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BIOLOGICAL AND MOLECULAR BASIS OF HUMAN BREAST CANCER

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1. ABSTRACT

Human breast cancer remains the most common malignancy in the American women. The ultimate cure of this disease relies on a better understanding of the mechanisms underlying the initiation and progression of this disease. The neoplastic transformation of HBEC *in vitro* represents a successful model for obtaining knowledge on the molecular and biological alterations that may contribute to the tumorigenic mechanisms. We have presented here a current understanding of chemically transformed HBEC in the following aspects: 1. Factors affecting the transformation of HBEC such as genetic predisposition and differentiation status and prior immortalization; 2. New targets for studying the mechanism of cell immortalization such as alterations in telomerase activity and differential expression of cell cycle dependent genes as well as others recently isolated through differential cloning such as H-ferritin, and a calcium binding protein; 3. Epigenetic and genetic mechanisms underlying cell transformation; 4. The association of microsatellite instability in specific loci on chromosomes 11, 13, and 16 with the progression of cell transformation; and 5. The application of microcell mediated chromosome transfer technique as an approach to testing the functional role of specific genes whose dysregulation or loss of function may contribute to the ultimate cell transformation. Further efforts in this cell system will be directed to determine the roles of identified molecular changes as well as the mapping/cloning of tumor suppressor or senescence genes such as those that may reside on chromosomes 11 or 17.

2. INTRODUCTION

Breast cancer is the most common neoplastic disease in women with 180, 200 new cases reported annually (1). In spite of earlier detection and better treatment largely due to recent technologic advances, breast cancer is still the second leading cause of cancer-related death claiming 46, 000 lives of American women annually (2). Progress has been made in defining some of the critical processes associated with the development of breast cancer. However, the specific biochemical and molecular mechanisms underlying many of these complex carcinogenic events still remain to be elucidated. It is generally accepted that malignant transformation involves genetic and epigenetic changes that derail common regulatory mechanisms and result in uncontrolled cellular proliferation and/or aberrant programmed cell death or

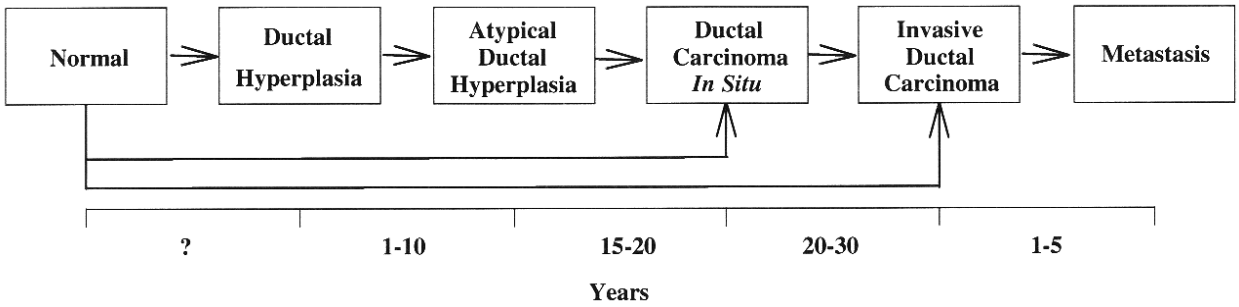

Figure 1. *In vivo* model of neoplastic progression of human breast carcinomas.

apoptosis (3, 4). These cellular abnormalities, hallmarks of a carcinogenic process, are frequently associated with molecular alterations involving activation of proto-oncogenes and inactivation of tumor suppressor genes as a result of genetic predisposition and/or exposure to physical (e.g., radiation), chemical (e.g., carcinogens, dietary components) and biological (e.g., viruses) environmental factors (5). A central challenge for cancer biology is to understand the cellular and molecular processes that drive normal human breast epithelial cells (HBEC) to neoplastic growth.

The vast majority of breast cancers are carcinomas, the malignant tumors of the epithelia. Based upon histological evaluations, development of breast cancer has been postulated to be a multi-step process and follows a defined sequence of qualitatively different events (6, 7) (Figure 1), as documented for a number of other malignancies (8, 9). In human breast, ductal hyperplasia and atypical ductal hyperplasia represent the initial stages of neoplastic growth and progress gradually to ductal carcinoma *in situ*, invasive ductal carcinoma and ultimate metastasis, even though normal cells could directly give rise to ductal carcinoma *in situ* or invasive ductal carcinoma (6, 10; Figure 1).

Analysis of pure populations of HBEC at various stages *en route* to malignancy would be the direct approach to understanding the cellular and molecular processes of breast carcinogenesis. However, primary cultures of HBEC from breast tissues at various neoplastic stages have been extremely difficult to establish and no cell lines at the intermediate stages of neoplastic transformation are available for mechanistic studies. The reasons for difficulties in establishing primary cultures of neoplastic breast cells are not fully understood and further refinements in culture techniques are clearly warranted for this experimental approach to be useful. On the other hand, primary culture of normal HBEC has been made possible by recent advances in tissue culture and, in some cases, normal HBEC lines have been successfully established (6, 11-14). Unlike rodent cells that are commonly used for oncogenic studies, normal HBEC do not exhibit spontaneous transformation *in vitro* and thus experimentally-induced transformation of normal HBEC *in vitro* has become a system of choice to elucidate the mechanism of breast carcinogenesis.

3. HUMAN BREAST EPITHELIAL CELLS IN CULTURE

It has been shown that the life span of HBEC cultured in adequate medium is comparable to that of adult human fibroblasts (30-40 doublings) (15, 16) and is profoundly affected by the concentration of calcium (Ca^{++}) in culture medium (13). Extended growth of HBEC for over 1000 days and more than 50 generations without expressing terminal differentiation has been maintained by lower Ca^{++} level in culture medium (13). HBEC cultured under low-Ca^{++} conditions maintain their normal diploid karyotype, form domes and duct-like structures in collagen, express specific keratin filaments and milk fat globule membrane antigen, and contain all the other structural features of breast epithelial cells (17-19). In addition, a higher number of doublings has been observed in HBEC derived from breast tissues with a lower differentiation grade and a higher proliferation rate (6), indicating that the growth characteristics of HBEC in primary culture reflect the *in vivo* degree of lobular development and the rate of cell proliferation *in vitro* (6).

Like all normal diploid and differentiated somatic cells, normal HBEC have a limited capacity to divide both *in vivo* and *in vitro*. Cellular mortality of normal HBEC is characterized by a progressive cessation of cell growth manifested in cell culture by senescence that typically occurs after 10-20 passages *in vitro* (approximately 100 to 200 population doublings) (13, 16, 20). In contrast, transformed or tumor cells are able to escape from senescence as a result of genetic and epigenetic changes that disrupt the regulatory mechanisms of limited growth potential and are thereby considered immortal (21). Induction of immortality or immortalization involves abrogation of cellular programs for limiting the rate and the number of cell replication (22) and is generally perceived as the key event of an oncogenic process (13, 23).

Spontaneous immortalization of HBEC is a rarely-occurring event. Numerous investigators have tried to induce immortalization of HBEC using various physical (e.g., radiation), chemical (e.g., benzo(a)pyrene) and biological (e.g., viruses, gene transfer) approaches, the last being the most consistent (20). Immortalization of HBEC has been successfully induced by introduction of the human papilloma virus 16 (HPV-16) oncogenes E6 and/or E7 (24-26), simian virus 40 (SV40) DNA (27). However,

Table 1. Expression of MDGI in relation to lobular differentiation in human breast

Case #	Age[a]	Lobule Type[b]	MDGI[c]
7	46	1	+/-
8	48	1	+/-
10	39	1	+/-
6	49	1/2	+
9	47	1/2	+/-
2	36	2	+
5	41	2/3	+
1	43	3	++
3	29	4	++++
4	35	4	++++

[a]Age of the donor at the time of reduction mammoplasty surgery.

[b]Lobules type 1, 2, 3, and 4, classified according to histopathogical criteria described previously (11).

[c]*In situ* hybridization with the antisense riboprobe of human MDGI. The intensity of the hybridization signal was scored as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++/++++).

immortalization of HBEC experimentally induced with viral oncogenes is often accompanied by expression of phenotypes indicative of neoplastic transformation, such as an increase in anchorage-independent growth and tumorigenesis in nude mice (27, 28). Clearly, a normal HBEC without expressing any transformed phenotypes is essential to any studies on experimentally-induced cell transformation.

We have reported that a mortal HBEC line #130, when cultured in medium containing 0.04 mM Ca^{++} (low Ca^{++}) for over 2 years, became immortalized spontaneously giving rise to MCF-10F (12, 17). Immortalization of these cells is characterized by their continuous growth in culture medium containing the conventional level of Ca^{++} (1.05 mM; also called high Ca^{++}) without entering senescence and expressing transformed phenotypes such as colony formation in agar or in agar-methocel (12, 13). Both the mortal and immortal cells are *bona fide* normal HBEC in nature, expressing genetic, cytogenetic, ultrastructural and phenotypic characteristics of normal human breast epithelia (12, 13) and represent cell lines closest to normal HBEC available.

The availability of normal HBEC in primary cultures, the mortal HBEC line (e.g., #130) and the immortal HBEC line MCF-10F provides us with a unique system to address some of the most important questions concerning the molecular mechanisms of cell transformation, such as: 1. What are the factors influencing susceptibility to cell transformation? 2. What are the molecular mechanisms responsible for cell immortalization? 3. What are the mechanisms associated with cell transformation?

4. FACTORS INFLUENCING SUSCEPTIBILITY OF HBEC TO CELL TRANSFORMATION

4.1. Lobular differentiation

Using our established *in vitro* system that allows us to efficiently culture HBEC whose growth characteristics *in vitro* reflect the *in vivo* degree of lobular development and the rate of cell proliferation (6), we tested on HBEC the transforming potential of chemical carcinogens known to be of etiological importance in various experimental models of mammary cancer (29). Our results indicate that HBEC of Lob 1, obtained from young nulliparous women, which are less differentiated, are more proliferative and more susceptible to be transformed by chemical carcinogens than those of the more differentiated Lob 3 from older and parous women (6). These results indicate that the susceptibility of HBEC *in vitro* is influenced by differentiation status of the breast *in vivo*. Whereas we do not have a complete elucidation of the genes involved in the process of mammary gland differentiation, we have recently cloned a gene, namely, mammary-derived growth inhibitor (MDGI), from the primary culture of HBEC (30). Using *in situ* hybridization techniques, we have demonstrated that the expression of MDGI is absent in the least differentiated Lob 1 and 2, low in the moderately differentiated Lob 3 and maximal in the most differentiated Lob 4 (30) (Table 1). In consideration of other observations that the MDGI locus, mapped to chromosome 1p21-23 (31), is frequently lost in breast tumors (32) and that MDGI expression is associated with tumor suppression in breast cancer cells (33), it is reasonable to speculate that MDGI might be a tumor suppressor and silenced in the very early stage of carcinogenesis. Overexpression of MDGI in the otherwise susceptible HBEC (e.g., MCF-10F cells) would confer resistance to chemical carcinogenesis in these cells. Experiments are currently in progress to test this hypothesis.

4.2. Genetic predisposition

The primary cultures obtained from women with no family history of breast cancer, when treated with the carcinogens *in vitro*, exhibit an increase in survival efficiency (11, 13), which is perceived to precede the acquisition of anchorage independence (34, 35). In contrast, treatment of HBEC from women with family history of breast cancer with the same carcinogens induces the formation of colonies in agar-methocel (14) (Figure 2). Colonies formed from the treated cells showed considerable anchorage-independent growth during the 21-day assay period (Figure 3). However, when individual colonies were isolated, they failed to grow. Since formation of colonies in agar is generally construed as indicating anchorage-independent growth, a hallmark of neoplastic cells, our results clearly showed that HBEC from women with familial history of breast cancer manifested phenotypic changes indicative of initial stages of neoplastic transformation in response to the carcinogen treatment. Since inherited and acquired (spontaneous and induced) genetic changes can predispose an individual to both premalignant and malignant transformation of a specific organ (36, 37), it is reasonable to speculate that genetic predisposition in women with familial history of breast cancer may confer inherited susceptibility to environmental chemical carcinogens.

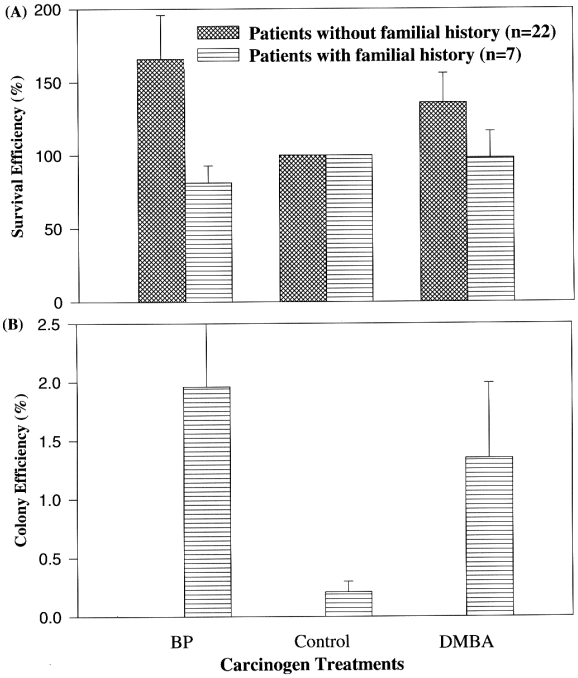

Figure 2. Survival and colony efficiencies in agar methocel of chemical carcinogen-treated HBEC *in vitro* (Reproduced with permission from Ref. 11).

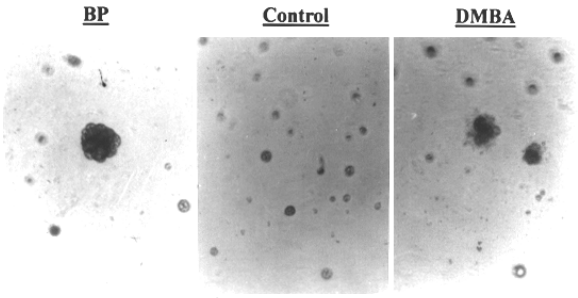

Figure 3. Colonies formed in agar methocel in chemical carcinogen-treated HBEC from women with familial history of breast cancer (Reproduced with permission from Ref. 11).

4.3. Cell immortalization

Since *in vitro* treatment of HBEC in primary cultures with chemical carcinogens failed to induce the full expression of malignant transformation, we decided to use the protocols developed for primary culture of HBEC with the immortalized cell line MCF-10F in order to elucidate whether immortalization is required for the expression of the full malignant phenotype. Upon treatment of MCF-10F cells with 7, 12-dimethylbenzo[α]anthracene (DMBA), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (NMU), or benzo[α]pyrene (BP), the treated cells showed altered morphology and altered pattern of growth, increased growth rate and anchorage independent growth in agar-methocel after 8-10 passages (around 157 days after the treatment). Control cells never developed colonies, whereas carcinogen-treated cells formed colonies from which the following clones were derived: D1, D2, and D3, from DMBA, M4 from MNNG, and BP1, BP2, BP5, BP6, BP7, and BP10 from BP treated cells (38) (Figure 4). All clones grew at a faster rate than their respective parental cells. Based upon this growth advantage, selected cell populations of clones BP1 and BP2 were isolated at approximately 446 days post-treatment; they were named BP1E and BP2B, respectively. BP1 and BP1E cells showed an increase in anchorage-independent growth, chemotaxis and invasiveness. From the D3 clone the D3-1 cell line was originated. It showed increased chemotactic and invasive capabilities, but to a lesser degree than BP1E. The tumorigenic potential of these cells was tested by inoculation into SCID mice. After 105 days after injection, mammary tumor developed from the BP1E cell line (Figure 4). None of the other BP clones or those derived from DMBA or MNNG-treated cells formed tumors (Table 2).

These results led us to conclude that chemical carcinogens induce the expression of the definitive neoplastic phenotype, tumorigenesis, as long as HBEC are immortalized prior to carcinogen exposure. This model allowed us to isolate clone of cells expressing different stages of progression to neoplastic transformation, which will certainly facilitate our studies on the molecular mechanisms of cell transformation. Specifically, our *in vitro* model will allow us to answer the following important questions: 1. Which is the molecular mechanism of cell immortalization? 2. Which are the genetic changes involved in the process of cell transformation? 3. Which are the genetic changes related to the process of tumorigenesis? 4. Which of the genes identified in the process of tumorigenesis are important to revert the process? and 5. Which of the genetic changes are relevant to the process of neoplastic development *in vivo*?

5. MOLECULAR MECHANISMS OF CELL IMMORTALIZATION

5.1. Activation of telomerase

There is evidence that the repetitive TTAGGG sequences located at the ends of human chromosomes (i.e., telomeres) may act as a molecular mitotic clock (39). It is generally believed that each successive genome replication is accompanied by gradual shortening of 50-200 bp due to incomplete replication of the 3' ends and cellular senescence occurs when telomeres reach a critically-short length that replication of the genome can not be maintained (22). Stabilization of the telomeric sequences at the ends of chromosomes, which is required for the continuous proliferation of immortal cells, involves the activation of the enzyme telomerase, which adds TTAGGG repeats to the 3' ends of chromosomes (40, 41). The genetic nature of cellular senescence implicates repression of telomerase as a key element of cell immortalization (41). Elevated levels of telomerase activities have been detected in a number of immortal cell lines and human tumor tissues (42, 43). Our observation indicates that cell telomerase is expressed in immortal MCF-10F cells but not in the mortal #130 cells, and telomere lengths that have become shorter in the #130 cells, have been maintained with no further shortening due to telomerase activation in the immortal MCF-10F cells (44), suggesting that telomerase reactivation may be one of

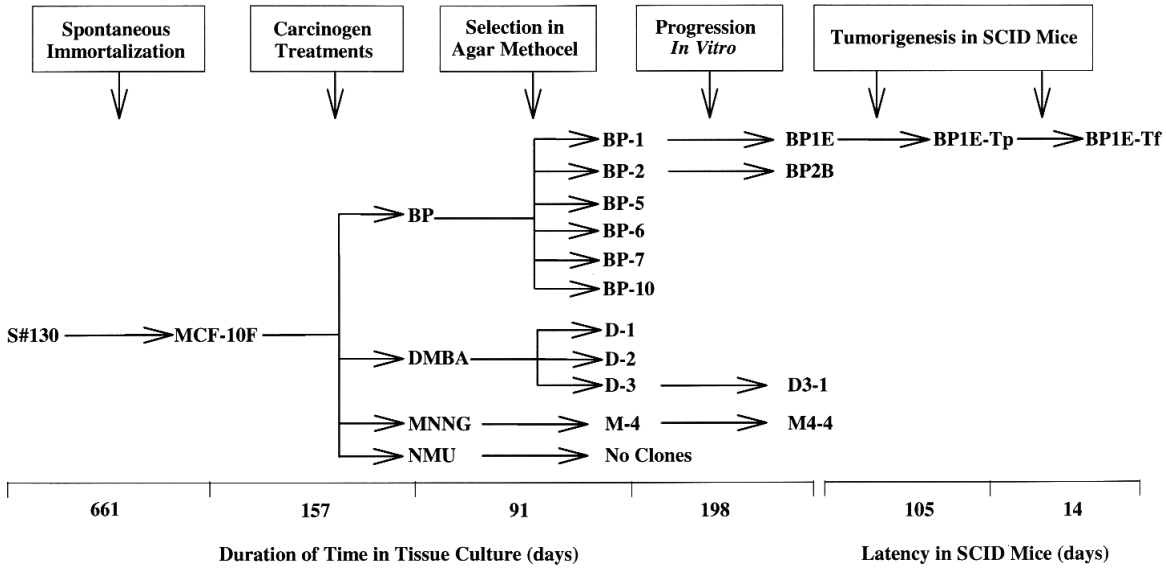

Figure 4. Diagrammatic representation of the evolution of MCF-10F cells treated with the chemical carcinogens.

the mechanisms leading to the spontaneous immortalization of MCF-10F cells. In addition, we have evidence that telomerase activity may be regulated by the Ca^{++} concentration in the medium (44).

5.2. Abrogation of cell cycle control

Result from cell fusion studies indicates that the phenotype of cellular senescence is dominant and immortality results from recessive changes in normal regulatory genes (45). Conceivably, inactivation of the genes that restrict cell cycle progression is essential to cell immortalization. Cyclin-dependent kinase (CDK) complexes and their inhibitors are essential components of cell cycle machinery, controlling cell cycle arrest in the G_1 phase of the cell cycle. Since p53 acts to regulate cell cycle progression through transcriptional activation of $p21^{WAF-1}$, an inhibitor of all G_1 CDKs (46), abrogation of p53 function has been implicated in the immortalization of HBEC. Insertional mutation at codon 247 of the p53 gene has been implicated in spontaneous immortalization of MCF-10F cells (47). Spontaneous immortalization *in vitro* has been observed in HBEC from a Li-Fraumeni patient with a point mutation in the p53 gene (41) and introduction of a single-amino acid deletion mutant (del239) of p53 gene abrogates wild-type p53-mediated cellular responses and induces immortalization of HBEC (48). A recent study indicates that alterations in p53 appear to be important in overcoming the M1 blockade (49). However, introduction of seven missense mutants of p53 genes failed to induce immortalization in the same cell line (48), even though all of these p53 mutants have been shown to abrogate p53-mediated transactivation in other cell types (50). In addition, the immortalized MCF-10F cells are still able to produce the wild-type p53 protein (7) and maintain wild-type p53-mediated functional responses, such as expression of $p21^{WAF-1}$ (51) and mdm2 (7). Therefore, the role of p53 in immortalization of HBEC needs further evaluation.

The CDK-4 inhibitor (CDKN2), commonly referred to as p16, is also an inhibitor of the cell cycle and has been localized to 9p21-22 (52). Homozygous deletion of this chromosomal subregion has been observed in the immortalized MCF-10F cells (53), which contain a balanced reciprocal translocation, t(3;9) (3p13;9p22) (ref 12). Similarly, loss of the 9p21 subregion has been correlated with the acquisition of an immortal phenotype of neoplastic human head and neck keratinocyte cell lines (54). Clearly, these results suggest a potential role of CDK2 in the control of immortalization of human breast epithelial cells.

Another inhibitor of the cell cycle, prohibitin, has been implicated in the process of cell immortalization (55, 56). Prohibitin gene is localized to chromosome 17q21 (57) where mutations have been reported in certain forms of breast cancer (58), suggesting that it may be a tumor suppressor gene. Lack of heterozygosity has been documented in immortalized cell lines (55). Expression of prohibitin gene produces a 30kD-protein that inhibits cell cycle transition and DNA synthesis in normal cells (59). The 3' untranslated region of prohibitin gene has been shown to function as a *trans*-acting regulatory RNA (i.e. riboregulator) crucial to its antiproliferative activity (56).

5.3. Genes preferentially expressed during cell immortalization

As further efforts to identify genes underlying the process of immortalization, we have performed subtractive hybridization and differential display analysis between immortal MCF-10F and its parental mortal #130 cells. Using a 10F(+)/130(-) subtractive cDNA library, we isolated more than 15 clones. Analysis of these clones showed that one of these clones contains sequences identical to H-ferritin (Figure 5) (60). Up-regulation of H-ferritin may be a source of iron necessary for growth and clonal expansion. Ferritin iron, once released, may

Table 2. Expression of transformed phenotypes in MCF-10F and its derivatives[1]

Phenotypes	#130	MCF-10F	BP1	BP1E
***In vitro* Growth**				
❑ Doubling time (hr)	N/A	93	80	44
Anchorage-Independent Growth				
❑ Survival Efficiency (%)	N/A	91.9 ± 35.0	N/A	N/A
❑ Colony Efficiency (%)	N/A	0	45 ± 12	88 ± 10
❑ Day 7 Colony Size (mm^2)	N/A	0	167 ± 26	687 ± 28
Duct-like Formation in Collagen Gel	N/A	+	+/-	-
Invasiveness Index in Matrigel	N/A	130 ± 7	457 ± 43	820 ± 181
Tumorigenesis in SCID Mice				
❑ No. Cells Injected (x 10^6)	N/A	7 - 30	20	10
❑ No. Animals w/ Tumors/Total No.	N/A	0/5	0/8	13/13
❑ Average Tumor Size (mm^3)	N/A	0	0	9.5
❑ Latency Period (days)	N/A	?	?	101

Notes: 1. This table is modified from ref. 38.

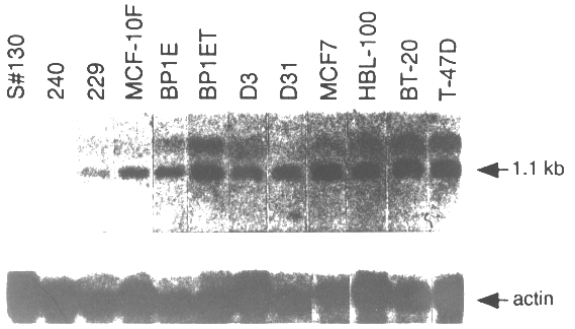

Figure 5. Differential expression of mRNA of H-ferritin in the transformed HBEC (Reproduced with permission from Ref. 60).

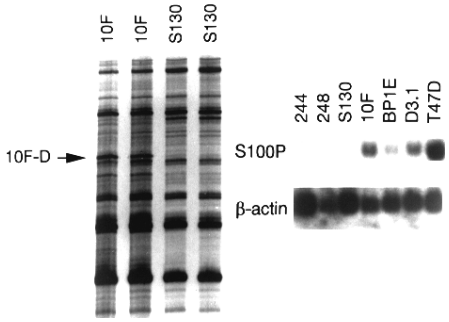

Figure 6. Northern blot hybridization using total RNA from immortal (MCF-10F), mortal (#130) and primary (#244) human breast epithelial cells showing a 0.6 kb transcript from the isolated calcium-binding protein gene (10F-D or S100P). The gene is expressed in the immortalized cells but not in the mortal or primary cells.

increase the level of reactive iron, leading to an increase in oxygen free radical generation, oxidative DNA damage and mutation. Amplification of H-ferritin gene (61) and overexpression of H-ferritin protein (62) have been associated with the progression of human breast cancer. The role of H-ferritin in the immortalization of human breast epithelial cells is unclear and will be fully evaluated by gene transfection studies.

Recently, we have observed an increase in the expression of a cDNA, namely, calcium binding protein (CaBP), in the immortalized cells by differential display analysis (Figure 6) (63). Sequence analysis revealed that the CaBP cDNA is S100P Ca^{++}-binding protein. Since Ca^{++} plays an important role in the spontaneous immortalization of MCF-10F human breast epithelial cells (13), it is conceivable that an increase in the expression of the S100P Ca^{++}-binding protein may facilitate the process of cell immortalization. Further characterization of other cDNA clones identified by differential analysis is still in progress.

6. MOLECULAR MECHANISMS OF CELL TRANSFORMATION

6.1. Epigenetic mechanisms

The support for epigenetic mechanisms mainly stems from the fact that the efficiency of cell transformation induced by chemical carcinogens far exceeds the rate of genomic mutations imposed by the treatment (64). This notion appears to receive further support from our observations that the expression of transformed phenotypes, such as increased colony efficiency in agar methocel, was maximal shortly after exposure to chemical carcinogens and declined gradually during subsequent passage of the treated cells (Figs 7 & 8). However, the possibility remains that the cells transformed via the genetic mechanisms were gradually overwhelmed by the surrounding non-transformed cells during prolonged culture. This possibility should be examined in a suitable system in the future.

6.2. Genetic mechanisms

While epigenetic mechanisms of cell transformation represent a valid alternative, genetic alterations are generally perceived as the cornerstone of neoplastic development. Conceivably, neoplastic transformation occurs as a consequence of cumulative genetic alterations in regulatory mechanisms influencing cellular proliferation and/or programmed cell death or apoptosis. To delineate the molecular mechanisms responsible for cell transformation, we studied the effects of the chemical carcinogens on the expression of bcl-2, an apoptosis inhibitor (65) that is highly expressed in breast carcinomas with a low apoptotic index (66, 67), and cyclin D1, a proliferation-associated gene which is frequently amplified or overexpressed in all forms of breast carcinoma (68-72). Our results indicated that the levels of bcl-2 and cyclin D1 expression were unaffected by chemical carcinogen treatments during the initial phases of cellular transformation *in vitro* (14). Therefore, the role of bcl-2 and cyclin D1 in the etiology of human breast cancer, if

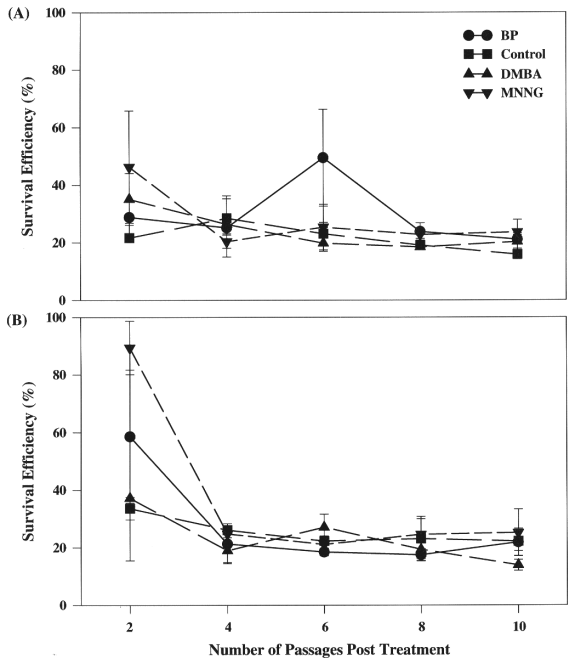

Figure 7. Survival efficiency of MCF-10F cells treated with the chemical carcinogens. MCF-10F cells were treated once (panel A) or twice (panel B) in a 7-day period. The treated cells were plated in agar methocel at every 2 passages after the treatment and allowed to grow anchorage-independently for 21 days.

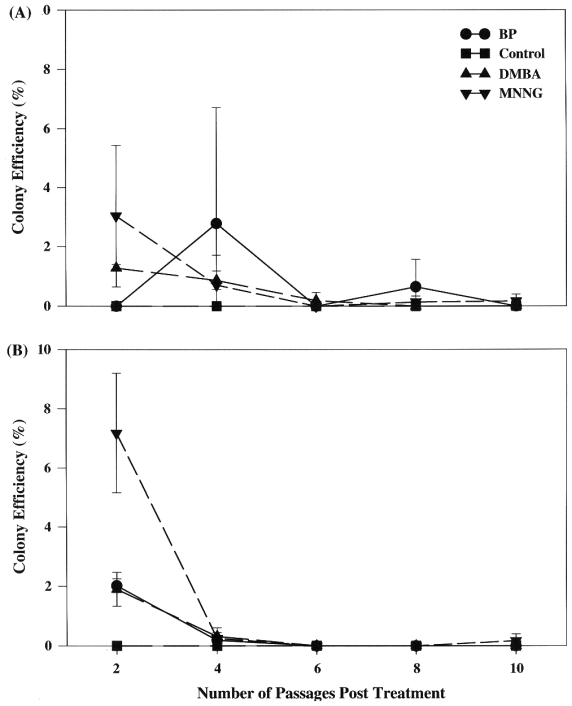

Figure 8. Colony efficiency of MCF-10F cells treated with the chemical carcinogens. MCF-10F cells were treated once (panel A) or twice (panel B) in a 7-day period. The treated cells were plated in agar methocel at every 2 passages after the treatment and allowed to grow anchorage-independently for 21 days.

any, appears to be subsequent to the initial stage of neoplastic transformation. The roles of other oncogenes and tumor suppressor genes in chemical carcinogenesis need to be evaluated.

7. GENOMIC CHANGES IN THE IMMORTALIZATION AND TRANSFORMATION OF HBEC

7.1. Genomic changes in cell immortalization

Senescence of human fibroblasts has been associated with genetic determinant(s) on chromosome 4 (73) and the long (q) arm of chromosome 1 (74). Karyotyping analyses of 5 spontaneously-immortalized human breast epithelial cell lines have identified several common chromosomal alterations including loss of chromosome 20p and gain of chromosome 1q (75). The most common genomic change in SV40-immortalized human cells is the loss of chromosome 6 (76). Recently, alterations at several other chromosomal loci (e.g. 20q13.2, 6q26-27) have been implicated in immortalization of various epithelial cells with viral oncogenes (77, 78). However, the nature and the function of genes located in these chromosomal loci remains to be defined. In our laboratory, we have determined that the immortalization of the MCF-10F is associated with the mutation at exon 7 of the *TP53* locus (47) (Figure 9), supporting the notion that inactivation or loss of function-mutation of the p53 gene is critical in the early stages of breast cancer progression (79, 80). In addition to further evaluation on the role of p53 in immortalization of HBEC, our laboratory is currently pursuing studies to determine further genomic changes such as microsatellite instability (MSI) and its underlying mechanisms that may play a role in the immortalization of HBEC.

7.2. Genomic changes in cell transformation

Genomic alterations have been recognized as a hallmark of cancer progression (81-85). The unstable changes of microsatellite, or very short simple repetitive sequences, designated as (CA)n, that are distributed throughout the genome (86, 87) represent such alterations. Its association with human malignancies has been extended from colorectal cancer (88-90) to neoplasms of the neck and head (91), lung (92), skeletal muscle (93), lympho-hematologic system (94), skin such as melanoma (95), prostate (96), gastrointestinal system (97), urinary bladder (98), liver (99), neurologic system (100, 101), cervix (102), endometrium (103), as well as breast (104-112). These findings indicate that MSI is associated with the general process of carcinogenesis.

MSI has been associated with the progression of breast cancer. However, its exact timing is controversial and its specific functional roles are not clear. It may be present as an early (105-108, 112), or a late event (113), or both (114), or not correlated (115, 116) during the breast carcinogenesis, dependent upon the markers (locus specific for each chromosome) used, and samples tested. This assumption is supported by the study of Aldaz et al (114) showing that some chromosomal loci might be involved early, while others late during the progression of human

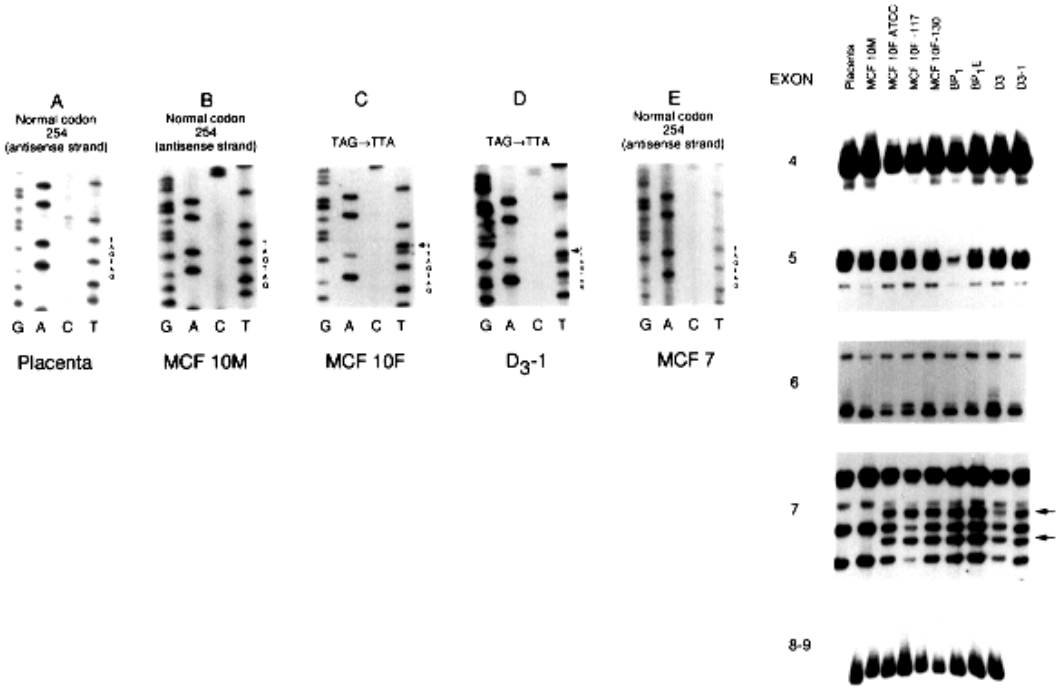

Figure 9. Figure 9 A-E. Direct DNA sequencing of the PCR amplified products generated from exon 7 of the p53 gene of A: placenta; B: MCF-10M cells; C: MCF-10F cells; D: D3-1 cells; and E: MCF-7 cells. An insertional mutation of a T at codon 254 was observed in MCF-10F (C) and its derivative D3-1 (D) cells, causing a frame shift following codon 254. Right-hand panel: PCR-SSCP analysis of exons 4-9 of the p53 gene. Exons 4, 5, 6 and 8-9 showed identical appearance in placenta and MCF-10M. A conformational shift in exon 7 was observed in three MCF-10F cell lines tested and the same shift was maintained in MCF-10F-derived transformed cell lines (BP1, BP1E, D3 and D3-1) (Reproduced with permission from Ref. 47).

breast cancers. Therefore, the elucidation of this genomic phenomenon can be further clarified by analysis with a comprehensive array of markers in an *in vitro* system such as ours that is free of affecting factors from variations among individuals and yet consists of various stages of cell transformation. More importantly, such studies may lead to the determination of underlying mechanisms such as defects of DNA mismatch repair genes that have been documented in colorectal, endometrial, ovarian, and prostate cancers (reviews in 117, 118), which have emerged as another type of factors as important as the tumor suppressor genes and oncogenes in breast carcinogenesis. Specifically, several questions exist: 1. Does MSI occur in preference to a particular chromosome(s) at a specific locus? 2. Is this instability really correlated with the phenotypic progression of human breast cancer and thus does occur in a sequential order? 3. What are the specific underlying mechanisms, such as mismatch repair gene defects, or DNA replication errors, or others?

We have pursued the first question by analyzing a total of 466 microsatellite loci on all the chromosomes in transformed HBECs representing the early and intermediate stages of cell transformation (119). These markers were selected to represent 38-96% the banded regions (according to the Human Genome Maps V) (120), taking into consideration of locations where tumor suppressor genes, oncogenes, DNA repair genes, and other cancer or cell growth regulation-associated genes are documented or postulated to be situated. Interestingly, we were able to detect MSI in only a very small number of loci; 0.64% (3/466), or 0.43% (2/466) of the markers analyzed were found in the BP-, or DMBA-transformed HBECs, respectively. These changes were exclusively found in the chromosomal regions of 11q25 at locus *D11S912* and 13q12-13 at loci *D13S260* and *D13S289* in the BP-cells, or the 13q12-13 region at loci *D13S260* and 16q12.1 at *D13S260* and 16q12.1 at *D16S285* in the DMBA-cells (119, 121) (Figure 10). Furthermore, the occurrence of MSI among these loci in the BP-cells seems to reflect a sequential order; i.e., 11q25 (*D11S912*) in the BP1 cells, followed by 13q12-13 (*D13S260*) and then another locus of 13q12-13 (*D13S289*) in the BP1E cells. However, this tendency is not seen in the DMBA cells. Our data have provided direct evidence that MSI is associated with the early and intermediate transformation of HBEC, during which only a very small proportion of loci are affected, and that the involvement of these loci on chromosomes 11 and 13 may be correlated with the progression of HBEC transformation *in vitro*. This finding supports that carcinogen-transformed HBECs, and presumably sporadic breast carcinomas, are also characterized with a mutator phenotype (122), that appears early as a driving force in tumor progression (review in 84, 123).

Table 3. Microsatellite instability in human breast lesions

MARKER	Type	TOTAL	%	DHP*	%	CIS	%	INV	%
D11S912	di	10/19	53	2/8	25	8/15	53	3/5	33
D11S940	di	2/20	10	0/9	0	1/16	6	1/5	20
D13S289	di	4/11	36	0/5	0	2/7	29	2/2	100
D13S260	di	5/19	23	0/7	0	5/16	31	3/4	75
D13S267	di	9/20	45	1/7	14	9/16	56	2/4	50
D16S285	di	1/16	6	0/4	0	1/15	7	0/3	0
D17S855	di	1/11	9	0/4	0	0/8	0	1/4	25

Notes: *DHP: ductal hyperplasia; CIS: carcinoma *in situ*; INV: invasive carcinoma; di: dinucleotide repeats.

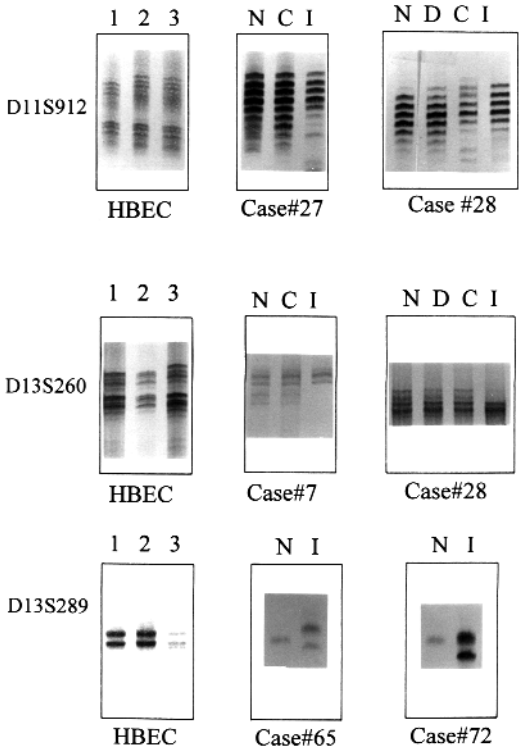

Figure 10. Microsatellite instability in the transformed HBECs (1: MCF-10F; 2: BP1; 3: BP1E) and human breast lesions (N: Normal; D: DHP; C: CIS; I: INV).

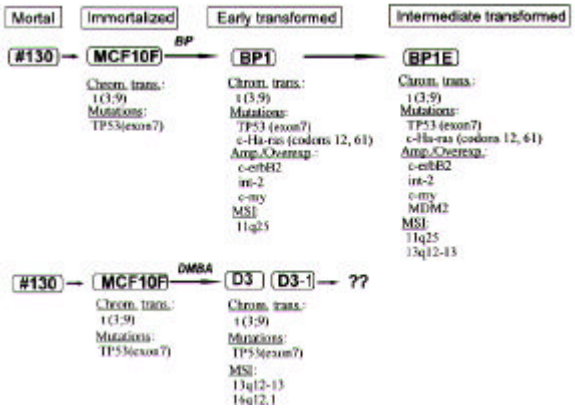

Figure 11. Genomic alterations in the evolution of immortalization and transformation of HBECs.

The carcinogenic mechanisms of benzo(a)pyrene are postulated to be related to its ability to produce G->T mutations, which affect nucleotides in GC-rich stretches of DNA (124), as showed by mutations of p53 at codons 154, 173, 248, 266, 173 and 277 (125, 126). We hypothesize that BP may cause additional mutations in TP53 and other target genes in the transformed cells, which hence contribute to the genomic instability detected in these cells. This is supported by the evidence that germline mutations of p53 are associated with the genomic instability with Li-Fraumeni syndrome (review in 127). Other mechanisms that may contribute to genomic instability include defects in DNA mismatch repair genes (reviews in 117, 118), DNA replication error and DNA repair defects (reviews in 84, 128, 129).

8. GENOMIC CHANGES IN HUMAN BREAST LESIONS

In order to test whether similar changes of microsatellits are present in human breast carcinogenesis *in vivo*, we have also done microsatellite polymorphism analysis in genomic DNA extracted from breast lesions of three different categories: ductal hyperplasia (DHP), carcinoma *in situ* (CIS), and invasive carcinoma (INV). Using the array of 7 markers including *D11S912*, *D11S940*, *D13S260*, *D13S289*, *D13S267*, *D16S285* and *D17S855*, we analyzed samples from 21 patients (Table 3) (119). Among these markers, D11S912 showed MSI in 10/19 (53%) of all samples including 2/8 of the preneoplastic lesion DHP, 8/15 of CIS, and 3/5 of INV (Figure 10; Table 3), in agreement with its early appearance in the transformed HBEC (i.e., BP1 cells) (see above). MSI of marker *D13S260*, which was absent in DHP, but present in 5/16 (31%) and 3/4 (75%) of CIS and INV, respectively (Figure 10; Table 3), suggesting a correlation with the progression and also a consistence with its alteration in the transformed HBEC (i.e., BP1E cells). In addition, the high MSI incidence in all samples for markers *D11S912* (53%), *D13S260* (23%), *D13S289* (36%) and *D13S267* (45%), as compared to lower rates of *D11S940* (10%), *D16S285* (6%) and *D17S855* (9%) (Table 3) may suggest that instability may prefer to occur in these loci during breast carcinogenesis, also in good agreement with the data from the transformed HBEC system (except for *D13S267*).

9. FUNCTIONAL ROLES OF CHROMOSOMES 11 AND 17 IN THE EXPRESSION OF TRANSFORMED PHENOTYPES OF HBEC

The functional role of specific genes on a chromosome can be determined by microcell fusion technique (130, 131), in which a monochromosome is introduced into the target cells. For example, tumor suppressor genes can exert a reversion of tumorigenic or

Table 4. Analysis of BP1E-11*neo* and BP1E-17*neo* Cells

Cells	G-418 Resistance[1]	pSV2neo Sequence[2]	Extra (Donor) Chromosome[3]	Growth Properties			
				Rate[4]	Anch. independence[5]	Ductulogenesis[6]	Ca^{++} Resistance[7]
BP1E control	No	No	No	100%	100%	-	Yes
BP1E-11neo	Yes	Yes	Yes	50%	42%	N/A	Yes
BP1E-17neo	Yes	Yes	Yes	10%	0%	+	No

Notes: 1. G-418 resistance: The BP1E cells could not survive a concentration of 100 μg/ml within a week; while the BP1E-11*neo* and BP1E-11*neo* cells were selected and routinely maintained in 400 μg/ml in the media**.**
2. *pSV2neo* sequence: The donor chromosomes 11 or 17 are tagged with vector *pSV2neo*. A partial sequence of 466 basepairs (GenBank Access number: U02434) of this vector was amplificed from the genomic DNA by PCR, transferred to nylon membrane, and hybridized with the *pSV2neo* probe to confirm the specificity of PCR products.
3. Extra (donor) chromosome: The detection of the donor chromosome 11 or 17 in the microcell hybrids BP1E-11*neo*, or BP1E-17*neo* was done by fluorescence *in situ* hybridization (FISH) using digoxigenin-labeled chromosome 11 or 17 coatsome (painting) probe (Oncor), together with a biotin-labeled *pSV2neo* probe. The presence of the donor chromosome 11 or 17 was judged by the co-presence of its distinct morphology in the metaphase from the host chromosome and the biotin signals on it.
4. Growth rate: It was determined by the total cell count at the end of a five-day growth on 24-well chambers (Costar). The growth rate of the control BP1E cells has been arbitarily set as 100%, and the tested as proportions of the control.
5. Anch. independence (anchorage independent growth): Colony formation of the cells were tested in 0.8% methocel semi solid gel on agar base for 21 days. The colony efficiency of the control has been arbitarily set as 100%, and the tested as a ratio of the control. The average colony size between the BP1E control and the BP1E-11*neo* cells was not different.
6. Dutulogenesis: This parameter was evaluated by growing cells in collagen matrix for 21 days. Although BP1E cells remained growing in cell clusters, the BP1E-17*neo* cells were able to regain the ability to form ductule-like structure in the matriz. "N/A" indicates data "not available."
7. Ca^{++} Resistance: The BP1E cells have adapted to grow in the high calcium contration (1.05 mM) in the medium. The introduction of chromosome 11 did not affect this growth property of the cells. However, the same calcium concentration in the medium induced senesent phenotypes of the BP1E-17*neo* cells, which is significantly reversed by replacing with low calcium concentration (0.04 mM) in the medium.

transformed phenotypes that can be demonstrated as a reduction in or a total loss of the neoplastic/transformed phenotypes, together with at various degree the reappearance of normal phenotype, as a result of the inhibition of activities of specific oncogenes or the replacement of intact tumor suppressor genes (132).

The application of microcell-mediated chromosome transfer (MMCT) technique (130) has led to the determination of genes on a candidate chromosome responsible for genetic disorders or neoplastic phenotypes (133-135). For example, growth/tumor suppressor activities have been detected in chromosomes 1, 3, 5, 6, 7, 9, 11, 17 and X in various human tumor cell lines (135-154). Senescence genes have been found on chromosomes 1, 2, 3, 4, 6, 7, 10, 11, 18 and X (for reviews, see 155). Putative tumor suppressor genes have been mapped to chromosomes 1q23-qter (156), 1 (157), 3p (153, 158, 159), 6q21-q23 and/or 6q26-q27 (147, 160, 161), 11 (160-162), 17 (163) and 17q (151, 154). While putative DNA repair genes have been localized to chromosomes 2 (164, 165), 3 (166), 8 (167) and 11 (168, 169), putative metastasis genes are found on chromosomes 8p23-q12 (170, 171), 10q (170), 11p13-p11.2 (170), 11 (172) and 17pter-q23 (173). Together, the application of this technique has allowed for a functional testing of genetic material residing in a specific chromosome that promises further characterization of specific genes affecting the phenotypes of a tumor cell.

As discussed above, genetic alterations in specific genes such as mutations of *TP53* and *c-H-ras genes*, amplification and/or overexpression of *c-erbB*, *c-myc* and *MDM2* genes, and instability of microsatellites in certain regions of chromosomes are associated with the phenotypic progression of HBEC during transformation (Figure 11). However, it is not clear whether any of these changes play a functional role in the ultimate expression of the transformed phenotypes of these HBECs.

In order to obtain such information, we have introduced normal human fibroblast-derived chromosome 11, or 17, respectively, into the transformed BP1E cells, through MMCT technique (174). We have found that BP1E cells transferred with chromosome 11 or 17, designated as BP1E-11*neo*, and BP1E-17*neo* cells, showed significant morphologic regression such as growth inhibition (e.g., contact inhibition, slow or no division) and characteristic of senescence in most or all clones, which lost the ability to divide and eventually died during a selection incubation period up to 6-12 months. Fluorescence *in situ* hybridization (FISH) analysis of expandable clones showed that a donor chromosome 11 was present in the BP1E-11*neo* cells, and that a donor chromosome 17 was present in the BP1E-17*neo* cells. Functional analyses showed that the BP1E-11*neo* cells exhibited a reduction in growth rate by 50% and in colony efficiency in agar-methocel by 55% (Table 4). Similarly, the BP1E-17*neo* cells lost its growth rate by 90% and its capacity to form colonies in agar-methocel completely, while regained the ability to form ductule-like structure in collagen-matrix and became intolerant to high calcium concentration in the medium (1.04 mM) (Table 4). These data indicate that both chromosomes 11 and 17 may play a functional role in the expression of transformed phenotypes

and that they may harbor tumor suppressor genes or senescence genes, whose normal functions/regulations may have been disrupted in the BP1E cells. Further studies should be directed towards the mapping and cloning of specific genes on these chromosomes and their roles in the progression of transformed phenotypes.

10. SUMMARY AND PERSPECTIVES

Human breast cancer remains the most common malignancy and second leading cause of cancer death in the American women. The ultimate cure of this disease relies on a better understanding of the mechanisms underlying the initiation and progression of this disease. The neoplastic transformation of HBEC *in vitro* represents a successful model for obtaining knowledge on the molecular and biological alterations that may contribute to the tumorigenic mechanisms (reviews in 4, 7, 20, 175, 176). We have presented here a current understanding of chemically transformed HBEC in the following aspects: 1. Factors affecting the transformation of HBEC such as genetic predisposition and differentiation status and prior immortalization; 2. New targets for studying the mechanism of cell immortalization such as alterations in telomerase activity and differential expression of cell cycle dependent genes as well as others recently isolated through differential cloning such as H-ferritin, and a calcium binding protein; 3. Epigenetic and genetic mechanisms underlying cell transformation; 4. The association of microsatellite instability in specific loci on chromosomes 11, 13, and 16 with the progression of cell transformation; and 5. The application of microcell mediated chromosome transfer technique as an approach to testing the functional role of specific genes whose dysregulation or loss of function may contribute to the ultimate cell transformation. Further efforts in this cell system will be directed to determine the roles of identified molecular changes as well as the mapping/cloning of tumor suppressor or senescence genes such as those that may reside on chromosome 11 or 17.

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Key words: Human Breast Epithelial Cells (HBEC); Cell Immortalization; Cell Transformation; Progression Of Neoplastic Phenotype; Carcinogens; Gene Expression; Calcium; Ca-Binding Protein; Mammary-Derived Growth Inhibitor; H-Ferritin; Gene Mutation; TP53; C-Erbb2; Int-2; C-Myc; Mdm2; Microsatellite Instability; D11S912; D13S260; D13S289; D16S285; Chromosome 11; Chromosome 13; Chromosome 16; Chromosome 17; Microcell-Mediated Chromosome Transfer; Senescence; Growth Inhibition; Anchorage Independent Growth

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