coamplification of a known amount of an allelic gene variant as an internal

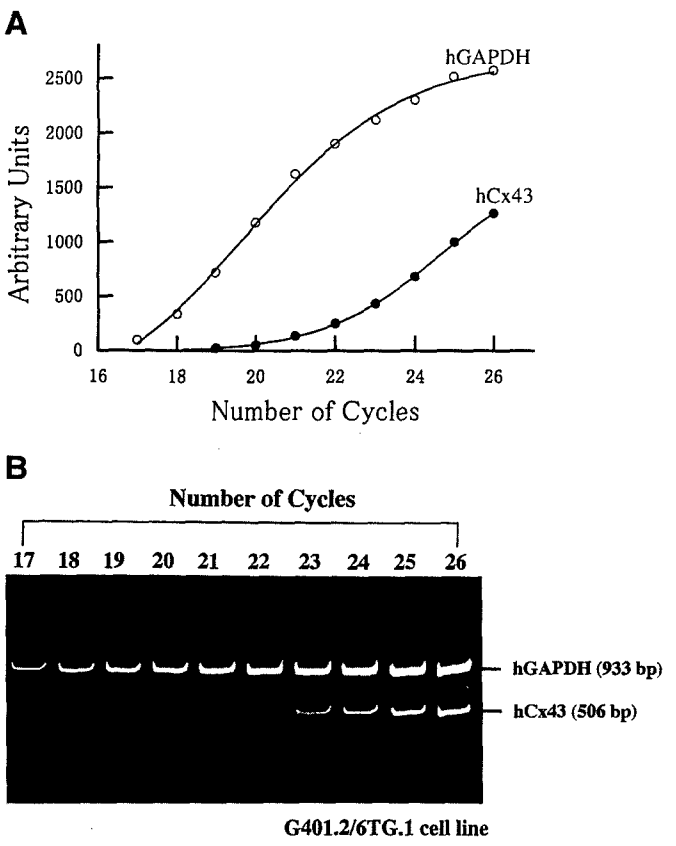


FIG. 6. Densitometric quantitation (A) of each band reflecting the GAPDH and connexin 43 genes in the gel shown in (B) of human G401.2/6TG.1 cell line.

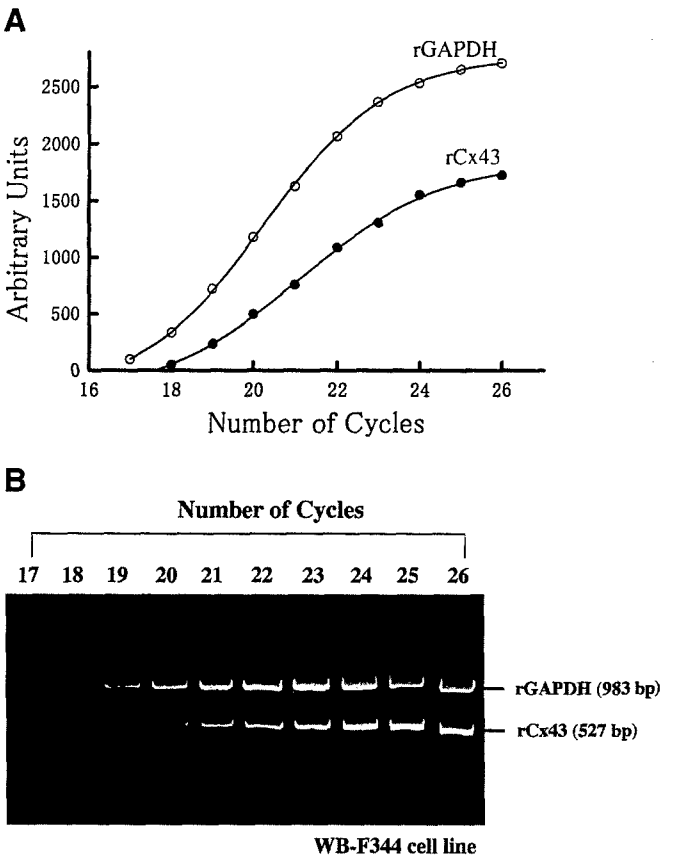


FIG. 7. Densitometric quantitation (A) of each band reflecting the GAPDH and connexin 43 genes in the gel shown in (B) of rat liver epithelial (WB-F344) cells.

standard (37, 38). These methods, however, are limited to measuring the mRNA level of a single gene. To measure the expression of more than one gene, an internal standard with the same sequences as the primers could be constructed and coamplified, but this would be costly and impractical. The multiple PCR has been shown to be a reliable method for the simultaneous amplification of two genes, and the results from this technique are in good agreement with the results of competitive PCR amplification of a target cDNA and an internal competitive template (39). This method is a nonradioactive multiplex RT-PCR technique for the rapid simultaneous amplification of two genes and quantitation of the relative levels of three kinds of connexins. Development of a new assay requires approximately 1 week.

A.11. Western Blot Analysis of Connexins

A.11.a. Background

Intercellular communication through gap junctions plays a critical role in maintaining the homeostasis of an organism by controlling the epigenetic expression of genes that establish a balance between cell proliferation, apoptosis, differentiation, and adaptive responses to environmental stimuli (3). Gap junction proteins form channels between contiguous cells, allowing the transfer of small signaling molecules up to a molecular mass of 1200 (40). A family of highly conserved genes codes for the gap junction proteins of which 13 mammalian gap junctions have been discovered thus far (3). Gap junctions in different tissues are not identical but they do share a basic structure. The gap junction consists of six protein subunits called connexins, which form a hexameric channel called a connexon. The connexon of one cell docks with the connexon of an adjacent cell to form a continuous channel between the two cells, thus forming a gap junction. However, the control of GJIC depends on the type of connexins that form the channels in the various types of cells found in each tissue. The connexon can be a homotypic–hexameric channel consisting of only one type of connexin, but a heterotypic channel consisting of two or more different types of connexins has been hypothesized since most cells express more than one type of connexin (41). Therefore, our understanding of the mechanism by which gap junctions are regulated must first depend on determining which connexins exist in the cell type being studied.

The most common method used in determining the type and size of connexins found in a cell is Western blot analysis. Western blot analysis of proteins is a well-established technique that entails electrophoretic separation of proteins in a gel matrix followed by electrophoretic transfer of the proteins from a gel to a paper matrix. The paper matrix is much more conducive to enzyme-linked immunosorbent assays than the gel. Specific antibodies can therefore be used to identify the particular connexins found in a given cell type. Western blot analysis can also be used to detect post-translational changes such as the phosphorylation status of the connexins.

A.11.b. Methods

Protein Extraction

Cells are normally grown between 90 and 100% confluency. Extractions are usually easier from cells grown in tissue culture plates than flasks. Usually 1×10^6 cells yield an adequate amount of protein and can be easily attained using 35-mm-diameter cell culture plates. Cells are rinsed three times with PBS and the plates are tipped on a paper towel to drain off all excess liquid. The lysis buffer (125 μl/10 cm^2) containing 20% SDS and 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) is added and the plates are shaken to evenly coat the cells with the lysis buffer. The cells are then scraped with a disposable cell scraper (Sarstedt, Newton, NC) and transferred to a 500-μl microcentrifuge tube and sonicated with three 10-s pulses at 35% maximal power using an ultrasonic probe to sheer the DNA. To avoid protein degradation, the samples are frozen and thawed only once for each Western blot analysis and protein determination. Therefore, the sample should be separated into several aliquots before freezing at −20°C. Also, to minimize the effects of phosphatases on the dephosphorylation of connexins, we have added 100 μM Na_3VO_4 to the PBS washes, and 100 nM aprotinin, 1.0 μM leupeptin, 1.0 μM antipain, 100 μM Na_3VO_4 and 5.0 mM NaF to the lysis buffer. Addition of these phosphatase inhibitors is not always

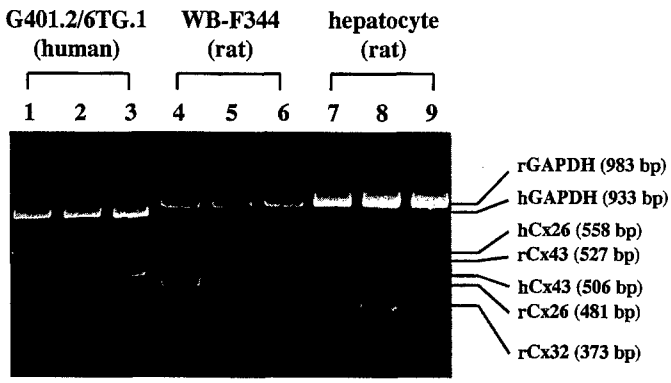


FIG. 8. RT-PCR of human and rat connexin genes. Primer pairs for human connexin 26 (lane 1), human connexin 32 (lane 2), and human connexin 43 (lane 3) were used to amplify RNA isolated from G401.2/6TG.1 cells. Primer pairs for rat connexin 26 (lanes 4, 7), rat connexin 32 (lanes 5, 8), and rat connexin 43 (lanes 6, 9) were used to amplify RNA isolated from rat liver oval cells (WB-F344) and primary hepatocytes. GAPDH was coamplified as a control. Expected PCR product sizes are indicated to the right.

necessary because comparative experiments in control and TPA-treated WB-F344 rat liver epithelial cells showed no difference between these two extraction procedures (data not shown). However, these results do not rule out the possibility that phosphatases could artifactually dephosphorylate connexins in other cell types.

SDS–PAGE

The proteins can be separated using discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (42). There are several commercially available gel boxes of varying sizes available. We use the Mini-PROTEAN system from Bio-Rad (Hercules, CA). A 12.5% acrylamide gel is used to separate the various isoforms of connexins. Electrophoresis-grade reagents should be used for all solutions. The separating gel consists of 12.5% acrylamide, 0.33% methylbisacrylamide, 0.1% SDS, 0.375 M Tris/pH 8.8, 0.05% ammonium persulfate, 0.05% *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and the stacking gel contains 4.0% acrylamide, 0.1% methylbisacrylamide, 0.1% SDS, 0.125 M Tris/pH 6.8, 0.05% ammonium persulfate, 0.10% TEMED. The running buffer contains 0.025 M Tris/pH 8.4, 0.1875 M glycine, 0.1% SDS. An adequate amount of protein for loading onto the gels is 15–20 μg. The protein samples are first mixed with an equal volume of 2× sample buffer containing 20% glycerol, 1.4 M 2-mercaptoethanol, 4% SDS, 0.125 M Tris/pH 6.8, 0.2% bromphenol blue. Unlike sample preparations for most SDS–PAGE runs, connexin samples are not boiled or heated to 60°C. Rainbow (Amersham International, Arlington Heights, IL) colored protein molecular weight markers are a convenient way of not only determining approximate molecular masses of the protein bands but also monitoring the electrophoretic run when there is a need to run beyond the bromphenol blue dye front. For connexin 43, gels are normally run at a constant potential of 200 V for 1 h 20 min, which is when the pink MW marker (lysozyme, 14.3 kDa) reaches the bottom of the gel. This will achieve the maximal resolution of the three connexin 43 bands.

Electrophoretic Transfer

After the electrophoretic run, the stacking gel is cut off and discarded and the separation gel is removed from the plates and soaked in transfer buffer for 10 min with no agitation. If PVDF transfer membranes (Millipore Corp., Bedford, MA) are used, membranes need to be soaked in 100% methanol for 60 s and then rinsed for 5 min in distilled water. The assembly of the gel transfer membrane sandwich consists of layering first the mesh pad followed by blotting paper, gel, transfer membrane, blotting paper, and mesh pad. To ensure good contact between the transfer membrane and gel and removal of air bubbles, a makeshift rolling pin should be passed over the sandwich with firm pressure before the last mesh pad is put on. All components should be premoistened and the transfer buffer (0.025 M Tris/pH 8.4, 0.1875 M glycine, 0.02% SDS, 20% methanol) should be chilled to 5°C. The sandwich must be placed in the transfer tank with the gel facing the anode side and the transfer membrane facing the cathode side. We normally run the transfer for 15–20 h at a constant potential of 20 V and use one to two ice packs throughout the run. Alternatively, shorter transfer times can be achieved with higher voltages.

Immunoblotting

The transfer membrane is removed from the sandwich and rinse once with distilled water. Ponceau protein staining dye is added to the transfer membrane (Sigma Chemical Co., St Louis, MO) for 30–60 s and then the membrane is rinsed with distilled water until there is a good contrast between the background and protein bands. The Ponceau-stained membrane can be recorded either by a photograph, benchtop scanner, or gel doc system. The latter two methods are more conducive for objective data analysis using computer software that can quantify densiometric scans. These data serve as a background measurement of total protein. After Ponceau staining, the membrane is treated with a blocking reagent for 1 h under gentle agitation. A suitable and economic blocking reagent is 5% Carnation nonfat dried milk dissolved in PBS + 0.1% Tween (PBS/Tween) that has been filtered through Whatman No. 1 filter paper. The primary and secondary antibodies are also dissolved in the PBS/Tween buffer. When blocking is complete, the primary antibody is added for 2–24 h under gentle agitation at a dilution of 1:500 to 1:3000. Treatment times and dilutions are highly dependent on the amount of connexin of interest present in the cell. For Cx43 in WB cells, a 1:2000 dilution of polyclonal anti-Cx43 (Zymed Inc., South San Francisco, CA) for 2 h is sufficient for good detection. Before the secondary antibody is added, the membranes are thoroughly washed to remove excess/unbound antibody. The wash consists of one 15-min rinse followed by two more 5-min rinses using fresh PBS/Tween for each wash. Following the washes a 1-h treatment under gentle agitation with a 1:1000 dilution of the secondary antibody is normally sufficient for good detection. Again the membranes are washed to remove excess/unbound antibody with one 15-min rinse followed by four more 5-min rinses using fresh PBS/Tween for each rinse. The choice of enzyme-conjugated secondary antibody depends on the detection technique. Horseradish peroxidase and alkaline phosphatases are the most commonly used secondary antibodies.

Detection

Chemiluminescent assays are very popular due to their high sensitivity and ease of use. Horseradish peroxidase-conjugated secondary antibodies are used for the chemiluminescent systems. We have used the ECL-detection kit (Amersham Life Science, Denver, CO); Supersignal, Supersignal Ultra, and Supersignal Blaze kits(Pierce, Rockford, IL); and Chemiluminescence Reagent Plus (NEN Life Sci, Boston, MA) but at present there are several more companies that have chemiluminescent systems. The three different Pierce kits vary in sensitivity from pico- to femtomoles. All these kits involve a 1:1 mixture of two different reagents that are pipetted onto the protein side of the membrane for 1 min. After 1 min, excess reagent is blotted off by touching the edge of the membrane to a paper towel and then the membrane is wrapped in clear food wrapping paper. The wrapped membrane can then be transferred to an X-ray film-developing cartridge or to a gel doc system. Care should be taken to have the protein side of the membrane facing the film or camera. We use ECL Hyperfilm (Amersham Life Science). Data analysis of the bands can be done using a desktop scanner or a gel doc system to acquire the data from the film and then process the data with computer software that can quantify densiometric data.

Membrane Stripping

Further experiments can also be done with the membranes by stripping them of the antibodies and reblocking and reprobing with another set of antibodies. We have had good success with the following stripping procedure. The blot is washed in 100 ml of 0.1 M citrate/pH 3.5 for 10 min and then rewashed in 100 ml of Tris-buffered saline (TBS) containing 0.5 M Tris/pH 7.5, 0.05 M NaCl, and 0.5% (v/v) IGEPAL (Sigma Chemical Co., St Louis, MO). These two wash steps are repeated two more times. After these wash steps, the membranes are rinsed another two times, 10 min each with TBS containing 0.15 M Tris/pH 7.5 and 0.015 M NaCl. Note the IGEPAL is not used in the final two washes and electrophoresis-purity reagents are used for all other ingredients. The membranes are now ready to be reblocked and reprobed.

TABLE 2

Some Commercial Sources of Various Connexin Antibodies

Connexin type	Source[a]	
	Polyclonal	Monoclonal
Cx30.3	ADI	
Cx31	ADI	
Cx31.1	ADI	
Cx32	ADI, CI, Biogen, Zym	CI, Zym
Cx33	ADI	
Cx37	ADI	
Cx40	ADI	
Cx43	ADI, CI, Zym	CI, TL, Zym
Cx45	ADI, CI	
Cx46	ADI	
Cx50	ADI	

[a] Alpha Diagnostics International, San Antonio, TX (ADI); Biogenesis LTD, Poole, United Kingdom; (Biogen); Chemicon International Inc., Temecula, CA (CI); Tranduction Laboratories, Lexington, KY (TL); Zymed Laboratories, South San Francisco, CA (Zym).

A.11.c. Discussion

Western blot analysis is an excellent way to screen cells for connexins. Currently several companies offer an assortment of primary antibodies to various connexins. Table 2 lists some of the commercially available antibodies. Western blot analysis should complement Northern blot analysis since the RNA message is not always translated. Western blot analysis can also be useful in determining some posttranslational changes. For example, the carboxy terminus of Cx43 has multiple phosphorylation sites that alter the electrophoretic mobility of the connexin. Figure 9 illustrates the three major isoforms of Cx43 typically seen in WB cells, which are normally termed P_0, P_1, and P_2. Pretreating the protein sample with phosphatase results in only the P_0 band, indicating that P_1 and P_2 are two different phosphorylation states of Cx43. Treatment with TPA results in hyperphosphorylation of Cx43 (Fig. 9) where the P_0 band disappears and multiple bands above P_2 appear. Treatment of the protein samples isolated from TPA-treated cells with phosphatase also results in only a P_0 band (data not shown). However, one must be cautious in the interpretation of the data. Although a correlation between GJIC and the change in phosphorylation exists, this does not necessarily indicate a cause and effect. Hyperphosphorylation of Cx43 has been observed to occur in cells with normal GJIC (43).

In conclusion, Western blots offer a sensitive and specific technique to identify various types and forms of

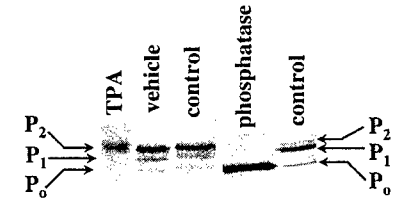


FIG. 9. Western blot analysis of Cx43 from WB-F344 rat liver epithelial cells. WB-F344 cells were treated with TPA (10 ng/ml, 37°C, for 15 min), or vehicle in the absence of TPA (vehicle), or calf intestinal alkaline phosphatase (10 units for 1 h, 37°C). Untreated cells were used as a control. P_0, Unphosphorylated; P_1 and P_2, phosphorylated species of connexin 43.

connexins. In the future, this technique should produce many more fruitful results concerning the biology of gap junctions and the relevance of GJIC to human diseases.

B. Isolation of Human Breast Epithelial Stem Cells

B.1. Background

Stem cells are undifferentiated cells capable of (a) proliferation, (b) self-maintenance, (c) production of a large number of differentiated functional progeny, and (d) regeneration of the tissue after injury and a flexibility in the use of these options (44). In essence, the most basic characteristics of stem cells seem to be the extensive capacity for self-renewal and the ability to generate differentiated daughter cells. Besides these two features, stem cells have been reported to be radiosensitive (45), slow cycling in cell division (46), and contact insensitive (47). Some specific genes have been shown to be expressed in certain stem cells (e.g., $\alpha_2\beta_1$ integrins with epidermal stem cells, α-fetoprotein with liver stem cells, and $CD34^+$ with hematopoietic stem cells). Stem cells may be identified by these markers or other methods such as colony-forming ability, extended time of label retention, and contact-insensitive growth (47, 48). The human breast epithelial cells (HBECs) with stem cell characteristics (Type I HBECs) to be described in this section were discovered by a distinguishable cell and colony morphology associated with these cells that is different from the conventional cell type (Type II HBECs). After characterization, Type I HBECs were found to possess major stem cell features, i.e., the ability to differentiate into other cell types by cyclic AMP-inducing agents and to form ductal and terminal end bud-like structures where mammary stem cells are believed to be located (49, 50).

B.2. Materials

B.2.a. MSU-1 Medium

This medium is a 1:1 mixture (v/v) of a modified Eagle's minimal essential medium (MEM) and a modified MCDB 153 supplemented with human recombinant epidermal growth factor (0.5 ng/ml), insulin (5μg/ml), hydrocortisone (0.5 μg/ml), human transferrin (5 μg/ml), 3,3′,5-triiodo-L-thyronine (1×10^{-8} M) and 17β-estradiol (1×10^{-8} M) (49).

The MSU-1 powder medium without growth factor/hormones and $NaHCO_3$ in 10-liter package prepared by commercial companies is first dissolved in 3 liters Milli-Q-purified water and titrated to pH 6.5 before adding 10.88 g of $NaHCO_3$ and the remaining water and filter-sterilized. The growth factors/hormones are prepared as follows:

Recombinant human EGF (Sigma): Dissolve in PBS as 100 μg/ml stock, then dilute to 10 μg/ml, aliquot in 25 μl/vial, and store in a freezer. Add 25 μl (10 μg/ml) to 500 ml medium.

Insulin (Sigma): Add 19.9 ml sterile H_2O and 0.1 ml glacial acetic acid into a 100-mg bottle; invert and shake to dissolve. Add 0.5 ml (5 mg/ml) to 500 ml medium.

Hydrocortisone (Sigma): Prepare 1 mg/ml in ethanol. Add 0.25 ml (1 mg/ml) to 500 ml medium.

Human transferrin (Sigma): Dissolve 2 mg/ml in PBS and filter-sterilize. Add 1 ml (2 mg/ml) to 500 ml medium.

3,3′,5-triiodo-DL-thyronine (Sigma): Prepare 1 mM (0.65 mg/ml in 0.05 N NaOH) stock, then dilute 100× to 1×10^{-5} M. Add 0.5 ml (1×10^{-5} M) to 500 ml medium.

17β-Estradiol (Sigma): Prepare 1×10^{-4} M stock (1 mg in 18.36 ml ethanol and 18.36 ml PBS). Add 50 μl (1×10^{-4} m) to 500 ml medium.

The MSU-1 medium can support the growth of both Type I and Type II HBECs. However, Type I HBECs may grow faster in MSU-1 medium supplemented with 5% fetal bovine serum (referred to as Type I medium) and Type II HBECs grow better in MSU-1 medium supplemented with 0.4% bovine pituitary extract (referred to as Type II medium).

B.2.b. Collagenase Solution

Collagenase (Sigma, Type 1A) is dissolved in the MSU-1 medium (2.5 mg/ml) and filter-sterilized.

B.3. Establishment of Normal Human Breast Epithelial Cell Culture from Reduction Mammoplasty Tissues

1. Transport tissues in Type I medium using 50-ml centrifuge tubes or other sterile containers on ice.
2. Scrape and remove most of the soft adipose tissues, then mince the remaining tissues into small pieces with a scalpel.
3. Digest tissues in collagenase solution in Erlenmeyer flasks at 37°C in a water bath overnight (1 g tissue in 10 ml collagenase solution).
4. Centrifuge to remove the collagenase solution.
5. Wash the cell pellet with MSU-1 medium and centrifuge to remove the medium.
6. Culture cells in Type I medium in 75- or 150-cm^2 flasks. Allow 2 h for fibroblasts to attach, then transfer cell and cell aggregates in medium to new flasks.
7. After overnight incubation, change to MSU-1 medium.
8. Change medium every 2 days for 1 week (floating tissues or cell aggregates in medium should be collected by centrifugation and replated during medium changes). Store cells in liquid nitrogen in PBS + 10% DMSO.

At Day 5 or 6 after the initiation of culture, fibroblasts may be selectively removed with 5× diluted

trypsin (0.002%) and EDTA (0.02%) from the culture containing both epithelial and fibroblast cells. This procedure works only for the initial culture but not for the subsequent subculture.

Example. Fifty grams of tissue digested in 500 ml collagenase solution overnight was plated in two 150-cm^2 flasks for 2 h for fibroblasts to attach. The floating tissue in Type I medium was transferred to two 150-cm^2 flasks (A) and incubated overnight. The floating tissue in flasks A was centrifuged and transferred to two new 150-cm^2 flasks (B). Both flasks A and B developed Type I and Type II HBECs in MSU-1 medium.

B.4. Starting Normal HBEC Culture from Passage I Cells Stored in Liquid Nitrogen

1. Thaw cells in a vial at 37°C.
2. Add cells in freezing solution (0.8 ml) to a plate with 10 ml Type I medium and inoculate in a 9-cm plate (plate A). Incubate for 2 h in a CO_2 incubator.
3. Transfer unattached cells to a 15-ml tube and centrifuge (1000 rpm, 8 min); add 8 ml Type I medium to plate A.
4. Suspend pelleted cells in 8 ml Type II medium and inoculate in a new plate (plate B); incubate both plates A and B in a CO_2 incubator.
5. After 1 day incubation, shake medium in plate B and immediately transfer the medium containing suspended cells to a 15-ml tube and centrifuge; add 8 ml Type I medium to the pelleted cells, disperse the cells, and transfer to a new plate (plate C). Add 8 ml Type II medium to plate B. Plate B may be washed the second time with agitation using Type I medium to remove loosely attached Type I HBECs (plate C^1).
6. Incubate in a 37°C, 5% CO_2 incubator; renew medium once every 3 days. Plates B and C (C^1) will contain Type II and Type I HBECs respectively, while plate A may be a source for stromal fibroblasts.

B.5. Characterization of Presumptive Human Breast Epithelial Stem Cells

The two types of HBECs have been extensively characterized and were found to differ substantially in phenotype (49, 51, 52). Besides morphological differences, the two types of cells can be separated and grown in pure cultures by two features: differential sensitivity to FBS and early or late attachment on plastic after trypsinization and subculture. Human breast cancers exhibit luminal epithelial cell markers (i.e., epithelial membrane antigen and cytokeratin 18) instead of basal epithelial cell markers (cytokeratin 14 and α_6-integrin) and are predominantly estrogen receptor (ER) positive and deficient in GJIC. These characteristics were shared by Type I but not Type II HBECs. Significantly, Type I HBECs show stem cell characteristics, i.e., the ability to differentiate into other cell types by cyclic AMP inducing agents and to form budding/ductal structures in Matrigel. According to the stem cell theory of carcinogenesis, stem cells give rise to cancer cells by blocking their differentiation and preserve the undifferentiated characteristics of stem cells in cancer cells (53).

TABLE 3

Major Phenotypic Differences between Type I and Type II HBECs

Parameter	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobblestone 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	−	+
α_6-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

We found that Type I HBECs were indeed more susceptible to neoplastic transformation than Type II HBECs. In response to an oncogenic stimulus (SV40 large T antigen), Type I HBECs acquired anchorage-independent growth which was not found for Type II HBECs (6). Furthermore, Type I HBECs were dramatically more susceptible to telomerase activation and immortalization after transformation by SV40 (54). These results support the concept of cancer as oncogeny as blocked or partially blocked ontogeny (14). The major phenotypic differences between Type I and Type II HBECs are listed in Table 3.

DISCUSSION

The hypothesis that the stem cell is a target cell for carcinogenesis was the motivating rationale to try to isolate human stem cells. In our quest to do so, we have isolated two human epithelial pluripotent stem cells [human kidney (47) and human breast (49)]. These two cell types are characterized by their lack of connexin expression and lack of functional GJIC. Additionally, one other presumptive pluripotent stem cell has been reported to lack functional GJIC (55). In addition, the totipotent stem cell, the fertilized egg, lacks GJIC (18). With the majority of evidence showing that most malignant cancer cells, which lack growth control, do not terminally differentiate and readily apoptose and lack functional homologous or heterologous GJIC (3), it seems that a normal stem cell ought to be similar to tumor cells, except for alterations in oncogenes and tumor suppressor genes.

Apparently, the major difference between the two is that the normal stem cell can be readily induced to express its connexin genes and form functional GJIC, whereas the carcinogenic process has rendered GJIC dysfunctional (3). Some cancer cells, such as HeLa, normally have their connexin genes suppressed at the transcription levels (56). However, given the right microenvironmental conditions, a connexin gene can be induced to restore GJIC and growth control (57, 58). Other tumor cells have GJIC disrupted either because of some mutation in their connexin or in some gene that regulates its synthesis, transport, assembly, and function (3).

With the isolation of pluripotent stem cells that appear to be characterized, in part, by the lack of expressed connexins and functional GJIC, it will now be possible to (a) study the mechanism by which the connexin genes are regulated and how the expression of those connexin genes might be related to pluripotent stem cell differentiation; (b) genetically engineer these stem cells for potential therapy of various human heredity diseases; (c) generate cells for tissue replacement of damaged or diseased organs; (d) screen for potential therapeutic or toxic chemicals that could alter the differentiation of stem cells; and (e) study the carcinogenesis of human stem cells when induced to form three-dimensional "organoids."

Finally, with the isolation of human stem cells that are characterized by the lack of connexin gene expression and functional GJIC, it should be possible to determine the controlling mechanisms that give a stem cell the ability to divide symmetrically to expand its numbers or to divide asymmetrically to differentiate yet retain one daughter cell with the ability to maintain stem cell ability. One hypothesis to explain these properties of stem cells seems to be emerging from our studies. These hypotheses can be viewed in Figs. 10A and 10B. In brief, our recent observations have shown that the pluripotent stem cells, when isolated and grown on traditional plasticware, can divide symmetrically for a number of divisions. Yet when these pluripotent stem cells are placed on a more "natural" substrate (i.e., Matrigel), they can form three-dimensional organoids that exhibit the original pluripotent stem cell and their differentiated daughter in a structure that mimics the structure found in the tissue from which it was originally isolated.

This implies that the stem cell–plastic surface interaction sends a signal within the stem cell to divide symmetrically (division plane being vertical) so that both daughters remain attached to the plastic surface. Both daughters receive the same signal as the mother stem cell before division. On the other hand, when the

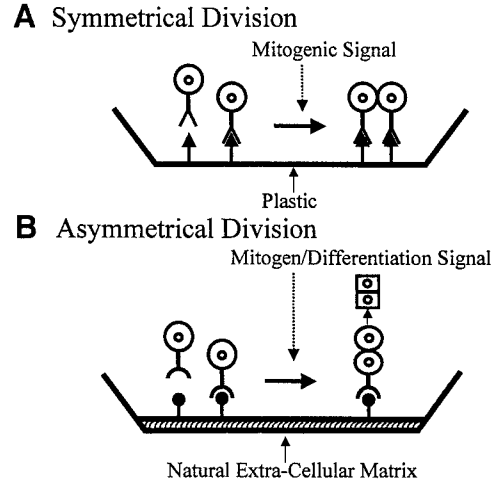


FIG. 10. Diagram illustrating how toti- or pluripotent stems might regulate symmetrical and asymmetrical cell division. (A) Stem cells attached to a particular matrix might receive a cell matrix signal to have its division plane vertical to the substrate giving rise to two cells, both of which are still attached to substrate. (B) The stem cell is attached to another substrate which sends a cell matrix signal to have the cell division plane horizontal to substrate. In this case, one daughter cell remains attached to substrate while the other is free of this signal as there is no GJIC between the daughters.

stem cell attaches to a more "natural" substrate such as collagen, laminin, or fibronectin, the cell divides asymmetrically (the division plane being horizontal to surface) so that one daughter still remains attached to the original surface and the other daughter is detached from that surface. This implies that the one cell still receives the original signal to remain "stem," while the other is now "free" of this restraint. Since there are no expressed gap junctions in the stem cells, there would be no transfer of these substrate–cell signals from one daughter to the sister daughter. This freedom from the gap junction suppression allows the daughter cell on top of its attached, but non-GJIC-coupled sister to express connexin genes and to differentiate.

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Roles of Ionising Radiation in Neoplastic Transformation of Human Breast Epithelial Cells

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Introduction

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

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

Ionising Radiation Affects Different Stages of Carcinogenesis

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

An immortal nontumourigenic cell line, derived from a normal human breast epithelial cell (HBEC) type with luminal and stem cell characteristics after transfection with SV40 large T antigen, can be converted by X rays (2 Gy $\times$ 2) into weakly tumourigenic cell lines which were selected as large fast-growing colonies in soft agar (11). These weakly tumourigenic cells, but not the parental immortal cells, responded to the ectopic expression of a mutated *neu* oncogene to enhance their tumourigenicity in nude mice. Although ERBB2 (also known as $p185^{C\text{-}erbB2}$) neu proteins are highly expressed in both immortal and X-ray transformed tumourigenic cell lines after the infection with the *neu* oncogene, only in the latter were the

ERBB2/neu proteins tyrosine phosphorylated (11). The mechanism for the differential response is not known. One possible mechanism is that a protein tyrosine phosphatase is present in the immortal cell line but not in the weakly tumourigenic cell lines transformed by X rays. The possible deletion of a protein tyrosine phosphatase gene such as the *PTEN* tumour suppressor (12) is to be investigated. In a different series of experiments, we found that normal human breast epithelial cells can be converted to cell lines with extended life span (i.e. bypassing senescence). It is clear that ionising radiation is capable of extending the life span, immortalising and developing tumourigenicity in HBECs.

Genetic and Epigenetic Effects of Ionising Radiation on Breast Carcinogenesis

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

Ataxia telangiectasia (AT) heterozygotes, who make up about 1% of the general population, have a 5.1-fold excess risk of breast cancer in women (15). The product of the *ATM* gene, which is mutated in patients with AT, has been shown to phosphorylate IKβ-α (16) and TP53 (17). This suggests a role of the *ATM* gene in NFKB activation and TP53 activation and cell growth control (16). Furthermore, AT cells were found to be more susceptible to the transcriptional activation of MYC and XRCC1 than other human cells after X irradiation (18).

Genes Affected by Ionising Radiation and Related to Neoplastic Transformation

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

There are only a few genes that are known to be mutated by ionising radiation and shown to be important for neoplastic progression. The *TP53* tumour suppressor gene appears to be the best known. Skin fibroblast cultures, derived from Li-Fraumeni syndrome, did not become immortal in culture but have been immortalised by X irradiation (one of six cultures) (19). These results suggest that loss of the wild-type *TP53* gene was necessary but not sufficient for immortalisation. It is interesting that the only human breast epithelial cell line immortalised by γ irradiation also lacked the TP53 tumour suppressor protein (10). However, the *TP53* mutation may not be the earliest tumour initiation event, since *TP53* mutations have been found in mammary ductal carcinoma in situ but not in epithelial hyperplasia (20).

Role of Stem Cell Differentiation in Breast Carcinogenesis

Epidemiological studies indicate that nulliparous or late parous increased the risk for breast cancer (21, 22); lifetime lactation reduced the risk (22). This could be due to the induced mammary gland differentiation (23). Furthermore, from the atomic bomb studies in Japan, younger women with undifferentiated mammary glands were found to be at higher risk for radiation-induced breast cancer (24).

Recently, it has been shown that normal HBEC cultures lacking the CDKN2A (also known as $p16^{INK4a}$) expression can be immortalised by ectopic expression of the

human telomerase gene (TERT), whereas HBEC cultures expressing the CDKN2A failed to do so (25). Using a different culture system, we also found that HBEC cultures (Type II cells) derived from reduction mammoplasty vary in the expression of CDKN2A. In our experiments to extend the life span of HBEC by X rays, we found that one out of four independent cultures was deficient in the expression of p CDKN2A. Interestingly, this CDKN2A-deficient culture is the only one able to bypass senescence after repeated X irradiation (average cumulative population doubling of 24 clones = 32) (Table 1 and Figure 1). These extended-life (EL) clones eventually stopped proliferating. The TP53 and CDKN1A were frequently and concomitantly elevated in these EL clones in Western blot analysis. However, they appear to contain the wild-type *TP53* since the cells showed radiation-induced G_1-phase arrest (C. J. Albert Liu, MS Thesis, Michigan State University, 1997).

TABLE I
Induction of Extended Life-span (EL) Clones from Different HBEC Cultures by X Irradiation

HBEC culture	CDKN2A	No. of experiments	X-ray treatment[a] (dose × times)	Independent EL clones obtained
HME-5	+[b]	2	2 Gy × 4	None
HME12	+	2	2 Gy × 4	None
HME14	+	3	2 Gy × 3	None
HME14	+	2	4 Gy + 2 Gy × 3	None
HME15	−	1	2 Gy × 4	11
HME15	−	1	2 Gy × 5	5
HME15	−	1	2 Gy × 6	6
HME15	−	1	4 Gy × 5	2

[a]Approximately 5×10^6 Type II HBECs were used in the initial treatment with X rays.
[b]CDKN2A expression determined by Western blot analysis shown in Figure 1.

Figure 1. Expression of CDKN2A ($p16^{INK4a}$) in different normal HBEC cultures.

We have previously developed two types of normal HBEC from reduction mammoplasty (26). Type I HBECs, in contrast to Type II cells that expressed a basal epithelial phenotype, were deficient in gap junctional intercellular communication, expressed oestrogen receptors and luminal epithelial cell markers, and showed stem cell characteristics (i.e. the ability to differentiate into another cell type and to form budding/ductal structures in Matrigel) (26–27). Although both cell types can be equally transformed by SV40 large T-antigen to acquire extended life span, they differ in the ability to become immortal (11/11 Type I EL clones became immortal compared to 1/10 for Type II EL clones). Both parental Type I and Type II cells, as well as their transformed EL clones at early passages (~30 cpd), showed a low level of

telomerase activity measured by the TRAP assay. For all the 11 Type I and 1 Type II EL clones that became immortal, telomerase activities were invariably activated at middle (~60 cpd) or late passage (~100 cpd). For the senescent EL clones, the telomerase activities were found to be diminished at mid-passage before the end of lifespan. Thus, Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalisation, a mechanism making them major target cells for breast carcinogenesis (W. Sun, et al. Cancer Res., in press). We plan to use this cell culture system to test if Type I HBECs with stem cell features are also more susceptible to neoplastic transformation by ionising radiation.

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A Human Breast Epithelial Cell Type with Stem Cell Characteristics as Target Cells for Carcinogenesis

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Chang, C. C., Sun, W., Saitoh, M., Tai, M-H. and Trosko, J. E. A Human Breast Epithelial Cell Type with Stem Cell Characteristics as Target Cells for Carcinogenesis. *Radiat. Res.* 155, 201–207 (2001).

Two types of human breast epithelial cells (HBEC) have been characterized. In contrast to Type II HBEC, which express basal epithelial cell phenotypes, Type I HBEC are deficient in gap junctional intercellular communication and are capable of anchorage-independent growth and of expressing luminal epithelial cell markers, estrogen receptors, and stem cell characteristics (i.e. the ability to differentiate into other cell types and to form budding/ductal organoids on Matrigel). A comparative study of these two types of cells has revealed a high susceptibility of Type I HBEC to immortalization by SV40 large T antigen, although both types of cells are equally capable of acquiring an extended life span (bypassing senescence) after transfection with SV40. The immortalization was accompanied by elevation of a low level of telomerase activity in the parental cells after mid-passage (~60 cumulative population doubling levels). Thus HBEC do have a low level of telomerase activity, and Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalization, a mechanism which might qualify them as target cells for breast carcinogenesis. The immortalized Type I HBEC can be converted to highly tumorigenic cells by further treatment with X rays (2 Gy × 2) and transfection with a mutated *ERBB2* (also known as *NEU*) oncogene, resulting in the expression of $p185^{ERBB2}$ which is tyrosine phosphorylated.

INTRODUCTION

Studies of carcinogenesis have shown that neoplastic transformation occurs in progressive stages (*1*). The process involves multiple pathways and multiple mechanisms[2] (*2*), and a tumor arises as a result of the accumulation of a series of genetic and epigenetic alterations in several oncogenes and tumor suppressor genes (*1*). Recent studies of chemopreventive agents (*3, 4*) and of *in vitro* transformation using different cell types (*5–7*) further confirm the role of differentiation in neoplastic transformation. Therefore, the primary goal of the study of carcinogenesis is not only to identify oncogenes/tumor suppressor genes that are frequently mutated in tumorigenesis but also to identify target cells that are more susceptible to neoplastic transformation. In this respect, the advantage of using the *in vitro* neoplastic transformation model to study the mechanisms of carcinogenesis is the feasibility of stepwise analysis of specific genetic and molecular alterations during the course of neoplastic transformation and the unambiguous identification of the roles of different cell types in carcinogenesis.

THE ROLE OF STEM CELL DIFFERENTIATION IN CARCINOGENESIS

Cancer cells are generally recognized as being in a relatively undifferentiated state (*8*). This undifferentiated state of tumor cells could be due to either dedifferentiation of a differentiated cell or the blocked differentiation of stem cells that give rise to tumor cells (*9*). The latter view is similar to the earlier concept of cancer as a disease of cell differentiation (*10*) or of "oncogeny as blocked or partially blocked ontogeny" (*11*). Furthermore, several cancers (i.e. chronic myelogenous leukemia, B-cell non-Hodgkin's lymphoma, and multiple myeloma) are known to contain markers [e.g. the t(9:22) Philadelphia chromosome] that can be traced to hematopoietic stem cells (*12–14*). Thus these cancers have been described as a disease of stem cells (*12*). The considerably higher cancer risk for the large intestine relative to the small intestine might be ascribed to the expression of BCL2 in the stem cell position in crypts of the large intestine but not the small intestine (*15*).

In breast cancer, there is evidence that stem cell proliferation and differentiation play an important role in carcinogenesis. First, epidemiological studies indicate that the lifetime risk of breast cancer developing in child-bearing women is linearly related to the age at which a woman has her first full-term pregnancy (*16*) and that breast cancer risk

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is higher in those who are nulliparous or late parous (*17, 18*). This has been hypothesized as being due to stem cell multiplication that commences at the time of puberty and occurs during each ovarian cycle until, but not after, the first pregnancy (*19*). Alternatively and more likely, pregnancy might induce full differentiation of the mammary gland, resulting in refractoriness of the gland to carcinogenesis (*20*). In animal experiments, the protective effect of pregnancy against chemical carcinogen-induced mammary cancer can be mimicked by short-term postcarcinogen treatment with pregnancy levels of estradiol and progesterone in nulliparous rats (*21*). The results suggest that these hormones eliminate or modify preneoplastic cells. Similarly, human chorionic gonadotropin (hCG) (*22*) and genistein can induce mammary gland differentiation in rats and reduce chemical carcinogen-induced mammary carcinogenesis (*23*). Second, in the studies of the effects of radiation from the atomic bombs in Japan, the frequency of ionizing radiation-induced breast cancers has been found to be inversely correlated with the age of the women at the time of exposure, indicating a higher breast cancer risk for the young undifferentiated mammary gland (*24*).

In *in vitro* studies, different HBEC types have been shown to respond differently to human papilloma virus (*5*), X rays (*25*), and the catalytic component of telomerase, TERT (*6*), in life-span extension (*25*) or immortalization (*5, 6*). A major determinant of whether these cells are rendered more susceptible to tumorigenic initiation has been found to be the nonexpression of CDKN2A/RB (*6, 25*).

GENE MUTATIONS IN HUMAN BREAST CANCER

For breast cancer, several oncogenes (i.e. *ERBB2*, *INT2*, *MYC*) and G_1-phase cyclins (D and E) were frequently found to be amplified or overexpressed (*26–30*). Loss of heterozygosity was frequently observed on many chromosome arms in breast cancer (i.e., 1p, 1q, 3p, 11p, 13q, 16q, 17p, 17q and 18q) (*31, 32*). Some hereditary breast cancer genes are known to be located on these chromosome arms (i.e. *BRCA1*, 17q; *BRCA2*, 13q; Li-Fraumeni syndrome and *TP53*, 17p). The FHIT (fragile histidine triad) at 3p14.2 and wnt-5a at 3p14-p21 are candidate breast tumor suppressor genes on chromosome 3p (*33, 34*). The gene encoding a cyclin kinase inhibitor, *CDKN1C* (also known as $p57^{KIP2}$), whose overexpression causes G_1-phase arrest, was cloned and mapped to chromosome 11p15.5 (*35*). The remaining breast cancer genes on chromosome 1, 16q and 18q have not been positively identified. In addition, four tumor suppressor genes are likely to be involved in breast carcinogenesis: *CDKN2A*, a cyclin-dependent kinase inhibitor (*36*); the maspin, a gene encoding a protein related to the serpin family of protease inhibitors (*37*); the *PTEN* on chromosome 10q23, a protein tyrosine phosphatase (*38*); and the *ATM* gene, which is mutated in ataxia telangiectasia, a breast cancer-prone syndrome (*39, 40*) and whose products phosphorylated NFKB1A (also known as IKBA) (*41*) and TP53 (*42, 43*). Similar to the observations for *BRCA1* and *BRCA2*, the *ATM* gene has been ruled out as a frequently mutated tumor suppressor gene in sporadic breast carcinomas (*44*). Yet loss of heterozygosity of these three genes has frequently been found in sporadic invasive ductal breast carcinoma (*45*), suggesting the presence of other putative tumor suppressor genes in their vicinity.

Since cancer cells may arise from different pathways[2] and may involve different combinations of oncogenes and tumor suppressor genes, the exact interaction and interdependency of the above-mentioned and other oncogenes and tumor suppressor genes in breast carcinogenesis are not known. However, *TP53* inactivation appears to play a key role in tumor initiation. Besides its frequent mutations in breast cancer, the three known breast cancer-prone syndromes, Li-Fraumeni syndrome, ataxia telangiectasia (*42*) and BRCA1 (*46*), are known to affect the function or regulation of *TP53* or *CDKN1A* (also known as $p21^{WAF1/CIP1}$). This is corroborated by *in vitro* studies that show that transfections with dominant-negative mutant *TP53* were able to immortalize HBEC (*47–49*). However, *TP53* mutation may not be the very first event in breast cancer initiation since the mutation was found in mammary ductal carcinoma *in situ* but not in epithelial hyperplasia (*50*). Alternatively, *TP53* may be inactivated by epigenetic mechanisms and trigger tumor initiation.

Besides alterations in oncogenes and tumor suppressor genes, recent studies have shown that telomerase activation (or a telomerase-independent alternative telomere lengthening mechanism) also plays a key role in immortalization, a critical and perhaps rate-limiting step in the development of most human cancers (*51*). Indeed, telomerase activity was detected in more than 95% of advanced-stage breast cancers (*52*).

All together, despite the complexity of the mechanism, the major events in carcinogenesis appear to involve (1) altered cell cycle regulation—bypassing cellular senescence → (2) telomerase activation—immortalization → (3) activation of a growth-promoting pathway—tumorigenic → (4) altered cell adhesion, mobility and protease/collagenase activity—invasion and metastasis. After tumor initiation, the accumulation of mutations or epigenetic changes could be facilitated by mechanisms of tumor promotion (*53*) and genomic instability (*54*).

IN VITRO NEOPLASTIC TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

HBECs have been immortalized by various methods using benzo(a)pyrene (*55*), SV40 large T antigen (*56* and references therein), human papilloma virus 16 E6 or E7 (*5*), radiation (*57*), mutant *TP53* (*47–49*), and the catalytic component of telomerase, TERT (*6*). In these studies, cell type differences (e.g. early compared to late passage) significantly affect the outcome of experiments (*5, 6*). Other than the viral oncogenes from SV40 or papilloma virus, the fre-

quencies of immortalization by other agents are generally very low. With the exception of experiments using our Type I HBEC (*7, 56*), none of the immortalized cell lines have been reported to express estrogen receptors and to be capable of anchorage-independent growth.

Spontaneously immortalized HBEC derived from fibrocystic mammary tissue or *in vitro* immortalized cell lines have been neoplastically transformed after benzo(a)pyrene treatment or *RAS* oncogene transfection (*58–60*). In our study, a Type I HBEC line immortalized by SV40 large T antigen and 5-bromodeoxyuridine/black light treatment was found to be tumorigenic in immune-deficient mice (*61*). The tumorigenicity of these cells can be further enhanced by the transfection with the *NEU* (now known as *ERBB2*) oncogene (*61*). In a different experiment, an SV40-immortalized cell line can be converted to weakly tumorigenic cells by X irradiation. The weakly tumorigenic cells became highly tumorigenic after transfection with a mutated *ERBB2* oncogene (*62*). These highly tumorigenic cells expressed an elevated level of $p185^{c\text{-}erbB2/neu}$ that was tyrosine phosphorylated. In contrast, the ectopic expression of $p185^{c\text{-}erbB2/neu}$ did not induce tumorigenicity in the immortal cell line. The $p185^{c\text{-}erbB2/neu}$ expressed in these cells was not tyrosine phosphorylated (*63*).

CHARACTERIZATION OF TWO TYPES OF HUMAN BREAST EPITHELIAL CELLS

We have previously developed a culture method for growing two morphologically distinguishable types of HBEC from reduction mammoplasty (*56*). These two types of cells are substantially different in many phenotypes (*7, 56, 61, 63*) as listed in Table 1. The most significant characteristics of Type I HBEC that are related to breast carcinogenesis are (a) the expression of estrogen receptors; (b) stem cell features (i.e. ability of Type I cells to differentiate into Type II cells and to form budding/ductal organoids on Matrigel); and (c) the high susceptibility to neoplastic transformation by an oncogenic stimulus (i.e. SV40 large T-antigen-induced anchorage-independent growth and a high frequency of immortalization) (*7, 56*).

Previously, we have found that Type I HBEC possess stem cell characteristics. The evidence came from the observation that Type I HBEC are deficient in gap junctional intercellular communication (GJIC) (*56*), similar to kidney and corneal stem cells (*64, 65*). Furthermore, Type I HBEC were capable of differentiating into Type II cells by cyclic AMP-inducing agents (i.e. forskolin and cholera toxin) (*56, 66*). The most convincing evidence, however, is the observation that Type I HBEC, in conjunction with Type II HBEC, form budding and ductal organoids in Matrigel (ref. *7* and Fig. 1) that are very similar to the human lobule Type I, as shown by Russo (*67*). These ductal structures can be formed overnight after the inoculation of a mixture of randomly distributed cells. After extended growth, lumen-like structures developed in these organoids. The Type II HBEC alone typically formed elongated and spherical organoids similar to the squamous metaplasia that developed in rat mammary cells in Matrigel (*68*).

Type I cells alone formed few budding (*7*) and acini structures similar to those formed by human luminal epithelial cells (*69*). Therefore, both luminal and stem cells are present in Type I HBEC cultures and are not morphologically distinguishable. Together with Type II HBEC, which

TABLE 1
Major Phenotypic Differences between Type I and Type II HBEC

	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobblestone appearance
Colony morphology	Boundary smooth and restricted	Boundary not smooth
Attachment on plastic surface after trypsinization	Late	Early
Effect of fetal bovine serum	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	Spherical and elongated structures
	Acini	
Anchorage-independent growth	+ (small colony/low frequency)	−
Response to SV40 large T antigen:	+ (large colony/high frequency)	−
Anchorage-independent growth	High frequency	Low frequency
Immortalization		

FIG. 1. Mammary organoid formed by mixing two types of human breast epithelial cells derived from reduction mammoplasty (Type I to Type II HBEC in 1:2 ratio) 1 day after inoculation of cells on Matrigel.

express basal epithelial cell phenotype, the three major breast epithelial cells in the mammary gland (stem, luminal, and basal or myoepithelial) are found in the two types of HBEC.

TYPE I HBEC WITH STEM CELL CHARACTERISTICS ARE MORE SUSCEPTIBLE TO TELOMERASE ACTIVATION AND IMMORTALIZATION

Since the two types of HBEC described above differ substantially in phenotypes and one of them shows stem cell characteristics, a comparative study was carried out to examine whether Type I HBEC are more susceptible to telomerase activation and immortalization after transfection with SV40 large T antigen (*7*). The results show that both types of cells acquire extended life span (i.e. bypassing senescence) at comparable frequencies. However, they differ significantly in their abilities to become immortal in continuous culture; i.e., 11 of 11 Type I extended life-span clones became immortal compared to 1 of 10 for Type II extended life-span clones. Both parental Type I and Type II cells as well as their transformed extended life-span clones at early passages (~30 cumulative population doubling levels) showed a low level of telomerase activity as measured by the TRAP assay. For all the 11 Type I and 1 Type II extended life-span clones that became immortal, telomerase activities were invariably activated at middle (~60 cumulative population doubling levels) or late passages (~100 cumulative population doubling levels). For the four Type II extended life-span clones selected randomly from the 9 Type II clones that did not become immortal, the telomerase activities were found to be diminished further at mid-passage before the end of their life span. Thus HBEC do have a low level of telomerase activity and Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalization, a potential mechanism to qualify these cells as major target cells for breast carcinogenesis.

The results of this study also provide some insight into and implications about breast carcinogenesis. First, unlike

previous reports that breast tissues (*70*) and HBEC (*6*) do not have telomerase activity, we found that both Type I and Type II HBEC do have a low level of telomerase activity, which needs to be elevated during immortalization. Second, a previous report concludes that telomerase activity may not be a biomarker for malignant transformation since it is present in both tumor and normal cells, including HBEC (*71*). This conclusion may be misleading since there was no quantitative comparison in that report and the comparison was made between tumor cells and unrelated normal cells. Using a well-characterized quantitative assay, we found that, although both primary breast epithelial cells and immortal or tumorigenic cells derived from them have telomerase activity, the activities in immortal and tumorigenic cells are significantly higher than that in primary cells. Third, the fact that SV40 large T antigen extends the life span (bypassing senescence) and telomerase activation effects immortalization further confirms that breast tumor initiation involves the tandem events of cell cycle deregulation and telomerase activation.

PERSPECTIVES

Our observations that Type I HBEC were more susceptible to telomerase activation and immortalization after transfection with SV40 large T antigen and that the resulting cells were capable of anchorage-independent growth and transformation to tumorigenic cells have implicated Type I cells as the major target cells for breast carcinogenesis. Furthermore, the similarity in phenotypes between Type I HBEC and breast carcinomas such as anchorage-independent growth, deficiency in GJIC, and expression of estrogen receptors and luminal epithelial cell markers (i.e. epithelial membrane antigen, cytokeratin 18, 19) supports the "oncogeny as blocked or partially blocked ontogeny" theory of carcinogenesis (*11*). However, our results based on transformation by SV40 need to be verified by experiments in which the cells are treated with other carcinogens. Recently, we found that co-transfection with a mutant *TP53* and the human *MYC* is a very effective method to extend the life span of HBEC. The resulting clones (some have more than 60 cumulative population doublings) express estrogen receptors and are capable of anchorage-independent growth. This method and other transformation methods will be used to test the hypothesis.

Although we have found the expression of variant and wild-type estrogen receptors in our Type I HBEC and in tumorigenic cell lines derived from them (*63*), the cell growth *in vitro* and tumor growth *in vivo* by these cells were not dependent on estrogen (our unpublished results). This may be because the cells had been transformed by SV40 large T antigen. Therefore, our goal is to obtain estrogen-responsive immortal cell lines by a different means of transformation. These cells will be very useful in other studies including testing the hypothesis that pregnancy levels of hormones (i.e. estradiol and progesterone) eliminate or modify premalignant mammary cells and mediate the protective effect of pregnancy against breast cancer (*21*).

The ability of the two types of HBEC to form mammary organoids in Matrigel similar to the human lobule Type I (*67*) suggests that these mammary organoids may be used as a surrogate for the human mammary gland to study the mechanisms of carcinogenesis as well as the regulation of mammary gland growth and differentiation in a more relevant cellular context. Furthermore, if Type I HBEC are target cells for breast carcinogenesis, they are also target cells for chemoprevention. Indeed, we have preliminary evidence that some potential chemopreventive agents such as genistein, vitamin D_3 and sphingosine are capable of inducing the differentiation of Type I HBEC into other cell types[3] (*72*). Therefore, Type I HBEC will be useful for studies of chemoprevention as well.

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Role of Stem Cells and Gap Junctional Intercellular Communication in Human Carcinogenesis

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Epidemiological data, experimental animal bioassays, studies of *in vitro* neoplastic transformation, and molecular oncology studies have implicated a multistage, multimechanism process in human carcinogenesis. From animal carcinogenesis studies, the operational concept of a single normal cell being irreversibly altered during the first step in carcinogenesis is called initiation. The subsequent interruptible or reversible clonal expansion of these initiated cells by non-cytotoxic mitogenic stimuli, compensatory hyperplasia due to cell death by necrosis, or inhibition of apoptosis is referred to as the promotion phase. Last, when one of these clonally expanded, initiated cells acquires sufficient genetic/epigenetic alterations to become neoplastically transformed and acquire the phenotypes of promoter independence, invasiveness and metastasis, it is referred to as the progression step of carcinogenesis. This report hypothesizes that the single normal cell that is initiated is a pluripotent stem cell. By assuming that the normal pluripotent stem cell is immortal and becomes mortal when induced to terminally differentiate, initiation would be viewed as the irreversible process by which a stable alteration in a finite number of proto-oncogenes and/or tumor suppressor genes could block terminal differentiation or "mortalization". Promotion would involve the reversible inhibition of gap junctional intercellular communication (GJIC) and while progression occurs with the stable down-regulation of GJIC.

INTRODUCTION

The use of information on mechanisms will be important for the estimation of risk from exposure to low-level radiations. Since detailed mechanisms of radiation-induced carcinogenesis in humans cannot be derived either from human epidemiological data (*1*) or from experimental whole-animal studies, *in vitro* neoplastic transformation systems using human cells will have to be developed which will approximate the epidemiological data and whole-animal results, since any human cell *in vitro* neoplastic transformation system will never completely mimic the situation *in vivo*. The objective of this speculative re-examination of concepts and hypotheses of carcinogenesis will be to try to put these different incomplete hypotheses into one new integrative theory. These hypotheses include: (a) the stem cell hypothesis or oncogeny as partially blocked ontogeny (*2*); (b) the multistage, multimechanism or initiation, promotion, progression hypothesis (*3*); (c) the oncogene/tumor suppressor gene hypothesis (*4*); and (d) the mutation/epigenetic hypothesis (*5*). The concept of gap junctional intercellular communication (GJIC) will be used to integrate these hypotheses (*6*). While there are additional cell–cell and cell–matrix interactions that do send messages that affect signal transduction in cells, this limited review will assume they can influence the effects of GJIC.

The fundamental idea is that there are only a few target cells that can give rise to a cancer, namely the pluripotent stem cells. If, after exposure to radiation, one of the genes that control the ability of a cell to multiply or to differentiate into a specialized cell (i.e. a proto-oncogene or tumor suppressor gene) is mutated, this stem cell is irreversibly changed in a way that it cannot terminally differentiate but has the potential to proliferate. This step is referred to as the initiation step. However, if this initiated stem cell is surrounded by, and communicating with, its normal neighbor by GJIC, it will be suppressed and will not form a tumor. At this stage, the stem cell, the oncogene/tumor suppressor gene, and the mutation hypotheses are integrated by the GJIC concept. If the suppressed, initiated stem cell, which is now partially differentiated, is exposed to agents or conditions that inhibit GJIC, these initiated cells can now proliferate. The mitogenic stimuli can be either non-cytotoxic mitogens (e.g. growth factors, cytokines, hormones) or the consequences of cytotoxic agents that induce compensatory hyperplasia through cell death by necrosis. In addition, the blockage of cell death by apoptosis could prevent the elimination of initiated cells and increase the initiated cell population (*7*). In other words, the clonal expansion of initiated cells (promotion) is probably the result of an increase in their cell birth and a decrease in cell death. Tumor promotion is the result of mitogenesis/blockage of apoptosis, not mutagenesis.

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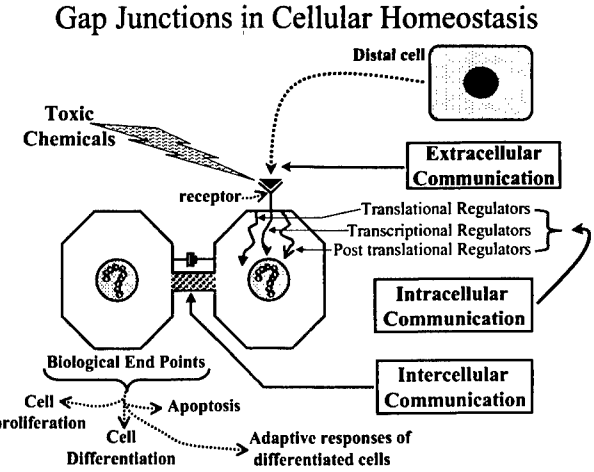

FIG. 1. Schematic diagram characterizing the postulated link between extracellular and intercellular communication through various intracellular transmembrane signaling mechanisms. Endogenous and exogenous extracellular signals which can trigger various intracellular signal transduction mechanisms can either increase or decrease gap junctional intercellular communication (GJIC) between cells in a multicellular organism. Cell proliferation, differentiation, apoptosis and adaptive responses of differentiated cells can occur as a consequence of the modulation of GJIC. (From Trosko *et al., Toxicol. Lett.* **102–103,** 71–78, 1998; with permission from Elsevier Science.)

Because these agents can alter gene expression at the transcriptional, translational or post-translational levels, they are referred to as epigenetic agents. After exposure to these promoters at threshold levels and above for long and regular periods (*8*), the initiated cells are multiplied into large clones of partially differentiated benign cells (e.g. papillomas of the skin, nodules of the breast, polyps in the colon, enzyme-altered foci of the liver). If one of these initiated cells in these benign tumors acquires additional changes in either oncogenes or tumor suppressor genes through either or both mutational and epigenetic changes that could lead to genomic instability, the benign initiated cell can become promoter-independent and be able to invade tissue and to metastasize to other organs. This would be referred to as the progression step of carcinogenesis.

The fundamental concept integrating these processes is the process of homeostasis. All 100 trillion cells of the human body (the few pluripotent stem cells, the progenitor stem cells, and terminally differentiated cells) talk to each other through hormones and growth factors (extracellular communication) which are secreted by one cell and travel over distance to bind to other cells. When this happens, various signal transduction systems are set off within the target cell (intracellular communication). These internal signals have at least two major functions; they alter the third form of communication (GJIC), and then they turn on or off various genes. The consequence of altering GJIC is either (a) cell proliferation, (b) cell differentiation, (c) programmed cell death or apoptosis, or (d) if terminally differentiated, alteration of its adaptive function (*6*) (Fig. 1).

Normal cells can control their growth, can terminally differentiate, can undergo apoptosis, and can perform their adaptive functions. They also have functional GJIC. Cancer cells do not have growth control, do not terminally differentiate, and cannot perform adaptive functions, nor do they undergo apoptosis normally. Cancer cells that have been tested do not have either functional homologous or heterologous GJIC.

EVOLUTION, GAP JUNCTIONS, AND MULTICELLULAR ORGANISMS

It was stated by T. Dobzhansky that "Nothing in biology makes sense except in the light of evolution" (*9*). This statement should serve as a framework to understand gap junctional intercellular communication and the roles it plays in the homeostatic regulation of cells in a multicellular organism and what happens when this fundamental biological process becomes dysfunctional (*10, 11*). Moreover, since the phenomenal advances in understanding some aspects of carcinogenesis have been made by highly specialized scientific endeavors of molecular biology, they have led many to believe that continued reductionalistic approaches to the study of living organisms (i.e. the Human Genome project) will eventually explain most, if not all, of the mechanisms that make humans human and will explain the basis for our many diseases, such as cancer. However, challenges to this philosophy (beyond reductionalism) (*12, 13*) suggest that there are processes not identified in the information found in the linear sequence of the 3 billion bases of the human genome.

When the transition from single-cell organisms to the first multicellular organism occurred, several new phenotypes appeared in these multicellular organisms which did not exist in single-cell organisms. These included (a) growth control, (b) cell differentiation, (c) programmed cell death or apoptosis, (d) synchronization of adaptive functions of terminally differentiated functions, and (e) appearance of a cell (the stem cell) that could divide either symmetrically or asymmetrically. Population control of single-cell organisms (cell proliferation) occurred through temperature and nutrient depletion. However, a new family of genes appeared in the early multicellular organism (*14*) to contribute to these new phenotypes. Included was the connexin family of genes that code for the proteins that self organize into a membrane-associated hemi-channel, the connexon, which links up to the neighboring cell's connexon to form a direct pathway for ions and small molecules (less than 1000 Da) to be shared by the cytoplasm of the coupled cells (*15*).

Most normal cells of multicellular organisms are coupled by one or more of the connexin proteins (the exception being the fertilized egg or totipotent cell; the single cells of the body such as the red blood cells, neutrophils, dividing cells and possibly pluripotent stem cells). The fertilized egg is the totipotent stem cell of a multicellular organism, and from studies of Drosophila, mouse and other multicel-

lular organisms, no GJIC is detected until the late blastula stage (*16*). Several human pluripotent epithelial stem cells (kidney, breast) (*17, 18*) and corneal epithelial stem cells (*19*) have been characterized as having no expressed connexins or functional GJIC. Normal cells expressing GJIC are characterized as having the ability to contact-inhibit, terminally differentiate, undergo apoptosis and, if differentiated, synchronize their adaptive functions.

On the other hand, cancer cells are characterized by not being able to contact-inhibit or have growth control, not being able to terminally differentiate, not having normal apoptosis function, and not having synchronized adaptive functions (*6*). Most if not all cancer cells lack functional homologous or heterologous GJIC (*20*). Therefore, the question that should arise is whether the appearance and function of connexin genes in the early multicellular organism is a cause or coincidence of the four cellular phenotypes (cell proliferation, differentiation, apoptosis, and adaptive responses of the differentiated cells) found in multicellular organisms, and whether the lack of functional GJIC is the cause or consequence of neoplastic transformation of human cells.

STEM CELLS, TELOMERASE AND IMMORTALITY

One of the major paradigms guiding research on transformation of human cells, using *in vitro* approaches within the paradigm of initiation/promotion/progression, is that there are two distinct hurdles that a normal, mortal cell must overcome before it becomes an immortal, malignant cell. In addition, two competing hypotheses relate to this paradigm of converting the mortal normal cell to an immortal, nontumorigenic cell. The stem cell hypothesis (*21*) and the de-differentiation hypothesis (*22*) relate to the idea that there exists a small pool of target cells which are susceptible to neoplastic transformation (the former hypothesis), while the latter assumes any and all cells, including all non-terminally differentiated cells, can de-differentiate (a universal characteristic found in all tumor cells of any organ). While the jury is still out in resolving this controversy, the evidence in lymphoreticular tumors seems to support the stem cell hypothesis (*23*). For tumors derived from mesenchymal or epithelial solid tissues, resistance to the stem cell hypothesis resides in the fact that pluripotent stem cells had not been isolated from individual tissues. Recently, with the isolation of stem cells from human kidney (*17*) and human breast (*18*) tissues, which have been characterized as not having expressed connexin genes or functional GJIC, as well as from various rodent tissues (*24*) and embryonic stem cells from early human embryos (*25, 26*), a new line of evidence can be used to support the stem cell hypothesis.

The new paradigm that emerges is that pluripotent stem cells are, by definition, immortal. They become mortal when they are induced to differentiate. Since tumors are characterized as having a clonal origin (*27*) and the cells of tumors are characterized as being immortal and lacking functional GJIC, which correlates with their inability to terminally differentiate and their inability to contact-inhibit, the tumor cells share many characteristics of the stem cell. Therefore, rather than a normal, mortal cell having to be immortalized first and then neoplastically transformed, the new paradigm views the process of carcinogenesis as first blocking the "mortalization" of a normal immortal pluripotent or progenitor stem cell. The initiation stage would then be seen as the irreversible alteration in a stem cell's ability to terminally differentiate or to become mortal (i.e., it stays immortal). At this time, it is not known how many, or which, proto-oncogenes or tumor suppressor genes must be activated or inactivated, respectively, to prevent a stem cell from terminally differentiating. The initiated cell is thus a stem cell that starts to differentiate but is partially blocked [oncogeny as partially blocked ontogeny (*2*)]. Clearly, assuming that functional GJIC is necessary for growth control and is necessary if not sufficient for terminal differentiation, then the fact that initiated cells are suppressed by surrounding and communicating normal cells indicates that promotion is the process by which the GJIC is reversibly inhibited to allow these cells to clonally expand (*28*). When additional genetic or epigenetic alterations stably inhibit GJIC (transcriptional down-regulation of connexin genes, prevention of connexin transport to membranes, altered phosphorylation of connexin proteins, altered gating of gap junctions), the cell can grow without inhibition, become genomically unstable, and acquire phenotypes needed for invasion and metastasis (the progression step) (Fig. 2).

To understand this hypothesis, the fundamental concept of a stem cell must be delineated. A stem cell is unique in that it must possess the property of having the ability to divide both symmetrically and asymmetrically (*29*). When a stem cell is stimulated [potentially from signals from the external environment (hormones, growth factors), cell–matrix interaction (fibronectin, collagen, etc.), cell–cell adhesion molecules, but not GJIC], depending on the net effect of those signals, the cell could divide either symmetrically to expand its numbers or asymmetrically to start one daughter cell down the pathway to terminal differentiation, while the other daughter maintains the property of being a stem cell. During the initiation phase of carcinogenesis, the immortal stem cell and various genes have been altered such that the signals needed for asymmetrical cell division have been inhibited but those for symmetrical division have not been. In other words, the cells can proliferate without terminally differentiating (Fig. 2). They remain immortal.

Recent observations that telomerase is needed to prevent telomere shortening and the loss of genes have led to the idea that senescence of the cell is the result of the inactivation of the telomerase gene (*30*). Reactivation of the telomerase activity in normal cells would lead to the immortalization process. Since in most normal somatic cells telomerase activity is low or nonexistent, and in most but not all tumor cells the telomerase activity is high and, of course, the tu-

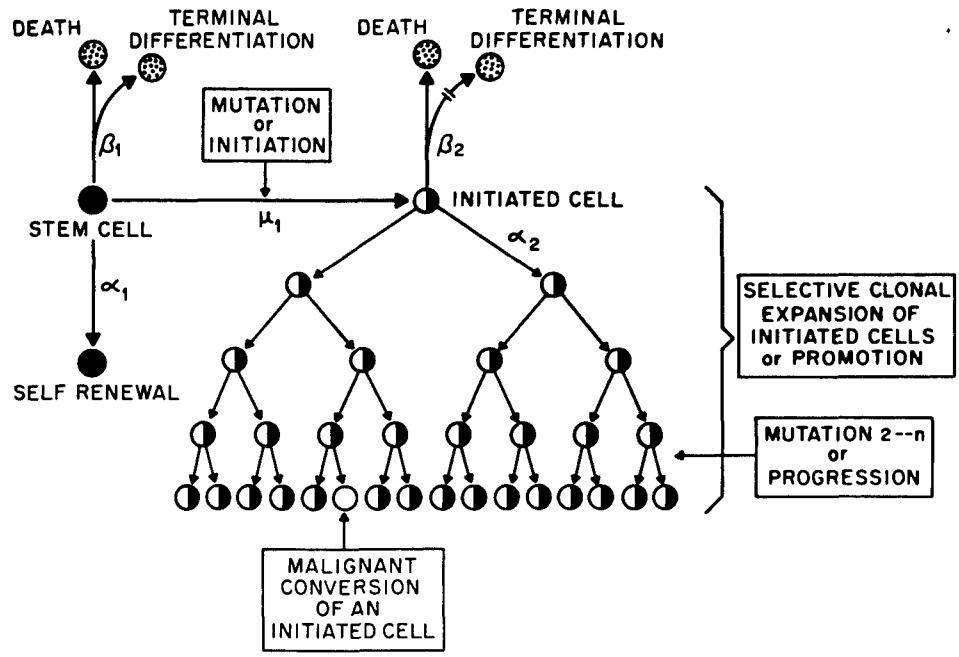

FIG. 2. The initiation/promotion/progression model of carcinogenesis. β_1 = rate of terminal differentiation and death of stem cell; β_2 = rate of death, but not of terminal differentiation of the initiated cell (—‖→); α_1 = rate of cell division of stem cells; α_2 = rate of cell division of initiated cells; μ_1 = rate of the molecular event leading to initiation (i.e. possibly mutation); μ_2 = rate at which second event occurs within an initiated cell. (From Trosko *et al.*, In *Modern Cell Biology*, Vol. 7, *Gap Junctions*, E. L. Hertzberg and R. G. Johnson, Eds., pp. 435–448, 1998; with permission from Alan R. Liss, New York.)

mor cell being immortal, leads one to believe that the process of carcinogenesis is one that fits the de-differentiation hypothesis. However, we have recently shown that the normal human breast epithelial pluripotent stem cells that we have developed have telomerase activity that is gradually lost in their differentiated daughter cells (*31*). These normal human breast epithelial pluripotent stem cells are readily immortalized (or more accurately, blocked from mortalization) and then are neoplastically transformed *in vitro* (*32*). These neoplastically transformed human breast stem cells share most of the phenotypic characterization of the original stem cell [lack of functional GJIC, estrogen receptors, telomerase activity etc. (*32*)]. In other words, the normal stem cell was not mortal. It was not immortalized during the initiation process by having its telomerase activated from an inactive state.

In general, all of the attempts to develop *in vitro* human fibroblast or epithelial neoplastic transformation systems by first immortalizing these cells by transfection with various viruses (SV40, E6, E7, papilloma genes) or with the catalytic telomerase gene (*33*), while generating interesting information, might not be relevant to the *in vivo* process of carcinogenesis in humans. When these techniques are used with primary cultures of human cells (which contain a few stem cells, many progenitor cells, and the terminally differentiated cells), it might be that these immortalizing viruses or genes rescue only the few stem cells in the population, not by immortalizing them (since they are, by definition, already immortal) but by preventing their mortalization. The progenitor and terminally differentiated cells might have their life span extended, but they are the ones going through crisis.

ROLE OF GJIC IN THE MODULATION OF RADIATION EFFECTS: ADAPTIVE RESPONSE AND THE BYSTANDER EFFECT

Leaving aside the differential radiation sensitivity/resistance intrinsic to the differentiated state of cells (stem cells in one tissue compared to another tissue, or stem cells compared to terminally differentiated cells) and the differential radiation sensitivity of the stages of the cell cycle (*34*), recent reports related to the adaptive response (*35*) and to bystander effects (*36*) have drawn attention to the role of gap junctional intercellular communication. In the former, exposure of cells to a low level of a radiation (or chemical) that was thought to damage DNA could, in many studies using different end points, reduce the effect of a subsequent higher dose than an exposure to a single high dose equivalent to the total of the two split doses. It is now well documented that low-level exposure to many types of radiation (UV light, ionizing radiation) can induce many signal transduction pathways (*37*), not necessarily by primary DNA damage triggering the response. In at least one case (*35*), it has been shown that gap junction intercellular communication was necessary for the adaptive response to occur, implying that radiation-induced signals in cells could

be transferred through gap junctions to neighboring cells. This suggests a damping effect of coupled cells.

Even more dramatic appears to be the phenomenon now known as the bystander effect. The phenomenon in radiation biology is where cells in a population that are not hit by a radiation particle (i.e. α particle) or photon (ionizing photon) but demonstrate signal transduction and specific gene activation. Evidence presented in this report suggests that this bystander effect can be transmitted either through extracellular release of molecules into the medium of irradiated cells that can affect nonirradiated cells or by direct transmission of signals from irradiated cells through gap junctions to unirradiated cells or from the irradiated cytoplasm to unirradiated nuclei of the same cell.

While not ignoring the extracellular bystander effect or the cytoplasm/nucleus transfer of radiation effects, the focus of future research will be on the gap junction-mediated bystander effects, because this might be relevant to the effect of low-level radiation exposures in tissues in which most cells are coupled by gap junction but stem cells do not gap junction-couple with their differentiated daughters. If low-level signal transduction signals can be transmitted to coupled neighboring cells, there could be either a damping effect of the radiation effect or possibly an opposite amplifying effect on non-hit cells. Most likely, the consequence of reducing or spreading signal transduction effects would be to alter gene expression (epigenetic effects). It should also be noted that the phenomenon of the bystander effect has implications in radio- and chemotherapy (*38–40*).

Moreover, while the term bystander effect is relatively new, the phenomenon on which the term is based is not. The fact that cells coupled by gap junctions could influence one another was first observed when normal cells with gap junctions could contact-inhibit cell division, while tumor cells without gap junctions could not (*41*). Furthermore, the concept of metabolic cooperation was observed when genetically different cells were cocultured (*42*). The concept that gap junctions could serve as a sink or point source between heterologous cells coupled by gap junctions (*43*) is old. The original observation that tumor-promoting chemicals could prevent metabolic cooperation between genetically different cells led to the idea that tumor promoters could block the suppression of initiated cells by their surrounding and gap junctionally coupled normal cells (*28*). Last, this could lead to the hypothesis that apoptosis could remove initiated cells from tissues (the strategy to develop new radiation and chemotherapies).

NEW STRATEGY FOR *IN VITRO* HUMAN TRANSFORMATION SYSTEMS

If the aforementioned ideas are correct (e.g. stem cell theory, multistep process of carcinogenesis; initiation blocks mortalization or terminal differentiation), then a strategy becomes clear. First, one should be able to isolate pluripotent stem cells from specific organs of consenting adults. Next, one should expose the stem cells to the initiating agent in question at various doses. After the exposure and time needed for mutation fixation, the exposed pluripotent stem cells should be forced to differentiate. The surviving noninitiated stem cells should be induced to become mortal (telomerase activity decreased) or to terminally differentiate. Those cells that remain capable of proliferating should be telomerase-positive and unable to become mortal (capable of maintaining telomeres). These immortal cells can then be used to identify additional genes needed to neoplastically transform these cells such that they ultimately lose GJIC and acquire invasive and metastatic phenotypes.

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