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MASPIN, A TUMOR SUPPRESSOR GENE, IS EXPRESSED IN HUMAN BASAL BREAST EPITHELIAL CELLS BUT NOT IN BREAST EPITHELIAL STEM CELLS AND BREAST CARCINOMA CELLS

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By

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ABSTRACT

MASPIN, A TUMOR SUPPRESSOR GENE, IS EXPRESSED IN HUMAN BASAL BREAST EPITHELIAL CELLS BUT NOT IN BREAST EPITHELIAL STEM CELLS AND BREAST CARCINOMA CELLS

By

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The phenotypes of cancer cells are very similar to stem cells as compared to differentiated somatic cells. Both are described as undifferentiated cells and share the common expression of many genes related to tumorigenesis, a basis for the concept that cancer cells are primarily derived from stem cells. Maspin, a protease inhibitor known to regulate not only invasion and metastasis, but also tumor growth and apoptosis, could be a tumor suppressor gene not expressed in stem cells and tumor cells. To test this hypothesis, I have examined the expression of maspin, using immunocytochemical and western blot techniques, in two types of normal human breast epithelial cells (HBEC), Type I HBEC show stem cell characteristics, whereas Type II HBEC expressed basal epithelial cell phenotypes. The results clearly show that Type I HBECs, indeed, did not express maspin in contrast to Type II HBECs which highly expressed the maspin. A series of Type I HBEC lines, neoplastically transformed at different stages (immortal, weakly and highly tumorigenic) and three breast carcinoma cell lines were also not found to express maspin. Thus, the silencing of maspin expression in breast cancer cells is very likely due to the continuous non-expression of a target stem cell phenotype. The results provide additional evidence for the stem cell theory of carcinogenesis and indicate that maspin could be used as a marker for early detection of breast cancer and for assessing the efficacy of chemopreventive or chemotherapeutic agents.

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LITERATURE REVIEW

I. The Role of Stem Cell in Carcinogenesis

Several lines of evidence suggests that cancer cells are derived from the adult stem cell pools of a tissue where initiating events of stem cells prevents cells from fully differentiating and resulted in uncontrolled clonal expansion of these cells. The stem cell theory of carcinogenesis proposes that stem cells are target cells in carcinogenesis and certain developmental or differentiating genes are blocked or partially blocked so the cells remain undifferentiated (Potter, 1978).

Stem cell research has received increasing attention because of its therapeutic application and potential role as a target cell in carcinogenesis. Its unlimited or extensive self-renewal capacity and ability to differentiate into multiple cell types ("pluri-potency") are highly valuable in various applications for medical treatments such as tissue engineering, transplantation and gene therapy. These two unique properties of stem cell are also important in understanding the role of stem cell in carcinogenesis.

By definition, "toti-potent" cells can give rise to all cell types within the organism. They can divide symmetrically or asymmetrically to produce partially committed organ specific stem cells, namely "pluri-potent" stem cells. These pluri-potent stem cells can give rise to several different cell types in different organs. Then, after further development, some cells are finally restricted to giving rise to one or fewer cell types to become "committed" progenitor cells. The committed progenitor cells eventually

develop and give rise to functionally and terminally differentiated cells. The stem cell theory of carcinogenesis states that the carcinogenic process starts from a stem cell (most likely a pluri-potent stem cell), assuming that a normal stem cell is "immortal" (Trosko, 2003).

As an alternative to the stem cell theory, the de-differentiation theory states that cancer cells are derived from de-differentiation of differentiated of cells to explain the undifferentiated state of tumor cells. The unlikelihood of dedifferentiation of differentiated somatic cells to develop into tumor favors the stem cell theory. For example, many differentiated hepatocytes, are tetraploid (Styles et al., 1985), while the nodule that give rise to tumors are diploid (Schwarze et al., 1984). It is a rare chance, therefore, that these cells can de-differentiate back to diploid state that characterize precancerous cells and this fact seriously undermines the de-differentiation theory as a possible explanation of less differentiated state of cancer cells. It has been documented that the stem cells share a number of tumor cell characteristics: contact insensitivity (Chang et al., 1987), extensive self-renewal capacity (Tang et al., 2001; Mathon et al., 2001), anchorage- independent growth (Chang et al., 2001; Chang et al., 2004; Lin et al., 2004), lack of gap junctional intercellular communication (GJIC), (Yamasaki et al., 1987; Chang et al., 1987; Kao et al., 1995; Matic et al., 2002; Grueterich and Tseng, 2002) and activated telomerase (Hiyama et al., 1995) or high susceptibility of telomerase activation (Sun et al., 1999) . This evidence also indicates the difficulty to de-differentiate back to undifferentiated state to regain those characteristics described.

Recently, more evidence suggests the role of stem cells as target cells in carcinogenesis. For example, McKay and his co-workers found a novel protein named nucleostemin, from a subtractive hybridization study, that was expressed in the nucleoli of CNS stem cells and embryonic stem cells, as well as in tumorigenic cells (Tsai and McKay, 2002). Structural analysis and mutation study of nucleostemin indicated a role of the gene in cell cycle regulation of cell growth, as well as the prevention of the cells from dying by apoptosis. The expression of nucleostemin decreased rapidly prior to cell cycle exit when the cells are differentiated. Furthermore, based on the evidence that activation or over-expression of nucleostemin reduces cell proliferation in CNS stem cells and transformed cells, nucleostemin seems to be correlated with cell proliferation or the lack of differentiation in these cells.

Recently, the role of myoepithelial cells as a host defense cell against cancer received some attention (Sternlicht and Barsky, 1997; Sternlicht et al., 1997). First, the mammary myoepithelial cells express protease inhibitors, including maspin, which have various tumor suppressive activities. Second, myoepithelial cells are rarely transformed and the myoepithelial tumor tends to be benign or low-grade neoplasms. Third, the myoepithelial cells promote differentiation and inhibit invasion in vitro. In terms of the stem theory of carcinogenesis, the properties of myoepithelial cell show that they are differentiated cells derived from stem cells and that they possess anti-cancer properties. The fact suggests that the differentiated breast epithelial cell type is unlikely to be a target cell and indirectly supports the stem cell theory of carcinogenesis. Evidence supporting the stem

cell theory of carcinogenesis has been previously reviewed (Chang et al., 2001) and mentioned in the Introduction.

II. Cancer Cell Phenotype

Hanahan and Weinberg summarized six essential capabilities of cancer cell or "hallmarks of cancer": 1) limitless replicative potential, 2) self-sufficiency in growth signals, 3) insensitivity to growth-inhibitory signals, 4) evasion of programmed cell death, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Now, in view of the stem cell theory, I will briefly summarize them to re-emphasize how the stem cell already possesses several important characteristics commonly found in the tumor cell.

1) Limitless replicative potential

Normal human cells have limited life spans (Hayflick, 1997), however, almost all malignant tumor cells can replicate indefinitely. In view of the stem cell theory, stem cells theoretically have extensive self-renewing capacity. More specifically, the stem cells or their partially differentiated daughter cells, that have not yet down-regulated their telomerase activity, but are exposed to a carcinogenic "initiator," will remain immortal or have limitless replication potential (Trosko et al., 2000). Thus, the first step of carcinogenesis is to prevent the stem cells to become mortal or to terminally differentiate.

Although the stem cell has extensive self-renewal capacity, it is not all clear whether stem cell maintains this capacity for an entire lifespan and if so, what type of intrinsic or extrinsic factors are involved to maintain this capacity. Several in vitro studies indicate that certain genetic alterations are required to maintain the self-renewal capacity. The induction of so-called "immortalization" of human mammary epithelial cell (HMEC) seems to involve two major events (Kiyono et al., 1998). First, the cell has to acquire an extended lifespan (to overcome that first stage senescence or mortality stage, M1) by altering the regulation of cell cycle genes especially the $p16^{ink4}$/Rb pathway (Kiyono et al., 1998). Inactivating this $p16^{ink4}$/Rb pathway to extend the lifespan of HMEC has been demonstrated by transfecting with SV40 large T-antigen (Kao et al., 1995) or papilloma virus E6 and E7 (Wazer et al., 1995). However, the cells that successfully evaded the senescence soon stop growing when they reach the next plateau, called crisis (Kiyono et al., 1998). As a second step in immortalization, the HBEC with extended lifespan may become immortal after the activation of telomerase (to overcome the M2, or crisis). HMEC with inactive p16/Rb may become immortal after transfecting with the human telomerase gene hTERT (Kiyono et al., 1998). However, HMECs, growth on feeder layers, have been immortalized without abrogating p16 (Herbert et al., 2002). It is interesting to note that estrogen is an agent known to activate telomerase on the hTERT promoter in a breast cancer cell line (Kyo et al., 1999). The capability of unlimited or prolonged replicative potential is believed to be established in early stage of carcinogenesis. This may be related to the characteristics of stem cells, the major target cells for carcinogenesis. The unlimited self-renewal ability of stem/precursor cells has been demonstrated for rodent cells (Tang et al., 2001; Mathon et al., 2001) but not for

human cells. However, breast epithelial stem cells have been shown to be more susceptible to telomerase activation (Sun et al., 1999).

Taken together, to preserve the limitless self-renewal capacity of stem cells, alteration of cell cycle gene regulation and activation of telomerase gene are required to occur during the development and growth of cancer

2) Self-sufficiency in growth signals

Normal cells require mitogenic growth factors, whereas tumor cells produce and express many of their own growth factors and growth factor receptors, thereby reducing their dependence on exogenous or endogenous growth factors from their normal tissue microenvironment. The most important growth factor for breast cancer cells appears to be the estrogen. In breast cancer patients, about two-thirds of tumors are ER-positive (Lippman et al., 1988). Fifty percent of these ER-positive tumors are estrogen-dependent and respond to endocrine therapy and anti-estrogen, Tamoxifen (Manni et al., 1980). The estrogen induction of proliferation of breast cancer required the expression of Myc (Watson et al., 1991) which is frequently amplified or overexpressed (Dickson, 1994). The over- expression of the neu oncogene might enable the cancer cell to become hyper-responsive to ambient level of growth factors that normally would not trigger proliferation in normal cells (Fedi, 1997). In fact, amplification of the EGF-receptor-related prooncogene c-erbB2/neu has been observed in 30% of primary human breast carcinomas (Slamon et al., 1987) and another 10% over-expressed c-erbB2 without

amplification of the gene (Kraus et al., 1987). It should be noted that, in contrast to breast epithelial cells, human breast epithelial stem cells express the estrogen receptors (Kang et al., 1997).

3) Insensitivity to growth-inhibitory signals

In normal cells, a number of anti-proliferative signals, including growth inhibitors (Hanahan and Weinberg, 2000), operate to maintain cellular quiescence and tissue homeostasis. However, cancer cells have an ability to evade these anti-proliferation signals and use various strategies to avoid being terminally differentiated. As an anti-growth factor, TGF-beta, prevents the phosphorylation of Rb, subsequently blocks the cell to advance through the G1 phase of the cell cycle (Hanahan and Weinberg, 2000). Endogenous TGF β signaling and growth suppression is lost during human mammary epithelial cell transformation by benzo(a) pyrene (Ge and Stanpfer, 2004). Down-regulation of GJIC is one of the mechanisms to prevent from being regulated by growth inhibitory signals, such as cyclic AMP, which is considered as a reverse transformation agent (Chan et al., 1989). It has been reported that Type I HBEC with stem cell characteristics and most breast carcinomas are deficient in GJIC (Kao et al., 1995). Another mechanism to avoid being terminally differentiated is the over-expression of the survival factor c-myc, which encodes a transcription factor. During the normal development, the growth stimulating action of Myc is associated with another factor, Max. The association can be supplanted by alternative complex of Max with a group of Mad transcription factors; the Mad-Max complexes elicit differentiation-inducing signals

(Foley and Eisenman, 1999). Overexpression of c-myc can reverse this process, shifting the balance back to favor Myc-Max complex, thereby impairing differentiation and promoting growth (Hanahan and Weinberg, 2000). In breast cancer cells, the abnormalities in the c-myc gene were reported in about 6-32% of breast cancer (Van de Vijver and Nusse, 1991). TGF-beta also suppresses the expression of the c-myc gene, which regulated the G1 cell cycle (Moses et al., 1990). ER-positive breast cancer cells are refractory to TGF-beta effects, whereas ER-negative breast cancer cells are often TGF- beta sensitive (Arteaga and Moses, 1996).

4) Evasion of programmed cell death

The balance between cell proliferation rate and cell attrition rate also determines the ability of tumor cell populations to expand in number. Apoptosis or programmed cell death is an active, energy-dependent process of cell death which occurs during development, in response to certain physiologic stimuli (Kerr et al., 1972). Unlike necrosis which is a passive osmosis-driven process due to the loss of ion-pumping activity, apoptosis shows characteristic morphologic changes i.e. cell shrinking and blebbing, nuclear and chromatin condensation, nuclear fragmentation, and packaging of cellular materials and organelles into membrane-bound vesicles termed apoptotic bodies (Hahm, 1998). Once cells are triggered by a variety of physiologic signals, the cells undergo apoptosis in a precisely choreographed fashion. There are two major protein families involved in apoptosis: antiapoptotic protein family (Bcl-2 relatives) and proapoptotic protein family (Bax relatives) during induction stage of apoptosis. The

tumor suppressor protein p53 can elicit apoptosis by upregulating the expression of Bax in response to sensing DNA damage.

5) Sustained angiogenesis

Angiogenesis, the growth of new capillary blood vessels, is critical for cancer cell to grow, invade and metastasize. New blood vessels bring in fresh nutrients and growth factors so that the tumor mass can expand. As a matter of fact, without forming new blood vessels, solid tumors cannot exceed a size of about $1mm^3$. Interaction between angiogenic proteins (i.e. VEGF, FGFs) and angiogenic proteins (i.e. thrombospondin) appear to be responsible for the angiogenic activity of malignant tumors. Interestingly, analyses of histologically distinct stages of human and rodent tumors suggest that the acquisition of an angiogenic phenotype occurs early in tumorigenesis and can be considered the rate limiting step during tumor progression (Hanahan and Folkman, 1996).

6) Tissue invasion and metastasis

In order for cancer cells to spread, the cells need to acquire the ability to invade the tissue and enter the blood stream to metastasize to other organs. These involve cell migration and the expression of proteases. It has been shown that increased expression of hepatocytes growth factor (HGF) and its receptor, met, occurs in invasive human breast cancer (Tuck et al., 1996; Wang et al., 1994; Jin, 1996) particularly at the migrating

tumor front (Tuck et al., 1996). The tissue invasion and metastasis involve the action of adhesion molecules, metalloproteases and cllageneses (Akiyama et al., 1996).

III. Genes Altered in Breast Cancer

As stated by Hanahan and Weinberg (2000), "tumors are the results of the accumulation of a series of genetic alterations. Genetic studies over the years have identified a number of oncogenes and tumor suppressor genes associated with the development of breast cancer **(See ref. In Chang et al., 2001)**.

i) Oncogenes

Three oncogenes (c-erb B2/neu, c-myc and int-2) and the G_1 phase cyclins (D and E) were frequently found to be amplified or overexpressed in breast cancers **(See ref. In Chang et al., 2001)**. In addition, many breast oncogenes have been identified. These include Brk (breast tumor kinase) (Barker et al., 1997; Mitchell et al., 1994), srk (Jacobs and Rubsamen, 1983; Luttrell et al., 1994), AKT3 (Nakatani et al., 1999) and ZNF217 (Nonet et al., 2001).

ii) Tumor Suppressor genes

As summarized (Chang et al., 2001), many tumor suppressor genes are involved in breast cancer including those responsible for breast cancer syndromes (i.e. BRCA1, BECA2, p53 in Li-Fraumeni syndrome, ATM gene in ataxia telangiectasia). In addition, PTEN (a lipid phosphatase), $P57^{klp2}$ (a cyclin kinase inhibitor), FHIT (fragile histidine triad), Wnt-5a, $p16^{ink4a}$ (a cyclin-dependent kinase inhibitor) and maspin are also likely to be involved in breast cancers. Loss of heterozygosity was frequently observed on many chromosome arms in breast cancer (i.e. 1p, 1q, 3p, 11p, 13q, 16q, 17p, 17q and 18q). Except for chromosome 1, 16q and 18q, potential tumer suppressor genes have been identified (Chang et al., 2001). Subsequently, a senescence gene, SEN16 (Reddy et al., 1999; Reddy et al., 2000)and a transcription repressor, CBFA2T3 (Kochetkova et al., 2002) have been located on 16q24 and the maspin gene has been mapped to 18q21.3 (Schneider et al., 1995).

IV. Maspin

The mammary serpin protease inhibitor, maspin, is a candidate for being a tumor suppressor gene whose expression is lost in many advanced breast tumors. Maspin has drawn the attention due to the various proposed functions in breast carcinogenesis, such as angiogenesis, metastasis, apoptosis and cell invasion.

1) Identification and location

The maspin gene was originally isolated from normal mammary myoepithelial cells by subtractive hybridization on the basis of its expression at the m-RNA level (Sager et al.,

1994). This maspin gene encodes a 42-kDa protein (Sager et al., 1994) and is mapped at 18q21.3 (Schneider et al., 1995) where the loss of heterozygosity is frequently observed in this region , not only breast cancer cells, but in a large number of human tumors (Schneider et al., 1995).

Maspin m-RNA and protein were found in normal mammary myoepithelial cells, but down-regulated in mammary carcinoma cells (Sager et al., 1994). The expression of maspin is tissue-type and cell-type specific and it is expressed in skin keratinocytes and prostate epithelial cells in addition to mammary epithelial cells (Futscher et al., 2002). Immunohistochemical studies showed that maspin is expressed in pancreatic cancer cells but not in normal pancreatic epithelium (Maass et al., 2001a). In contrast, in mammary tissue, maspin is expressed in normal epithelial cells, but significantly less in mammary tumor cells (Maass et al., 2001a). In contrast to p53, the maspin expression was higher in ER-negative tissue than ER-positive tissue (Martin et al., 2000). Based on immunohistochemical studies, the maspin is expressed in the nucleus, as well as in the cytoplasm of breast myoepithelial cells (Reis-Filho et al., 2001; Lele et al., 2000).

2) Function

Although it has not been clearly understood the role of maspin in breast cancer, there are many functions of maspin suggested since the discovery of the maspin gene in 1994. Unlike the other serpins, maspin does not act as an inhibitor to an array of serine proteases, including urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Martin et al., 2000; Pemberton et al., 1995). However,

there is evidence that tPA is a target of the tumor suppressor gene maspin (Sheng et al., 1998). Exogenously introduced maspin gene expression in breast tumors rendered the inhibition of cell invasion in vitro and metastasis in vivo (Sager et al., 1994). Recombinant maspin protein blocks the motility of carcinoma cells (Sheng et al., 1996). Neutralization of maspin by an anti-maspin antibody abolished the invasion suppressive effect and increased apoptosis of conditioned medium from cultured breast myoepithelial cells on tumor cells (Shao et al., 1998). Maspin overexpression increased a rate of apoptosis and block angiogenesis in vivo and in vitro (Jiang et al., 2002; Zhang et al., 2000a; Zhang et al., 2000b). Maspin expression in breast tumor cells reduces tumor induction and metastasis in nude mice (Sager et al., 1994; Shi et al., 2001; Shi et al., 2003; Streuli, 2002), and also invasion of the basement membrane in vitro (Sager et al., 1994). Moreover, treatment of human breast cancer cells with recombinant maspin (rMaspin) inhibits cell motility (Sheng et al., 1996).

3) Transcriptional Regulation

The molecular and biological mechanisms of the functions of maspin still remain unknown. However, there is evidence that maspin interacts with the p53 tumor suppressor pathway (Maass et al., 2000). Zhou reported that p53 regulates the expression of maspin in breast cancer cell lines (Zou et al., 2000). Maspin expression was up-regulated when wild-type p53 gene was introduced through an adenoviral vector. P53 activates the maspin promoter by binding directly to the p53 consensus-binding site present in the maspin promoter (Zou et al., 2000). Furthermore, they demonstrated that

DNA-damaging agents and cytotoxic drugs induced endogeneous maspin expression in cancer cells containing wild-type p53, but not in cells containing mutant p53 (Zou et al., 2000). These results indicate that the function of maspin in apoptosis might be regulated through the p53 pathway.

Although the maspin gene is silenced in cancer cells, maspin gene deletions and mutations have not been found (Barsky et al., 1997), indicating the involvement of an epigenetic mechanism. The mechanism of the maspin gene regulation has not been clearly understood. However, maspin is up-regulated by the treatment of gamma linoleic acid, an essential fatty acid with anticancer properties (Jiang et al., 2002). Maspin reacts with tissue type plasminogen activator in vivo (Sheng et al., 1998). Interestingly, recombinant maspin exerts a biphasic effect on the activity of single-chain tissue plasminogen activator, acting as a competitive inhibitor at low concentrations (<0.5 mM) and as a simulator at higher concentrations (Sheng et al., 1998). Maspin gene expression was induced by MnSOD (Manganese-containing superoxide dismutase cDNA) transfectants of MCF-7. (Li et al., 1998).

The involvement of DNA methylation was first suggested by Domann in 2000 (Domann et al., 2000). Maspin promoter is unmethylated and is an open chromatin structure in normal, maspin-positive HMECs. In contrast, this chromatin structure is often aberrantly methylated and associated with a closed chromatin structure in maspin-negative human breast cancer cell lines (Domann et al., 2000). Methylation-associated silencing is found with a high frequency, as high as 80% in human breast cancers (Domann et al., 2000).

Additional experiments confirmed that maspin expression is regulated by DNA methylation and/or histone deacetylation in maspin-negative breast cancer cell lines (Domann et al., 2000; Futscher et al., 2002; Maass et al., 2002).

INTRODUCTION

Cancer cells are generally known to be similar to stem cells in phenotypes. The extreme case is exemplified by human embryonic stem cells (ES) which formed teratomas in SCID-beige mice (Thomson et al., 1998). Of all the common characteristics, the most notable description is the undifferentiated state of cancer and stem cells. The undifferentiated state of cancer cells could be due to the de-differentiation of differentiated somatic cells or due to the blocked differentiation of stem cells which give rise to tumor cells (Varmus and Weinberg, 1993; Sell, 1993). The latter is similar to other concepts of cancer as a disease of cell differentiation (Markert, 1968), a disease of stem cells (Sawyers et al., 1991) or as an "oncogeny as blocked or partially blocked ontogeny" (Potter, 1978). Cancer cells are known to be deficient in homologous or heterologous junctional gap intercellular communication (GJIC) (Yamasaki et al., 1987). Several human adult stem cells were also found to be deficient in GJIC (Chang et al., 1987; Kao et al., 1995; Matic et al., 2002; Grueterich and Tseng, 2002; Chang et al., 2004). Some characteristic tumor cell phenotypes such as contact-insensitive growth and the ability of anchorage independent growth (AIG) are shared by stem cells. The former has been reported for human kidney epithelial stem cells (Chang et al., 1987) and the latter were shown in human breast epithelial, human liver and mesenchymal stem cells (Chang et al., 2001; Chang et al., 2004; Lin et al., 2004). Nucleostemin, a nucleolar protein that interacts with p53, is present in CNS stem cells, ES cells and cancer cells but not in the differentiated cells of adult tissues (Tsai and McKay, 2002). Similarly, Oct-4, a transcription factor previously reported to be exclusively expressed in pluripotent early

embryo cells, ES cells and undifferentiated tumor cells, has been recently shown to be expressed in many human adult stem cells (Trosko et al., 2004). Furthermore, the expression of α-fetoprotein and vimentin, collectively termed the "oval cell phenotypes" (Alison et al., 1997), was found in adult human liver stem cells and hepatomas (Chang et al., 2004).

Mammary cancers primarily originated from the relatively undifferentiated mammary gland, i.e. the rodent terminal end bud (TEB) or the human lobule 1 where most mammary epithelial stem cells reside. For example, it has been demonstrated that the carcinogen acts on TEB and that the structure is the one that evolves to intraductal proliferation, carcinoma in situ and invasive carcinoma (Russo and Russo, 1996). The study of pathogenesis of breast cancer in relation to the lobular composition of breast also identified lobule 1 as the site of origin of the most frequent breast malignancy, the ductal carcinoma (Russo and Russo, 1997). Consistent with this correlation is the observation that lobule 1 contains high frequency of estrogen-receptor (ER) positive cells (14%) compared to lobule 2 (4%) and lobule 3 (0.5%) (Russo and Russo, 1998), and the fact that two thirds of breast cancer is ER-positive (Lippman and Allegra, 1980).

At the cellular level, the mammary gland contains two types of epithelial cells, the luminal and the basal (or myoepithelial in alveoli) epithelial cells besides the epithelial stem cells. Our laboratory has developed a cell culture method to grow two types of human breast epithelial cells (HBEC) from reduction mammoplasty tissues (Kao et al., 1995). Type II HBEC expressed basal epithelial cell markers whereas Type I HBEC

showed luminal epithelial and stem cell phenotypes. The stem cell characters of Type I cells include 1) deficiency in GJIC; 2) the ability to differentiate into Type II HBEC; 3) the expression of Oct-4 (Trosko et al., 2004) and 4) the ability to form ductal and budding or lobule 1-like structures on Matrigel (Chang et al., 2001). Furthermore, Type I HBEC have been shown to be more susceptible to telomerase activation, immortalization and neoplastic transformation (Sun et al., 1999; Kang et al., 1998). When the phenotypes of breast carcinomas or in vitro neoplastically transformed Type I HBEC are compared with Type I and Type II HBEC, it is clear that tumorigenic breast cells are similar to Type I HBEC rather than Type II HBEC. These similarities include deficiency in GJIC, the expression of estrogen receptor, Oct-4 and luminal epithelial cell markers (i.e. epithelial membrane antigen, cytokeratin 18) and ability of AIG (Kao et al., 1995; Kang et al., 1997; Chang et al., 2001; Trosko et al., 2004).

Since stem cells and differentiated cells are substantially different in phenotypes as shown in Type I and Type II HBEC, and if cancer cells are derived from and preserve many stem cell characteristics, it is expected that the phenotypes of the early stage cancer cells are already very different from differentiated normal cells. Indeed, it was found that the most dramatic and consistent phenotypic change occurred at the normal-to-in situ carcinoma transition (Porter et al., 2001). Another implication is the doubt about genetic alterations in cancer cells based on differential display or microarray studies of cancer and normal cells since the great majority of the cells in normal tissues are not the stem cells from which most cancers arise. For example, Cx26 and α6 integrin and maspin have been considered as tumor suppression genes based on differential display studies

showing their expression in normal cells but not in tumor cells (Lee et al., 1991; Sager et al., 1993; Zou et al., 1994). In fact, our results show that Cx26 and α6 integrin were expressed in Type II HBEC but not in Type I cells (Kao et al., 1995; Chang et al., 2001). Bernards and Weinberg (Bernards and Weinberg, 2002) hypothesized that certain combination of genetic alterations that are selected relatively early in tumorigenesis for proliferation advantage they confer will, incidentally, also confer invasive/metastatic phenotype. Since maspin is known to be a protease inhibitor which regulates not only invasion and metastasis but also tumor growth, angiogenesis and apoptosis (Zhang et al., 2000b; Jiang et al., 2002; Streuli, 2002; Shi et al., 2003), it is hypothesized that this gene could be the kind of tumor suppressor gene not expressed in stem cells and tumor cells. The experiments of this study were carried out to investigate this hypothesis.

MATERIALS and METHODS

Cell Culture

a) Normal Human Breast Epithelial Cells

Breast tissues were obtained from healthy women who underwent reduction mammoplasty. After removing most adipose tissues, the tissues were minced into small pieces and digested with collagenase for overnight before culturing in the MSU-1 medium (Kao et al., 1995). The initial primary culture developed in one week were aliquoated in small vials and stored at –80°C liquid nitrogen storage until use.

To recover cells from liquid nitrogen storage, the cells were thawed at 37°C and transferred to a 15 ml centrifuge tube containing MSU-1 medium. The tube was then centrifuged at 1000 rpm for 10 min. After removing the freezing solution and the medium from the tube, the cell pellet was plated in 6 ml of MSU-I medium with 5% fetal bovine serum (FBS) (the Type I medium) in a 10 cm cell culture dish (Plate A) and incubated for two hours. After the incubation, the supernatant containing unattached cells was removed, transferred to a 15 ml centrifuge tube and centrifuged at 1000 rpm for 10 min. The Plate A containing Type I HBEC and fibroblast cells was further incubated after adding 6 ml of fresh MSU-I medium. The cell pellet was suspended in 6 ml of MSU-I medium with bovine pituitary extract (BPE) (50 µl/ml) (the Type II medium), plated in a 10 cm cell culture dish (Plate B) and incubated for overnight. Next day, the supernatant containing unattached cells was carefully removed and transferred to a 15 ml centrifuge tube and centrifuge at 1000 rpm for 10 min. After discarding the medium, the

cell pellet was suspended in 6 ml of MSU-I medium with 5% FBS and plated in a 10 cm dish (Plate C), which contains mostly Type I HBEC and few fibroblasts. The few colonies of fibroblasts that developed in a week can be marked and removed by scraping with a rubber policeman. The Plate B after further incubation in the Type II medium contains Type II HBEC. The procedure of obtaining these 2 types of HBEC is diagrammatically depicted in Fig. 1. For this study, the primary HBEC cultures HME 29, 30 and 31 derived from 3 different women (age 26, 23, 21 respectively) were used. The phenotypic difference between Type I and Type II HBEC from previous studies (Kao et al., 1995; Chang et al., 2001) are listed in Table 1.

The culture medium was changed once every three days. Subculture of HBEC was carried out by cell removal with 0.01% trypsin and 0.01% EDTA followed by the inactivation of trypsin with 10% FBS.

Table 1 Characteristics of Type I cells and Type II HBEC (Modified from (Kao et al., 1995)

Plastic	Type I	Type II
Cell Morphology in MSU-1 Medium	Variable in shape	Uniform in shape, cobble-stone appearance
Colony Morphology in MSU-1 Medium	Boundary smooth and restricted	Boundary not smooth, unrestricted
Attachment on Plastic After Trypsinization	Late	Early
Effect of FBS on Cell Growth	Promoted	Inhibited
EMA Expression	+	-
Keratin 18 Expression	+	-
Keratin 14 Expression	-	+
α6 Integrin Expression	-	+
Connexin 43	-	+
Connexin 26	-	+
Effect of Cholera Toxin	Induction of Type I into Type II HBEC	Growth promotion
AIG on Soft Agar	+	-
Telomerase Activation and Immortalization by SV-40 Large T Antigen	High Frequency	Low Frequency
Organoids Structures Formed on Matrigel	Acini Budding/ductal structure in conjunction with Type II HBEC	Hollow Spheres
Gap Junctional Intercellular Communication	Deficient	Proficient

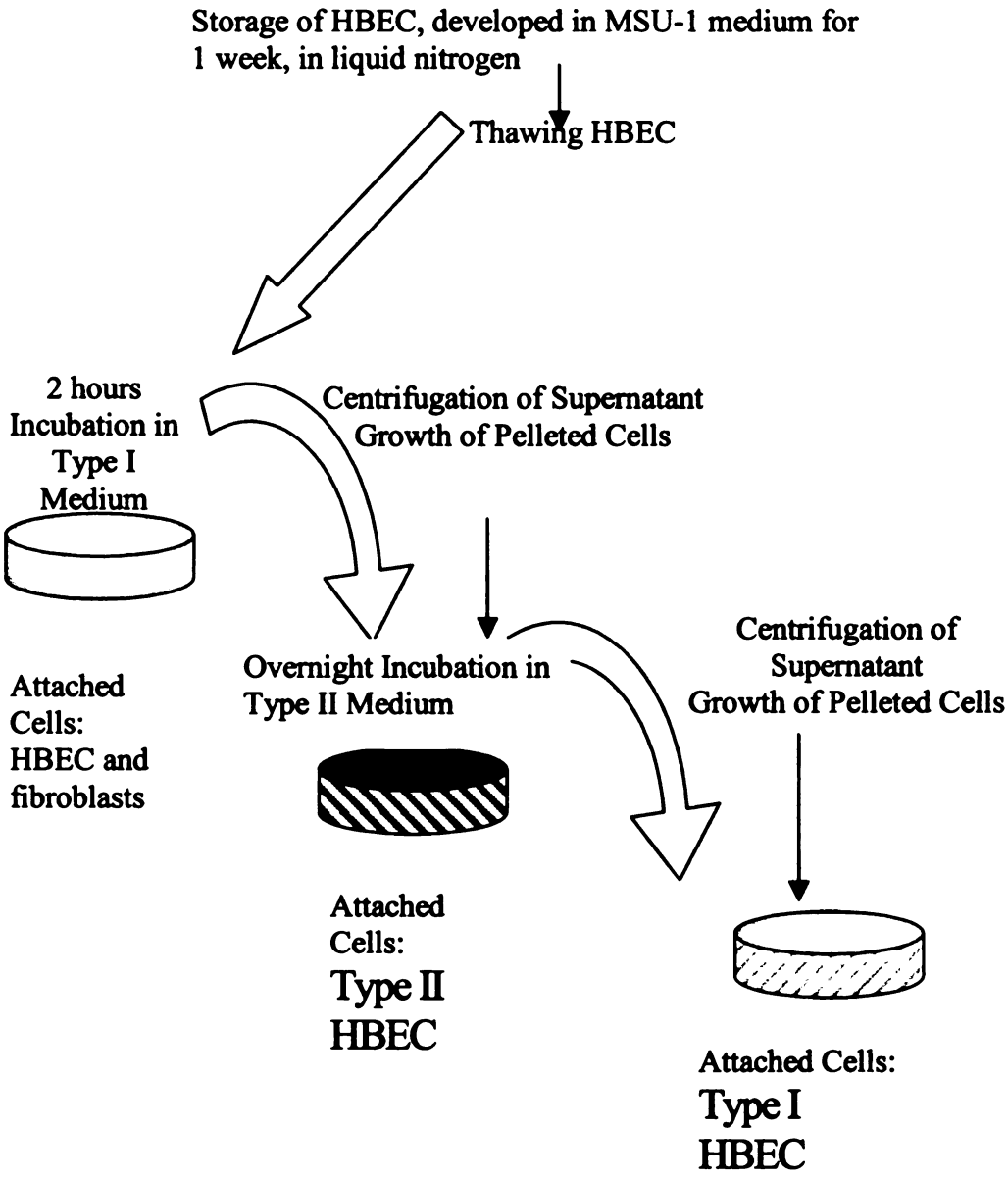
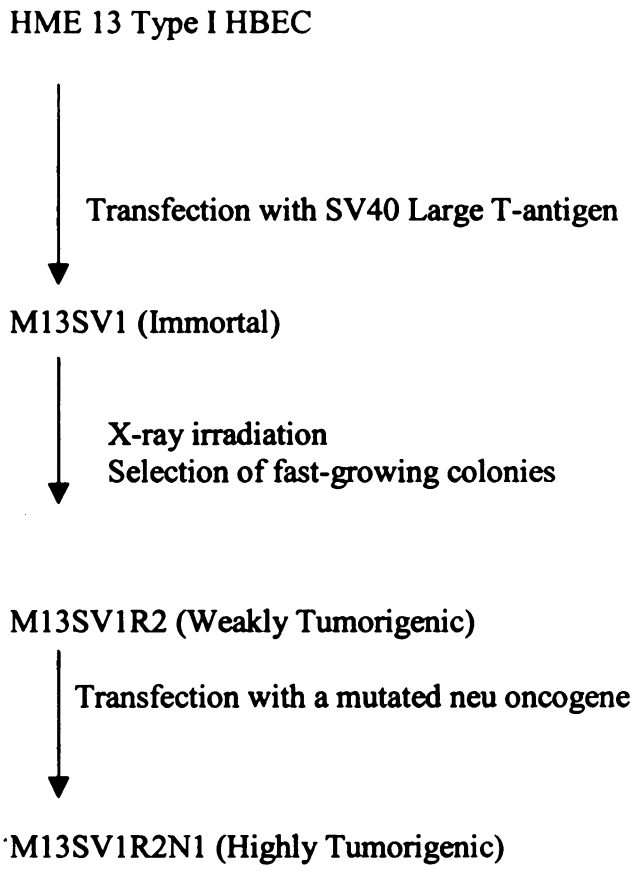


Figure 1. Isolation scheme to obtain 2 types of Normal Human Breast Epithelial Cells

b) Tumorigenic Breast Epithelial Cells

Immortal, weakly tumorigenic and highly tumorigenic breast epithelial cell lines have been derived from Type I HBEC after step-wise treatment with SV40 large T-antigen, X-rays and c-erbB2/neu oncogene (Kang et al., 1998) (Fig. 2). These in vitro derived cell lines together with the well known breast carcinoma cell lines, MCF-7 (ER-positive) and MDA-MB-231 (ER-negative), were also used in this study.



Figures 2 In vitro immortalization and neoplastic transformation of Type I HBEC (Kang et al., 1998)

Western Blot Analysis of Maspin Protein Expression

Cells cultured in 10 cm dish, were washed with phosphate buffered saline (PBS) three times, and lysed with 20% SDS lysis solution (500 µl) containing protease and phosphatase inhibitors (Trosko et al., 2000). The viscous lysate was carefully collected with a scraper and transferred to a tube on ice. The lysate in the tube was sonicated with a sonicator (range~33) for 30 seconds 3 times. The proteins were aliquated in smaller tubes and stored in –20°C freezer until use.

Protein concentrations were measured using Biorad Protein Quantification kit (Biorad, California). Equal amount of protein (20 µg/lane) were separated by 12% SDS-PAGE and transferred from the gel to PVDF membranes (Millipore Corp, Bedford, MA). Immunoblotting was carried out using anti-human maspin antibodies (BD Biosciences-PharMingen, San Diego, CA, 1:1,000 dilution). Horseradish peroxidase-conjugated secondary antibody was used for the chemiluminescent assay. The immunoreactive protein complexes were detected by ECL-detection kit (Amersham, Life Science, Denver, CO).

When the membrane was needed to be reblotted, the membrane was washed in 100 ml of 0.1 M citrate, pH=3.5, for 10 minutes. Then, the membrane was further washed in 100 ml Tris-buffered Saline (TBS) containing 0.5 M tris (pH=7.5), 0.05M NaCl and NP40 (IGEPAL) (0.5% v/v) for 20 minutes. After repeating the above two steps twice, the membrane was washed twice with 100 ml TBS without IGEPAL for 10 minutes. After

this step, the membrane was reblocked with 5% milk in PBS with 0.1% Tween before initiating the re-probing.

Immunocytochemical detection of maspin expression

Cells were plated into 8-well chamber slide (LAB-TEK, Nalge Nunc Int., Naperville, IL; 12,000 cells/well) for 2-3 days. The cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min. After rinse with PBS, the cells were permealized with 3% H_2O_2 in methanol at room temperature for 10 min and treated with 10% FBS in PBS for 30 min for blocking. These cells were then treated with primary antibody (a mouse antihuman maspin monoclonal antibody from BD Biosciences-Pharmingen, Cat. #554292, 1:100 dilution) for overnight at 4°C on a rocking platform, following rinse with PBS, the cells were incubated with the secondary antibody (i.e. FITC-conjugated sheep anti-mouse IgG, 1:200 dilution in 1% FBS in PBS) for 45 min at 4°C. The cells were thoroughly washed with PBS and mounted with aqua-poly mount (Polysciences Inc. Cat#18606) and a cover glass. The image and fluorescence of cells were observed and recorded using a Nikon Eclipse TE 300 microscope connected to a digital camera and computer. Images in this thesis are presented in color.

RESULTS

Differential expression of maspin in Type I and Type II HBEC

Type I and Type II HBECs were obtained from reduction mammoplasty tissues using the scheme described in Fig. 1 by taking advantage of the difference in time required for cell attachment onto the plastic dishes after cell plating. Furthermore, these two types of HBEC can be selectively grown using different media, i.e. Type I MSU-1 medium with FBS promoted the growth of Type I HBEC while inhibited the growth of Type II HBEC; Type II MSU-1 medium with BPE promoted the growth of Type II HBEC and the conversion of Type I to Type II HBEC.

Type I and Type II HBEC display a wholesale difference in phenotypes (Kao et al., 1995; Chang et al., 2001) as shown in Table 1 and can be recognized morphologically as shown in Fig. 3.

Before studying the expression of maspin in these cells, Type I and Type II HBEC were immunocytochemically stained with cytokeratin cell markers to reconfirm their cell types. As shown in a representative figure (Fig.4), Type I HBEC is positive for cytokeratin 18, but not cytokeratin 14, whereas Type II HBEC is positive for cytokeratin 14, but not cytokeratin 18.

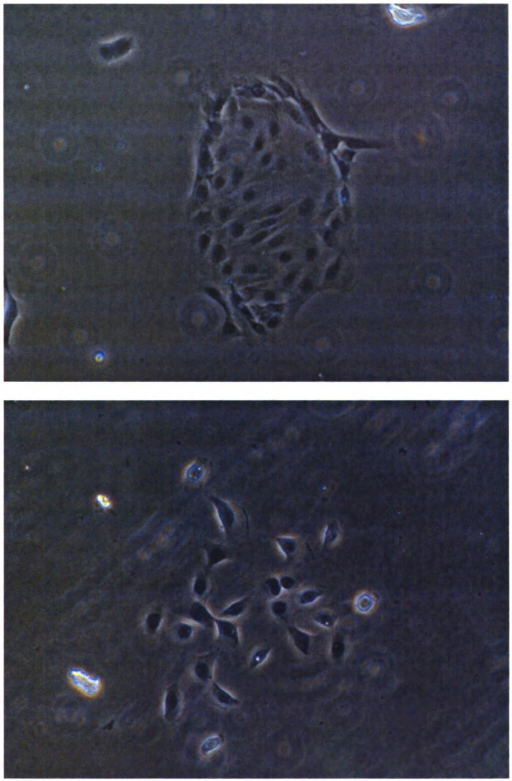


Figure 3 **Morphologies of Type I (Top) and Type II (Bottom) HBECs of HME30**

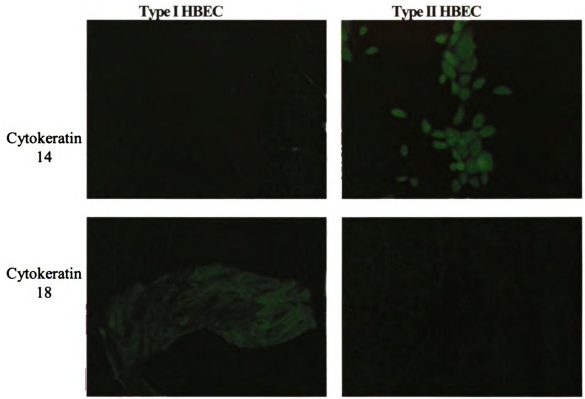
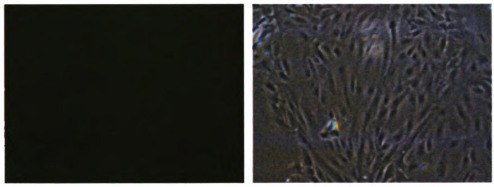


Figure 4 **Expression of Cytokeratin 14 and 18 in Type I and Type II HBECs**
Top Right: **Cytokeratin 14 Expression in Type I HME 30**
Bottom Right: **Cytokeratin 18 Expression in Type I HME 30**
Top Left: **Cytokeratin 14 Expression in Type II HME 30**
Top Right: **Cytokeratin 18 Expression in Type II HME30**

To examine the expression and cellular localization of maspin protein of the 2 types of HBEC, immunocytochemical stainings of these cells were carried out. As shown in Fig. 5, maspin expression was not observed in Type I HBEC, but was clearly seen in the cytoplasm of Type II HBEC. All Type I and Type II cells from the two different HBECs tested (HME 30 and 31) similarly showed that the maspin was expressed in Type II HBEC (Fig. 5a, 5b), but not in Type I HBEC (Fig. 5a, 5b).

For confirmation, western blot analysis was also carried out to examine the protein expression of maspin in Type I and Type II HBEC derived from three different individuals. The results show that maspin (42 kD) was highly expressed in Type II HBECs, but not in Type I HBECs (Fig. 6). Although the levels of expression may vary among different cell cultures, the contrasting opposite expression of maspin in Type I and Type II HBEC was consistent in these cell cultures.

Type I



Type II

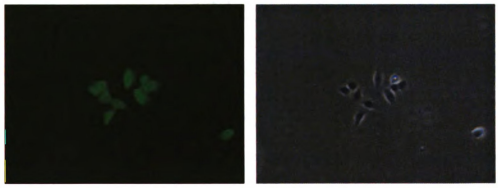


Figure 5a **Maspin Expression by Immunostaining in Type I (Top) and Type II (Bottom) HBEC of HME 30**
The phase contrast images of cells are shown on the right.

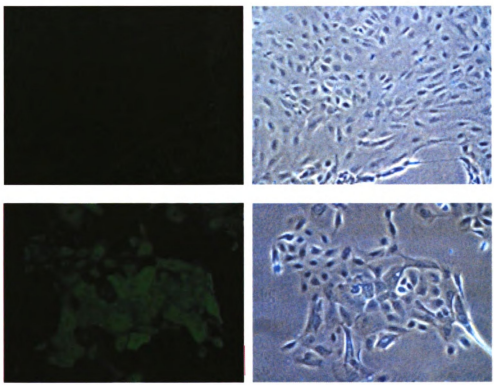


Figure 5b Maspin Expression by Immunostaining in Type I (Top) and Type II (Bottom) HBEC of HME 31
The phase contrast images of cells are shown on the right.

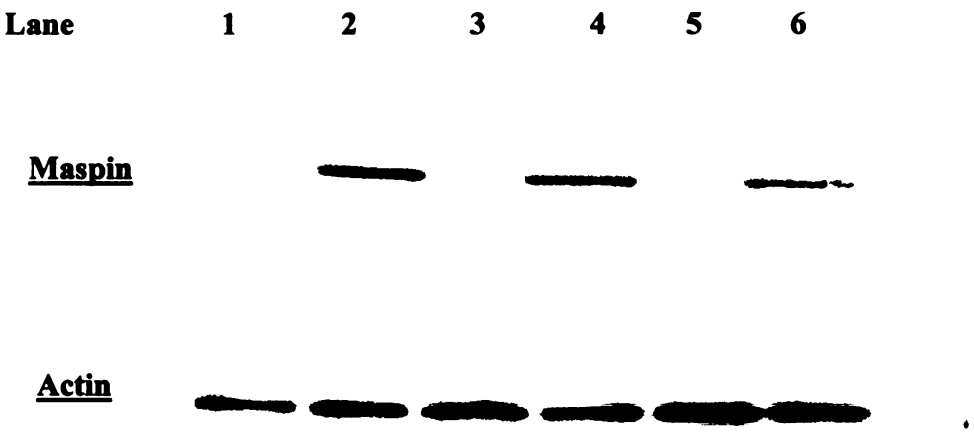


Figure 6 **Western Blot Analysis of Maspin Expression in Type I and Type II HBEC of HME29 (lanes 1, 2), HME30 (lanes 3, 4) and HME31 (lanes 5, 6)**

Expression of maspin in *in vitro* transformed HBEC and cancer cell lines

A series of in vitro immortalized and neoplastically transformed Type I HBEC lines (M13SV1, M13SV1R2, M13SV1R2N1) were characterized for maspin expression. As shown in Fig. 7, all these Type I HBEC neoplastically transformed at different stages (immortal M13SV1, weakly tumorigenic M13SV1R2 and highly tumorigenic M13SV1R2N1) were negative in maspin expression by immunocytochemical studies similar to normal Type I HBEC. As expected, the breast cancer cell lines, MCF-7 (ER+) and MDA-MB-231 (ER-) were negative in maspin expression (Fig. 8)

In western blot analysis, all the in vitro transformed Type I HBEC lines (i.e. immortal M13SV1, weakly tumorigenic M13SV1R2 and highly tumorigenic M13SV1R2N1) (Kang et al., 1998) as well as the MCF-7, MDA-MB-231 and T47D breast cancer cell lines were found not to express the maspin protein (Fig. 9).

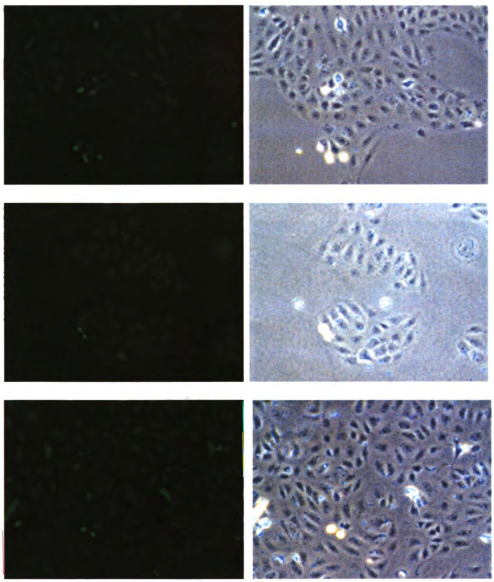


Figure 7 **Maspin Expression of In vitro Transformed Type I HBEC Lines at Different Stages by Immunocytochemical Analysis**
The phase contrast images of cells are shown on the right.

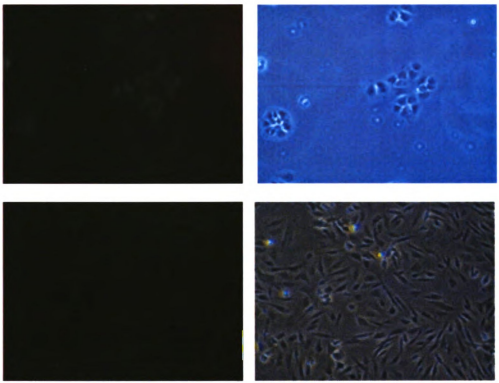


Figure 8 **Maspin Expression in Breast Carcinoma Cell Lines, MCF-7 (top), MDA-MB-231 (bottom) by Immunostaining**
The phase contrast images of cells are shown on the right.

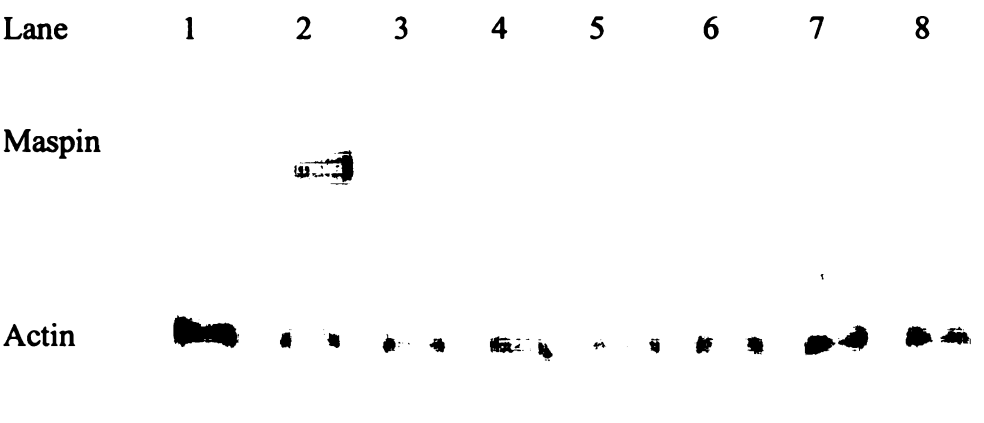


Figure 9 Western Blot Analysis of Maspin Expression in normal HBEC, in Vitro Immortalized or Neoplastically Transformed Type I HBEC and Breast Carcinoma Cell Lines

Lane 1: Type I HME 30
Lane 2: Type II HME 30
Lane 3: M13SV1
Lane 4: M13SV1R22
Lane 5: M13SV1R2N1
Lane 6: MCF7
Lane 7: MDA-MB-231
Lane 8: T47D

DISCUSSION

This study using immunocytochemical staining and Western blot analysis has demonstrated that the human breast epithelial cell type (Type I HBEC) with stem cell characteristics did not express maspin, whereas Type II HBEC with basal epithelial phenotypes expressed maspin. Furthermore, a series of step-wise neoplastically transformed cell lines derived from a Type I HBEC (Kang et al., 1998), i.e. immortal M 13SV1, weakly tumorigenic M 13SV1R2 and highly tumorigenic M 13SV1R2N1, did not show maspin expression. Previously, normal human breast epithelial cells (myoepithelial or basal epithelial) were shown to express maspin (Zou et al., 1994; Maass et al., 2001a; Maass et al., 2001b) whereas the majority of human breast cancer cell lines including MCF-7 and MDA-MB-231 did not show maspin expression (6/7 or 6/8) (Zou et al., 1994; Maass et al., 2002). Our experiment also confirmed that these two well-known breast cancer cell lines, MCF-7 and MDA-MB-231, did not express maspin. Therefore, the results are not consistent with the idea that maspin is expressed in all normal breast epithelial cells and that the expression of this serine proteinase inhibitor is down-regulated or mutated during tumor progression (Maass et al., 2001b).

These results have several implications. First, similar to Cx 26, α-6 integrin and Oct-4 (Lee et al., 1991; Sager et al., 1993; Trosko et al., 2004) maspin is a breast epithelial stem cell marker whose expression is preserved from stem cells to tumorigenic cells as shown in the different Type I HBEC lines transformed at different stages. Although maspin has been clearly shown to possess tumor-suppressing activity (Zou et al., 1994), its down regulation in breast cancer cell lines (Zou et al., 1994; Maass et al., 2001a) or loss of

expression in tumor tissues, in contrast to cells in normal tissues such as myoepithelial cells (Maass et al., 2001b), does not mean a real change has occurred in gene expression from the target cells (i.e.Type I HBEC) to tumor cells. The results provide an alternative origin of a tumor suppressor gene and an additional mechanism explaining how stem cells could be target cells for carcinogenesis.

Second, since maspin disappears when Type I HBECs differentiate into other cell types, maspin expression might be used to screen for chemopreventive agents. Indeed, sphingoid bases (sphinosine and sphinganine), which are potential chemopreventive agents, have been shown to induce the differentiation of Type I HBEC into Type II HBEC with the concomitant emergence of maspin expression in Type II HBEC (Ahn, 2003). The effect of this type of agent could reduce the target breast epithelial stem cells for carcinogenesis.

Thirdly, since down-regulation of maspin could be a marker for breast cancer and its target precursor cell, the non-expression of this gene is expected to be early in precancerous cells, i.e., ductal carcinoma in situ. This gene could be used as a marker for early detection of precancerous cells.

When Type I HBECs differentiate into Type II HBEC, there is a wholesale change in the expression of may genes and functions {Kao, Nomata, et al. 1995 24865 /id} {Chang, Sun, et al. 2001 19479 /id} {Chang, Tsai, et al. 2004 31265 /id} {Chang, Sun, et al. 2001 19479 /id} {Chang, Olson, et al. 2004 31314 /id}. These conversions are clearly due to

epigenetic changes during cell differentiation. For maspin, the re-expression of maspin in maspin-negative breast cancer lines (MCF-7, T-47D and MDA-MB-231) can be effected by treatment with 5-aza-2'deoxycytidine and/or trichostatin A, indicating that DNA methylation and/or histone deacetylation is/are partially responsible for the silencing of the maspin gene in breast cancer cells (Futscher et al., 2002). Furthermore, it has been shown that, in normal cells expressing maspin, the maspin promoter is unmethylated and the promoter region has acetylated histones (Maass et al., 2002; Futscher et al., 2002). In contrast, normal cells that do not express maspin have a completely methylated maspin promoter with hypoacetylated histones (Maass et al., 2002). These mechanisms are very likely to be involved in the silencing of maspin expression in Type I HBEC and can be examined in future studies.

Bernards and Weinberg (Bernards and Weinberg, 2002) hypothesized that the metastatic behavior of cancer cells seems to be determined relatively early in tumorigenesis. They suggest that a subset of the mutant alleles acquired by incipient tumor cells early in tumorigenesis confer not only the selected replicative advantage, but also, later in tumorigenesis, the proclivity to metastasize. Since maspin has been shown not only to inhibit the proteinase activity but also to reduce tumor growth through a combination of reduced angiogenesis and increased apoptosis (Shi et al., 2003), it is a gene fitting that description. However, the silencing of maspin in tumor cells might not be due to mutation or epigenetic alteration during tumor progression, but rather due to continuous expression of a target stem cell phenotype.

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