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Two Types of Normal Human Breast Epithelial Cells Derived From Reduction Mammoplasty: Phenotypic Characterization and Response to SV40 Transfection

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TWO TYPES OF NORMAL HUMAN BREAST EPITHELIAL CELLS DERIVED FROM REDUCTION MAMMOPLASTY : PHENOTYPIC CHARACTERIZATION AND RESPONSE TO SV40 TRANSFECTION

By

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ABSTRACT

TWO TYPES OF NORMAL HUMAN BREAST EPITHELIAL CELLS DERIVED FROM REDUCTION MAMMOPLASTY : PHENOTYPIC CHARACTERIZATION AND RESPONSE TO SV40 TRANSFECTION

By

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A culture method has been developed to grow two morphologically and cytochemically distinguishable normal human breast epithelial cell types derived from reduction mammoplasty. Type I cells are characterized by a deficiency in gap junctional intercellular communication (GJIC), the expression of epithelial membrane antigen (EMA), keratin 18, and the non-expression of keratin 14 and α6 integrin, in addition to growth stimulation by fetal bovine serum (FBS). Type II cells are characterized by a proficiency in GJIC, the expression of keratin 14, α6 integrin and non-expression of EMA and keratin 18, in addition to growth inhibition by FBS. Thus, Type I cells exhibited some luminal epithelial cell characteristics and Type II cells expressed some basal

epithelial cell phenotypes of the human mammary gland. Furthermore, Type I cells can be induced by cholera toxin to change their morphology to a Type II cell morphology.

Type I and Type II cells were transfected with SV40 DNA to determine if one of the two cell types is more susceptible to neoplastic transformation. The results showed that clones of extended life (EL) can be obtained from both cell types by SV40 transfection. Type I cell-derived SV40 transfected EL clones become immortal spontaneously or are immortalized by treatment with 5-bromodeoxyuridine (BrdU)/black-light (2/9). None of the Type II cell-derived SV40 transfected EL clones (0/8) have been immortalized thus far. The Type I and Type II cell-derived EL and immortal cell lines resemble their parental cells with respect to EMA, keratin 18 and keratin 14 expression and GJIC. All Type I cell-derived SV40 transfected clones formed colonies in soft agar (9/9), whereas Type II cell-derived clones did not (0/8). One Type I immortal line (immortalized by SV40 and BrdU/black light) was weakly tumorigenic. The infection and expression of a mutated neu oncogene greatly enhanced the tumorigenicity of this line. Additionally, the growth factor/hormonal requirements of these normal and transformed human breast epithelial cells were determined. Our results suggest that Type I breast epithelial cells might be the major target cells for *in vivo* neoplastic transformation.

To:

My Wife, Ching-Fen Lin

My Parents, My Sister and My Brother

For Their Love and Support

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LIST OF ABBREVIATIONS

AIG	Anchorage independent growth
bFGF	Basic fibroblast growth factor
B[a]P	Benzo[a]pyrene
BSA	Bovine serum albumin
BrdU	5-bromodeoxyuridine
DDT	1,1'-(2,2,2-trichloroethylidene)bis(4-chlorobenzene)
CT	Cholera toxin
CALLA	Common acute lymphoblastic leukemia antigen
Cx	Connexin
CS	Contact-sensitive
CPDL	Cumulative population doubling level
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EMA	Epithelial membrane antigen
E_2	17 β-estradiol
EDTA	Ethylenediamine tetraacetic acid
EL	Extended life
FBS	Fetal bovine serum
GJIC	Gap junctional intercellular communication

HBEC	Human breast epithelial cell
HMEC	Human mammary epithelial cell
HPV	Human papilloma virus
HT	Human transferrin
HC	Hydrocortisone
INS	Insulin
K-14	Keratin 14
K-18	Keratin 18
LOH	Loss of heterozygosity
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MFGM	Milk fat globule membrane antigen
4NQO	4-nitroquinoline-1-oxide
PNA	Peanut agglutinin
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
T_3	3,3'-5-triiodo-DL-thyronine
SV40	Simian virus 40
UV	Ultraviolet light

CHAPTER 1

Introduction

Breast cancer is a major malignancy of American women. Approximately 180,000 new cases of breast cancers and 46,000 deaths due to this disease are estimated to occur in American women in 1992 (Boring et al., 1992). The etiology and detailed mechanism of human breast cancer is poorly understood (Marshall , 1993). The current evidence from experimental and epidemiological studies of animal and human carcinogenesis indicates that carcinogenesis is a complex, multi-step, multi-mechanism process involving genetic, and possibly epigenetic changes of different sets of oncogenes and tumor suppressor genes (Fialkow, 1977; Vogelstein et al., 1988; Callahan et al., 1990; Wainfan and Poirier, 1992).

Tumor cells are believed to arise from stem cells or early precursor cells and have a phenotype similar to normal undifferentiated cells at that stage (Sigal et al., 1992) or have combined phenotypes of different cell types of common lineage [e.g. leukemia cells express both lymphoid and myeloid cell antigens (Sawyers et al., 1991)]. Therefore, cancer has been termed a disease of the pluripotent stem cell (Sawyers et al., 1991), a disease of cell differentiation (Markert , 1968) or oncogeny as blocked or partially blocked ontogeny (Potter, 1978). In human beings, the life time risk of breast cancer developing in child-bearing women seems to be linearly related to the age at which a woman has her first full-term pregnancy (MacMahon et al., 1973). This has been explained by John Cairns (1975) 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 induces full differentiation of the mammary

gland, with elimination of terminal end buds, resulting in refractoriness of the gland to carcinogenesis (Russo et al., 1990).

Over the past few years, several laboratories have analyzed primary human breast tumors for evidence at the molecular level of frequently occurring mutations (Callahan and Campbell, 1989; Callahan et al., 1990). To date, twelve such mutations have been detected. Three of these represent the amplification of cellular proto-oncogenes (c-myc, int-2/hst and c-erbB2). The remainder of the mutations represent losses of heterozygosity (LOH) at specific regions of the human genome (chromosomes 1p, 1q, 3p, 11p, 13q, 16q, 17p, 17q and 18q). In addition, germ line mutations of the tumor suppressor gene, p53, have been associated with a dominantly inherited familial breast cancer syndrome (Malkin et al., 1990). The Ataxia-telangiectasia gene predisposes those carrying it to cancer, particularly breast cancer in women (Swift et al., 1991). The breast cancer susceptibility gene on ch. 17q is expected to be cloned soon (Roberts, 1993).

A different approach to understand the mechanism of breast carcinogenesis is to develop an *in vitro* neoplastic transformation system. The major advantage of this approach is the ability to analyze genetic alterations at different stages of neoplastic transformation, in addition to developing an *in vitro* assay for carcinogenic and chemopreventive agents. The development of consistent *in vitro* neoplastic transformation of human breast epithelial cells, however, has not been successful in the past except for the use of viral oncogenes (Chang et al., 1982; Stampfer and Bartley, 1985; Rudland et al., 1989; Band et al., 1990; Bartek et al., 1990; Bartek et al., 1991; Garcia et al., 1991; Berthon et al., 1992; Van Der Haegen and Shay, 1993; Shay et al., 1993).

The human mammary gland contains two major epithelial cell types that form the ductal tree (i.e. luminal and basal epithelial cells). These two cell types show different expression in keratins and epithelial membrane antigen. The human breast epithelial cells in culture were either derived from lactational fluids (milk cells) or from reduction mammoplasty tissues. Milk cells, although are of luminal origin, are considered highly differentiated. The cell cultures derived from reduction mammoplasty using the commonly used MCDB 170 (Hammond et al., 1984) or DFCI-1 (Band and Sager, 1989) medium exhibit predominantly basal epithelial cell phenotypes (Taylor-Papadimitriou et al., 1989; Trask et al., 1990) instead of the phenotypes of luminal epithelial cells. The luminal epithelial cells are considered more relevant to breast carcinogenesis since breast cancer cells are found to show luminal epithelial cell phenotypes (Taylor-Papadimitriou et al., 1989; Trask et al., 1990; Koukoulis et al., 1991).

We have recently developed a defined culture medium which supports the growth of two types of normal human breast epithelial cells derived from reduction mammoplasty exhibiting either luminal or basal epithelial specific antigens. These two types of cells are morphologically and cytochemically distinguishable and can be separated as pure cultures. The characterizations of these two types of cells was the first objective of this research. The human breast epithelial culture system also provides an excellent opportunity to test if one of the two cell types is more susceptible to neoplastic transformation. The comparative study of immortalization and malignant transformation by SV40 large T-antigen using these two types of cells was the second objective of this study.

CHAPTER 2

Literature Review

I. Differentiation and Tumorigenesis

The present evidence from experimental and epidemiological studies of human and animal carcinogenesis indicates that carcinogenesis is a complex, multi-step, multi-pathway process. It has been shown that neoplastic transformation involves genetic changes of different sets of a series of oncogenes and tumor suppressor genes (Vogelstein et al. , 1988; Callahan et al., 1990). The initiation/promotion/progression model appears to be a useful model to understand the multiple steps of carcinogenesis (Boutwell, 1974; Weinstein et al., 1984; Trosko and Chang, 1989). Initiation and progression seem to involve irreversible genetic alterations in a single cell which has the capacity to proliferate (i.e., stem cells or initiated cells), whereas the process of promotion, which involves the clonal amplification of initiated cells, appears to be potentially reversible and interruptible (Boutwell , 1974). Thus, mutagens are good initiators and progressors (Hennings et al., 1983). Tumor-promoting conditions, which are caused by chemicals, cell removal, cell death or oncogenic gene products affecting cell proliferation, seem to be mitogens, rather than mutagens (Slaga et al., 1978; Trosko and Chang, 1980, 1986). Both genetic (e.g., Li-Fraumeni's syndrome) and environmental factors (exogenous mutagen/carcinogens) have been shown to contribute to tumorigenesis (Ames, 1989; Loeb, 1989; Malkin et al., 1990).

Carcinogenesis is now known as a disease with a long latency period, often 20 years or more, between the initiation of carcinogenesis and the onset of the terminal, invasive, and metastasis phase of the disease (Sporn, 1991). For example, the individuals, who

carried the germ line p53 mutations (the dominantly inherited Li-Fraumeni syndrome), were usually diagnosed with tumors at the age of 15-44 years (Malkin et al., 1990). The developmental factors which affect cell proliferation and differentiation might play a very important role in neoplastic transformation. The carcinogenesis process has been described to involve some disruption of normal differentiation (Pierce, 1974; Mintz, 1975; Potter, 1978). Malignant stem cells may be derived from normal stem cells and have a capacity for proliferation and differentiation by a process equivalent to a postembryonic differentiation (Pierce, 1974). Blockage of differentiation at different stages, from stem cells to differentiated cells, might result in different grade of tumors, benign or malignant tumors (Mintz, 1975). The indirect evidence for the stem cell and differentiation in carcinogenesis can be obtained by the observation of induction of some cancer cells to differentiate and become non-tumorigenic (Friend et al., 1971; Dexter, 1977; Collins et al., 1978; Breitman et al., 1980; Bloch, 1984; Harris, 1990). For example, the human promyelocytic leukemia cell line (HL-60) can be induced to differentiate to morphologically and functionally mature granulocytes by incubation with retinoid acid, dimethyl sulfoxide and other polar compounds (Collins et al., 1978; Breitman et al., 1980). Friend leukemia virus-induced leukemic cells involve blockage of erythroid differentiation of parental cells and can be induced to synthesize hemoglobin and to become terminally differentiated by DMSO and ouabain (Friend et al., 1971; Bernstein et al., 1976). In addition to the fact that cancer cells can be induced to differentiate and become non-tumorigenic, the evidence for the role of differentiation in carcinogenesis also include 1) many protooncogenes and tumor suppressor genes have been shown to be involved in development and differentiation (Bar-Sagi and Feramisco, 1985; Muller, 1986;

Dmitrovsky et al., 1986; Rijsewijk, 1987; Liotta et al., 1991), and 2) a subpopulation of less differentiated contact-insensitive (CS^-) cells, found in early passage primary syrian hamster embryonic cell culture, was considerably more susceptible to neoplastic transformation than a population of cells depleted of CS^- cells (Nakano et al., 1985). Furthermore, the stem cell theory of carcinogenesis has been proposed by several investigators (Cairns, 1975; Nowell, 1976; Lajtha, 1983; Steel and Stephens, 1983; Kondo, 1983; Trosko and Chang, 1989). Stem cell has been defined as an undifferentiated cell capable of 1) proliferation, 2) high capacity for self-renewal, 3)ability to produce a large number of differentiated, functional progeny, 4) regenerating the tissue after injury, and 5) a flexibility in the use of these options (Lajtha, 1979; Potten and Loeffler, 1990). The clonal origin of tumors has been demonstrated (Nowell, 1976). According to the stem cell theory of carcinogenesis, a tumor is believed to result from the accumulation of mutations occurred in a particular cell which is an "immortal" stem cell followed by clonal amplification during the life of an animal. As pointed out by Steel and Stephens (1983), a neoplastic stem cell is one that had become unresponsive to the controls that normally limit the size of the stem-cell population. However, these tumor stem cells still can produce descendants that differentiate. Besides, from the study of liver stem cells and liver carcinogenesis, Sigal et al. (1992) have concluded that tumor cells are transformed stem cells or early precursor cells, and that most of the so-called "tumor markers" are not genes activated or induced by a transformation event but rather normal genes expressed in the normal stem cell population. The incidence of breast tumors in atomic bomb survivors increase linearly with the radiation dose at a rate of about 4×10^{-6} per rad except in the following groups: women of ages 40 to 49 years old

at the time of the bombing show a linear decrease in the incidence with increase in the dose and girls of ages 0 to 9 years old show no significant increase in incidence above the control level (Tokunaga et al., 1979). The latter observation suggests that immature mammary gland is resistant to carcinogenesis probably due to the lack of cancer-prone stem cells (Kondo, 1983). Therefore, carcinogenesis of an organ is initiated by mutation of its stem cells formed during organogenesis (Kondo, 1983).

II. Etiology of Human Breast Cancer

The lifetime risk of human breast cancer has been reported to be linearly related to the length of the interval between puberty and the first full-term pregnancy (MacMahon et al., 1973). This has been explained by John Cairns (Cairns, 1975) as related to stem cells proliferation. Stem cells of human breast epithelium increase in number commencing at the time of puberty and then undergo a certain fluctuation in number with each ovarian cycle until, but not after, the first full-term pregnancy, whereupon the population builds up to a final level that is no longer influenced by the ovarian cycle or by additional pregnancies (Cairns, 1975). The risk of breast cancer is increased from 30% to 130% for women who never have children (Cairns, 1975). These observations imply that the stem cells might be the target cells of the neoplastic transformation and these target cells may be influenced by differentiation. In addition, a number of genetic, developmental and environmental risk factors for breast cancers has been identified. Among the genetic factors are : 1) the Li-Fraumeni's Syndrome involving the mutation of p53 gene on ch. 17p (Malkin et al., 1990), 2) the Ataxia-telangiectasia Syndrome (Swift et al., 1991), 3)

the breast cancer tumor suppressor gene BRCA1 on ch. 17q (Smith et al., 1992) , and 4) family history of breast cancer (Mettlin et al., 1990). The developmental factors beside the old age at first birth and nulliparity (MacMahon et al., 1973) also include early menarche (Bouchardy et al., 1990), late menopause (Ewertz and Dutty, 1988), and benign breast disease (Dupont and Page, 1987). Hormones, especially estrogens, appear to play a key role in these developmental factors (Henderson et al., 1993).

There are only few established environmental factors known to effect human breast cancer. While dietary factors are believed to play some role, as might be predicted from animal studies, in human breast carcinogenesis, clarification still need to be forthcoming (Howe et al.,1991, Kushi et al., 1992). Alcohol and cholesterol epoxide (El-Bayoumy , 1992), in addition to many lipophilic environmental pollutants (i.e. PCB, DDT, heptachlor) (Wolff et al., 1993; Davis et al., 1993) might be considered as potential contributors. However, ionizing radiation is the best established exogenous environmental agent known to cause human 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 (Land et al., 1991), as well as from radiation therapy of Hodgkin's disease (Pollner, 1993) and other nonmalignant conditions (Hildreth et al., 1989; Shore et al., 1977).

III. Epithelial Cell Types Derived from Breast Tissue

The mammary gland contains two major epithelial cell types, i.e. the luminal cells and basal epithelial cells (Taylor-Papadimitriou et al., 1989; O'Hare et al., 1991). Both types of epithelial cells express specific antigenic markers which permit their identification in

mammary tissues or dissociated cells. The markers for normal luminal epithelial cells are: milk fat globule membrane antigen (MFGM), keratin 7, keratin 8, keratin 18, keratin 19, casein, α-lactalbumin, lactoferrin (review by Russo, 1990), epithelial membrane antigen (EMA) (Sloane and Ormerod, 1981) and peanut agglutinin (PNA) (Newman et al., 1979). The heterogeneous staining of keratin 19, MFGM, EMA and PNA has been observed for luminal epithelial cells as well as carcinomas; they are mainly expressed in differentiated luminal epithelial cells (Taylor-Papadimitriou et al., 1989). On the other hand, the markers for normal basal or myoepithelial cells are actin, myosin, common acute lymphoblastic leukemia antigen (CALLA), keratin 5, keratin 14 and α6 integrin (Taylor-Papadimitriou et al., 1989; Trask et al., 1990; O'Hare et al., 1991; Koukoulis et al., 1991). Comparing their specificity for the two types of cells, the antibodies to keratin 8 and 18 are specific for the luminal layer and keratin 5 and 14 are excellent basal cell (myoepithelial) markers (Russo et al., 1990). Moreover, the primary breast carcinomas show a keratin profile (keratin 7, 8, 18 and 19), corresponding to that of the dominant luminal cell (Taylor-Papadimitriou et al., 1989; Trask et al., 1990), and are believed to be originated from terminal ductal lobular units (Wellings et al., 1975).

Several *in vitro* culture systems for normal human breast epithelial cells have been reported in literature. The normal human breast tissues are obtained either from reduction mammoplasty (Stampfer et al., 1980; Peterson and Deurs, 1988; Band and Sager, 1989; Emerman and Wilkinson, 1990; Ethier et al., 1990) or from lactation fluids (Buehring, 1972; Taylor-Papadimitriou et al., 1977). The most commonly used medium for human breast epithelial cells appears to be the MCDB 170 (Hammond et al., 1984), DFCI-1 (Band and Sager, 1989) and RPMI 1640 (Bartek et al., 1991), supplemented with EGF,

transferrin, insulin, hydrocortisone, cholera toxin, fetal bovine serum or bovine pituitary extract. Studies of antigenic marker expression in those normal human breast epithelial cell cultures derived from reduction mammoplasty reveal that these cells express mainly keratin 5, 6, 7, 14 and 17. Therefore these media appear to support selectively the growth of basal or myoepithelial cells instead of luminal epithelial cells (Taylor-Papadimitriou et al., 1989; Trask et al., 1990). On the other hand, cells derived from lactation fluids continue to express keratin 7, 8, 18, and 19. Around 70% of the colonies show homogeneous expression of keratin 7, 8, and 18, with 85% of these colonies expressing keratin 19 (Taylor-Papadimitriou et al., 1989). Meanwhile, 30% of the colonies express keratin 14 (Taylor-Papadimitriou et al., 1989). This implies that the cells derived from milk contains both luminal and basal epithelial cells. However, the cells derived from lactation fluids usually represent more differentiated epithelial cells. Therefore, the available breast epithelial cell cultures using the existing methods are either the predominant basal or myoepithelial cells (reduction mammoplasty) or a mixture of luminal and myoepithelial cells (milk cell). Since most breast carcinomas are of ductal luminal origin, a normal human breast epithelial cell culture with pure or predominantly undifferentiated luminal epithelial cells would be preferred for the carcinogenesis study of the mammary gland.

Normal primary human breast epithelial cells have been shown to communicate with homologous cells (Fentiman and Taylor-Papadimitriou, 1977). In contrast, most of human breast cancer cell lines are deficient in gap junctional intercellular communication (GJIC) (Fentiman et al., 1979). Lee and Tomasetto (1992) have shown that normal human mammary epithelial cells derived from reduction mammoplasty express two connexin

genes, Cx26 and Cx43, whereas neither gene is transcribed in a series of mammary tumor cell lines (Lee et al., 1992; Tomasetto et al., 1993). As mentioned previously, the major type of normal human breast epithelial cells, derived from reduction mammoplasty using existing methods, is the basal or myoepithelial cell. Therefore the normal human breast epithelial cells used by Lee and Tomasetto are very likely the myoepithelial cells. We have recently developed a culture system which support the growth of two types of cells, and the cells express either the luminal or basal epithelial cell markers. The former was deficient in gap junctional intercellular communication (GJIC) and the latter was proficient in GJIC. Furthermore, the cell expressing luminal cell markers did not express any gap junction gene, whereas cells expressing basal epithelial cell markers expressed mRNA for connexin 26 and connexin 43 (Yang et al., 1993).

Stem cells of the mammary gland have been described and discussed by several groups. Using monoclonal antibodies which can identify various epithelial cell types within the rat mammary gland, Dulbecco et al., (1986) have followed the evolution of cell types from the emergence of mammary ducts in the fetus to adulthood. They showed that stem cells are present in end buds and in the myoepithelial layer of ducts. From the observations of the differentiation ability of benign and malignant mammary tumors, Rudland (1987) has suggested that stem cells in rat and human mammary glands are capable of differentiating to the other major cell types of the mammary parenchyma, and during the carcinogenesis process they generate genetically unstable cells which lose their ability to differentiate. Medina and Smith (1990) have shown that mammary gland stem cells were present in all parts of mammary parenchyma at all stages of differentiation by the transplantation experiments. Kim and Clifton (1993) have recently identified and

isolated a subpopulation of cells with clonogenic ability by fluorescence-activated flow cytometric analyses and sorting. These clonogenic cells produce alveolar units in the recipient rats. According to these reports mentioned above, the stem cells of mammary gland do exist and can be identified.

IV. Oncogenes and Tumor Suppressor Genes Involved in Mammary Gland Carcinogenesis

Recently, significant progress has been made in the identification and characterization of specific mutations which frequently occur in primary breast tumor DNA by several laboratories (see review by Callahan, R. and G. Campbell 1989). To date, three cellular proto-oncogenes (c-myc, c-erbB2 and int-2/hst) have been found to be amplified in breast tumors. Several groups have found that 6-56% of primary breast tumors contain an amplification of the c-myc (Callahan, 1989). The c-erbB2 proto-oncogene (also known as c-neu or HER-2) encodes a transmembrane glycoprotein ($gp185^{erbB-2}$) which is an epidermal growth factor receptor-related protein (Bargmann et al., 1986). About 10-40% of primary breast tumors are found to bear the amplification of the c-erbB2 gene (Callahan, 1989). The c-erbB2 amplification has been shown to be correlated with poor wurvival in lymph node-positive breast cancer (Slamon et al., 1987; Borg et al., 1990). In one study, the c-erbA-1 proto-oncogene, which is a member of the steroid/thyroid hormone receptor gene family, is found to be frequently co-amplified with the c-erbB2 (van de Vijver et al., 1987). The c-erbB2 overexpression in breast cancer has been hypothesized to contribute to a high growth rate of tumor cells, but not to increase the metastatic potential (see review van de Vijver and Nusse, 1991). In addition, a recently

identified gene, erbB3, the third member of the erbB/EGF receptor gene is found constitutively activated in some human breast tumors (Kraus et al., 1993), but it is not known whether the gene is rearranged or amplified. Amplification of the int-2/hst oncogene, which exhibits basic fibroblast growth factor (bFGF) activity (Merlo et al., 1990) , has been observed in 9-23% of primary breast carcinomas (Callahan, 1989). Recently, the involvement of c-src protooncogene product with signalling pathways in human breast cancer has been demonstrated (Luttaell et al., 1994). The c-src may play an important role in breast carcinogenesis since the protein kinase activity derived from $pp60^{c\text{-}src}$ was shown to be elevated in breast cancer specimens (Ottenhoff-Kalff et al., 1992).

Another class of mutations resulting in the inactivation of "tumor-suppressor" gene or loss of heterozygosity (LOH) at specific regions of the human genome has been identified (chromosomes 1p, 1q, 3p, 11p, 13q, 16q 17p, 17q and 18q) (Callahan et al., 1990; Sato et al., 1991). The LOH in breast carcinomas might involve tumor suppressor genes which are located on these chromosome arms: small cell lung carcinoma gene (3p), Wilm's tumor gene (11p), Rb-1 (retinoblastoma) (13q), p53 (17p) and the DCC (18q) gene. The involvement of p53 mutations in human breast cancer is also supported by the Li-Fraumeni's syndrome that is caused by germ line p53 gene mutation and that predisposes the carriers to breast cancer (25%) and other neoplasms (Malkin et al., 1990). The breast-cancer-suppressor gene, BRCA1, located on chromosome 17q21 appears to be involved in the largest proportion of inherited breast cancer (Smith et al., 1992). Ataxia-telangiectasia is an autosomal recessive syndrome. The carriers, especially homozygote, have a higher risk of cancer than that in the general population, particularly breast cancer

in women (Swift et al., 1991). Patients with ataxia-telangiectasia and cells derived from homozygote and heterozygote are unusually sensitive to ionizing radiation which is the best known environmental carcinogen for breast cancer as mentioned previously.

Additionally, it is now known that SV40 large T-antigen protein is responsible for the immortalization of rodent and human cells (Chang, 1986). The major mechanism of regulation of cellular senescence might be due to the binding of tumor suppressor genes Rb and p53 by SV40 large T-antigen (Shay et al., 1991).

V. Neoplastic Transformation of Human Cells *In Vitro*

Neoplastic transformation of human cells *in vitro* has been reviewed before (Chang, 1986; Rhim, 1989; Paraskeva, 1990; Shay, 1991). Normal human fibroblasts or epithelial cells have a finite lifespan. The most difficult and critical step in neoplastic transformation *in vitro* appears to be the acquisition of cellular immortality, since immortal human cells from various tissues have been easily converted to tumorigenic cells by oncogene transfection or further treatment with chemical or physical mutagens/carcinogens. These neoplastic transformations of immortal human cells have been demonstrated in human fibroblasts (Namba et al., 1986; Wilson et al., 1989; Hurlin et al. 1989), keratinocytes (Koukamp et al., 1986; Rhim et al., 1985, 1986), urothelial cells (Reznikoff et al., 1988), bronchial epithelial cells (Amstad et al., 1988), kidney epithelial cells (Haugen et al., 1990) and breast epithelial cells (Clark et al., 1988). Paraskeva et al. (1990) provided the first experimental evidence for the neoplastic transformation of adenoma to carcinoma of human colonic epithelial cells by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

Human cells *in vitro* can be neoplastically transformed by physical carcinogen (e.g., UV, and ionizing radiation), chemical carcinogens or viruses (see review by Chang, 1986; Rhim, 1989; Paraskeva, 1990; Shay, 1991). The immortalization of human cells *in vitro* by chemical of physical carcinogens/mutagens is rarely successful. So far the most successful approach to the transformation of human cells *in vitro* is the use of viral agents, either as infectious particles or DNA. The most widely used viral agent is SV40, a DNA tumor virus. The transformation of mammalian cells by SV40 is known to require the expression of the early region of the viral genome, which encodes the large T-antigen. The expression of this gene is required for the maintenance of the transformed phenotype. Many researchers have also shown that the SV40 transformed human epithelial cell lines are usually non-tumorigenic in athymic nude mice (Chang, 1986). To date, the best known cellular effect of SV40 is lifespan extension followed by immortalization of rare survivors after the "crisis", a period of no net increase in cell number.

Rhim has reported that normal human epidermal keratinocytes could be immortalized by Ad12-SV40 hybrid virus (see review by Rhim, 1989). The immortal human cell line (RHEK-1) is non-tumorigenic. After treatment with chemical carcinogens [MNNG or 4-nitroquinoline-1-oxide (4NQO)] or ionizing irradiation, the cells become tumorigenic in nude mice (Thraves et al., 1990). This immortal cell line can also be neoplastically transformed by a variety of retroviruses containing H-ras, bas, fes, fms, erbB and src oncogene (Rhim, 1989). Beside human keratinocytes, immortal cell lines can be successfully established by SV40 from, human bronchial epithelial cells, human salivary gland epithelial cells, nasal polyp epithelial cells from cystic fibrosis patients, and cystic

fibrosis bronchial epithelial cell lines (Rhim , 1989). The *in vitro* multistep process of neoplastic transformation also has been demonstrated in human urinary tract epithelial cells (Reznikoff et al., 1988), colon mucosal epithelial cells (Moyer and Aust, 1984), human prostate epithelial cells (immortalized by SV40 or Human papillomavirus type 18, respectively) (Bae et al., 1993; Rhim et al., 1993), and human kidney epithelial cells (immortalized by Adenovirus Type 12 or Nickel) (Whittaker et al., 1984; Haugen et al., 1990).

VI. Neoplastic Transformation of Human Breast Epithelial Cells *In Vitro*

A. Chemical carcinogen

Chemical carcinogens and ionizing radiation are better choices of carcinogens for neoplastic transformation *in vitro* than viral oncogenes. However, immortalization of human breast epithelial cells by chemical mutagens/carcinogens is extremely difficult and has not been consistently demonstrated before, with only one successful report in literature (Stampfer and Bartley, 1985). In this report, the rapidly growing primary cultures of normal human mammary epithelial cells (HMEC), derived from reduction mammoplasty, were exposed to benzo[a]pyrene for two or three 24-hr periods. Cells with extended life (EL) were first obtained after the treatment. Subsequently, two continuous cell lines emerged from the EL culture. The two immortal lines do not form tumors in nude mice and are incapable of anchorage-independent growth. Immunochemical studies of these cell lines (express K-14 but not K-18 or K-19) indicate that these immortal cell lines primarily express basal cell or myoepithelial cell markers (Taylor-Papadimitriou et al., 1989). Introduction and overexpression of a point-mutated or truncated erbB-2 gene

in the immortal mammary epithelial cell line caused the cells to form colonies in soft-agar, to induce transient nodules frequently in athymic mice and to produce progressive tumors *in vivo* at a low frequency (Pierce et al., 1991). This particular immortal cell line after transfection by SV40 large T-antigen, produces no tumors in nude mice. The v-ras transformants and the transformants bearing both the large T-antigen and ras oncogene were weakly and strongly tumorigenic, respectively (Clark et al., 1988).

Additionally, a spontaneously immortalized human breast epithelial cell line, MCF-10, was derived from the human fibrocystic mammary tissue (Soule et al., 1990). This immortal line with near diploid karyotype was non-tumorigenic in nude mice, but was able to grow three dimensional in collagen. This cell line, however, lacked the ability of anchorage-independent growth. Immunocytochemical study indicates that MCF-10 expressed both luminal and myoepithelial cell markers (expression of MFA-breast, MC-5, EMA and keratin-14, but not keratin 19) (Tait et al., 1990). Altered cell morphology, increased growth rate and acquired anchorage-independent growth can be observed in this cell line in response to treatment with chemical carcinogens (i.e., MNNG, B[a]P, 7,12-dimethylbenz[a]anthracene, N-methyl-N-nitrosourea, B[a]P, MNNG) (Calaf and Russo, 1993). One clone treated with B[a]P became tumorigenic in nude mice. Furthermore, MCF-10, after transfection with c-Ha-ras oncogene, exhibited anchorage-independent growth, loss of requirement for hormones and epidermal growth factor, and was tumorigenic in nude mice (Basolo et al., 1991).

B. Viral agents

a. Human papilloma virus (HPV)

Band and Sager (1989) have developed a medium (DFCI-1) which supports long-

term culture of normal human breast epithelial cells derived from reduction mammoplasty. The epithelial cells growing in this medium express keratin 5, 6, 7, 14 and 17 (Trask et al., 1990). This indicates that the normal human breast epithelial cells developed by this method represent myoepithelial cells of mammary tissue (Trask et al., 1990). These normal epithelial cells can be immortalized by human papilloma virus (HPV) types 16 and 18 *in vitro* (Band et al., 1990). These immortal cells do not form tumors in nude mice, but reduce their growth factor requirement compared to the parental normal cells. However, human papilloma virus types 16 and 18 are most commonly associated with cervical carcinoma and have not been found to be associated with breast cancer.

b. SV40

Like other human cells, SV40 has been found to be an efficient agent to immortalize normal human breast epithelial cells derived from reduction mammoplasty and lactation fluids (Chang et al., 1982; Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Berthon et al., 1992; van der Haegen and Shay, 1993; Shay et al., 1993). These immortal cell lines are incapable of anchorage-independent growth, and are mostly non-tumorigenic in nude mice. The clones, SIT2, 3, derived from reduction mammoplasty, showed weak tumorigenicity with a long latent period (Berthon et al., 1992). One series of SV40 immortalized clones (MTSV, MRSV) was transfected by v-Ha-ras oncogene (Bartek et al., 1991). The results indicated that most of the drug resistant clones became senescent, except one clone which entered a crisis phase and emerged as an immortal cell line that formed colonies in soft agar and induced tumors in the nude mice (Bartek et al., 1991). From this review, all the *in vitro* neoplastic transformation of normal human breast epithelial cell experiments were performed using

a population of mammary epithelial cells without separation of the two major epithelial cell types (i.e., luminal and basal epithelial cells). Most of the normal human breast epithelial cell cultures, especially those derived from reduction mammoplasty, predominantly contained basal epithelial cells. Since most of the breast tumors are believed to originate from the luminal cell lineage, the luminal cells seem to play a very important role in the carcinogenesis of the mammary gland. Therefore, it is imperative to use undifferentiated luminal epithelial cells for *in vitro* neoplastic transformation studies.

CHAPTER 3

Two Types of Normal Human Breast Epithelial Cells Derived from Reduction Mammoplasty : Phenotypic Characterization and Response to SV40 Transfection

Abstract

A culture method to grow two morphologically distinguishable normal human breast epithelial cell types derived from reduction mammoplasty has been developed. Type I cells were characterized by a more variable cell shape, smooth cell colony boundaries, the expression of epithelial membrane antigen (EMA), keratin 18 and the non-expression of keratin 14 and α6 integrin. In addition, the Type I cells were growth stimulated by fetal bovine serum (FBS) and were deficient in gap junctional intercellular communication (GJIC). In contrast, Type II cells were characterized by a uniform cell shape, expression of keratin 14, α6 integrin and the non-expression of EMA and keratin 18. In addition, Type II cells were growth inhibited by FBS and were proficient in GJIC. A proportion of Type I cells can be induced by cholera toxin to change their morphology to a Type II cell morphology. Since EMA and keratin 18 are antigenic markers for breast luminal epithelial cells while keratin 14 and α6 integrin are antigenic markers for breast basal epithelial cells, the Type I cells antigenically resemble luminal epithelial cells while the Type II cells more closely resemble basal epithelial cells. Type I and Type II cells were transfected with SV40 DNA. Clones with extended life (20-50 cumulative population doubling level) were obtained from both Type I and Type II cells by SV40 transfection. Some (2/9) of the SV40 transfected Type I cell clones became immortal (>100 cumulative population doubling level), whereas none (0/8) of the SV40 transfected Type II cell clones

became immortal. The SV40 transfected Type I and Type II cell-derived extended life clones and immortal cell lines phenotypically resembled their parental cells with respect to EMA, keratin 14 and keratin 18 expression and GJIC. Each (9/9) of the SV40 transfected Type I cell clones grew in soft agar; none (0/8) of the SV40 transfected Type II cell clones were capable of growing in soft agar. These results provide evidence that normal human breast epithelial cells, derived from reduction mammoplasty, can be separated into two morphologically and antigenically different cell types and that these two different cell types significantly differ in their response to an oncogenic (SV40) stimulus.

Introduction

The human breast contains a variety of cell types including luminal and basal epithelial cells that form the ductal tree. These two types of epithelial cells are immunocytochemically distinguishable in tissue sections (Taylor-Papadimitriou et al., 1989) or in enzymatically dissociated single-cell suspensions (O'Hare et al., 1991). Antigenic markers that can distinguish these two cell types would include the epithelial membrane antigen (EMA) and keratin 18 which are predominantly expressed in luminal epithelial cells (Taylor-Papadimitriou et al., 1989; O'Hare et al., 1991) and keratin 14 and α6 integrin which are specifically expressed in basal epithelial cells (Taylor-Papadimitriou et al., 1989; Koukoulis et al., 1991). When the expression of these antigenic markers were examined in primary human breast carcinomas, it was found that the carcinoma cells were similar to the luminal epithelial cells in their expression of antigens (Taylor-Papadimitriou et al., 1989; Trask et al., 1990; Koukoulis et al., 1991).

This evidence can be interpreted as indicating that breast carcinomas are primarily derived from luminal epithelial cells or their precursor cells with similar phenotypes. Most normal human breast epithelial cell cultures were derived either from lactational fluids, which contained cells primarily of luminal origin, or were derived from reduction mammoplasty. Cells from reduction mammoplasty, cultured in the commonly used MCDB170 (Hammond et al., 1984) or DFCI-1 (Band and Sager, 1989) media, exhibit predominantly basal epithelial cell phenotypes (Taylor-Papadimitriou et al., 1989; Trask et al., 1990). In the present study, a culture method has been developed to grow these two types of normal human breast epithelial cells, i.e., luminal epithelial cells and basal epithelial cells, from reduction mammoplasty tissues. The detailed characterization of these two types of cells and their response to SV40 transfection is presented in this communication.

Materials and Methods

Culture media. The medium used in these studies, MSU-1 medium, is a 1:1 mixture (v/v) of a modified Eagle's MEM (GIBCO BRL Life Technologies, Inc., Grand Island, NY) and a modified MCDB 153 (Sigma Chemical Co., St. Louis, MO) supplemented with human recombinant epidermal growth factor (0.5 ng/ml) (E-1264 Sigma), insulin (5 ug/ml) (I-1882, Sigma), hydrocortisone (0.5 ug/ml) (H-0888, Sigma), human transferrin (5 ug/ml) (T-7786, Sigma), 3, 3', 5-triiodo-D.L.-thyronine ($1x10^{-8}$ M) (T-2627, Sigma), and 17 β-estradiol ($1x10^{-8}$ M) (E-2257, Sigma). The modified Eagle's MEM (Chang et al., 1981) contains Earle's balanced salt solution with 1 mg/ml sodium bicarbonate and 7.64 mg/ml sodium chloride, a 50% increase in all vitamins and essential amino acids

(except glutamine), and a 100% increase in all nonessential amino acids and 1 mM sodium pyruvate (pH adjusted to 6.5 before the addition of sodium bicarbonate). The modified MCDB 153 was prepared from commercial MCDB 153 (Boyce and Ham, 1983) powdered medium (M-7403, Sigma), supplemented with 0.1 mM ethanolamine (E-6133, Sigma), 0.1 mM phosphorylethanolamine (P-0503, Sigma), $1.5x10^{-4}$ M calcium and amino acids, i.e., isoleucine ($7.5x10^{-4}$ M), histidine ($2.4x10^{-4}$ M), methionine ($9x10^{-5}$ M), phenylalanine ($9x10^{-5}$ M), tryptophan ($4.5x10^{-5}$ M) and tyrosine ($7.5x10^{-5}$ M) (Pittelkow and Scott, 1986) (Sigma). The pH of this medium was also adjusted to 6.5 before the addition of sodium bicarbonate ($1.4 x10^{-2}$ M).

Acquisition, processing and culturing of human breast epithelial cells (HBEC). All reduction mammoplasty tissues were obtained from female patients of 21-29 years of age. A total of 7 reduction mammoplasty tissue specimens, from 7 different patients, were examined in these studies. The HBEC that were obtained from the 7 reduction mammoplasty tissue specimens are designated HME-5, 6, 8, 11, 12, 13 and 14. The tissue specimens were minced into small pieces with scalpels, then digested in collagenase-Type IA (C-9891, Sigma) solution (1 g tissue / 10 mg of collagenase in 10 ml medium) at 37°C in a waterbath overnight (16-18 hr). The next morning, the solution containing the digested tissues was centrifuged to remove the collagenase solution. The cellular pellet was washed once with MSU-1 medium before being suspended in the MSU-1 medium supplemented with 5% fetal bovine serum (FBS) (GIBCO). Subsequently, the cells were plated into two flasks (150 cm^2). After a 2 hour incubation, the cells (or cell aggregates) which remained in suspension were transferred to 4-6 flasks

(75 cm^2) for the purpose of reducing the number of attached fibroblasts. After an overnight incubation, the medium was changed to the FBS-free MSU-1 medium. The MSU-1 medium was changed once every 2 days for 1 week. Subsequently, the cells were removed with solutions of trypsin (0.01%) (Sigma) and ethylenediaminetetraacetic acid (EDTA) (0.02%) (Sigma) and stored in solution [phosphate buffered saline (PBS) containing 10% dimethyl sulfoxide] in liquid nitrogen. During this week period, virtually all of the fibroblasts can be removed by treatment (1-2 times) with diluted trypsin (0.002%) and EDTA (0.02%) solution.

To start a culture from stored frozen cells in liquid nitrogen, the cells were thawed and placed in the MSU-1 medium supplemented with 5% FBS for 4 hours for the attachment of residual fibroblasts. The epithelial cells in suspension were transferred to new flasks and cultured in the FBS-free MSU-1 medium. All cultures were incubated at 37°C in incubators supplied with humidified air and 5% CO_2.

Separation of the 2 types of HBEC. The first passage of HBEC, recovered from liquid nitrogen storage, was plated in MSU-1 medium supplemented with 5% FBS. After overnight culture, the cells which remained in suspension, were transferred to new plates. Continued culture of these cells in the FBS-containing medium gave rise to one morphological type of cell. The attached cells, in the overnight culture, cultured in the FBS-free MSU-1 medium supplemented with cholera toxin (1 ng/ml) (Sigma) and 0.4% bovine pituitary extract (Pel-Freez, Rogers, AR) gave rise to a second morphological type of cell. The rare contaminants of the other cell type in these cultures can be removed by mechanically scraping the unwanted small colonies once they were

morphologically recognizable.

Assessment of the characteristics of cultured HBEC.

1. Morphology. The normal HBEC with the 2 different types of morphology can be observed and easily distinguished under a Nikon phase contrast microscope without any treatment.

2. Immunocytochemical analysis. HBEC, grown on 35 mm culture dishes for colony development or until 50% confluent, were rinsed with PBS and fixed with formaldehyde (3.7% in PBS) for 10 min. After treatment with 95% ethanol for 5 min, the cells were incubated with bovine serum albumin (BSA) (Sigma) (1% in PBS) for 30 min. This was followed by treatment with the primary antibody against the target molecules (prepared in PBS containing 0.1% BSA) for 30 min. The cells were then incubated with biotin-conjugated bridging antibody (Sigma) and then with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Sigma) (in PBS with 0.1% BSA) for 30 min. All treatments were carried out at room temperature. Following each treatment, the cells were thoroughly rinsed with PBS (5-6 times). The monoclonal antibodies against keratin 14 (C-8791) and keratin 18 (C-8541) were obtained from Sigma. The monoclonal antibody against EMA was a gift from Dr. M. G. Ormerod of the Institute of Cancer Research, Royal Cancer Hospital (Sutton, Surrey, UK). The monoclonal antibodies to integrin $\alpha 6$ (BQ16) and $\beta 4$ (UM-A9) (Liebert et al., 1993) were gifts from Dr. M. Liebert of the University of Michigan, Ann Arbor, MI. Cells for integrin immunostaining were treated differently than those for keratin 14, 18 and EMA. These cells were fixed with paraformaldehyde (4% in PBS) for 30 min. Subsequently, the cells were placed in ice-

cold methanol for 30 sec. After permeablization, the cells were washed with PBS and incubated in a 3% BSA and 0.1% Tween 20 solution for 1.5 hr to block non-specific binding. Subsequently, the cells were incubated with the primary antibodies against integrin α6 and β4 for 1.5 hr at room temperature. Without using the bridging antibody, the primary antibody-treated cells were incubated for 1.5 hr in the dark with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab., Inc. West Grove, PA) (in PBS containing 1% BSA and 0.01% Tween 20) . The fluorescence was detected and analyzed by a ACAS 570 cell analyzer (Meridian Instruments, Okemos, MI).

3. Gap junctional intercellular communication (GJIC). GJIC was studied by the scrape loading/dye transfer technique previously developed in this laboratory (El-Fouly et al., 1987). HBEC were grown to 80% confluency in 35 mm culture dishes, rinsed several times with PBS, and exposed to 0.05% Lucifer yellow (Sigma) dye solution in PBS. Several cuts were made on the monolayer of cells with a scalpel in the presence of the dye mixture to load the dye into cells. The cells remained in the dye solution at room temperature for 4 min. After removing the dye solution, the cells were rinsed several times with PBS and examined using a Nikon phase contrast epifluorescence microscope to assess the extent of dye transfer.

Treatment of cultured HBEC with SV40 DNA.

SV40 viral DNA was purchased from GIBCO BRL Life Technologies. The transfection was mediated by lipofectin (GIBCO). The two cellular types of HBEC were plated on 60 or 90 mm plates. When the cells reached 50 ~ 70% confluency (each plate contained approximately 2-3 x 10^6 cells), they were washed with the FBS-free MSU-1

medium twice, then incubated with 3 ml of FBS-free MSU-1 medium containing SV40 DNA (0.67 ug/ml) and lipofectin (10 ug/ml) at 37^{0}C, for 16 hours. The next day, the lipofectin containing medium was replaced with the MSU-1 medium (with or without FBS, for the two different types of HBEC) and the cells were cultured for 3 days. The SV40 treated cells were subcultured and replated in 9 cm plates at a lower cell density to provide more room for cell growth and colony formation. The cells which were able to form large colonies could be easily distinguished from the non-transfected cells which senesced after subculture. Developing colonies from the 2 cellular types of HBEC after SV40 transfection were isolated by the trypsin/glass ring method for further characterization. HBEC from 3 reduction mammoplasty specimens (from 3 different patients, i.e., HME-11, HME-13 and HME-14) were used for the SV40 transfection. The expression of SV40 large T-antigen in the SV40 transfected HBEC was examined by immunostaining using the monoclonal antibody AB-2 (Oncogene Science, Inc., Uniondale, NY). The immunostaining procedure for SV40 large T-antigen is similar to the method used for the keratin 14 , 18 and EMA antibodies described previously.

Anchorage independent growth (AIG). 0.5% Agarose (Type I, low EEO, Sigma), prepared in MSU-1 medium at 39^{0}C, was added to 60 mm culture dishes and allowed to solidify in the incubator. HBEC cells ($2x10^4$), suspended in the medium at 39^{0}C with 0.33% agarose, were overlaid on top of the hard agar layer. Plates were incubated at 37^{0}C and liquid medium was added 3 days after HBEC inoculation and renewed every 3 days. After 3~4 weeks of incubation, the AIG colonies were observed between the two agarose layers and the size of the colony was determined

Assessment of cell proliferation potential. The cumulative population doubling level (cpdl) was determined by calculating the initial (Ni) and final (Nf) HBEC number using the formula: pdl = ln (Nf / Ni) / ln 2, where ln is the natural log. SV40 transfected HBEC, which exhibited higher proliferative activities than non-SV40 transfected HBEC, are referred to as having extended life. SV40 transfected HBEC which were propagated continuously (a cpdl of 100 or greater) are referred to as being immortal.

Results

Characteristics of HBEC in culture

Morphology of the 2 types of HBEC. Single HBEC or HBEC aggregates from reduction mammoplasty tissues began to proliferate in MSU-1 medium within 2 days. These initial cell cultures were subcultured and stored in liquid nitrogen at day 7. The first passage cells, subcultured from the initial culture or thawed from the preserved cells in liquid nitrogen, when cultured in the FBS-free MSU-1 medium, formed 2 morphologically distinguishable colonies (Fig. 1A). The first colony type contained cells that were elongated and variable in shape, less reflective and less distinctive in cell boundary (Fig. 1A-right and 1B). The cells in this colony are herein referred to as Type I cells. The second colony type, herein referred to as Type II, contained cells that were more uniform in cell shape (cobble stone-shaped) and have a conspicuous cell boundary (Fig. 1A-left). The edge of Type I cell colonies is smooth and appears to be bounded by a layer of elongated cells in contrast to the non-constrained outline of Type II cell colonies. The presence of these 2 types of colonies/cells has been consistently observed in all 7 HBEC primary cultures examined, i.e., HME-5, 6, 8, 11, 12, 13 and 14. The

Figure 1 Representative first passage of HBEC in MSU-1 medium for 5 days (X~90). Both Type I (A-right, and B) and Type II (A-left) colonies develop in this medium.

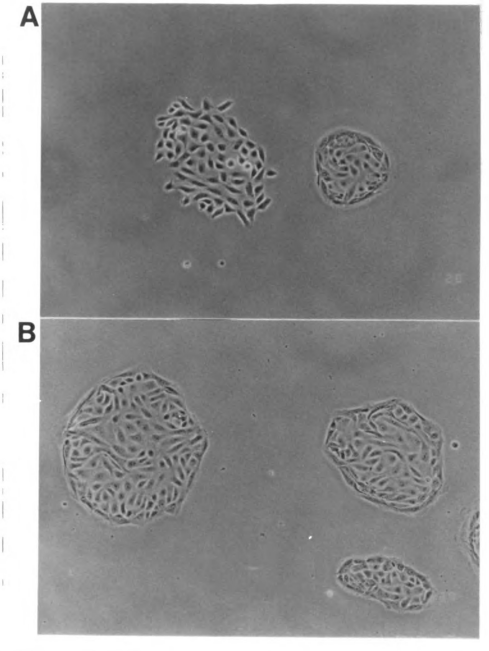

Figure 1

frequencies of these 2 types of colonies/cells vary among the 7 different HBEC cultures, e.g., the frequencies of Type I/Type II colonies at early passage for HME-5, HME-6 and HME-8 are 45%/55%, 9%/91% and 72%/28%, respectively. In addition to Type I and Type II cell colonies, some colonies containing both Type I and Type II cells were also observed (Fig. 2B and C-left). In these colonies, Type I cells were invariably situated at the center of the colony and were completely or partially surrounded by Type II cells. This spontaneously morphological change of Type I cells into Type II cells has been observed in each of the 7 HBEC cultures.

Development of enriched Type I HBEC cultures. One major difference between Type I and Type II cells is their growth response to FBS. Type I cells are stimulated to grow by FBS whereas Type II cells are growth inhibited by FBS (data not shown). A second difference between Type I and Type II cells is that after subculture, Type II cells attach to plastic plates earlier than do Type I cells. Many Type I cells still remain in suspension in the medium one day after trypsinization and replating. These cells can be transferred to a new plate to obtain a culture enriched in Type I cells. Adding FBS to the culture medium (inhibits the growth of Type II cells) can further enrich the cultures for Type I cells.

Induction by cholera toxin of Type I HBEC into Type II HBEC. The first passaged HBEC (HME-5 and 8) were inoculated in MSU-1 medium in the presence or absence of cholera toxin (1 ng/ml). After 10-12 days, the frequencies of 3 types of colonies (Type I cells only, Type II cells only and both Type I and Type II cells) were determined. The results from these experiments consistently showed that cholera toxin significantly ($P<0.01$) increased the frequency of colonies containing both types of cells (Table 1).

Figure 2 HBEC colonies grown in MSU-1 medium for 8 days (X~90). One type of colony contains only Type I cells (A and C-right), the other colonies contain both Type I and Type II cells (B and C-left). In these latter colonies, the Type I cells were either partially or completely surrounded by Type II cells.

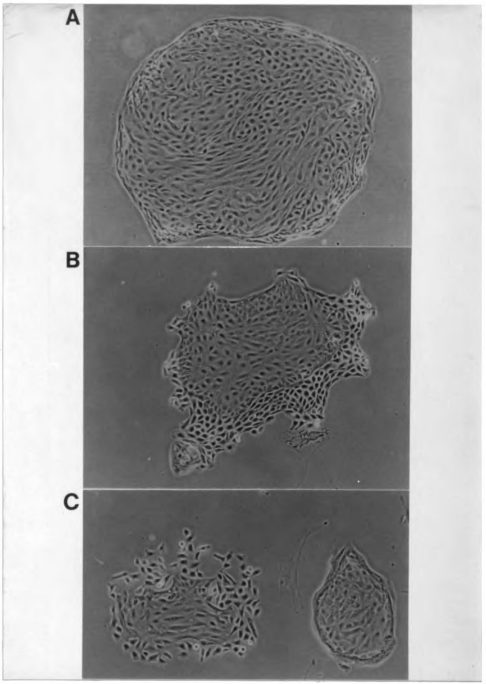

Figure 2

Table 1 Effect of cholera toxin on frequencies of Type I, Type II and Type I/ Type II colonies developed from early passage HBEC

HBEC	Cholera Toxin (1ng/ml)	Total Colonies (No. of plate)	Colony Type		
			Type I cells only	Type I cells surrounded by Type II cells	Type II cells only
HME-5[a]	--	301 (4)	95 (31%)	83 (28%)	123 (41%)
	+	362 (4)	44 (12%)	155 (43%)[c]	163 (45%)
HME-8[a]	--	1477 (4)	940 (64%)	161 (11%)	376 (25%)
	+	1941 (4)	1147 (59%)	315 (16%)[c]	479 (25%)
HME-8[a]	--	1918 (10)	1421 (74%)	54 (3%)	443 (23%)
	+	1716 (10)	1249 (73%)	150 (9%)[c]	317 (18%)
HME-14[b]	--	723 (3)	652 (90%)		71 (10%)
	+	666 (3)	547 (82%)		119 (18%)[c]

[a] Early passage HBEC (HME-5 and 8) were innoculated in MSU-1 medium supplemented with 5% FBS for 1 day. The medium was changed to MSU-1 with or without cholera toxin the next day, and the cells were incubated for a total of 10 days for colony development. The colony-forming efficiencies were 15% and 18% in the absence and presence of cholera toxin, respectively for HME-5.

[b] HBEC (HME-14) enriched for Type I cells were inoculated in MSU-1 medium supplemented with 5% FBS for 1 day. The medium was change to MSU-1 with or without cholera toxin the next day, and the cells were incubated for a total of 10 days for colony development.

[c] The frequencies of Type I/Type II cell colonies (HME-5 and 8) or Type II cell colonies (HME-14) are significantly higher upon cholera toxin treatment ($P < 0.01$) (Baildy, 1959).

The frequency of colonies exemplified by Type I cells surrounded by Type II cells, in the presence of cholera toxin, was increased from 28% to 43%, 11% to 16% and 3% to 9%, respectively. The total number of colonies was not significantly influenced by cholera toxin. In the second experiment, HBEC (HME-14) enriched for Type I cells were inoculated in MSU-1 medium in the presence or absence of cholera toxin (1 ng/ml). After 10 days, in the presence of cholera toxin, the frequency of colonies with Type II cells was found to be significantly ($P<0.01$) increased (from 10% to 18%), while the total number of colonies was not significantly changed (Table 1). These results provide evidence that treatment with cholera toxin enhances the transition of Type I cells into Type II cells.

GJIC in Type I and Type II HBEC. Type I cells and Type II cells were examined for their ability to perform GJIC using the scrape loading/dye transfer technique. The results show that Type I cells were deficient in GJIC (Fig. 3A), while Type II cells were efficient in GJIC (Fig. 3B). This difference in GJIC in Type I and Type II cells was observed in each of the 7 HBEC (HME-5, 6, 8, 11, 12, 13 and 14) and was observed in both early and late passage cells.

Expression of EMA, keratins 14 and 18 and α6/β4 integrins in Type I and Type II HBEC. Three HBEC (HME-5, 11 and 12) were examined for their antigenic marker expression. The results showed that Type I cells consistently expressed EMA and keratin 18 (luminal epithelial cell markers) but not keratin 14 (basal epithelial cell marker), while Type II cells consistently expressed keratin 14 but not EMA and keratin 18 (Fig. 4). Type I cells did not express α6 (basal epithelial cell marker) or β4 integrins (Fig. 5A and B) whereas Type II cells consistently expressed α6 integrins (Fig. 5C) but did not express

Figure 3 Representative gap junctional intercellular communication (GJIC) in Type I and Type II HBEC as examined by the scrape loading/dye transfer technique. Type I cells were deficient in GJIC (A). Type II cells were efficient in GJIC (B) (X~90).

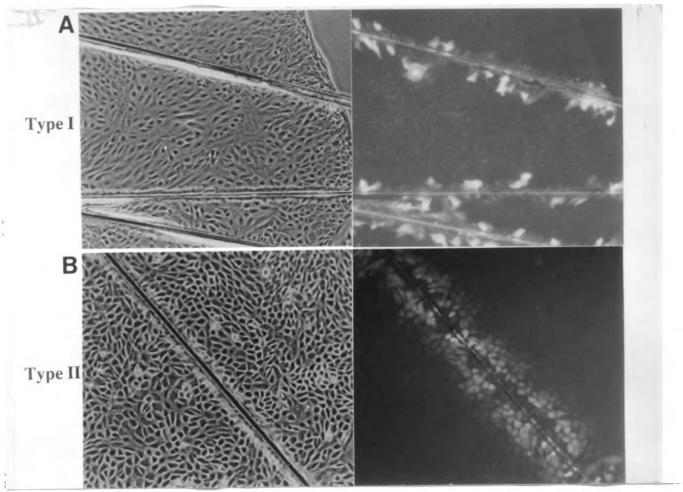

Figure 3

Figure 4 Representative expression of EMA (top), keratin 18 (middle) and keratin 14 (bottom) in Type I (left) and Type II (right) HBEC as revealed by immunofluorescence staining and detected by the ACAS-570 laser cytometer. (X~200).

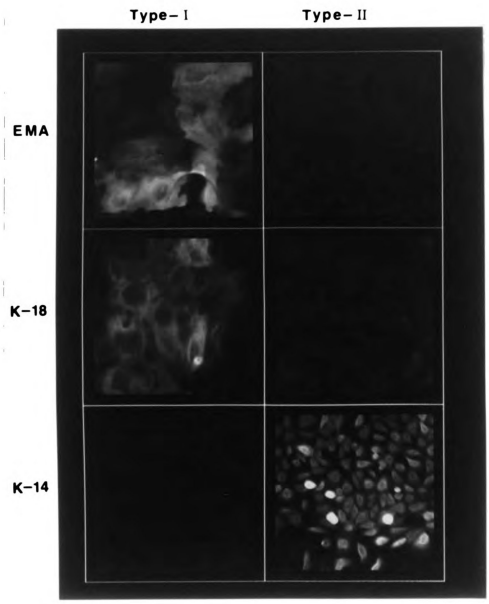

Figure 4

Figure 5 Representative immunofluorescence staining of Type I (A, B) and Type II (C, D) HBEC using antibodies against α6 integrin (A, C), β4 integrin (B, D). (X~200).

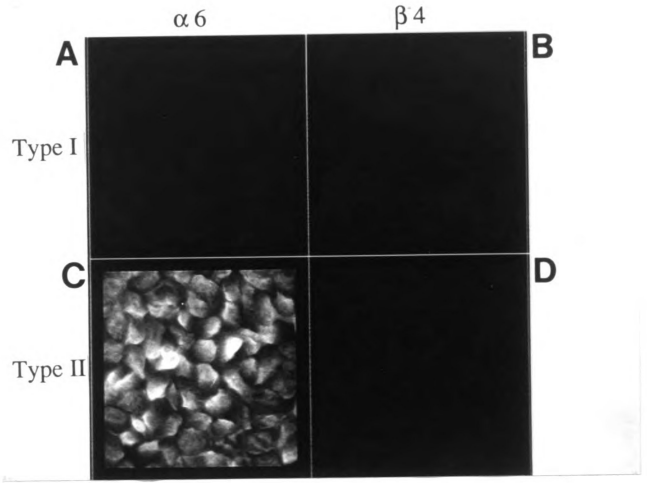

Figure 5

β4 integrins (Fig. 5D). That Type I and Type II cells are distinctly different in their antigenic expression has been demonstrated in these studies. A summary of the differences between Type I and Type II cells is provided in Table 2.

Effect of transfection of Type I and Type II HBEC with SV40 DNA.

Isolation of SV40 transfected HBEC clones. Type I and Type II cells, from 3 HBEC (HME-11, 13 and 14), were transfected with SV40 DNA. After SV40 transfection, the majority of the cells became senescent and stopped proliferating within a week. Against the background of senescent cells, a few colonies containing proliferating cells became evident. These colonies (clones) were isolated for continual growth expansion and further characterization. A total of 9 independent Type I cell-derived SV40 transfected colonies were isolated from HME-11 (referred to as M11SV-1, 2, 3, 4 and 5), HME-13 (referred to as M13SV-1 and 2) and HME-14 (referred to M14SV-1 and 2). A total of 8 independent Type II cell-derived SV40 transfected colonies were isolated from HME-11 (referred to as M11SV-21 and 22), HME-13 (referred to as M13SV-21, 22 and 23) and HME-14 (referred to as M14SV-21, 22 and 23). Immunocytochemical analysis, using antibodies against SV40 large T-antigen, showed that each of the isolated clones were positive for the expression of SV40 large T-antigen whereas the non-transfected parental cells were negative (Fig. 6, Table 3). The expression of SV40 large T-antigen in the SV40 transfected Type I and Type II cells were restricted to the nuclei.

Proliferation characteristics of SV40 transfected Type I and Type II HBEC. The cpdl was determined for each of the clones of SV40 transfected Type I and Type II cells.

Table 2 Summary of 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	boundary not smooth
Attachment on plastic surface after typsinization	late	early
Effect of fetal bovine serum	growth promotion	growth inhibition
Effect of cholera toxin	induces Type I cells to change into Type II cell morphology	
Gap junctional intercellular communication	deficient	efficient
Expression of: Epithelial membrane antigen keratin 18 keratin 14 α 6 integrin	 + + -- --	 -- -- + +

Figure 6 Representative immunofluorescent staining of SV40 transfected Type I HBEC (B) and SV40 transfected Type II HBEC (D). Non-transfected parental Type I HBEC (A) and Type II HBEC (C) did not show staining. (X~200).

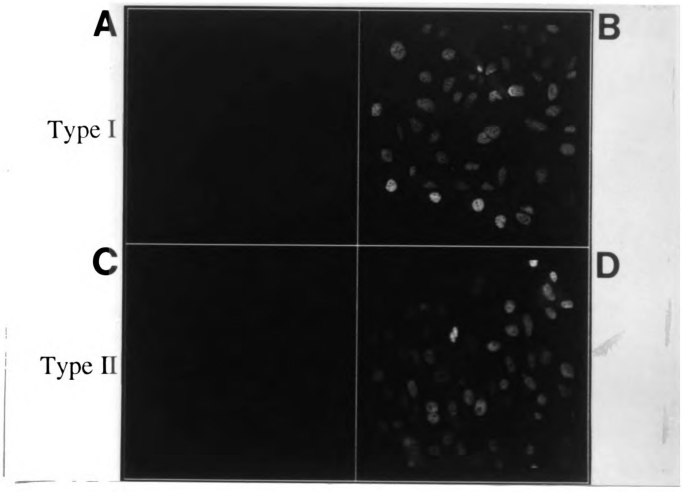

Figure 6

Table 3 Summary of characteristics of SV40 transfected Type I and Type II HBEC

Type I	SV-40 T-antigen	EMA	K-18	K-14	GJIC	AIG
M11SV-1	+	+	++	--	--	+
M11SV-2	+	+	++	--	--	+
M11SV-3	+	+	++	--	--	+
M11SV-4	+	+	++	--	--	+
M11SV-5	+	+	++	--	--	+
M13SV-1	+	+	++	--	--	+
M13SV-2	+	+	++	--	--	+
M14SV-1	+	+	++	--	--	+
M14SV-2	+	+	++	--	--	+
Type II						
M11SV-21	+	--	--(+)	+	N.D.	--
M11SV-22	+	--	--(+)	+	+	--
M13SV-21	+	--	--	+	N.D.	--
M13SV-22	+	--	--	+	+	--
M13SV-23	+	--	--	+	+	--
M14SV-21	+	--	--	+	N.D.	--
M14SV-22	+	--	+	+	N.D.	--
M14SV-23	+	--	+	+	N.D.	--

* abbreviations: EMA, epithelial membrane antigen; K-18, keratin 18; K-14, keratin 14; GJIC, gap junctional intercellular communication; AIG, anchorage independent growth; N.D., not done; +, positive; ++, strong positive; --, negative; --(+), heterogeneous.

Almost all of the SV40 transfected clones had an extended lifespan, a phenomenon independent as to whether or not the clones were derived from Type I or Type II cells, maximum cpdl ranging from 20~50, i.e., Type I, cpdl, mean ± S.E. = 34.4 ± 2.7; Type II, cpdl, mean ± S.E. = 27.2 ± 2.1). Two of the clones (2/9) (M13SV-1 and M13SV-2), both derived from SV40 transfected Type I cells, became immortal (with and without crisis for M13SV-1 and M13SV-2, respectively), having a cpdl greater than 100. None of the Type II cell-derived SV40 transfected clones (0/8) became immortal.

Characterization of SV40 transfected Type I and Type II HBEC. The expression of EMA, keratin 18, keratin 14 and the ability to perform GJIC in the SV40 transfected Type I cell clones and Type II cell clones were found to mimic their parental counterparts,i.e., the expression of EMA, keratin 18 and GJIC deficiency was observed in Type I cell clones and the expression of keratin 14 and GJIC proficiency was observed in the Type II cell clones. These data are summarized in Table 3.

Anchorage independent growth (AIG) of SV40 transfected Type I and Type II HBEC.

SV40 transfected Type I and Type II cells had markedly different abilities to grow in soft agar (AIG). Each of the SV40 transfected Type I cells formed colonies in soft agar (AIG) (Fig. 7, Table 3). The colony-forming frequency was, mean ± S.E., 7.2 ± 2.8 %. The largest colonies observed ranged from 0.5 mm to 1 mm in diameter. In contrast, none of the SV40 transfected Type II cells formed colonies in soft agar.

Discussion

As summarized in Table 2, that Type I and Type II HBEC are quite different

Figur

I HBE

Figure 7 Representative anchorage independent growth (AIG) of SV40 transfected Type I HBEC (A, X~36) (B, X~90).

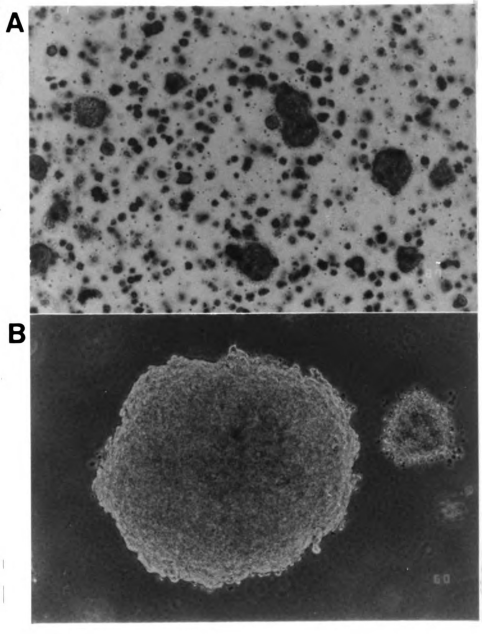

Figure 7

phenotypically. Type I cells express EMA and keratin 18 but do not express keratin 14 and α6 integrin. EMA and keratin 18 expression are markers for luminal epithelial cells (Taylor-Papadimitriou et al., 1989; O'Hare et al., 1991). Type II cells express keratin 14 and α6 integrin but do not express EMA and keratin 18. Keratin 14 and α6 integrin expression are markers for basal epithelial cells (Taylor-Papadimitriou et al., 1989; Koukoulis et al., 1991). In previously published reports in which cultures of primary HBEC were examined, it appears that the vast majority of the cultured cells, derived from reduction mammoplasty, more closely resembled, phenotypically, our Type II cells as judged by cell morphology, antigenic expression, and growth inhibition by FBS (Hammond et al., 1984; Band and Sager, 1989; Taylor-Papadimitriou et al., 1989; Trask et al., 1990). Cultures of primary HBEC, in which the predominant phenotype is similar to our Type I cells, heretofore has not been reported. The reason for this may be due to the type of culture media used in these studies. The media most often used to culture primary HBEC, (e.g., MCDB 170, DFCI-1) (Hammond et al., 1984; Band and Sager, 1989) are most often supplemented with cholera toxin and/or bovine pituitary extract. In our study, these supplements favored the conversion of Type I to Type II cells (cholera toxin, this study and bovine pituitary extract, unpublished results).

An important observation in our studies is that we can accelerate the spontaneous change of Type I cells into Type II cells by the addition of cholera toxin to the culture media. This not only results in a change in cellular morphology, but, in addition, results in a striking change in gene expression, e.g., the switch from EMA and keratin 18 expression to keratin 14 and α6 integrin expression. Furthermore, these cells become altered in their responsiveness to serum, i.e., Type I cells are growth stimulated by FBS

while the growth of Type II cell is inhibited by serum supplementation. This conversion may be triggered by an increase in the cellular levels of cyclic AMP induced by cholera toxin. The culture medium developed in our studies (MSU-1) allows for the separation and/or enrichment of Type I and Type II cells upon supplementing the medium with FBS or cholera toxin/bovine pituitary extract.

The induction of immortalization of HBEC has been reported previously (Chang et al., 1982; Stampfer and Bartley, 1985; Band and Sager, 1990; Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Berthon et al., 1992; Van Der Haegen and Shay, 1993; Shay et al., 1993). In these studies , either HBEC derived from lactational samples (milk cells) or HBEC derived from reduction mammoplasty were utilized. Cultured milk cells most often express keratin 18 (luminal epithelial cells); on occasion, colonies of cultured milk cells will also express keratin 14 (Taylor-Papadimitriou et al., 1989). Cells from reduction mammoplasty contain predominantly basal epithelial cells when cultured in MM or MCDB 170 medium (Taylor-Papadimitriou et al., 1989). Utilizing the MSU-1 culture medium with or without FBS, we can enrich 'for these 2 types of cells (luminal and basal epithelial cells) and determine their differential response to a potential oncogenic stimulus.

SV40 is an oncogenic agent that has been reported by a number of laboratories to induce immortalization of primary HBEC (Chang et al., 1982; Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Berthon et al., 1992; Van Der Haegen and Shay, 1993; Shay et al., 1993). After transfection of SV40 DNA into Type I and Type II cells, clones with extended lifespan were derived from Type I and Type II cells at comparable frequency. However, 2 of 9 of the SV40 transfected Type I cell-

derived extended life clones converted to immortal cell lines, while none (0/8) of the SV40 transfected Type II cell-derived extended life clones became immortalized. Although we have never obtained an immortal cell line from a Type II cell-derived extended life clone, we do not exclude this possibility. More impressively, however, is the difference in anchorage independent growth (AIG) between SV40 transfected Type I and Type II cells. None (0/8) of the SV40 transfected Type II cell-derived extended life clones displayed AIG, while each (9/9) of the SV40 transfected Type I cell-derived extended life clones showed AIG. AIG is often used to identify tumor cells (Hamburger and Salmon, 1977) and is frequently described as a marker for neoplastic transformation (Stoker, 1968; Macpherson, 1973; Marshall et al., 1977). In past reports, in which SV40 was effective in the immortalization of HBEC, such immortalized cells were incapable of AIG (Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Berthon et al., 1992; Van Der Haegen and Shay, 1993; Shay et al., 1993), with the possible exception of one cell line which was selected in soft agar (Chang et al., 1982). Thus, our results demonstrate that Type I cells and Type II cells differ substantially in their response to an oncogenic stimulus (SV40), particularly with regard to AIG.

The SV40 transfected Type I and Type II cells closely resembled the phenotypes of their parental cells, such as the expression of EMA and keratins. In addition, they maintained the parental phenotype with regard to GJIC. The Type I cell-derived SV40 transfected clones also are similar in many respects to human breast carcinoma cell lines. For example, MCF-7 and T47D human breast carcinoma cell lines, which express the antigenic markers keratin 18 and EMA but not keratin 14 (data not shown and Taylor-Papadimitriou et al., 1989), are deficient in GJIC and have AIG (data not shown). These

observations support the hypothesis that the origin of human breast carcinomas is the luminal epithelial cell and suggest that the Type I cells described in this communication might be the major target cell for neoplastic transformation.

Parenthetically, we should note that our SV40 transfected Type I cell clones (extended life or immortal) were shown to be non-tumorigenic when inoculated into athymic nude mice. However, one Type I cell-derived SV40 transfected extended life clone (M11SV-1) was treated with a combination of BrdU and black light, a potent mutagenic treatment (Chu et al., 1972). An immortal cell line has been obtained from this treatment and this cell line has been shown to be weakly tumorigenic in athymic nude mice. Infection of the BrdU/black light immortalized cell line with a mutated rat neu oncogene (Dotto et al., 1989) greatly enhanced the tumorigenicity of these cells upon inoculation into athymic nude mice (Kao et al., 1994). Currently we are examining our SV40 transfected extended life clones and immortalized cell lines for their responsiveness to additional oncogenic stimuli.

Cancer cells are believed to arise from stem cells or early precursor cells and often have a phenotype similar to normal undifferentiated cells (Sigal et al., 1992) or have a combined phenotype of different cell types of a common lineage (e.g., leukemia cells often express both lymphoid and myeloid cell antigens) (Sawyers et al., 1991). Therefore, cancer has been termed a disease of the pluripotent stem cell (Sawyers et al., 1991), a disease of cell differentiation (Markert, 1968) or oncogeny as blocked or partially blocked ontogeny (Potter, 1978). For human tissues, except for peripheral blood stem cells (Gabbianelli et al., 1990), stem cells of solid tissues have rarely been characterized. Attempts to characterize subpopulations of cells with stem cell characteristics in solid

tissues have been reported for fetal kidney epithelial cells (Chang et al., 1987) and for epidermal cells (Jones and Watt, 1993). In our study, the phenotype of our Type I cells is suggestive of stem cells as they have the ability to give rise to another type of cell with a different phenotype, i.e., Type I cell (expresses luminal epithelial cell markers) to a Type II cell (expresses basal epithelial cell markers). In addition, our Type I cells are deficient in GJIC; GJIC deficiency has been reported to be a characteristic of putative stem cells (Chang et al., 1987).

In summary, 2 types of HBEC were derived and cultured from human breast reduction mammoplasty. Type I cells have antigenic characteristics of luminal epithelial cells while Type II cells have antigenic characteristics of basal epithelial cells. Importantly, these 2 types of cells differ substantially in their response to an oncogenic (SV40) stimulus, i.e., the SV40 transfected Type I cells have a greater tendency to become immortal, and most strikingly, have the ability to grow in soft agar (AIG); SV40 transfected Type II cells totally lack the ability to grow in soft agar (AIG). The ability to separate HBEC into cell types that vary in their sensitivity to oncogenic stimuli will facilitate our ability to consistently and reproducibly transform normal HBEC by oncogenic agents.

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CHAPTER 4

Characterization of Two Types of Normal Human Breast Epithelial Cells Derived from Reduction Mammoplasty: Growth Requirements in Defined Culture Media and Neoplastic Transformation

Abstract

A chemically defined culture medium was developed to support the growth of two distinctly different types of normal human breast epithelial cells (HBEC), Type I and Type II cells, derived from reduction mammoplasty. Type I cells expressed the epithelial membrane antigen (EMA) and keratin 18, expression markers of luminal epithelial cells. Type II cells expressed keratin 14 and α6 integrin, expression markers of basal epithelial cells. Type I cells are deficient in gap junctional intercellular communication (GJIC) while Type II cells are proficient in GJIC. In this study we examined and compared the growth factor and hormone requirements of these two types of cells *in vitro*. In addition, a series of cell lines originated from the Type I cell cultures with different degrees of tumorigenicity were obtained by sequential transfection with SV40 DNA (extended lifespan and non-tumorigenic), treatment with 5-bromodeoxyuridine (BrdU)/black light (immortal and weakly tumorigenic) and infection of a virus carrying the neu oncogene (immortal and highly tumorigenic). These cell lines were also examined for their growth factor and hormone requirements *in vitro*. Since BrdU/black light treatment failed to convert Type II cell-derived SV40 transfected clones to immortal cell lines, these cells were not examined for growth factor/hormonal requirement *in vitro*.

Growth of Type I cells (passage 2) was consistently inhibited by withdrawing epidermal

growth factor (EGF), hydrocortisone (HC) or insulin (INS) from the culture media; the majority of these cells became senescent after 10 days of culture. Growth of Type II cells (passage 2) was consistently inhibited by withdrawal of only INS from the culture media. After 10 days of culture, the passage 2 Type II cells were successfully subcultured to obtain the passage 3 Type II cells; their growth was inhibited by withdrawal of INS, EGF and HC from the culture media. Growth of Type I cells was consistently enhanced by adding fetal bovine serum (FBS) or cholera toxin (CT) to the culture media; growth of Type II cells (passage 2 and 3) was consistently inhibited by FBS supplementation. The growth promoting effect of CT was observed in the second passaged Type II cells in only one of two HBEC cultures tested and not detected in the third passaged Type II cells. The requirement of 3,3',5-triiodo-DL-thyronine (T_3) for maximal growth of Type I and Type II cells was observed only in one of the two HBEC cultures tested (i.e., HME-12, Type I and second passaged Type II cells). Withdrawal of human transferrin (HT) or 17β-estradiol (E_2) from the culture media did not alter the growth of Type I or Type II cells.

SV40 transfected Type I cells, whether or not they were treated with BrdU/black light or the neu oncogene still required EGF, HC or INS for optimal growth. However, rapidly growing tumor cells obtained from athymic nude mice, derived from Type I cells treated with SV40, BrdU/black light and the neu oncogene, did not show a growth dependency for EGF, HC or INS but did appear to require HT and T_3 for optimal growth. FBS, but not CT stimulated the growth of these cell lines. The results of this study provide insight into the growth factor/hormonal responsiveness of two distinctly different HBEC (Type I and Type II cells), and growth factor/hormonal requirements of a series of immortal and

/or neoplastic cell lines derived from one of these types of cells (Type I). Type I cells and the immortal and tumorigenic cell lines derived from these cells were shown to be consistently growth stimulated by FBS, the growth of Type II cells was inhibited by FBS. Thus, the serum requirements for primary cultures of normal HBEC will vary and is dependent, at least in part, on the predominant type of cell in the culture.

Introduction

Normal human breast epithelial cell (HBEC) cultures have been developed from human lactational fluids and from reduction mammoplasty tissues (Buehring, 1972; Taylor-Papadimitriou et al., 1977; Stampfer et al., 1980; Hammond et al., 1984; Petersen and Deurs, 1988; Band and Sager, 1989; Emerman and Wilkinson, 1990; Ethier et al., 1990). HBEC derived from lactational fluids were usually grown in short term cultures and were predominantly luminal epithelial cells (Buehring, 1972; Taylor-Papadimitriou et al., 1977). HBEC derived from reduction mammoplasty tissues have been predominantly basal epithelial cells (Taylor-Papadimitriou et al., 1989; Trask et al., 1990). Reduction mammoplasty derived cells have been grown in long term cultures in MCDB 170 medium (Hammond et al., 1984) or in DFCI-1 medium (Band et al., 1989) supplemented with insulin (INS), hydrocortisone (HC), epidermal growth factor (EGF), human transferrin (HT), ethanolamine, phosphoethanolamine, and bovine pituitary extract (BPE). BPE may be replaced by prolactin and prostaglandin E_1 for a completely chemical defined culture medium (Hammond et al., 1984).

Recently, we have developed a chemically defined culture medium which supported the growth of two phenotypically distinct types of HBEC (Type I and Type II), derived

from reduction mammoplasty tissues (Kao et al., 1994). The Type I cells expressed keratin 18 and the epithelial membrane antigen (EMA), expression markers of luminal epithelial cells (Taylor-Papadimitriou et al., 1989; O'Hare et al., 1991). In addition, these cells were deficient in gap junctional intercellular communication (GJIC). In contrast, the Type II cells expressed basal epithelial cell (or myoepithelial cell) specific markers, i.e., keratin 14 and α6 integrin (Taylor-Papadimitriou et al., 1989; Koukoulis et al., 1991); these cells were efficient in GJIC. Furthermore, the Type I cells, after transfection with SV40 DNA, were able to form colonies in soft agar (anchorage independent growth) while the SV40 DNA transfected Type II cells did not exhibit growth in soft agar (Kao et al., 1994). In this communication, we examine and report the *in vitro* growth requirements of these two distinct types of HBEC and a series of neoplastically transformed cell lines derived from the Type I HBEC.

Materials and Methods

Culture media. The culture medium used in these studies, MSU-1 medium, is a 1:1 mixture (v/v) of a modified Eagle's MEM (GIBCO BRL Life Technologies, Inc., Grand Island, NY) and a modified MCDB 153 (Sigma Chemical Co., St. Louis, MO) supplemented with EGF (0.5 ng/ml)(E-1264, Sigma), INS (5 ug/ml)(I-1882, Sigma), HC (0.5 ug/ml) (H-0888, Sigma), HT (5 ug/ml) (T-7786, Sigma), 3, 3', 5-triiodo-D.L.-thyronine (T_3)($1x10^{-8}$ M) (T-2627, Sigma), and 17β-estradiol (E_2)($1x10^{-8}$ M) (E-2257, Sigma). The modified Eagle's MEM (Chang et al., 1981) contains Earle's balanced salt solution with 1 mg/ml sodium bicarbonate and 7.64 mg/ml sodium chloride, a 50% increase in all vitamins and essential amino acids (except glutamine), and a 100% increase

in all nonessential amino acids, and 1 mM sodium pyruvate (pH adjusted to 6.5 before the addition of sodium bicarbonate). The modified MCDB 153 was prepared from the commercial MCDB 153 (Boyce and Ham, 1983) powdered medium (M-7403, Sigma), supplemented with 0.1 mM ethanolamine (E-6133, Sigma), 0.1 mM phosphorylethanolamine (P-0503, Sigma), $1.5x10^{-4}$ M calcium and amino acids, i.e., isoleucine ($7.5x10^{-4}$ M), histidine ($2.4x10^{-4}$ M), methionine ($9x10^{-5}$ M), phenylalanine ($9x10^{-5}$ M), tryptophan ($4.5x10^{-5}$ M) and tyrosine ($7.5x10^{-5}$ M) (Pittelkow and Scott, 1986). All chemicals were purchased from Sigma. The pH of this medium was adjusted to 6.5 before the addition of sodium bicarbonate ($1.4 x10^{-2}$ M).

Acquisition, processing and culturing of human breast epithelial cells (HBEC).

Reduction mammoplasty tissues were obtained from 3 female patients of 21-29 years of age. The HBEC obtained from the 3 reduction mammoplasty tissue specimens were designated HME-5, HME-11 and HME-12. The tissue specimens were minced into small pieces with scalpels, then digested in collagenase-Type IA (C-9891, Sigma) solution (1 gram tissue per 10 mg of collagenase in 10 ml medium) at 37°C in a waterbath overnight (16-18 hr). The next morning, the solution containing the digested tissues was centrifuged to remove the collagenase solution. The cellular pellet was washed once with MSU-1 medium before being suspended in the MSU-1 medium supplemented with 5% fetal bovine serum (FBS)(GIBCO). Subsequently, the cells were plated in two flasks (150 cm^2). After a 2 hour incubation, the cells (or cell aggregates) which remained in suspension were transferred to 4-6 flasks (75 cm^2) for the purpose of reducing the number of attached fibroblasts. After an overnight incubation, the medium was changed to the

FBS-free MSU-1 medium. The MSU-1 medium was changed once every 2 days for 1 week. Subsequently, the cells were removed with solutions of trypsin (0.01%) (Sigma) and ethylenediaminetetraacetic acid (EDTA) (0.02%) (Sigma) and stored in solution [phosphate buffered saline (PBS) containing 10% dimethyl sulfoxide] in liquid nitrogen. During this one week period, almost all of the fibroblasts can be removed by treatment (1-2 times) with diluted trypsin (0.002%) and EDTA (0.02%) solution.

To start a culture from stored frozen cells, the frozen cells in liquid nitrogen were thawed and placed in MSU-1 medium supplemented with 5% FBS for 4 hours for the attachment of residual fibroblasts. The epithelial cells in suspension were transferred to new flasks and cultured in the FBS-free MSU-1 medium. All cultures were incubated at 37^{0}C in incubators supplied with humidified air and 5% CO_2.

Separation of Type I and Type II HBEC. The first passage of HBEC, recovered from liquid nitrogen storage, was plated in the MSU-1 medium supplemented with 5% FBS. After overnight culture, the cells which remained in suspension were transferred to new plates. Continued culture of these cells in the FBS-containing medium gave rise to Type I cells. The attached cells, in the overnight culture, cultured in the FBS-free MSU-1 medium supplemented with cholera toxin (CT) (1 ng/ml)(Sigma) and 0.4% BPE (Pel-Freez, Rogers, AR) gave rise to Type II cells. The rare contaminants of the other cell type in these cultures were removed by mechanically scraping the unwanted small colonies once they were morphologically recognizable.

Establishment of cell lines from HBEC treated with SV40 DNA, 5-bromodeoxyuridine (BrdU)/black light and the neu oncogene.

Treatment of the Type I and Type II HBEC with SV40 DNA. SV40 viral DNA was purchased from GIBCO. The transfection was mediated by lipofectin (GIBCO). The Type I and Type II HBEC were plated on 60 or 90 mm plates. When the cells reached 50 ~ 70% confluency (each plate contained approximately 2-3 x 10^6 cells), they were washed with the FBS-free MSU-1 medium twice, then incubated with 3 ml of FBS-free MSU-1 medium containing SV40 DNA (0.67 ug/ml) and lipofectin (10 ug/ml) at 37°C, for 16 hours. The next day, the lipofectin containing medium was replaced with the FBS-free MSU-1 medium and the cells were cultured for 3 days. The SV40 treated cells were subcultured and replated in 9 cm plates at a lower cell density to provide more room for cell growth and colony formation. The cells which were able to form large colonies could be easily distinguished from the non-transfected cells which senesced after subculture. Colonies from the Type I and Type II HBEC after SV40 transfection were isolated by the trypsin/glass ring method for further characterization. HBEC from reduction mammoplasty specimen HME-11 were used for the SV40 transfection.

Treatment of SV40 transfected Type I and Type II HBEC with 5-Bromodeoxyuridine (BrdU) and black light. The combined treatment of BrdU and black light has been shown to induce high frequency of auxotrophic mutants in mammalian cells (Chu et al., 1972). SV40 transfected Type I and Type II cells were cultured in medium containing $1x10^{-4}$ M BrdU in 9 cm plates for 3 days. After incorporation of the BrdU into DNA, these cells were irradiated by black light (313 nm) for 1 hr and the treated cells were washed with PBS, then incubated in FBS-containing or FBS-free MSU-1 medium for

Type I or Type II cells, respectively. Most of the cells died and detached from the plate after the treatment and only a few cells survived and formed colonies (clones) with sustained growth. These clones were isolated and propagated.

Treatment of SV40 transfected, BrdU/black light treated Type I HBEC with the neu oncogene. The Glu664-neu virus-producing cell was a gift of Dr. G. Paolo Dotto (The Cutaneous Biology Research Center, Boston, MA) (Dotto et al., 1989). The virus carries a complete cDNA copy of the rat neu oncogene with a point mutation at amino acid position 664. This resulted in a substitution of glutamine residue for valine. The point mutation at 664 position leads to full oncogenic activation of the neu oncogene. The cells were plated in 9 cm plates and exposed to an undiluted viral stock in culture medium with 8 ug/ml polybrene (Sigma). After 2-3 hr of virus exposure, the virus-containing medium was replaced by normal culture medium. These infected cells were continuously cultured for 3 days before they were exposed to G418-containing medium (400 ug/ml) (GIBCO) for the selection of G418-resistant clones. The negative controls were the cells transfected by pC6M-neo virus (originally constructed by Stocking et al., 1985) which carries only the G418 resistance gene. The virus-producing cells for the control were also kindly provided by Dr. Dotto (Dotto et al., 1989).

Tumorigenicity assay. Cells derived from the different cell lines were injected s.c. into 21-30 day old female athymic nude mice (Harlan Sprague-Dawley Inc. Indianapolis, IN) for the tumorigenicity assay ($6x10^6$ cells/site, 2 sites/mouse). The mice were examined weekly for the presence of tumors. Some of the tumors were excised and reestablished in cell culture for further characterization; cells from these cultures also were inoculated

into athymic nude mice.

Assessment of HBEC proliferation *in vitro.* To determine growth factor and hormone requirements, HBEC were grown in various media, each of them lacking one growth factor or hormone as compared to the complete MSU-1 medium. The growth requirements of HBEC for EGF, HT, HC, INS, E_2 and T_3 were examined in this study. In addition, FBS (5%) or CT (1 ng/ml) was added individually to the MSU-1 medium to determine their effects on growth. The growth of HBEC in various media was measured by quantitation of total nucleic acid extracted from the cell culture (Li et al., 1990). Briefly, Type I and Type II HBEC (passage 2) ($1x10^4$ cells) were plated in 6 cm plates in triplicate in the complete FBS-containing MSU-1 medium. The next day, the medium was replaced by the medium to be examined. One plate of Type II cells growing in the complete FBS-free MSU-1 medium for 10 days was subcultured and replated once again. The cells used in this latter experiment were designated passage 3 Type II HBEC. All cells were incubated for 10 days at 37°C (media changed twice), the cells were washed twice with PBS and lysed with 2 ml of 0.1 N sodium hydroxide. The lysate was transferred into 2.2 ml Eppendorf tubes and centrifuged for 2~3 min. The absorbance of the clear lysate at 260 nm was measured using a Gilford spectrophotometer. Each treatment was done in triplicate plates and a t-test was used to determine if the treatment was statistically different from the control.

Results

The growth factor and hormonal requirements of 2 HBEC, derived from two different

reduction mammoplasty tissue specimens (HME-5 and HME-12), were examined in this study. The Type I and Type II HBEC derived from these specimens are morphologically distinguishable (Fig. 8). The results from studies involving Type I cells (passage 2) are presented in Fig. 9. HME-5 and HME-12 cultures responded similarly to media supplementation and growth factor/hormone withdrawal. The results can be summarized as follows: 1) the growth of Type I cells was greatly enhanced by FBS and CT supplementation; 2) the growth of Type I cells was substantially inhibited by withdrawing EGF, HC or INS and completely prevented by withdrawing all of the growth factor/hormone supplements from the culture medium. The requirement of T_3 for maximal growth of Type I cells was observed in only one of the two HBEC cultures tested (HME-12). No apparent effect of HT or E_2 on the growth of Type I cells was observed. For Type II cells (passage 2), the results are presented in Fig. 10 and summarized as follows: 1) the growth of Type II cells was substantially inhibited by supplementation of the medium with FBS, while CT supplementation promoted the growth only in one of two HBEC (HME-12); 2) the growth of Type II cells was substantially reduced by withdrawing INS from the culture medium, or by withdrawing all of the growth factor/hormone supplements from the medium; 3) withdrawing of EGF from the culture medium reduced the growth of one of two HBEC (HME-5). The requirement of HC or T_3 for maximal growth was observed only in one of two HBEC cultures (HME-12). No apparent effect of HT or E_2 on the growth of these cells was observed.

The growth responsiveness of passage 3 Type II cells (HME-12) to growth factor/hormone withdrawal was slightly different than the responsiveness of passage 2

Figure 8. Normal HBEC cultured in MSU-1 medium contained two types of epithelial cells : Type I cells (A) (X~90), Type II cells (B) (X~90).

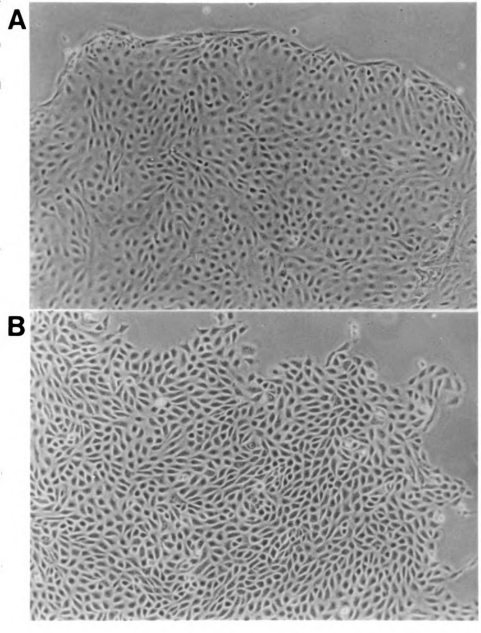

Figure 8

Figure 9. Growth factor and hormonal requirements of passage 2 Type I HBEC derived from human breast specimens HME-5 and HME-12 were examined in this study. The relative growth is the average total nucleic acid content of cells grown in triplicate dishes. All treatments were compared with MSU-1 medium supplemented with human transferrin (HT), insulin (INS), hydrocortisone (HC), epidermal growth factor (EGF), 17β-estradiol (E_2), and 3, 3', 5-triiodo-D.L-thyronine (T_3). The treatments are: 1, MSU-1 with FBS; 2, MSU-1 with CT; 3, MSU-1 without HT; 4, MSU-1 without INS; 5, MSU-1 without HC; 6, MSU-1 without EGF; 7, MSU-1 without E_2; 8, MSU-1 without T_3; 9, MSU-1; 10, MSU-1 without all supplements (All S.). *, significantly different from the control (MSU-1) ($0.01 < P < 0.05$); **, highly significantly different from the control (MSU-1) ($P < 0.01$).

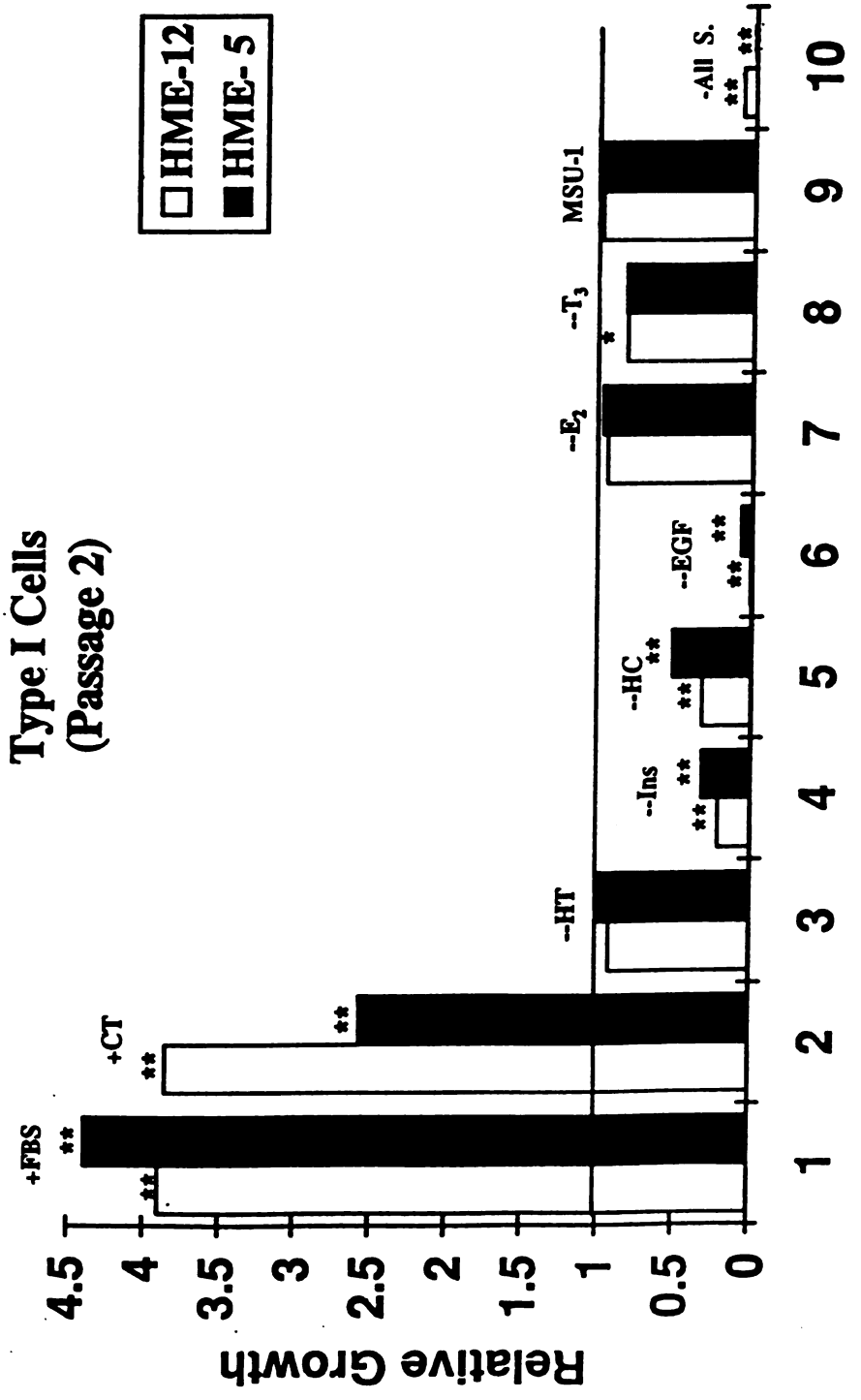
Type I Cells
(Passage 2)
HME-12
HME- 5
Relative Growth
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
+FBS
+CT
--HT
--Ins
--HC
--EGF
--E2
--T3
MSU-1
-All S.

Figure 9

Figure 10. Growth factor and hormonal requirements of passage 2 Type II HBEC derived from human breast specimens HME-5 and HME-12 were examined in this study. The relative growth is the average total nucleic acid content of cells grown in triplicate dishes. All treatments were compared with MSU-1 medium supplemented with human transferrin (HT), insulin (INS), hydrocortisone (HC), epidermal growth factor (EGF), 17β-estradiol (E_2), and 3, 3', 5-triiodo-D.L-thyronine (T_3). The treatments are: 1, MSU-1 with FBS; 2, MSU-1 with CT; 3, MSU-1 without HT; 4, MSU-1 without INS; 5, MSU-1 without HC; 6, MSU-1 without EGF; 7, MSU-1 without E_2; 8, MSU-1 without T_3; 9, MSU-1; 10, MSU-1 without all supplements (All S.). *, significantly different from the control (MSU-1) ($0.01<P<0.05$); **, highly significantly different from the control (MSU-1) ($P<0.01$).

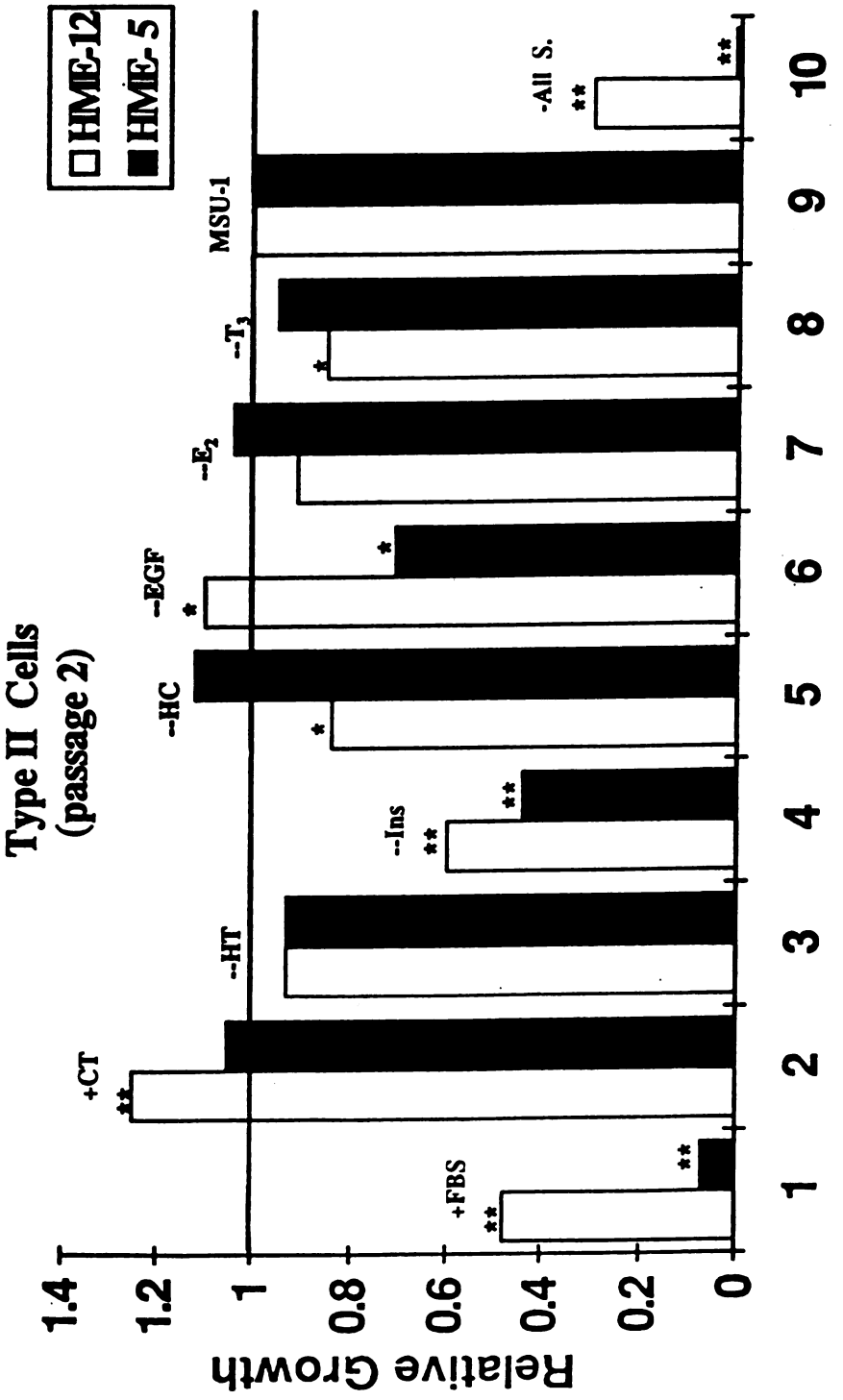
Type II Cells
(passage 2)
HME-12
HME-5
Relative Growth
1.4
1.2
1
0.8
0.6
0.4
0.2
0
+FBS
+CT
--HT
--Ins
-HC
-EGF
--E2
--T3
MSU-1
-All S.
1
2
3
4
5
6
7
8
9
10

Figure 10

Type II cells (HME-12). These results are shown in Fig. 11 and indicate that passage 3 Type II cells had a greater growth requirement for INS, HC and EGF when compared to passage 2 Type II cells (Fig. 10). Like passage 2 Type II cells, the growth of passage 3 Type II cells was substantially inhibited by the addition of FBS to the culture medium. No apparent effect of CT, HT, E_2 or T_3 on growth of these cells was observed.

Normal Type I and Type II HBEC derived from human breast specimen HME-11 were used for SV40 DNA transfection in this study. A total of 5 Type I cell-derived SV40 transfected clones (M11SV-1,2,3,4 and 5) and 2 Type II cell-derived SV40 transfected clones (M11SV-21 and 22) were isolated. These SV40 transfected extended life clones derived from HME-11 cells did not become immortal. To induce immortalization, these cells were further treated with BrdU/black light. After the treatment, most of the cells died. Among the survivors, one clone of cells (derived from M11SV-1) having immortal characteristics (cpdl >100) was obtained. This cell line was designated M11SV-1B1. The M11SV-1B1 cell line, when inoculated into athymic nude mice, formed slow-growing palpable tumors (4 of 7 mice developed tumors, the size of the tumors were 0.3 to 0.5 cm in diameter one month post-inoculation), whereas the parental M11SV-1 cells were non-tumorigenic in athymic nude mice (0/6). The cells derived from a tumor formed by M11SV-1B1 cells were designated M11SV-1B1T. M11SV-1B1 cells were also infected with the neu oncogene. One cell line expressing the neu oncogene (designated M11SV-1B1N) was also tumorigenic in athymic nude mice (2 of 7 mice developed tumors, the size of the tumors was about 0.5 cm in diameter after one month post-inoculation). A cell line derived from one of the two tumors formed by M11SV-1B1N cells in athymic nude mice was highly tumorigenic (5 of 5 mice developed

Figure 11. Growth factor and hormonal requirements of passage 3 Type II HBEC derived from human breast specimen HME-12. The relative growth is the average total nucleic acid content of cells grown in triplicate dishes. All treatments were compared with MSU-1 medium supplemented with human transferrin (HT), insulin (INS), hydrocortisone (HC), epidermal growth factor (EGF), 17β-estradiol (E_2), and 3, 3', 5-triiodo-D.L-thyronine (T_3). The treatments are: 1, MSU-1 with FBS; 2, MSU-1 with CT; 3, MSU-1 without HT; 4, MSU-1 without INS; 5, MSU-1 without HC; 6, MSU-1 without EGF; 7, MSU-1 without E_2; 8, MSU-1 without T_3; 9, MSU-1; 10, MSU-1 without all supplements (All S.). *, significantly different from the control (MSU-1) ($0.05<P<0.01$); **, highly significantly different from the control (MSU-1) ($P<0.01$).

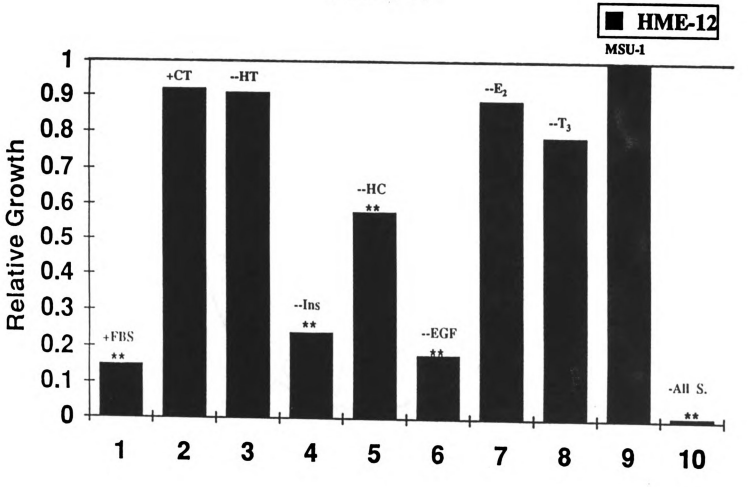
Type II Cells
(Passage 3)
HME-12
MSU-1
Relative Growth
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
+FBS
**
+CT
--HT
--Ins
**
--HC
**
--EGF
**
--E2
--T3
-All S.
**
1
2
3
4
5
6
7
8
9
10

Figure 11

tumors, the size of the tumors were 1.5 cm in diameter in less than three weeks). This cell line was designated M11SV-1B1NT. The tumorigenicity of pC6M-neo virus (vector control for neu oncogene) infected G418-resistant M11SV-1B1 cells was similar to the parental M11SV-1B1 cells (3 of 6 mice developed tumors, 0.3 to 0.5 cm in diameter one month post-inoculation). In contrast, no immortal clone was obtained from the Type II cell-derived SV40 transfected clones (i.e., M11SV-21 and M11SV-22), after treatment with BrdU/black light.

The growth requirements of these cell lines, i.e., M11SV-1, M11SV-1B1, M11SV-1B1T, M11SV-1B1N and M11SV-1B1NT, were examined for their growth responsiveness to media supplementation and growth factor/hormone withdrawal. The results of this study are shown in Fig. 12 and can be summarized as follows. SV40 transfected Type I cells with an extended lifespan (M11SV-1), similar to non-SV40 transfected Type I cells (Fig. 9), required EGF, HC and INS for optimal growth. However, the degree of growth dependency of the SV40 transfected cells on these growth factors was slightly reduced as compared to the non-SV40 transfected Type I cells. The M11SV-1 cells which became immortalized by BrdU/black light treatment (M11SV-1B1), the tumor cells (M11SV-1B1T) derived from the M11SV-1B1 cells and the neu oncogene infected cells (M11SV-1B1N) still required EGF, HC and INS for maximum growth. The M11SV-1B1 and M11SV-1B1N cells also expressed a dependency for HT for optimal growth; M11SV-1B1N cells additionally required T_3 for optimal growth. The most highly tumorigenic cells (M11SV-1B1NT), in contrast, did not require INS, HC or EGF for optimal growth but did require HT and T_3 for optimal growth. The growth of all five of these cell lines was severely inhibited in medium deleted of all growth factors and hormonal supplements.

Figure 12. Growth factor and hormonal requirements of Type I cell-derived SV40 transfected cell lines derived from human breast specimen HME-11. The relative growth is the average total nucleic acid content of cells grown in triplicate dishes. The cell lines used in the study are: 1) M11SV-1, Type I cell extended life by SV40; 2) M11SV-1B1, an immortal cell line derived from M11SV-1 after treatment with BrdU/black light; 3) M11SV-1B1T, a cell line reestablished from a tumor derived from M11SV-1B1 cells after inoculation into athymic nude mice; 4) M11SV-1B1N, neu oncogene infected M11SV-1B1 cells and 5) M11SV-1B1NT, a cell line reestablished from a tumor derived from M11SV-1B1N cells after inoculation into athymic nude mice. All treatments were compared with MSU-1 medium supplemented with human transferrin (HT), insulin (INS), hydrocortisone (HC), epidermal growth factor (EGF), 17β-estradiol (E_2), and 3, 3', 5-triiodo-D.L-thyronine (T_3). The treatments are: 1, MSU-1 with FBS; 2, MSU-1 with CT; 3, MSU-1 without HT; 4, MSU-1 without INS; 5, MSU-1 without HC; 6, MSU-1 without EGF; 7, MSU-1 without E_2; 8, MSU-1 without T_3; 9, MSU-1; 10, MSU-1 without all supplements (All S.). *, significantly different from the control (MSU-1) ($0.05<P<0.01$); **, highly significantly different from the control (MSU-1) ($P<0.01$).

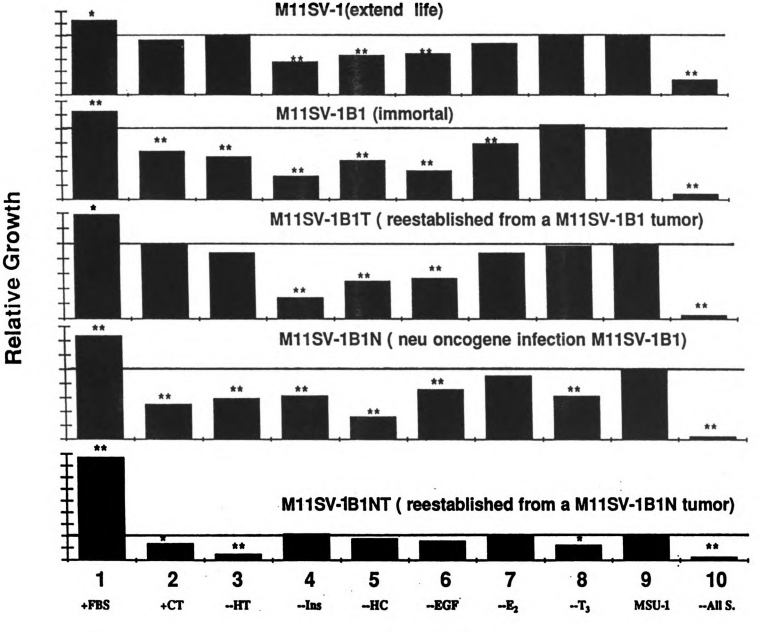
Relative Growth
M11SV-1(extend life)
M11SV-1B1 (immortal)
M11SV-1B1T (reestablished from a M11SV-1B1 tumor)
M11SV-1B1N (neu oncogene infection M11SV-1B1)
M11SV-1B1NT (reestablished from a M11SV-1B1N tumor)
1 +FBS
2 +CT
3 --HT
4 --Ins
5 --HC
6 --EGF
7 --E2
8 --T3
9 MSU-1
10 --All S.

Figure 12

FBS supplementation enhanced the growth of each of these cell lines as seen with non-SV40 transfected Type I cells. In contrast, there was no evidence that CT supplementation was growth stimulating to any of the SV40 transfected cell lines. CT was shown to be growth stimulatory to the non-SV40 transfected Type I cells (Fig. 9).

Discussion

Development of a culture system that will permit sustained growth of primary HBEC is indispensable to an understanding of the biology of normal and cancerous breast cells as well as the mechanisms involved in neoplastic transformation of these cells. While progress has been made in growing HBEC by several groups (Buehring, 1972; Taylor-Papadimitriou et al., 1977; Stampfer et al., 1980; Hammond et al., 1984; Petersen and Deurs, 1988; Band et al., 1990; Emerman and Wilkinson, 1990; Ethier et al., 1990), the cell cultures developed to date from reduction mammoplasty have been predominantly basal epithelial cell cultures. In the studies provided in this communication, we report on the development of a chemically defined culture medium (MSU-1) that permits the growth and separation of two phenotypically distinct types of HBEC, i.e., Type I and Type II cells. Our Type I cells possess antigenic characteristics of luminal epithelial cells, i.e., they express keratin 18 and EMA but do not express keratin 14 and α6 integrin (Taylor-Papadimitriou et al., 1989; O'Hare et al., 1991; Kao et al., 1994). These cells are more variable in shape, have smooth cell colony boundaries and are deficient in GJIC (Kao et al., 1994). Our Type II cells possess antigenic characteristics of basal epithelial cells as they express keratin 14 and α6 integrin but do not express EMA or keratin 18 (Taylor- Papadimitriou et al., 1989; Koukoulis et al., 1991; Kao et al., 1994). These cells

are more uniform in shape (cobble-stone like), do not have smooth colony boundaries and are proficient in GJIC (Kao et al., 1994). The development of culture methodologies in which to separate and subsequently propagate these two distinctly different human breast epithelial cell types has heretofore not been reported.

In studies recently completed, we showed that our Type I cells (with luminal epithelial cell characteristics) and our Type II cells (with basal epithelial cell characteristics) differ markedly in their responsiveness to an oncogenic stimulus (SV40 DNA transfection), i.e., the SV40 transfected Type I cells have the capability of growing in soft agar (anchorage independent growth) while the SV40 transfected Type II cells are unable to grow in soft agar (Kao et al., 1994). In addition, Type I cells gave rise to Type II cells upon treatment with cholera toxin (Kao et al., 1994). Human breast carcinoma cell lines, such as MCF-7 and T47D and primary human breast carcinomas, possess antigenic characteristics similar to our Type I cells, e.g., expression of EMA and keratin 18 (our unpublished results and Taylor-Papadimitriou et al., 1989), non-expression of keratin 14 (our unpublished data and Taylor-Papadimitriou et al., 1989), α6 integrin (our unpublished data) and gap junction genes Cx 26 and Cx 43 (Yang et al., 1994). Such data suggests that our Type I cells may be target cells in neoplastic transformation of the human breast. Prerequisite to successful and consistent neoplastic transformation of specific epithelial cell types of human breast tissue is an understanding of the growth factor/hormonal responsiveness of the cell type in question. Thus, one of the major objectives of this study were to assess growth factor/hormonal responsiveness of our Type I and Type II HBEC.

The growth factor/hormone responsiveness of Type I cells (passage 2) and Type II

cells (passage 2 or 3) was not identical. The most striking difference between these two types of cells was that FBS promoted the growth of Type I cells (passage 2), whereas the growth of Type II cells (passage 2 and 3) was inhibited by FBS. In addition, the growth of Type I cells (passage 2) was sharply enhanced by CT, whereas CT did not consistently affect the growth of Type II cells (passage 2 and 3). Deletion of EGF, HC and INS from the culture medium substantially reduced the growth of Type I cells. Only INS was important for optimal growth of the passage 2 Type II cells, whereas third passaged Type II cells required INS, EGF and HC for optimal growth. The requirement of T_3 for maximal growth of Type I and Type II cells was observed only in one or the two HBEC cultures tested (i.e., HME-12, Type I and the second passage Type II cells). No apparent effect of HT or E_2 on the growth of both Type I and Type II cells was observed. Few reports have described the growth of HBEC in a chemically defined culture medium. Hammond et al. (1984) provided evidence that EGF, INS and HC are important medium supplements for optimal growth of HBEC. Balakrishnan et al. (1989) reported that both HC and CT were important for optimal growth of HBEC. The studies by Ethier et al. (1990) indicate that EGF, INS and CT are essential for optimal HBEC growth. Clearly, the differences in growth responsiveness of HBEC to growth factor/hormone supplementation and/or withdrawal, seen in the above studies, at least in part, may be due to the different proportion of Type I (luminal) and Type II (basal) epithelial cells in the cell cultures.

Our studies clearly show that two types of HBEC cells, obtained from reduction mammoplasty, can be grown in a chemically defined culture medium and that these two types of cells also differ in their responsiveness to FBS supplementation. In the report

by Hammond et al., (1984), HBEC that were grown in MCDB 170 medium were growth inhibited by FBS; these cells express keratin 14 but not keratin 18 (Taylor-Papadimitriou et al., 1989). These characteristics indicate that the cells grown under the culture conditions of Hammond et al., (1984) are phenotypically similar to our Type II cells. In addition, BPE (Hammond et al., 1984; Band and Sager, 1989) is commonly supplemented to MCDB 170 and DFCI-1 media for growing HBEC. With our medium (MSU-1), we found that BPE promoted the conversion of Type I cells to Type II cells and significantly promoted the growth of passage 2 but not passage 3 Type II cells (data not shown). These observation provide further evidence that prior studies using MCDB 170 or DFCI-1 media were examining primarily HBEC characteristic of our Type II cells.

SV40 has been reported to transform primary HBEC into HBEC with extended lifespan; some of these cells ultimately become immortal (Chang et al., 1982; Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Berthon et al., 1992; Van Der Haegen and Shay, 1993; Shay et al., 1993). The SV40 transfected HBEC used in this study (M11SV-1) did not become immortal. These cells, however, were immortalized by combined BrdU/black light treatment. This SV40/BrdU-black light immortalized cell line (M11SV-1B1) differs from most other SV40 immortalized HBEC lines reported in the literature (Chang et al., 1982; Rudland et al., 1989; Bartek et al., 1990; Garcia et al., 1991; Bartek et al., 1991; Van Der Haegen and Shay, 1993) in being tumorigenic, albeit weakly, in athymic nude mice. After infection of this cell line (M11SV-1B1) with an activated neu oncogene, such cells formed slow growing tumors in athymic nude mice. The cell line derived from these tumors cells (M11SV-1B1NT) formed rapidly growing tumors in athymic nude mice. A comparative study of cell line

M11SV-1 (extended life), M11SV-1B1 (immortal), and M11SV-1B1NT (highly tumorigenic) should facilitate our understanding of the mechanisms involved in immortalization , BrdU/black light and neu oncogene neoplastic transformation.

Growth factor/hormonal requirements of several immortal human breast epithelial cell lines have been reported before. The growth of SV40 immortalized HBEC, derived from lactational fluids (milk cells), was stimulated by INS and HC; these cells grew better in medium with 10% FBS than with 1% FBS (Chang et al., 1982). Garcia et al., 1991, however, reports that the addition of INS, HC or EGF was not required for the growth of SV40 immortalized HBEC lines derived from milk cells. Van Der Haegen and Shay (1993) have reported that the growth of SV40 immortalized HBEC derived from reduction mammoplasty was EGF independent in the presence of BPE; without BPE, the cells were growth promoted by EGF. They also showed that the growth of these cells was not affected by 5% FBS, in contrast to normal HBEC which were growth inhibited by FBS. Band et al. (1990) have reported that normal and human papilloma virus immortalized HBEC did not show any growth in DFCI-1 medium not supplemented with FBS, BPE, EGF, HC, INS, T_3 and CT. This minimal medium, when supplemented with EGF, was able to support the growth of human papilloma virus immortalized HBEC but not normal HBEC.

Although these immortal HBEC lines did not show a uniform growth factor/hormonal requirement, it is clear, however, that they show different growth factor/hormonal requirements than do normal HBEC. First, SV40 immortalized HBEC lines can be grown in FBS containing media (Chang et al., 1982; Van Der Haegen and Shay, 1993). It is also known that a number of human breast carcinoma cell lines, isolated from metastases

or pleural effusions, are grown in media supplemented with FBS (Soule et al., 1973; Engel and Young, 1978; Smith et al., 1987). In our study, we found that SV40 transfected HBEC lines, with different degrees of tumorigenicity (extended lifespan, immortal, tumorigenic), were growth stimulated by FBS. The growth-promoting effects of FBS on our SV40 transfected Type I HBEC lines and on those immortal or tumorigenic HBEC lines reported in literature are similar to the FBS induced growth characteristics of our Type I HBEC rather than our Type II HBEC. These observations suggest that our Type I cells may be target cells for neoplastic transformation in human breast tissue. Secondly, the immortal HBEC cell lines described in the literature (Band et al., 1990; Garcia et al., 1991) were found to have reduced growth dependence on certain growth factors/hormones e.g., INS, HC or EGF. Similar results were found in our most pronounced tumorigenic cell line (M11SV-1B1NT). In addition, our M11SV-1B1NT cell line required HT and T_3 for optimal growth *in vitro*.

In summary, two types of normal HBEC, i.e., Type I cells (with luminal epithelial cell characteristics) and Type II cells (with basal epithelial cell characteristics), obtained from reduction mammoplasty, were separately cultured in a chemically defined medium. FBS differentially effected the growth of these two types of cells; it enhanced the growth of Type I cells while it inhibited the growth of Type II cells. Treatment of the Type I cells sequentially by SV40, BrdU/black light and the neu oncogene resulted in cells that formed rapidly growing tumors when inoculated into athymic nude mice. The tumor cells had growth factor/hormonal requirements that differed substantially from their parental cells. The ability to separate HBEC into different cell types and to separately propagate these cells in a culture system is an important step toward successful and consistent

neoplastic transformation of HBEC *in vitro*.

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CHAPTER 5

Summary and Conclusions

The objectives of this thesis research are: 1) to characterize two morphologically distinguishable cell types of normal human breast epithelial cells (Type I and Type II), derived from reduction mammoplasty tissues using a defined medium and 2) to determine if one of the two cell types is more susceptible to neoplastic transformation by SV40 DNA.

The major results from this study related to the characterization of the two cell types are listed in Table 2. These may be summarized as follows. 1) The two morphologically distinguishable cell types (Type I, cell shape more variable and elongated, cell boundary less distinctive, colonies with smooth and bounded outlines and Type II, cell shape more uniform and cobble-stone like, colony outline appears unrestrained) were found in all the 7 primary human breast epithelial cultures (derived from reduction mammoplasty tissues obtained from 7 patients)examined in this study. 2) The Type I cells were deficient in gap junctional intercellular communication (GJIC); in contrast, Type II cells were efficient in GJIC; 3) Type I cells could be promoted to growth by fetal bovine serum (FBS), whereas Type II cells were growth inhibited by FBS; 4) Type I cells expressed EMA and keratin 18, the expression markers of luminal epithelial cells, but not keratin 14 and $\alpha 6$ integrin, the expression markers of basal or myoepithelial cells, Type II cells showed the opposite phenotypes; 5) Type I cells remained insuspension and unattached on plastic surface after trypsinization for an extended period of time; 6) Epidermal growth factor, insulin and hydrocortisone were essential for supporting the growth of both passage 2 Type I and passage 3 Type II cells; for passage 2 Type II cells, only insulin was

important for cell growth and 7) Type I cells could be induced to change their morphology to a Type II cell morphology by cholera toxin.

These characterizations indicate that Type I cells exhibit many luminal epithelial cell phenotypes, whereas Type II cells express several basal epithelial cell phenotypes. Characterization of breast carcinoma cell lines such as MCF-7 and T47D, indicate that these cancer cells share many phenotypes of Type I cells but not Type II cells (Table 4, i.e., deficiency in GJIC, and lack of expression of gap junction genes for Cx26 and 43, expression of EMA, keratin 18 but not keratin 14 and α6 integrin). Therefore, human breast cancers are likely to be derived from Type I cells (with luminal epithelial cell characteristics). The suggestions that Cx26 and α6 integrin as potential breast tumor suppressor genes (Lee et al., 1991; Sager et al., 1993), based on their expression in normal breast epithelial cells (most likely, these cells resemble Type II cells rather than Type I cells) and their absence in breast tumor cells need to be reevaluated in light of our data.

The results from the comparative study of neoplastic transformation by the SV40 DNA in Type I and Type II cells indicate that clones of extended life (EL) (20-50 cpdl) can be obtained from both cell types. Type I cell-derived EL clones can be converted to immortal cell lines (2/9) spontaneously or by further treatment with BrdU and black light. One cell line immortalized after BrdU/black light treatment was tumorigenic in immune-deficient mice. None of the 8 Type II cell-derived EL clones, derived from three individuals, have been immortalized thus far. Type I and Type II cell-derived EL and immortal cell lines resembled their parental cells with respect to EMA, keratin 14 and keratin 18 expression and GJIC. Each (9/9) of the SV40 transfected Type I cell clones

Table 4 Some Major Differences of Type I HBEC, Type II HBEC, MCF-7 and T47D Cells

	Type I	Type II	MCF-7 & T47D
Cell and colony morphology			
Attachment on plastic surface after typsinization			
Effect of cholera toxin			
Effect of fetal bovine serum	**promotion**	**inhibition**	**promotion**
Gap junctional intercellular communication	**deficient**	**efficient**	**deficient**
Expression of			
Epithelial membrane antigen	+	--	+
Keratin-18	+	--	+
Keratin-14	--	+	--
α6 integrin	--	+	--

grew in soft agar; non (0/8) of the SV40 transfected Type II cell clones were capable of growing in soft agar. Furthermore, infection and expression of a mutated neu oncogene greatly enhanced the tumorigenicity of the immortal cell line with weak tumorigenicity (M11SV-1B1). Therefore, Type I cells responded differently to an oncogenic (SV40) stimulus and might be the major target cells for neoplastic transformation. The function of the neu oncogene appears to enhance tumorigenicity of SV40 immortalized, weakly tumorigenic Type I human breast epithelial cells. The neoplastically transformed Type I HBEC were promoted to grow by FBS similar to their parental cells, but appears to has reduced growth dependency on INS, HC and EGF.

The significance of this study appears to be the development of a cell culture method to grow a human breast epithelial cell type with some luminal (and perhaps some stem cell) characteristics and the demonstration that this cell type (Type I) might be the target cell for neoplastic transformation. These ideas were based on the observations that this type of cell appears to be more susceptible to neoplastic transformation and the resulting transformed cells resembled the phenotypes of primary breast cancer cells. Furthermore, the results demonstrate that the phenotype of the neoplastically-transformed cells was dependent on the differentiation state of the parental cells. This is most impressively shown by SV40 transfected Type I cells which were capable of AIG in contrast to the SV40 transfected Type II cells. Furthermore, most of the SV40 immortalized human breast epithelial cell lines reported in literature, which failed to show AIG. Finally, the Type I human breast epithelial cells are deficient in GJIC, similar to a subpopulation of normal human fetal kidney epithelial cells with stem cell characteristics (Chang et al., 1987). Therefore, we speculate that the deficiency in GJIC might be a feature of stem

cells and could play an important role in tumorigenesis.

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