STEM CELL-LIKE PROTEIN EXPRESSION IN BREAST CANCER CELL LINES

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FOR THE DEGREE OF MASTER OF SCIENCE

BY
NURETTİN İLTER SEVER
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TO MY PARENTS, AND TO MY GRANDMOTHER WHOM I LOST LAST DECEMBER

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Mehmet Öztürk

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Şevket Ruacan

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assist. Prof. Tamer Yağcı

Approved for the Institute of Engineering and Science

Prof. Dr. Mehmet Baray
Director of Institute of Engineering and Science

ABSTRACT

Stem cell-like protein expression in breast cancer cell lines

N.İlter Sever

M.Sc. in Molecular Biology and Genetics

Supervisor: Prof. Dr. Mehmet Öztürk

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Breast cancer is the most common type of cancer and the second leading cause of cancer deaths among women. Most of breast carcinoma arises from the breast ductal epithelium. Recent studies in the past decade have displayed that many organs in our body possess organ-specific stem cells which have the capacity of self-renewal and differentiation toward specialized cell types which are specific for that organ. Breast is also one of these organs and breast epithelium contains stem/progenitor cells which give rise to two types of differentiated breast epithelial cell types: luminal epithelial and myoepithelial. Due to their property of division-competence for self-renewal, stem cells are more prone to malignant transformation than more differentiated cell types. Therefore, it is considered that breast cancer may have arisen from a stem/progenitor cell found in mammary epithelium and recent studies support this hypothesis. In the study described in this thesis, breast cancer cell lines have been demonstrated to display stem/progenitor cell-like protein expression. Breast cancer cell lines have been characterized according to their marker protein expression related to the differentiation status of the mammary epithelial cell type by using immunofluorescence and Western blotting techniques. Also, single cell-cloning of GI-101 cell line has been performed. It has been demonstrated that a single cell could give rise to different cell populations, further supporting the hypothesis that breast cancer cell lines display stem/progenitor cell-like protein expression.

ÖZET

Meme kanseri hücre hatlarında kök hücre protein ekspresyonu

N.İlter Sever

Master Tezi, Moleküler Biyoloji ve Genetik Bölümü

Tez yöneticisi : Prof. Dr. Mehmet Öztürk

2003, 70 sayfa

Meme kanseri kadınlar arasında en yaygın ve en çok ölüme neden olan ikinci kanser türüdür. Meme kanserinin önemli bir kısmı meme kanal epiteli kökenlidir. Son on yıldaki çalışmalar vücudumuzdaki pekçok organın kendilerini yenileme ve o organa özel hücre tiplerine farklılaşabilme kapasiteleri olan kök/öncül hücrelere sahip olduklarını göstermiştir. Meme de bu organlardan biridir ve yapılan çalışmalar meme epitelinde farklılaşmış luminal epitel ve miyoepitel hücre tiplerine dönüşebilen kök/öncül hücrelerin varlığını ortaya koymuştur. Kendini yenilemek için bölünme potansiyelinin fazla olması kök/öncül hücreleri malignant dönüşüme farklılaşmış hücre tiplerine göre daha açık hale getirmektedir. Bu nedenle, meme kanserinin kök/öncül hücre kaynaklı olduğu düşünülmektedir ve yakın dönemdeki çalışmalar bu hipotezi desteklemektedir. Bu çalışmada, meme kanseri hücre hatlarında kök/öncül hücre protein ekspresyonu gösterilmiştir. Meme kanseri hücre hatları immünofloresan ve Western blotting metodları kullanılarak meme epiteli farklılaşma statüsünü belirleyici proteinlerin ekspresyonuna göre sınıflandırılmıştır. Ayrıca, GI-101 hücre hattının tek hücre klonlaması yapılmış ve tek bir hücrenin birden fazla hücre tipine dönüşebildiği gösterilmiştir ve bu çalışma meme kanseri hücre hatlarında kök/öncül hücre protein ekspresyonunun var olduğu hipotezini desteklemektedir.

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ABBREVIATIONS

APS	Ammonium Persulphate
ASMA	Alpha Smooth Muscle Actin
BSA	Bovine Serum Albumin
CALLA	Common Acute Lymphoblastic Leukemia Antigen
Cdk	Cyclin-dependent kinase
CK	Cytokeratin
DAPI	4'6'-Diamidino-2-Phenylindole
DCIS	Ductal Carcinoma in situ
DLLC	Differentiated Large Light Cell
DMEM	Dulbecco's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
ESA	Epithelial Specific Antigen
FCS	Fetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
Het	Heterogenous
HRP	Horse Radish Peroxidase
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LCIS	Lobular Carcinoma in situ
LDC	Large Dark Cell
LOH	Loss of Heterozygosity
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
MUC1	Sialomucin
NaCl	Sodium Chloride
Na_2HPO_4	Sodium Monohydrogen Phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PVDF	Polyvinyl Difluoride
Rb	Retinoblastoma
SDS	Sodium Dodecyl Sulphate
SLC	Small Light Cell
TBS-T	Tris Buffered Saline with Tween-20
TDLU	Terminal Ductal-Lobular Unit
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
TGF	Transforming Growth Factor
Tris	Tris (Hydroxymethyl)-Methylamine
ULLC	Undifferentiated Large Light Cell
UV	Ultraviolet

CHAPTER I INTRODUCTION

1.1 OVERVIEW OF HUMAN BREAST DEVELOPMENT:

Breasts are assumed to arise as the result of reciprocal epithelial and mesenchymal interactions. The mammary epithelium is an ectodermal derivative. Both male and female show the same pattern of development *in utero.* The prenatal breast development includes primarily condensation of epithelial cells in the thoracic/pectoral region where the breast bud forms. The nipple primordium forms as a narrow collection of epidermal cells. This nipple grows to a nodule which then gives rise to the formation of the breast bud. Mesenchymal cells condenses deeply around the breast bud and blood vessels form. The following event is branching of buds. Later, secondary buds appear, elongate and secondary buds branch and the breast bud invades into mesenchymal tissue and ductal morphogenesis occurs. At this stage, there is high expression of Epidermal Growth Factor Receptor (EGFR) and Transforming Growth Factor-alpha (TGF-α), suggesting an autocrine stimulation of proliferation (Howard and Gusterson, 2000; M.T.Lewis, 2000).

At the infant stage, both male and female show the same pattern of development. Wide variation is observed in the degree of glandular development and in the functional differentiation of the cells lining the ducts. At birth, the ductal system opens onto the surface through the breast pit, a small depression on the surface of the skin. The underlying mesenchyme proliferates to form a nipple. Whole-mount analyses at birth demonstrate the diversity in the degree of development present, which can range from simple blunt-ended tubular structures to well-developed branching. The infant breast is able to respond to the secretory stimuli that arise from maternal hormones and the production of milk by 80-90% of both sexes is due to the effects of prolactin on withdrawal of the sex steroids. Development of

interlobular and intralobular stroma containing fibroblast populations is observed at infant stage. During the first 2 years of life, the epithelium lining the ducts differentiates and involutes sequentially. The infant breast appears to undergo involution (death of epithelial cells by apoptosis) once it is removed from the influence of maternal hormones. By 2 years of age, only small ductal structures in a fibroblastic stroma are observed. The breast remains in this state until puberty (Howard and Gusterson, 2000).

At puberty, in females, ovarian hormones stimulate rapid and invasive ductal elongation driven by growth of a structure called the terminal end bud, which consists of four to six layers of relatively undifferentiated 'body cells' and a surrounding single layer of 'cap cells'. Body cells differentiate into **luminal epithelial cells**, while cap cells differentiate into **myoepithelial cells.** Also, an increase in the amount of fatty and fibrous tissue (80%) is observed. The primary ducts branch leading to segmental and subsegmental ducts, which lead the terminal ducts that give rise to blind-ended ductules called acini. A collection of acini arising from one terminal duct and embedded in intralobular stroma is referred to as terminal duct lobular unit (TDLU), which is the functional unit of the breast. Upon reaching the limits of the fat pad at ductal maturity, ductal elongation ceases and terminal end buds regress to leave a branched system of differentiated ducts. These ducts will remain relatively quiescent as long as the animal remains virgin (Howard and Gusterson, 2000; M.T.Lewis, 2000).

Each pregnancy leads the breast to a cyclical growth pattern which contains lobuloalveolar differentiation, lactation, involution and gland remodeling processes. Hormonal changes during pregnancy lead to this cyclical phase of development in which there is a dramatic transition from a predominantly ductal to a predominantly lobuloalveolar gland morphology. Lobuloalveolar progenitor cells located within the ducts proliferate to form alveolar buds, which further differentiate to form the alveoli. Near midpregnancy, the alveolar epithelium acquires the capacity to produce milk proteins (the stage I transition of lactogenesis) but secretory function is inhibited. At parturition, inhibition of secretory function is released and these cells begin to secrete large quantities of milk (the stage II transition of lactogenesis). Upon weaning, milk secretion ceases and the gland involutes. During involution,

most alveolar cells undergo apoptosis (programmed cell death), while a residual epithelial population remodels itself back into a ductal tree to await the next pregnancy. During successive rounds of pregnancy and involution, the TDLUs expand and decrease in size with an increase and subsequent decrease in the number of acini (M.T.Lewis, 2000).

At the postmenopousal involution stage, both lobules and ducts are reduced in number. The intralobular stroma is replaced by collagen, whereas the glandular epithelium and the interlobular connective tissue regress and are replaced by fat. The remainings are a few acini and ducts embedded in thin strands of collagen that are widely dispersed throughout the fat (Howard and Gusterson, 2000).

1.2 BREAST CANCER

1.2.1 EPIDEMIOLOGY OF BREAST CANCER

1.2.1.1 Incidence of Breast Cancer

Breast cancer is the second leading cause of cancer deaths in women today (lung cancer is the first one) and is the most common cancer among women, excluding nonmelanoma skin cancers. According to the World Health Organization, more than 1.2 million people would be diagnosed with breast cancer in 2001 worldwide (www.imaginis.com). The American Cancer Society estimates that in 2001 approximately 192,200 new cases of invasive breast cancer would be diagnosed among women in the United States. Another 46,400 women would be diagnosed with ductal carcinoma in situ (DCIS), which will be introduced in part 1.2.2.2.2, a non-invasive breast cancer. DCIS is the earliest form of breast cancer, confined to the milk ducts of the breast. It is estimated that 40,600 deaths will occur from breast cancer (40,200 among women, 400 among men) in the United States (www.imaginis.com). Estimated breast cancer incidences and deaths are displayed in Table 1.1. The incidence rate of breast cancer (number of new breast cancers per 100,000 women) increased by approximately 4% during the 1980s but leveled off to 100.6 cases per 100,000 women in the 1990s. The death rates from breast cancer also declined significantly between 1992 and 1996, with the largest decreases among younger women. Medical experts attribute the decline in breast cancer deaths to earlier detection and more effective treatments (www.imaginis.com).

Table 1.1 Estimated Breast Cancer Cases/Deaths Worldwide

Region	New Cases (2000)	Deaths (2000)
Eastern Africa	13,615	6,119
Middle Africa	3,902	1,775
Northern Africa	18,724	8,388
Southern Africa	5,537	2,504
Western Africa	17,389	7,830
Caribbean	6,210	2,310
Central America	18,663	5,888
South America	69,924	22,735
Northern America	202,044	51,184
Eastern Asia	142,656	38,826
South-Eastern Asia	55,907	24,961
South Central Asia	129,620	62,212
Western Asia	20,155	8,459
Eastern Europe	110,975	43,058
Northern Europe	54,551	20,992
Southern Europe	65,284	25,205
Western Europe	115,308	40,443
Australia/New Zealand	12,748	3,427
Melanesia	470	209
Micronesia	62	28
Polynesia	127	58

Source: J. Ferlay, F. Bray, P. Pisani and D.M. Parkin. GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. IARC CancerBase No. 5. Lyon, IARCPress, 2001

It has been estimated that approximately 50% of women who develop breast cancer have no identifiable risk factor beyond increasing age and female gender (Madigan et al., 1995). Age plays a major role in breast cancer risk (Ries et al., 1999). In women under the age of 30, breast cancer is extremely uncommon.

From 1992 to 1996, the incidence of breast cancer in women aged 35 to 39 was 59 per 100.000 cases; however, in women aged 55 to 59, the incidence was 296 per 100.000 cases. The annual incidence continues to rise, albeit more gradually, as a women enters her 60s and 70s (Ries et al., 1999). Breast cancer probability related with age of woman is displayed in Table 1.2. (www.imaginis.com)

Table 1.2 Breast Cancer Probability Related with Age of Woman

By age 30	1 out of 2,212
By age 40	1 out of 235
By age 50	1 out of 54
By age 60	1 out of 23
By age 70	1 out of 14
By age 80	1 out of 10
Ever	1 out of 8

Source: Feuer EJ, Wun LM. DEVCAN: Probability of Developing or Dying of Cancer. Version 4.0. Bethesda MD: *National Cancer Institute,* 1999.

Breast cancer in men is infrequent and in the year 2000, it was estimated that 1500 men would develop breast cancer and 400 of these men would die from the disease (Greenlee et al., 2000). The lifetime risk of being diagnosed with breast cancer in men is 0.11% compared with 13% in women (Brewster and Helzlouser, 2001). The genetic syndrome, Klinefelter syndrome, which affects 1 in every 1000 men, is responsible for 3 to 8% of male breast cancer cases (Hultborn et al., 1997). Other genetic causes of male breast cancer include inherited mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 (Friedman et al., 1997; Couch et al., 1996). Nongenetic causes of male breast cancer include radiation exposure, liver disease, gynecomastia, obesity, and exposure to estrogen medication (Lynch et al., 1999).

1.2.1.2 Risk Factors of Breast Cancer in Women

Multiple factors are associated with an increased risk of developing breast cancer, including increasing age, family history-inherited predisposition to breast cancer (BRCA1 and BRCA2), exposure to female reproductive hormones (both endogenous and exogenous), dietary factors, benign breast disease, and environmental factors (such as ionizing radiation). The majority of these factors convey a small to moderate increase in risk for any individual women (Dickson and Lippman, 2001). Attention will be concentrated on the endogenous hormonal effects on breast cancer since these effects relate to our study.

1.2.1.2.1 Endogenous Hormonal Effects on Breast Cancer

The development of breast cancer in many women appears to be related to female reproductive hormones. Epidemiologic studies have consistently identified a number of breast cancer risk factors, each of which is associated with increased exposure to endogenous estrogens. Early age at menarche, nulliparity or late age at menopause increase the risk of developing breast cancer (Gail et al., 1989; Rosner et al., 1996).

As described earlier, the age-specific incidence of breast cancer increases steeply with age, but the rate of this increase, in postmenopausal period, decreases to one-sixth of that of in premenopausal period. This decrease suggests that ovarian activity plays a major role in the etiology of breast cancer. The relative risk of developing breast cancer for a woman with natural menopause before age 45 is one-half that of a woman whose menopause occurs after age 55 (Trichoupoulos et al., 1972).

Age at menarche and the establishment of regular ovulatory cycles are strongly linked to breast cancer risk. Earlier age at menarche is associated with an increased risk of breast cancer (Kelsey et al., 1993); there appears to be a 20% decrease in breast cancer risk for each year that menarche is delayed (MacMahon et

al., 1973). It must be noted that hormone levels through the reproductive years in women who experience early menarche may be higher than in women who undergo a later menarche (MacMahon et al., 1982). Additionally, late onset of menarche results in a delay in the establishment of regular ovulatory cycles, although there is some controversy over whether this delay confers any additional protective effect (MacMahon et al., 1982).

Age at first full-term pregnancy clearly influences breast cancer risk. Based on epidemiologic studies, women whose first full-term pregnancy occurs after age 30 have a two- to fivefold increase in breast cancer in comparison with women who have a first full-term pregnancy before approximately age 18 (MacMahon et al., 1970, Trichoupoulos et al., 1983). Nulliparous women are at greater risk for the development of breast cancer than parous women (MacMahon et al., 1970). During pregnancy, mammary cells differentiate into mature breast cells prepared for lactation. After this differentiation, these cells have a longer cell cycle, allowing more time for DNA repair in G_1 (Russo et al., 1982). Breast cancer risk increases transiently after a pregnancy. The increased risk lasts approximately 10 years (Bruzzi et al., 1988). The reason for the increased risk has been hypothesized to be the increase in proliferation, growth, and maturation of breast cells preparing for lactation, leading to the development of mutations. Also, studies have suggested that a long duration of lactation reduces breast cancer risk in premenopausal women (Newcomb et al., 1994).

1.2.2 MOLECULAR BIOLOGY OF BREAST CANCER

1.2.2.1 Somatic Mutations in Breast Cancer

Breast cancer, like other forms of malignancy, is thought to progress by accumulation of a series of genetic and resulting phenotypic changes in the pathways regulating cellular proliferation, differentiation, death (apoptosis or necrosis), DNA repair, tissue compartmentalization, and responses to therapy (Dickson and Lippman, 2001). Classical cytogenetic studies have revealed that genetic regions, which are frequently rearranged, amplified, deleted, or altered, have been commonly detected

on chromosomes 1,3,6,7,8,9,11,13,15,16,17,18, and 20 (Schrock et al., 1996; Ried at al., 1995).

The most common genetic abnormality in the progression of sporadic breast cancer is the loss of heterozygosity (LOH). An LOH event uncovers the functional consequences of a mutation in an allele of a tumor suppressor gene by removal of the dominant, normal allele. For the sporadic breast cancer, LOH on chromosomes 17p, 13q, 9p, and 16q lead to the inactivation of the tumor suppressor genes *TP53* (encoding p53 protein), *RB-1* (encoding Rb protein), *CDKN2* (encoding the p16 protein), and *CDH1* (encoding the E-cadherin protein), respectively. *TP53*, *RB-1*, and *CDKN2* regulate the cell cycle, on the other hand *CDH1* primarily regulates differentiation and tissue compartmentalization (Dickson and Lippman, 2001). Before LOH of the one allele, the other allele for these genes is inactivated by two different ways: Point mutation is more commonly observed for *TP53* and *Rb* genes in contrast to *CDKN2* and *CDH1* genes, where gene methylation more commonly occurs (Brenner et al., 1995).

The second most common type of cytogenetic alteration in breast cancer appears to be gene amplification. Gene amplification involves the formation of extra-chromosomal, self-replicating units and then the permanent incorporation of these genetic elements into chromosomal regions (Dickson and Lippman, 2001). The best-established amplified and functional genes for mammary tumorigenesis detected to date are the growth factor receptor *c-ERBB*$_2$ (encoding c-erbB-2 protein), the nuclear transcription factor *c-MYC* (encoding c-Myc protein), and the cell-cycle kinase regulator *CCND1* (encoding cyclin D1 protein) (Ried at al., 1995; Knuulila et al., 1998).

An important class of genes frequently altered in sporadic breast cancer is members of the epidermal growth factor receptor (EGFR) family of growth factor receptors. The members of this family of protooncogenes (EGFR, erbB-2 or Her-2/neu, erbB-3, and erbB-4) all share extensive homology and encode transmembrane glycoproteins with tyrosine kinase activity. They become oncogenic through gene amplification or overexpression at the mRNA and protein levels, leading to aberrations in signal transduction pathways and deregulation of cellular proliferation (Bacus et al., 1994). All the members of this family have been described as

overexpressed in breast carcinoma; the most extensively studied receptors, EGFR and c-erbB-2 are known to be overexpressed in 20 to 40 percent of breast cancers (Slamon et al., 1989; Lewis et al., 1990). Also, the growth factor TGF-α is a member of the EGF family and is a ligand for EGFR. Elevated expression of TGF-α has been consistently associated with neoplastic transformation, with transgenic mouse models providing direct evidence for the role of TGF-α in malignant transformation of breast epithelium (Sandgren et al., 1990). Another family, the fibroblast growth factors (FGFs) and their receptors are thought to play a role in breast cancer. In a study by Penault-Llorca et al. (1995), FGFR1 and FGFR4 were found to be expressed at high levels in the breast cancer cell lines and in the breast tumour samples studied.

Also, the anti-apoptotic protein *bcl-2* was found to be overexpressed in 30 to 45 percent of breast carcinoma cases (Joensuu et al., 1992). *Bcl-2* expression in human breast tissue varies dramatically throughout the menstrual cycle, suggesting that *bcl-2* regulation is hormone-dependent (Sabourin et al., 1994).

The *E-cadherin* protein is a calcium-dependent cell adhesion molecule, involved in homophilic cell-cell interactions, thus it is important for the maintenance of differentiation and tissue compartmentalization. Loss of function of *E-cadherin* seems to facilitate malignant invasive growth of breast cancer cells (Ingvarsson, 1999). The *E-cadherin* gene, *CDH1*, has been found mutated in a large portion of lobular breast tumours (Berx et al., 1995; Vos et al., 1997). As described earlier, LOH is observed for *E-cadherin* gene. No mutations have been detected in the *CDH1* gene in the ductal breast tumours. On the other hand, reduced expression of *E-cadherin* has been found in both lobular and ductal breast cancer (Oka et al., 1993).

Cyclins are the molecules which are crucial for the maintenance of the cell cycle and which complex with cyclin-dependent kinases (cdks) to proceed the cell cycle. Upon formation of the complex, Cdks phosphorylate downstram targets to propel the cellular machinery into the next phase of the cell cycle (Marx et al., 1994). Overproduction of cyclins and cdks lead to the deregulation of the cell cycle and therefore uncontrolled cell division. In the study by Buckley et al. (1993), among 20 breast cancer cell lines which were analyzed for the expression of cyclin A, B1, C,

D1, D2, D3, and E, it was demonstrated that 7 of 20 cell lines displayed increased expression of cyclin D1. Five of the seven displayed increased overexpression of cyclin D, which is important for G1-S transition of the cell cycle. In the same study, 45 percent of 124 primary breast tumors were displayed to have increased expression of Cyclin D1.

Most of the somatic changes in breast cancer are summarized in Table 1.3 (Couch and Weber, 1997).

Table 1.3 Somatic Changes in Breast Cancer

Gene/Region	**Modification**	**Frequency**
EGFR	Overexpression	20-40
HER-2/neu	Overexpression	20-40
FGF-1/FGF-4	Overexpression	20-30
TGF-α	Overexpression	ND
Ha-*ras*	Mutation	5-10
c-*src*	Overexpression	50-70
TP53	Mutation/ Inactivation	30-40
RB1	Inactivation	20
Cyclin D1	Overexpression	35-45
E-cadherin	Reduced/absent expression	60-70
MMPs	Increased expression	20-80
bcl-2	Overexpression	30-45
c-*myc*	Amplification	5-20

1.2.2.2 Histology of Breast Cancer

The breast is composed of two main types of tissues: glandular tissues and stromal (supporting) tissues. Glandular tissue possesses the milk-producing lobules and the ducts (milk passages), while the stromal tissue contains the fatty and fibrous connective tissue (www.imaginis.com)

Breast carcinoma arises from the epithelium of the mammary gland, which includes the milk-producing lobules and the ducts that carry milk to the nipple. Malignant transformation of the stromal, vascular, or fatty components of the breast is extremely rare. These facts may largely explain why breast size is not a risk factor for breast cancer, since all women have a similar amount of breast epithelium while breast size is determined largely by the amount of stromal and fatty tissue (Couch and Weber, 1997).

The breast tissue, during lifetime of a women, experiences some changes. These changes can include an increase in the number of breast cells (hyperplasia) or the emergence of atypical breast cells (atypical hyperplasia). In some instances, a portion of breast tissue that exhibits abnormal characteristics can eventually develop into a cancerous tumor. While the appearance of atypical hyperplasia increases the risk of breast cancer, not all women with abnormal breast cells go on to develop breast cancer.

The typical progression of breast cancer from a normal breast tissue is summarized below:

Normal $\rightarrow$ Hyperplasia (increase in the number of cells) $\rightarrow$ Atypical hyperplasia (increase in the number of abnormal cells, a marker for breast cancer) $\rightarrow$ Carcinoma in situ (cancer exists but it is limited to the ducts or lobules where it originally developed) $\rightarrow$ Invasive Cancer (cancer exists and has spread beyond the ducts or lobules where it originally developed)

Some breast tumours may skip one or more intermediate steps. In general, anything farther along than atypical hyperplasia is considered as a cancer.

The benign, non-cancerous breast conditions include fibrocystic breast condition, cysts, galactoceles, fibroadenomas, phyllodes tumors, intraductal papillomas, granular cell tumors, duct ectasia, fat necrosis and breast inflammation-mastitis (www.imaginis.com)

1.2.2.2.1 Lobular Carcinoma *in situ:*

Literally meaning "in place," the term "in situ" refers to a very early form of cancer. In general, the term "in situ" is used to indicate that abnormal cancer cells are present but have not spread past the boundaries of glandular tissues where they initially developed. Lobular carcinoma in situ (LCIS) refers to a sharp increase in the number, appearance, and abnormal behavior of cells contained in the milk-producing lobules of the breast (found within the lobes) (www.imaginis.com)

LCIS is not a preinvasive lesion but appears to be a marker of increased risk for the development of invasive cancer. Clinical diagnosis of LCIS is not possible, since LCIS does not form a palpable lesion and therefore cannot be identified on physical examination and is not visible on mammography. Thus, the diagnosis of LCIS is made as an incidental finding on a breast biopsy obtained for diagnosis of an adjacent lesion. Evidence that LCIS is a marker lesion and not a true malignant lesion comes from studies demonstrating that the invasive cancer that may develop subsequently is likely to occur distant from the known focus of LCIS and is more often of ductal than lobular histology (Kinne et al., 1991).

The histologic features of LCIS show little variation and are usually easily recognized. LCIS is most often characterized by a solid proliferation of small cells, with small, uniform, round-to-oval nuclei, and variably distinct cell borders. LCIS is typically present in the terminal duct-lobular units and distends and distorts the involved spaces. In some instances, LCIS involve extralobular ducts. The growth within these ducts may be either solid or pagetoid (i.e., the LCIS cells are insinuated between the duct basement membrane and the native ductal epithelial cells (Winer et al., 2001).

LCIS has a low proliferative rate, is typically positive for estrogen receptor (ER), and rarely shows overexpression of HER-2/neu, or accumulation of the p53 protein (Albonico et al., 1998; Rudas et al., 1997; Bur et al., 1992). In addition, LCIS is characterized by loss of expression of the adhesion molecule E-cadherin (Vos et al., 1997).

1.2.2.2.2 Ductal Carcinoma *in situ*:

Ductal carcinoma *in situ* refers to the most common type of noninvasive breast cancer in women. In DCIS, the cancer cells are limited to milk ducts in the breast and have not spread into the fatty breast tissue.

The term, ductal carcinoma in situ (DCIS), refers to a family of cancers that occur in the breast ducts. There are two categories of DCIS: non-comedo and comedo. The term, comedo, describes the appearance of the cancer. When comedo type breast tumors are cut, the dead cells inside of them (necrosis) can be expressed out just like a comedo or blackhead on the skin.

The most common non-comedo types of DCIS are:

- Solid DCIS: cancer cells completely fill the affected breast ducts.
- Cribriform DCIS: cancer cells do not completely fill the affected breast ducts; there are gaps between the cells.
- Papillary and micropapillary DCIS: the cancer cells arrange themselves in a fern-like pattern within the affected breast ducts; micropapillary DCIS cells are smaller than papillary DCIS cells.

Comedo type DCIS (also referred to as Comedocarcinoma) tends to be more aggressive than the non-comedo types of DCIS. Pathologists are able to easily distinguish between comedo type DCIS and other non-comedo types when examining the cells under a microscope because comedo type DCIS tends to plug the center of the breast ducts with necrosis (dead cells). When necrosis is associated with cancer, it often means that the cancer is able to grow quickly. Necrosis is often seen with microcalcifications (tiny calcium deposits) (www.imaginis.com).

A number of biological markers in DCIS lesions have been evaluated. These studies have generally shown that comedo-type DCIS lesions lack estrogen and progesterone receptors, overexpress the HER-2/neu (c-erbB-2) oncogene, show mutations of the p53 tumor suppressor gene with accumulation of its protein product (Albonico et al., 1998; Rudas et al., 1997) and demonstrate angiogenesis in the surrounding stroma (Guidi et al., 1994; Engels et al., 1997).

1.2.2.2.3 Invasive Breast Cancer

Invasive breast cancer may be ductal or lobular in histologic type, and while there are a few distinguishing clinical features, the natural history and treatment of the two lesions are virtually identical. About 80 percent of invasive breast cancers are ductal carcinomas, in which carcinoma has first originated in the milk ducts of the breast and then spreads to the other parts of the body. Infiltrating lobular carcinoma, in which carcinoma has first originated in the lobules of the breast and then spreads to the other parts of the body, is less common, representing 5 to 10 percent of the breast cancers (Couch and Weber, 1997). The remaining types of invasive breast cancer are very rare; some of these are tubular cancer, characterized by prominent tubule formation; medullary carcinoma, a lesion that appears poorly differentiated under the microscope but is thought to have a more favorable prognosis than other breast cancers (Fisher et al, 1990); mucinous (or colloid) carcinoma, characterized by the abundant accumulation of extracellular mucin, bulky tumors, and a good prognosis (Harris et al, 1992); and inflammatory breast cancer, in which the breast appears swollen and inflamed because cancer cells block the lymphatic vessels in the skin of the breast, preventing the normal flow of the lymph fluid and leading to reddened, swollen and infect-looking breast skin (Gunhan-Bilgen et al., 2002).

1.3 STEM CELLS AND CANCER, POSSIBLE ROLE OF STEM CELLS IN BREAST CANCER

1.3.1 STEM CELL CONCEPT

Cell biologists refer to a stem cell in a tissue whose undifferentiated characteristics are conserved while it serves as a source of cells that undergo change, lose their division-competence and acquire specialized functions. Therefore, a stem cell has two important properties to be determinant: 1) high capacity of self renewal, 2) capability of differentiation into cells which have specialized functions. The potential of a stem cell differs depending on the tissue, the stage of morphogenesis (embryonic, pubertal, or post-pubertal) and the kind of population dynamics required to maintain tissue function. For instance, an embryonic stem cell is capable of producing the set of seed cells for embryonic body parts and whole organ systems, on the other hand, the potential of the stem cells of adult tissues is restricted to the production of the cell types for the tissue in which they reside, therefore, they are referred to as tissue-specific stem cells. Organs as diverse as liver (Michalopoulos et al., 1997), brain (McKay et al., 1997), intestine (Cosentino et al., 1996), skin (Jones et al., 1997), lung (Emura et al., 1997) and muscle (Ferrari et al., 1998) were described to contain organ-specific stem cells. More than one type of multipotent stem cell may be present in an organ system such as blood (Boll et al., 1980), intestine (Cosentino et al.,1996), cerebellum (Snyder et al., 1992), and retina (Turner et al., 1990). In all of the systems studied, it has been shown that the immediate progeny of a stem cell are division-competent. Therefore, there exists a hierarchy of division-competent cells. This consists of:

1. stem cells, which can produce all the cell types of a tissue including new stem cells,
2. progenitor cells, which are multipotent, but produce only a subset of the cell types of an organ
3. lineage-limited or committed cells which produce one cell type before differentiating.

Tissue-specific stem cells are capable of both symmetric and asymmetric mitosis. Symmetric mitosis yield two new stem cells when a stem cell divide, on the other hand, asymmetric mitosis yield two cells with alternate fates, one is a new stem cell and the other is a committed progenitor. This is the beginning of the generation of cell diversity. The capacity of asymmetric mitosis is not limited to primary stem cells as multilineage progenitors must also produce multiple cell types (Chepko and Smith, 1999).

1.3.2 STEM CELLS AND CANCER

Since cancer is thought to be a clonal disease originating in changes in the regulation of cell replication, it seems likely that those cells in a tissue that are still capable of mitosis are the ones that sustain genetic changes that lead to tumorigenesis. Tissue-specific stem cells remain division-competent for the life of the individual. Because of their possible susceptibility to genetic mutations and the probability of their passing on such changes to their progeny, tissue specific stem cells and their division-competent progeny are highly implicated in the processes of neoplasia. (Chepko and Smith, 1999).

For a stem cell to persist for a lifetime of an animal, self-renewal is a very crucial property. Since cancer can be considered to be a disease of unregulated self-renewal, understanding the regulation of normal stem cell self-renewal is also fundamental to understanding the regulation of cancer cell proliferation. Due to the common property of self-renewal, newly arising cells can be assumed to appropriate the machinery for self-renewing cell division that is normally expressed in stem cells. It has been demonstrated that many pathways that are classically associated with cancer may also regulate normal stem cell development. Wnt (Zhu et al., 1999), Shh (Wechsler-Reya et al., 1999) and Notch (Varnum-Finney et al., 2000) pathways have been shown to contribute to the self-renewal of stem cells and/or progenitors in a variety of organs, including the hemapoietic and nervous systems. When dysregulated, these pathways can contribute to oncogenesis. Mutations of these pathways have been associated with a number of human tumours, including colon carcinoma (Polakis et al., 2000) and epidermal tumours (Chan et al., 1999) (Wnt),

medulloblastoma (Wechsler-Reya et al., 2001) and basal cell carcinoma (Gailani et al., 1999) (Shh) and T-cell leukemias (Ellisen et al., 1991) (Notch). Stem cells are most probably the target of transformation in most types of cancers. Since stem cells have the machinery for self-renewal already activated, maintaining this activation may be simpler than turning it on *de novo* in a more differentiated cell.; that is; fewer mutations may be required to maintain self-renewal than to activate it ectopically. Also, unlike mature cells which die after short periods of time, stem cells often persist for long periods of time, leading to a much greater opportunity for mutations to accumulate in individual stem cells than in most mature cell types.

Many analogies can be considered between normal stem cells and tumorigenic cells, both have extensive proliferative potential and the ability to give rise to new (normal and abnormal) tissues. Both are composed of heterogenous combination of cells, with different phenotypic characteristics and different proliferative potentials. Since most tumours have a clonal origin, tumorigenic cancer cells must give rise to phenotypically diverse progeny, including cancer cells with indefinite proliferative potential, as well as cancer cells with limited or no proliferative potential. Although some of the heterogeneity in tumors arises as a result of continuing mutagenesis, it is likely that aberrant differentiation of cancer cells leads to this heterogeneity. In other words, both normal stem cells and tumorigenic cells give rise to phenotypically heterogenous cells that exhibit various degrees of differentiation. Thus, tumorigenic cells can be considered as cancer stem cells that undergo an aberrant and poorly regulated process of organogenesis analogous to what normal stem cells do.

There are two general models of heterogeneity currently discussed in solid cancer cells. One model proposes that cancer cells of many different phenotypes have the potential to proliferate extensively, but any one cell would have a low probability of exhibiting this potential in an assay of clonogenicity or tumorigenicity, while the other model proposes that although most cancer cells have only limited proliferative potential, only a subset of cancer cells consistently proliferate extensively in clonogenic assays and can form new tumours. Conventional therapies of cancer may shrink tumours by killing mainly cells with limited proliferative potential. If the putative cancer stem cells are less sensitive to these therapies, then

they will remain viable after therapy and reestablish the tumour. On the other hand, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow (Reya et al., 2001).

1.3.3 MAMMARY EPITHELIAL STEM CELLS

An important feature of the mammary gland is the regenerative capacity of its epithelium, demonstrated by successive reproductive cycles. During pregnancy in the mouse, a massive increase (25-27 fold) in the number of mammary epithelial cells occurs within the inguinal mammary fat pad (Kordon and Smith, 1998). As described earlier in part 1.1, this massive epithelial population is subsequently reduced following lactation through apoptotic cell death during involution. The subsequent events of cell growth and apoptotic cell death occur during and after each pregnancy period. This observation can be interpreted as an indication of the presence of a mammary epithelial stem cell (Chepko and Smith, 1999).

Serial passage of tissue fragments or dispersed cells transplanted to the proper tissue site in successive hosts is considered as the most stringent test for the existence of stem cells. The earliest serial transplantation experiments demonstrated that the self-renewal capability of the mammary epithelial cells persists for up to seven generations (Gardener and Hoshino, 1967). Daniel and Young (1971) serially transplanted epithelial ductal fragments into successive nulliparous hosts successfully until they had lost the ability to undergo branching morphogenesis, thus displayed the generative potency of mammary epithelial cells. The same researchers also displayed that when fragments from such senescent ducts were again transplanted into a host subsequently made pregnant, they were unable to produce branching ducts, however, developed secretory lobules along their length.. On the other hand, while carrying clonally-derived mammary outgrowths to senescence in breeding hosts, it was observed that certain fragments from randomly sampled outgrowths produced a complete ductal tree, but failed to produce secretory lobules. These experiments demonstrate that a mammary epithelial stem cell can produce at

least two kinds of limited progenitors and the competence of production of these progenitors is controlled separately.

It was shown that any fragment from lactating or involuted glands faithfully reproduces full lactating outgrowths in pregnant mice (Kordon and Smith, 1998; Smith and Medina, 1988). Therefore, any part of the epithelium has the capacity to generate full complement of functional mammary epithelium. Whether one stem cell could produce an entire mammary gland was demonstrated by using mouse mammary tumor virus (MMTV) as an experimental model (Kordon and Smith, 1998). The researchers transplanted random fragments of MMTV-infected Czech mouse mammary epithelium. Since Czech mice have no endogenous nucleic acid sequences related to MMTV, nucleic acid homology with MMTV, demonstrated by using Southern blotting, in the epithelium of the MMTV-infected Czech mice from these outgrowths has pointed out the fact that an entire mammary gland may comprise the progeny of a single cell.

Based on their observations under the electron microscope, two researchers (Chepko and Smith, 1999) could manage to characterize mammary cell morphotypes structurally and functionally. The researchers have taken “pale” or “light”-staining cells, which had been noticed before (Smith and Medina, 1988) in mouse mammary explants and which were the only cells that entered mitosis, as a beginning point of their studies. The basic properties of stem cells; division- competence, symmetric and asymmetric mitosis and a possible undifferentiated cytology were the guidelines for the researchers to characterize the mammary cell types. The presence of mitotic chromosomes referred to mitotic competence, while the appearance of side-by-side pairs of cells referred to symmetric cell division and the presence of one-above-the-other pairs of cells referred to asymmetric cell division. The cytological differentiation of the cells were based on how many organelles they contain, how well developed the organelles are, whether they contain specialized proteins, and whether they demonstrate polarity (apical (facing the lumen) or basal (facing the stroma) orientation). Presence of milk protein granules, presence of Golgi vesicles and rough endoplasmic reticulum (RER), polar distribution of organelles, and luminal contact, which is a further indication of polarization, are prominent in the luminal cells of the mammary gland. On the other hand, myoepithelial cells are

flattened, elongated cells located at the basal surface of the epithelium, and their cytoplasmic feature is the presence of an apically oriented nucleus and many basally distributed myofibrils (Smith and Chepko, 2001).

The same researchers (Chepko and Smith, 1999) extended the classification of mammary cell morphotypes from two to five. The five morphotypes in mammary epithelium are:

1. a morphologically primitive small light cell (SLC)
2. a cytologically more complex, but still undifferentiated large light cell (ULLC)
3. a cytologically very differentiated large light cell (DLLC)
4. the classic cytologically complex luminal cell (large dark cell or LDC)
5. the myoepithelial cell

The structural and functional characteristics of mammary cell morphotypes (excluding myoepithelial cell) are displayed in Table 1.4 (Taken from Chepko and Smith, 1997). According to this classification, ultrastructurally, the putative stem cells reside in mammary epithelium basally (in contrast to apically) and also are distinct from the basally oriented myoepithelial cells.

These researchers then proposed a model of cell proliferation and differentiation in mammary epithelium based on morphological markers. According to this model, SLC (stem cells), devoid of differentiated characteristics, generate daughter cells morphologically similar to the mother cell. One of the daughters retains its undifferentiated nature and remains a stem cell. The sister cell, although remaining morphologically identical to the stem cell, becomes a primary progenitor cell with the capacity to undergo multiple divisions. All daughters of a primary progenitor cell divide, and, depending on the orientation of the cleavage furrow at the time of determination, differentiate into secondary progenitor cells (ULLC) that are committed either to secretory or to myoepithelial differentiation. ULLC can divide multiple times to produce large numbers of ULLC which differentiate into DLLC and then LDC. Alternatively, they can produce myoepithelial cells, but these are not produced in very large numbers. The model is summarized on Table 1.5 (Taken from Chepko and Smith, 1997).

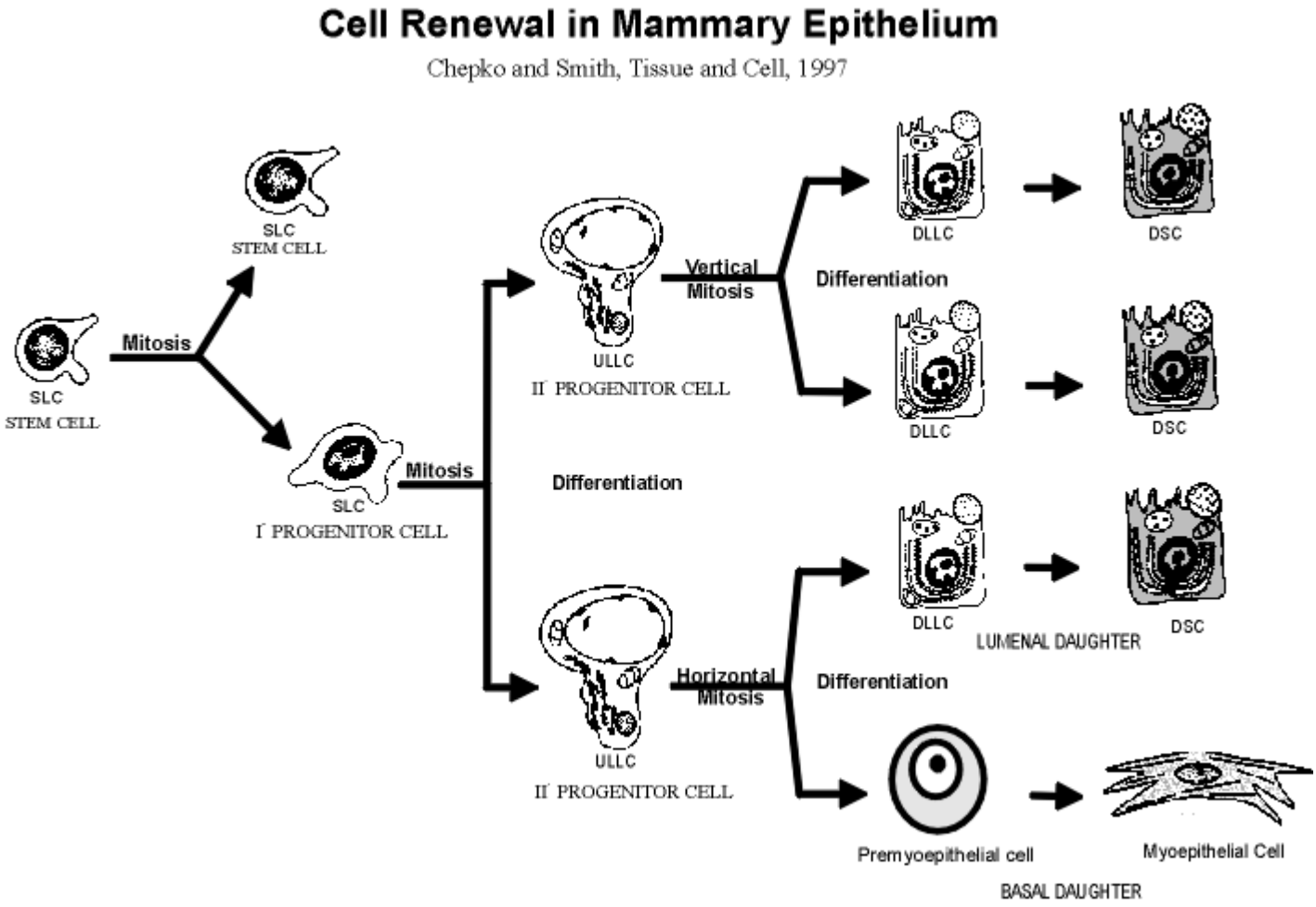
Table 1.4 Structural and Functional Characteristics of Mammary Cell Morphotypes

Structural and Functional Characteristics of Mammary Cell Morphotypes[a]

Characteristic	Small Light Cell	Large Light Cells		Large Dark Cell
		Undifferentiated	Differentiated	
Percent Total Cells	~3%	~5% (9% in virgin)		~70-75% (with myoepithelial cells comprising the difference)
Location	No lumenal contact	May or may not contact lumen	Contacts lumen	Contacts lumen
Nuclear Morphology	Small; pale nucleoplasm with hetero-chromatin	Very large; round; pale, fibrillar, nucleoplasm; no heterochromatin	Large; round; pale nucleoplasm, with heterochromatin	Medium; oval, round, or irregular; light to dark nucleoplasm with heterchromatin
Cytoplasmic Characteristics	Pale with few organelles	Pale with some RER, secretory granules, small lipid droplets	Pale; well developed golgi and RER; secretory granules and lipid droplets	Deeply stained; well developed golgi and RER; secretory granules and lipid droplets
Mitosis	yes	yes	?	?
Grouping	1. Singles 2. Like pairs 3. Mixedpairs 4. Clusters	1. Singles 2. Like pairs 3. Mixed pairs 4. Clusters	1. Singles 2. Like pairs 3. Large arrays	Comprised most areas of epithelium
Function	Division-competent; mixed population of hypothesized stem cells and I°progenitor cells	Division-competent; probable II°progenitor cell; makes some milk products	Synthesis and secretion of proteins and lipids; continues to differentiate	Synthesis and secretion of proteins and lipids; fully differentiated secretory cell

a. Excluding myoepithelial cell
Adapted from Chepko and SmithTissue and Cell, 1997 **29** (2) 239-253

Table 1.5 Cell Renewal in Mammary Epithelium



Normal human breast epithelium expresses an array of marker proteins that is indicative of the two lineages of the luminal and myoepithelial cells in the mature mammary gland. In the mature gland, the luminal epithelial cells express **Cytokeratin 18** (**CK18**), **Cytokeratin 19** (**CK19**), **Epithelial Specific Antigen** (**ESA**), and **sialomucin** (**MUC1**) (ESA is mostly expressed on the basal side of the luminal compartment on the other hand, MUC1 is expressed on the apical side of the luminal compartment), while the myoepithelial cells express **Cytokeratin 14** (**CK14**), **vimentin**, **β4-integrin**, **common acute lymphoblastic leukemia antigen (CALLA)**, and **α-smooth muscle actin** (**ASMA**) (Jones et al., 1997; Petersen and van Deurs, 1988; Taylor-Papadimitrou et al., 1989). Also, the putative stem cells of the mammary gland are believed to express markers such as the basal cytokeratin **Cytokeratin 5** (**CK5)** (Boecker et al., 2002) and **Epithelial Specific Antigen** (**ESA**)

Balzar et al., 1999). Balzar et al. (1999) suggested that ESA is related to increased epithelial proliferation and negatively correlates with cell differentiation. Also, Gudjonsson et al. (2002) demonstrated that CK19 may be a potential marker for stem cell compartment of the mammary gland (described in detail below). These markers are used in the studies of mammary epithelial cells for the characterization of their differentiation status and constitute the basis of the study described in this thesis.

Two- or three-dimensional culture systems such as collagen or laminin in combination with flow cytometry and single cell sorting were used to demonstrate whether an epithelial cell population gives rise to any elaborate structure resembling *in vivo* such as terminal duct lobular unit (TDLU). Stingl et al. (1998) demonstrated that when cultured on Collagen gel as a basement membrane system, ESA-positive and MUC1-negative human breast epithelial cells (HBECs) give rise to mixed colonies of luminal and myoepithelial cells, therefore displaying stem/progenitor cell property. Dontu et al. (2003) has developed an in vitro cultivation system for propagation of human mammary stem/progenitor cells. In this study, mammary epithelial cells were cultivated as nonadherent mammospheres in suspension and the differentiation potential of these mammospheres were tested in reconstituted two-dimensional culture systems such as Matrigel or collagen, it was demonstrated the nonadherent mammospheres have the capability of differentiation into luminal or myoepithelial cells. Gudjonsson et al. (2002) proposed that MUC1-negative/ESA-positive cells exist *in vivo*. In the study, by using these cell surface markers and immunomagnetic sorting, MUC-negative/ESA-positive epithelial cell population was isolated and also immortalized by transduction of E6/E7 genes from human papilloma virus type 16 (HPV). In clonal cultures, this population gave rise to both differentiated ESA-positive/MUC1-positive luminal epithelial cells and differentiated ASMA-positive myoepithelial cells. Also, when cultured in laminin gel, this population formed branching structures resembling terminal duct lobular units (TDLU). Therefore, this population can be assumed as the stem/progenitor cell compartment of the breast. This population was further characterized by the expression of Cytokeratin 19 (CK19) by immunofluorescence, therefore it was proposed that CK19 can be assumed as a marker for stem/progenitor cells, also CK19 was demonstrated not to be expressed in terminally differentiated ESA-positive/MUC-positive luminal epithelial cells, in contrast to former studies (Bartek

et al., 1985; Paine et al., 1992). In the study described in this thesis, CK19 was used as a marker for stem/progenitor cells.

The mammary epithelium developmental model described above by Chepko and Smith is further supported by the studies of Pechoux et al. (1999) and Stingl et al. (2001). Pechoux et al. (1999), devised two media specific for both luminal and myoepithelial cell lineages, respectively and grew the lineages in these media separately. When the media is switched to the other type, the luminal epithelial cells displayed conversion to myoepithelial cells, while the vice versa did not occur. This result suggests that human mammary luminal epithelial cells contain progenitors to myoepithelial cells. By using a combination of flow cytometry and *in vitro* colony assay procedures such as single cell culturing for human breast epithelial cells, Stingl et al. (2001) characterized bipotent mammary epithelial progenitor cells in normal adult human breast tissue when they observed colonies containing a central core of cells expressing luminal markers surrounded by CK14-positive myoepithelial-like cells.

1.3.4 MAMMARY EPITHELIAL STEM CELLS AND THEIR POSSIBLE ROLE IN BREAST CANCER

Colin R. Sharpe (1998) proposed a developmental hypothesis to explain the evolution of breast cancer. According to her hypothesis, if a cell in a breast bud (the narrow collection of cells in the thoracic/pectoral region, described earlier in Part 1.1) acquires a genetic mutation that is not repaired, the mutation is inherited to its progeny, thus carrying the mutation into multiple developing ductal systems. If a similar mutation occurs in a cell in the tip of one of the ducts, the daughter cells in that duct could inherit the genetic hit, but adjacent ducts might not, leading to "genetic mosaicism". Therefore, earlier genetic mutations should result in larger altered areas.

The studies performed by Tsai et al. (1996) support this hypothesis. It has been shown that the mammary epithelium is a mosaic of discrete regions in which all cells have the same inactive X chromosome, suggesting that each region developed

from the same stem cell. Entire lobules and large ducts had the same X chromosome inactivated. Therefore, it can be assumed that the lesions could have arisen within a population of mutated cells that arose from one stem cell, as the result of mutations that occurred after X-chromosome inactivation.

As described earlier in Part 1.2.1.2, the epidemiologic studies have indicated that breast cancer risk for a child-bearing women is linearly related to the age at which a women has her first full-term pregnancy and is higher in those who are nulliparous or late parous (MacMahon et al., 1970; Love et al., 1997; Canty, 1997). This increased risk has been attributed to the stem cell multiplication that commences at the time of puberty and occurs during each ovarian cycle until the first pregnancy (Cairns et al., 1975) and to the induction of full differentiation of the mammary gland by pregnancy, which results in the refractoriness of the gland to carcinogenesis (Russo et al., 1990). Also, in the studies of the effects of radiation from the atomic bombs in Japan, the frequency of ionizing radiation-induced breast cancers has been inversely correlated with the age of the women at the time of exposure, indicating a higher breast cancer risk for the young undifferentiated mammary gland (Thompson et al., 1994). These studies are evidences about the role of the stem cell proliferation and differentiation in breast carcinogenesis.

Also, studies about loss of heterozygosity (LOH) in breast cancer further supported the role of stem cells in breast cancer. O'Connell et al. (1994) found that 50% of benign proliferative breast lesions and 80% of DCIS lesions shared specific LOH patterns with more advanced lesions from the same breast, which pointed out an early LOH, most probably occurred in a stem cell, expanded by normal breast growth. In another study by Deng et al. (1996), DNA from breast cancer tissue was compared with DNA from adjacent, phenotypically normal terminal ductal-lobular units (TDLUs). In 8 of 30 cases LOH was found in both the cancerous tissue and the adjacent TDLUs, in which the same allele was missing in both types of tissue. Also, in the study by Lakhani et al. (1999), 'normal' breast tissues from patients with carcinoma were examined. Normal luminal and myoepithelial cell clones from cancerous patients were studied for LOH. LOH was identified in 5 of 10 breast cancer cases, in normal luminal and myoepithelial cells in regions both adjacent to and also distant from the tumour. The demonstration of genetic alteration in normal

luminal and myoepithelial cells points out that these genetic alterations probably occur very early in breast development, further supporting the role of stem cells in breast cancer.

Another study by Smith and Boulanger (2002) demonstrated that local regions of serially transplanted mammary epithelium (transplantation in MMTV-infected mice) occasionally manifested regions of hyperplastic lobular development. These lesions were proved to be clonal in origin by restriction enzyme (RE) digestion and repeatedly produced hyperplastic lobular mammary outgrowths on transplantation. Also, in their study, one normal fragment, a fourth-generation transplant, generated an aggressive mammary neoplasm and spawned several metastases to the lung. Both mammary and metastasized lung lesions were demonstrated to be clonal in origin. This study further confirms that premalignant and malignant clones may constitute a lineage potential of aging mammary stem cells.

CHAPTER II AIM OF THE STUDY

2.1 AIM

Organ-specific stem cells were described for most of the organs in our body. These stem cells are responsible for constituting the entire cellular population of the tissue, which are differentiated to perform specialized functions attributed for the organ. Recent studies have demonstrated the existence of stem/progenitor cells in mammary epithelium which give rise to the differentiated lineages of the mammary epithelium: luminal and myoepithelial cell lineages. Mammary carcinoma arises from the mammary epithelium. Studies have displayed that the differentiated mammary gland is less susceptible to breast carcinogenesis, therefore the putative stem/progenitor cells are more prone to acquire genetic alterations, leading to malignant transformation. Recent studies have supported this hypothesis, therefore it is proposed that the putative stem cells play an important role in breast carcinogenesis.

Our aim is to investigate whether our established breast cancer cell lines have stem cell-like property, that is, to demonstrate that the established breast cancer cell lines have derived from a stem/progenitor cell, in other words, that they are clonal in origin. Established breast cancer cell lines have been used in our study since they are devoid of any contamination of cells such as fibroblasts or blood cells.

2.2 STRATEGY

We used six breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100 and 734-B) to study their marker protein expression of the breast epithelium by using indirect immunofluorescence. Five marker proteins (Cytokeratin 19 (CK19), Cytokeratin 18 (CK18), Sialomucin (MUC1), Alpha-Smooth Muscle Actin (ASMA) and Epithelial Specific Antigen (ESA)) were used to specify the epithelial differentiation status of the cell lines in order to investigate any stem/progenitor cell-like property. Western Blotting was performed to further confirm the protein expression profile of the cell lines. To display the clonal origin of the cell lines, single cell culture of GI-101 cell line was performed and marker protein expression was demonstrated by using indirect immunofluorescence.

CHAPTER III MATERIALS AND METHODS

3.1 Tissue Culture

All cells (listed in Table 3-1) were grown as monolayer culture in 100-mm or 150-mm culture dishes. The cells were grown in Dulbecco's Medium (DMEM) supplemented with 10%FCS, 1% non-essential amino acids and 1% penicillin/streptomycin and were kept at 37°C and 5% CO_2.

3.1.1 Thawing of Cells

The vial of the frozen cell line from the liquid nitrogen tank was taken and immediately placed on ice. The vial was then placed at 37°C water bath until the frozen cell suspension was thawed. Then, the thawed cell suspension was transferred into 10 ml fresh medium (DMEM) in 15-ml Falcon tube. The cells were centrifuged at 1500 rpm for 5 minutes at 4°C. Supernatant was discarded and the pellet was resuspended in 10 ml fresh DMEM and the cell suspension was placed in 100-mm dish. Cells were left overnight in culture. The medium was refreshed on the following morning.

3.1.2 Subculturing of Cells

Culture renewal was performed every two or three days. Cells were splitted when they reached 80-90% confluency. For splitting, the medium was

aspirated and the cells were washed with sterile PBS (pH: 7.4) for twice. PBS was removed and trypsin was added to the plates. Plates were incubated in the incubator for 5-10 minutes until the cells were detached. Cells were plated in the desired dilution into new plates.

3.1.3 Freezing of Cells

The cells to be frozen were grown in a 150-mm cell culture dish to 70-80% confluency. The cells were washed twice with PBS, trypsinized and placed in the incubator until they were detached. The unattached cells were collected with 10 ml fresh DMEM. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The cells were resuspended in 1.5 ml freezing medium containing 20% FCS, 10% DMSO, 70% DMEM and the cell suspension was placed in a vial. The vial was immediately placed in -20°C, and was kept for 3 hours at this temperature. Then, the vial was moved to -80°C and was kept for o/n at this temperature and transferred to liquid nitrogen tank on the following day.

Table 3.1 The cell lines used in this study (All the cell lines studied were of human origin and were grown in DMEM medium)

NAME	TISSUE	DESCRIPTION
MCF-7	Breast Cancer	From a 69-year-old Caucasian female, differentiated adenocarcinoma origin, estrogen receptor-(+)
CAL-51	Breast Cancer	From the pleural effusion metastasis of a 45-year-old female, adenocarcinoma origin, no viral infection
MDA-MB-175	Breast Cancer	From a 56-year-old Black female, pleural effusion ductal carcinoma origin, tumor formation in nude mice
GI-101	Breast Cancer	Established in 1985, recurrent infiltration ductal carcinoma origin, poorly differentiated, estrogen receptor-(+)
HBL-100	Breast	From the milk of a 27-year-old Caucasian nursing female, viral infection by SV40
734-B	Breast Cancer	Established subclone of MCF-7 cell line

3.2 Single Cell Cloning

The cells grown in 50-mm dishes were washed with PBS twice and then trypsinized. The detached cells were collected by using 10 ml fresh DMEM and were counted by using cytometer. Serial dilutions were performed to approximate the optimum concentration of 1 cells/1 well by using fresh DMEM. Then cells were seeded in 24- or 96-well plates in desired amounts and carefully examined after seeding and in the following days whether single cell is present in each well. Medium of cells were refreshed once a week. Upon reaching the desired confluency, cells were transferred to 24-, 6-well plates and 60-mm dish in order. When cells were grown until desired confluency in 60-mm dish, they were seeded on the autoclaved-sterilized coverslips which were placed in the wells of 6-well plates. After 2 days, indirect immunofluorescence of the cells was performed, as described below.

3.3 Indirect Immunofluorescence Staining

Cells were seeded on the autoclaved-sterilized coverslips which were placed into the well of 6-multiwell plates at the concentration of 100.000 cells/ml after washing with PBS, trypsinization and counting and were grown for 4 days in 3 ml DMEM. Cells were washed with PBS three times after medium aspiration and then were fixed with ice-cold methanol at +4°C for 5 min, and blocked against non-specific binding with PBS containing 10% FCS for 30 min. Then cells were incubated with monoclonal mouse anti-CK19 (1: 500) (Santa Cruz/ sc-6278), mouse anti-CK18 (1: 500) (a gift from D.Bellet, France), mouse anti-ESA (1: 500) (Sigma / E-6011), mouse anti-ASMA (1: 100) (ABCAM / ab7817-500) or mouse anti-MUC (1: 100) (ABCAM / ab8607-250) antibody diluted in PBS containing 1.5% FCS for 60 min. After washing with PBS three times with 5 min intervals, cells were incubated with FITC-conjugated anti-mouse (DAKO / F-0479) (1: 200) secondary antibody diluted in PBS containing 1.5% FCS for 45 min. Beginning from this step, the procedure was performed under dark. After washing with PBS three times with 5 min intervals, nuclear DNA was stained with DAPI or Hoechst 33258 for 5 min.

DAPI or Hoechst 33258 was aspirated and destaining was performed in double-distilled water for 5 min. Coverslips were taken out from the well and excess water was removed by tissue paper, coverslips were mounted onto slides containing 12 µl 80% glycerol. Each immunostaining experiment was done using negative controls (no first antibody). Stained cells were examined under fluorescence microscope (ZEISS) and pictures were captured in a digital Kodak Camera (DC290, Eastman Kodak Co.), using Adobe Photo Deluxe (Adobe Systems Inc.) software. Pictures were processed using Adobe Photoshop 5.0 (Adobe Systems Inc.) software.

Phosphate-Buffered Saline (PBS)

10 X Stock Solution (1 lt)	Working Solution (pH: 7.3-7.6)
80 g NaCl, 2g KCl,	10X PBS diluted to 1X with
11.5 g $Na_2HPO_4.7H_2O$, 2 g KH_2PO_4	ddH_2O

DAPI Staining

- 1 mg DAPI was dissolved in 1 ml double-distilled water (Stock Solution – kept at -20°C)
- 10 µl stock solution was diluted in 100 ml 1X PBS. (Working Solution – kept at room temperature)

Hoechst Staining

- Hoechst 33258 stock solution was diluted 250 times with ddH_2O

3.4 Immunoblotting

3.4.1 Crude Total Protein Extraction from Cultured Cells

Cells were grown to 70-80% confluency and were washed with ice-cold PBS once. Then, cells were scraped in ice-cold PBS and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was discarded, cell pellets were stored at -80°C for

further analysis. Pellets were then lysed immediately in NP-40 lysis buffer, which was prepared freshly just before use. For lysis, the pellets were resuspended in 4 volume of NP-40 lysis buffer and were incubated on ice for 30 minutes by stirring the cell within 10 minutes periods. The lysate was then centrifuged at 13000 rpm for 5 minutes at 4°C. Supernatant was then aliquoted into fresh Eppendorf tubes, stored at -80°C and some amount was also taken for protein quantitation.

NP-40 Lysis Buffer (1 ml)

50 µl 1 M Tris (pH 8.0)

125 µl 2 M NaCl

10 µl NP-40

40 µl 25X Protease Inhibitor (Tablet dissolved in 2 ml sterile ddH_2O) (Sigma)

775 µl ddH_2O

3.4.2 Bradford Assay for Protein Quantification

Stock Bovine Serum Albumin (BSA) was prepared as in concentration 1.0 mg/ml and was kept at -20°C. Different dilutions of BSA was prepared as described below:

Tube numbers	1	2	3	4	5	6	7
BSA stock (µl)	0	1	2	4	8	16	32
ddH_2O (µl)	100	99	98	96	92	84	68
Bradford Working(µl)	900	900	900	900	900	900	900

Protein samples were prepared as described below:

Tube numbers	1	2	3	4	5	6	7
Sample (µl)	0	2	2	2	2	2	2
ddH_2O (µl)	98	98	98	98	98	98	98
Bradford Working(µl)	900	900	900	900	900	900	900
Lysis Buffer (µl)	2	-	-	-	-	-	-

(1 = blank in both tables)

Blanks, BSA standards, test samples were prepared in disposable plastic cuvettes. The absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer. The absorbance of each BSA standard was plotted linearly as a function of its theoretical concentration and best straight line was drawn in the form of the equation "y = mx + b" where y = absorbance at 595 nm and x = protein concentration. This equation was used to calculate the concentration of the protein samples based on the measured absorbance.

Bradford Stock Solution	**Bradford Working Solution**
17.5 mg SERVA Blue G	85 ml ddH_2O
10 ml 88 % phosphoric acid	3 ml 95 % ethanol
5 ml 95 % ethanol	6 ml 88 % phosphoric acid
250 ml ddH_2O	6 ml Bradford stock solution

Both solutions were kept in a brown bottle at +4°C.

3.4.3 SDS-Polyacrylamide Gel Electrophoresis of Proteins

The glass plates were assembled according to manufacturer's instructions (CBS). The volume of the gel mold was determined according to the information provided by the manufacturer (CBS). In a Falcon tube, the appropriate volume of

solution containing the desired concentration of acrylamide for the resolving gel was prepared. Effective range of separation of SDS-PAGE gels due to different acrylamide concentrations are summarized in Table 3.2. In this study, 10% resolving gel was used and the concentrations of its components are described in Table 3.3.

Table 3.2 Effective range of separation of SDS-PAGE gels

Acrylamide concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

TEMED was added to the gel solution for polymerization just before the gel was cast. The mixture was swirled rapidly without delay. Then, the resolution SDS-PAGE gel solution was poured into the gap between the glass plates. Sufficient space for stacking gel was left. Immediately, 100% isopropanol was overlaid onto resolution SDS-PAGE gel using a Pasteur pipette. The gel was placed in a vertical position to polymerize at room temperature. An aliquot of resolution gel was kept to check polymerization. After polymerization was complete, the overlaying isopropanol was poured off and the top of the gel was washed several times with distilled water. Any remaining water was removed with the edge of a paper towel. Then, 5% stacking SDS-PAGE gel solution was prepared in a Falcon tube, TEMED was added for polymerization just before the gel was cast. The concentrations of the components of the 5 % stacking gel are described in Table 3.4. The mixture was swirled rapidly without delay. Then, the stacking SDS-PAGE gel solution was poured directly onto the surface of the polymerized resolution SDS-PAGE gel. A clean comb was inserted immediately into the SDS-PAGE gel solution, it was avoided trapping air bubbles. To fill the spaces of the comb completely, more stacking SDS-PAGE gel solution was added. The gel was placed in a vertical

position at room temperature. An aliquot of stacking gel was kept to check polymerization.

While the stacking gel was polymerizing, the samples to be loaded were prepared by heating them to 100°C for 5 minutes in 1X SDS gel-loading buffer to denaturate the proteins. Then, the heated protein lysate/SDS gel loading buffer mixtures were immediately placed on ice and were incubated for 5 min. After polymerization was complete, the comb was removed carefully. The wells were washed with distilled water to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus. 1X Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. Then 30-50 μg of the protein lysates and the marker proteins of known molecular weight were loaded in a predetermined order into the bottom of the wells. The electrophoresis apparatus was attached to an electrophoresis supply and the gel was run at 80 Volts until the dye front has moved to the resolving gel, later the voltage was increased to 120 Volts, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off. The glass plates were removed from the electrophoresis apparatus and were placed on a paper towel. By using a spatula, the plates were separated from each other. Orientation of the gel was marked by cutting a corner from the top of the gel.

Tris-Glycine Electrophoresis Buffer (5X)

22.5 g Tris

108 g Glycine

7.5 g SDS

750 ml ddH_2O.

Volume was then adjusted to 1500 ml with ddH_2O, stored at 4°C, diluted to 1X with ddH_2O when used.

30 % mix (Acrylamide and bis-acrylamide solution)

29.2 g acrylamide

0.8 g Bis-acrylamide

70 ml ddH_2O

Volume was adjusted to 100 ml with ddH_2O, filter sterilized, stored at 4°C in dark bottles.

10% SDS

A 10% (w/v) stock solution was prepared in ddH_2O.

APS

A small amount of 10% stock solution was prepared freshly in ddH_2O.

5X Gel Loading Buffer

3.8 ml ddH_2O

1.0 ml 0.5 M Tris-HCl

0.8 ml glycerol

1.6 ml 10% SDS

0.4 ml 0.05% BPB fluid

400 µl β-Mercaptoethanol (added freshly)

Table 3.3 Components of 10% resolving gel for Tris-Glycine SDS-PAGE

Solution components	Component Volumes (ml)							
10%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
ddH_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Table 3.4 Components of 5% stacking gel for Tris-Glycine SDS-PAGE

Solution components	Component Volumes (ml)							
10%	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
ddH_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% mix	0.17	0.33	0.50	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.50	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

3.4.4 Transfer of Proteins from SDS-polyacrylamide Gels to Solid Supports

When the SDS-polyacrylamide gel was approaching the end of its run, four pieces of Whatman 3MM paper and one piece of transfer membrane (PVDF) was cut as the exact size of the SDS-polyacrylamide gel by wearing gloves. The membrane was left in methanol for 1 minute and then was soaked into transfer buffer for 10-15 minutes. Meanwhile, the Whatman 3MM papers were soaked into a shallow tray containing a small amount of transfer buffer and were kept shaking for 1 minute.

The transfer apparatus was set as follows:

1. 2 pieces of Whatman 3MM paper that have been soaked in transfer buffer were placed onto the plate which will be positively charged (anode). All air bubbles were squeezed.
2. The PVDF membrane was placed onto the Whatman 3MM papers. (The transfer membrane should be exactly aligned and the air bubbles trapped between it and the Whatman 3MM paper were squeezed out.)

3. The glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank, and the gel was transferred to a tray of transfer buffer and was kept there for 10 minutes.
4. Then SDS-polyacrylamide gel was placed onto the transfer membrane. Any trapped air bubbles were squeezed out.
5. 2 pieces of Whatman 3MM paper were placed to the top of the sandwich (this side will be negatively charged during the transfer (cathode side)).

The electrical leads of the apparatus were connected to the power supply and the transfer was carried out at a current of 3 mA/cm^2 of the gel for 45 minutes. The electric current was turned off at the end of the run time and the transfer apparatus was disassembled from top downward, peeling off each layer in turn. The positions of wells and pre-stained marker were marked using a needle or a pin. Also, the top left-hand corner of the membrane was cut for orientation purpose.

Transfer Buffer

2.9 g Glycine Volume was then adjusted to 1 lt with ddH_2O

5.8 g Trisma base

0.37 g SDS

200 ml methanol

3.4.5 Immunological Detection of Immobilized Proteins (Western Blotting)

The membrane was then immersed in the blocking solution which contains 3% milk powder in 0.1% Tween-TBS solution for one hour on a rotating platform. Then the membrane was incubated with primary antibody (Table 3.5), which has been diluted according to instructions in blocking solution, for one hour at room temperature or at 4°C overnight on a slowly rotating platform. Later, the membrane was washed for four times, once for 10 minutes, and three times for 5 minutes, with TBS-T. After the washing, the membrane was incubated with a secondary antibody HRP-conjugated anti-mouse Ig (ABCAM / ab6728-1), which is diluted as 1:3500 in blocking solution for 1 hour at room temperature on a slowly rotating platform. The

membrane was then washed with TBS-T for four times, once for 10 minutes, and three times for 5 minutes. The membrane becomes ready for incubation with substrate and development. After the detection of proteins, membrane was further used for loading control. The primary antibody rabbit anti-calnexin (Sigma/C-4731) diluted in blocking solution was used as the loading control. The HRP-conjugated anti-rabbit Ig (DAKO / P-0448) (1:2500 diluted in blocking solution) was used as the secondary antibody. All primary and secondary incubations and washings were performed as described above.

Table 3.5 The primary antibodies used in this study for Western blotting

Company	Origin	Against/Clone name	Clonality	Dilution
Santa Cruz	Mouse	Cytokeratin 19/A53-B/A2	Monoclonal	1:1000
*	Mouse	Cytokeratin 18/JAR13	Monoclonal	1:5000
Sigma	Mouse	ESA/VU-1D9	Monoclonal	1:1000
Sigma	Rabbit	Calnexin	Monoclonal	1:5000

* *Kindly provided by D.Bellet, France*

For detection, either ECL (**E**nhanced **C**hemi**L**uminescence; Amersham Pharmacia Biotech. Cat. No. RPN 2106) or LumiLight (Roche Diagnostics GmbH. Cat No. 2 015 200) detection systems were carried out according to manufacturer's instructions.

Tris-Buffered Saline (TBS) (10X)

24.2 g Tris-Base
80 g NaCl

Volume was adjusted to 1lt with ddH_2O. TBS was diluted to 1X with ddH_2O before use (pH: 7.6).

CHAPTER IV RESULTS

4.1 Characterization of Breast Cancer Cell Lines by Marker Antibodies

To investigate whether breast cancer cell lines display stem cell-like property, the 6 breast cancer cell lines were characterized in terms of marker protein expression which describes the epithelial differentiation status of the cell. Immunofluorescence of 6 breast cancer cell lines with 5 marker antibodies was performed as described in "Materials and Methods" section. Western Blotting was done to further confirm the protein expression. The marker proteins that were used in our study and their expression in terms of mammary epithelial differentiation status are described in Table 4.1.

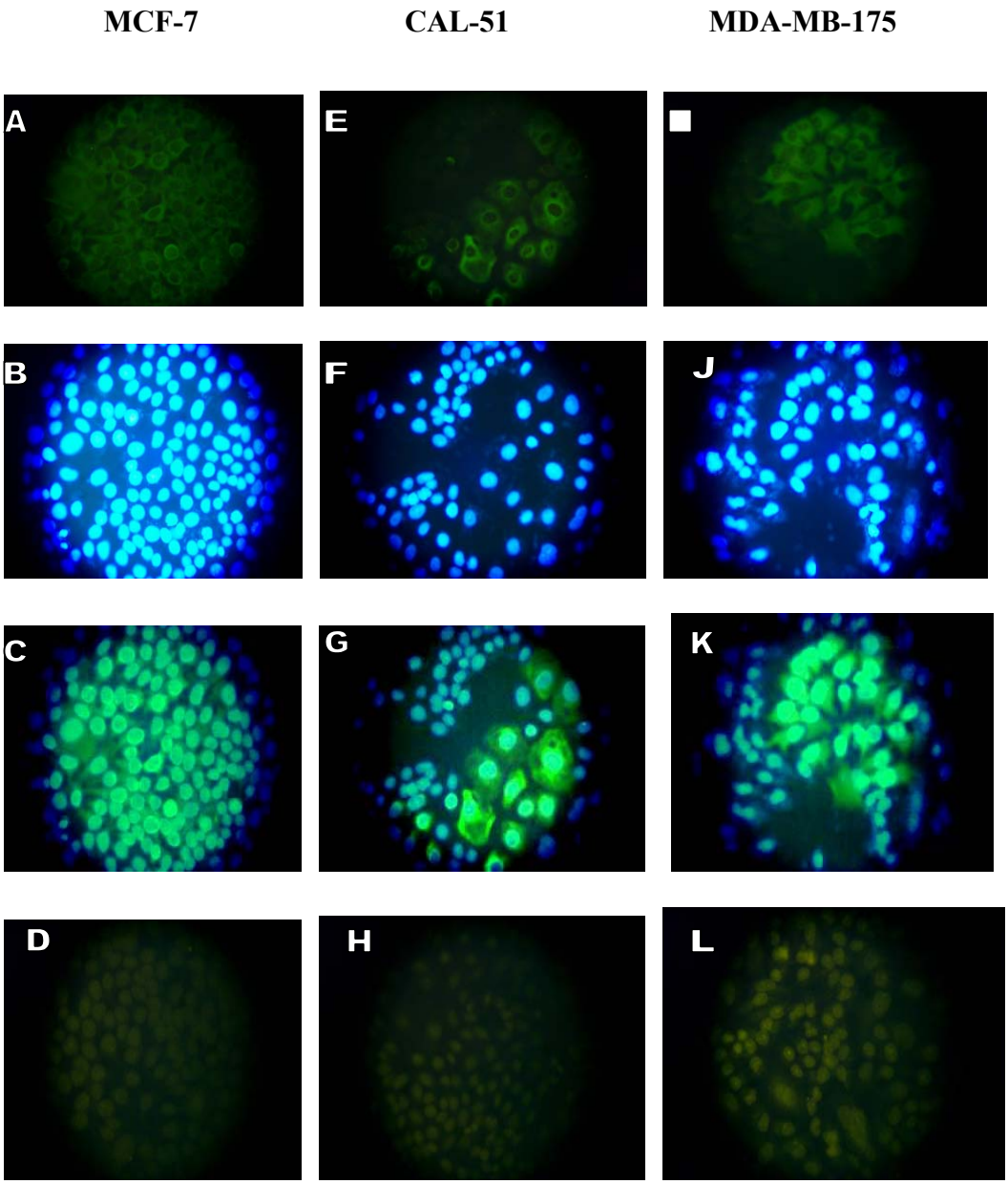
Table 4.1 Marker proteins of mammary epithelial cells used in this study and their expression in terms of differentiation status

Marker Proteins	Differentiation Status			References
	Stem/ Progenitor Cell	Differentiated Luminal Epithelial Cell	Differentiated Myoepithelial Cell	
Cytokeratin 19 (CK19)	+	+/-	-	Gudjonsson et al., (2002), Taylor-Papadimitriou et al., (1989)
Cytokeratin 18 (CK18)	-/+	+	-	Taylor-Papadimitriou et al., (1989), Pechoux et al. (1999)
Epithelial Specific Antigen (ESA)	+	+	-	Gudjonsson et al., (2002), Stingl et al., (1998)
Sialomucin (MUC1)	-	+	-	Gudjonsson et al., (2002), Stingl et al., (1998)
Alpha-Smooth Muscle Actin (ASMA)	-	-	+	Gudjonsson et al., (2002), Petersen and van Deurs, (1988)

4.1.1 Indirect Immunofluorescence of Breast Cancer Cell Lines

4.1.1.1 Staining of Cell Lines with anti-CK19 antibody

6 breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown on coverslips and immunofluorescence of these cell lines was performed by using anti-CK19 antibody. Counterstaining with DAPI displayed that MCF-7 and 734-B were positive; CAL-51, MDA-MB-175 and GI-101 were heterogenous; HBL-100 was negative for cytoplasmically expressed CK19 antigen (Figure 4.1).



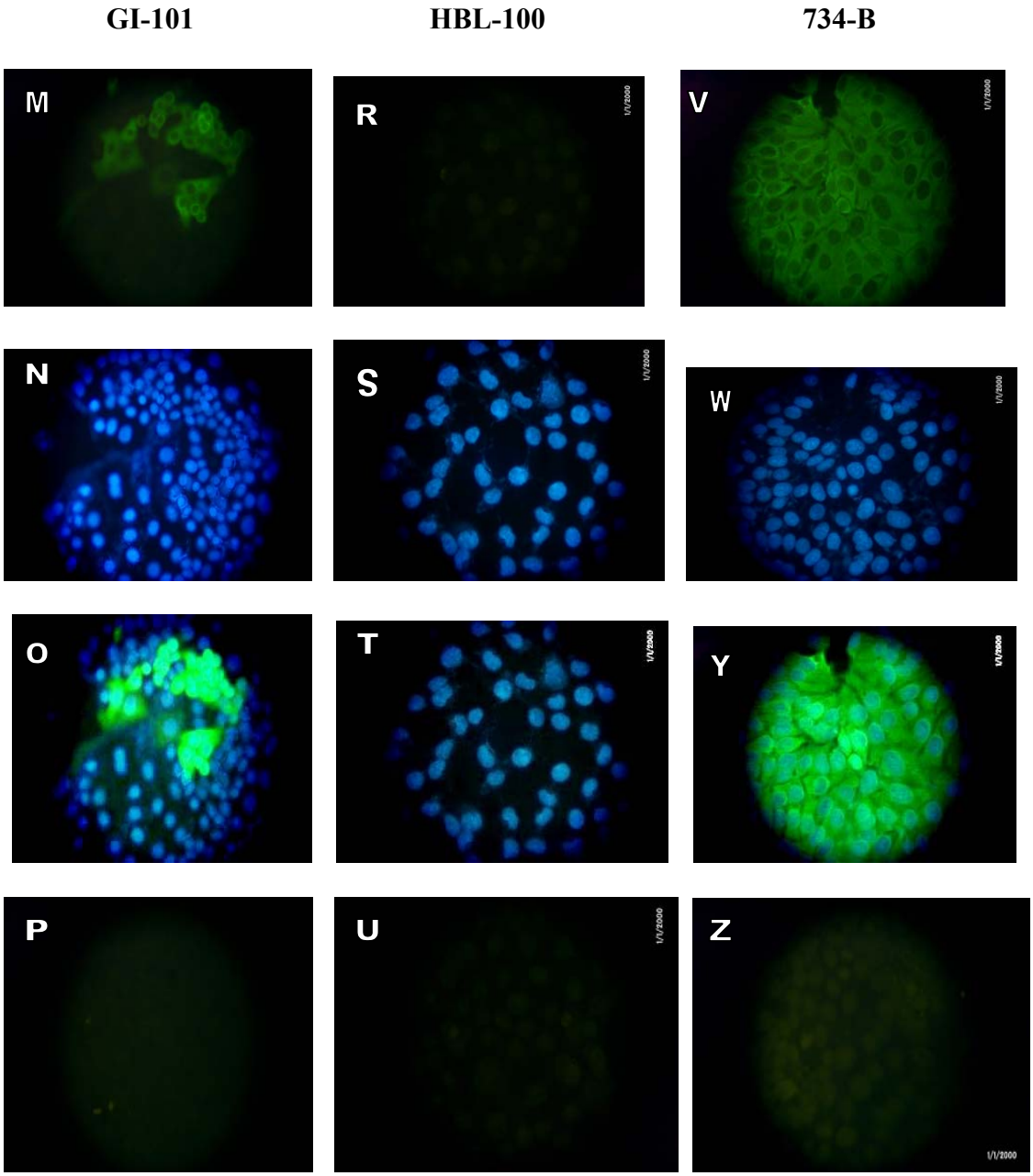
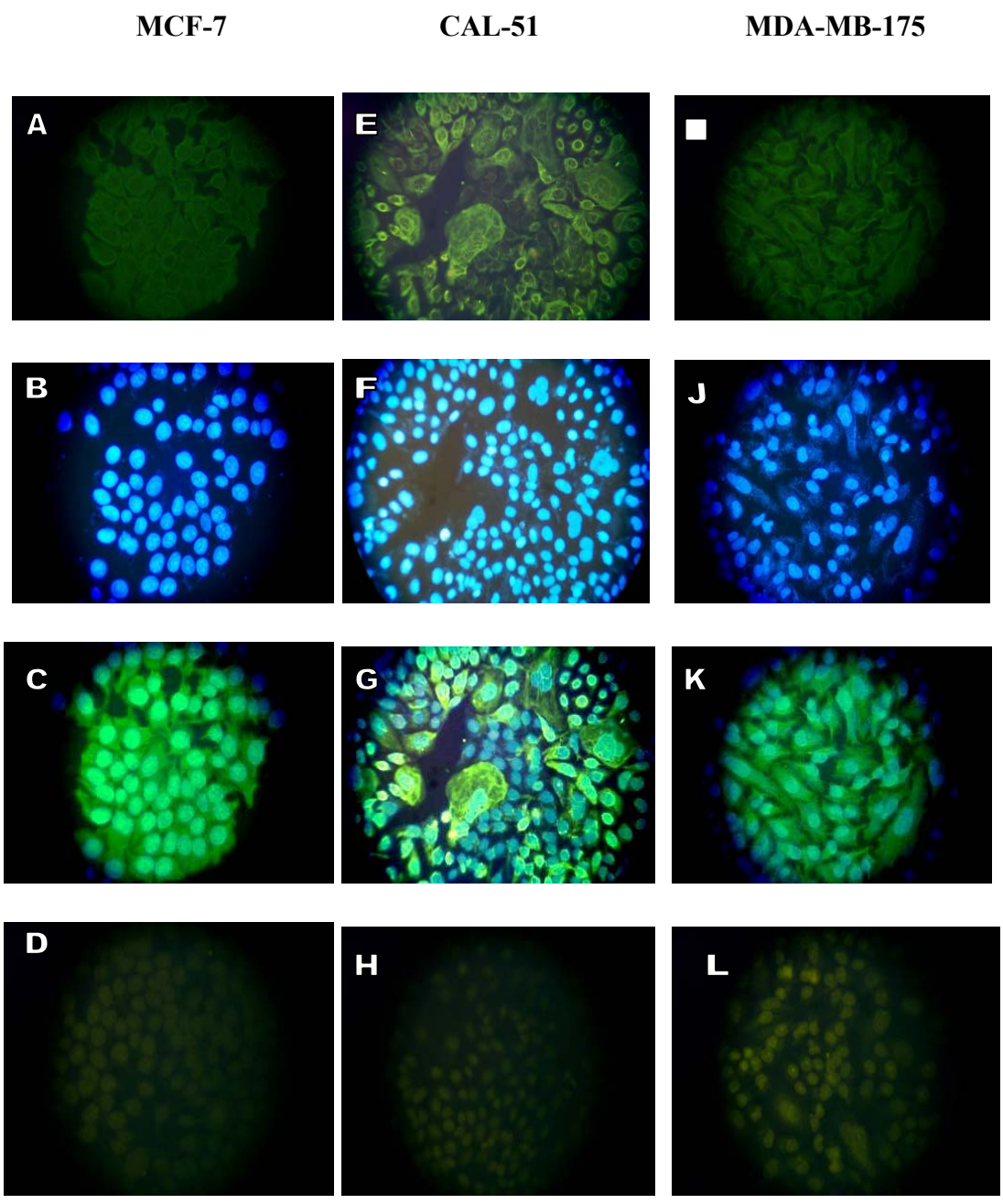


Figure 4.1 Immunofluorescence staining of breast cancer cell lines with anti-CK19 antibody. (CK19 staining: MCF-7 (A), CAL-51 (E), MDA-MB-175 (I), GI-101 (M), HBL-100 (R), 734-B (V). DAPI staining: MCF-7 (B), CAL-51 (F), MDA-MB-175 (J), GI-101 (N), HBL-100 (S), 734-B (W). Merged images: MCF-7 (C), CAL-51 (G), MDA-MB-175 (K), GI-101 (O), HBL-100 (T), 734-B (Y). Negative control (with no primary antibody): MCF-7 (D), CAL-51 (H), MDA-MB-175 (L), GI-101 (P), HBL-100 (U), 734-B (Z).)

4.1.1.2 Staining of Cell Lines with anti-CK18 antibody

6 breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown on coverslips and immunofluorescence of these cell lines was performed by using anti-CK18 antibody. Counterstaining with DAPI displayed that all cell lines except CAL-51 was positive for cytoplasmically expressed CK18, while CAL-51 was heterogenous for CK18 (with approximately 95% positivity) (Figure 4.2).



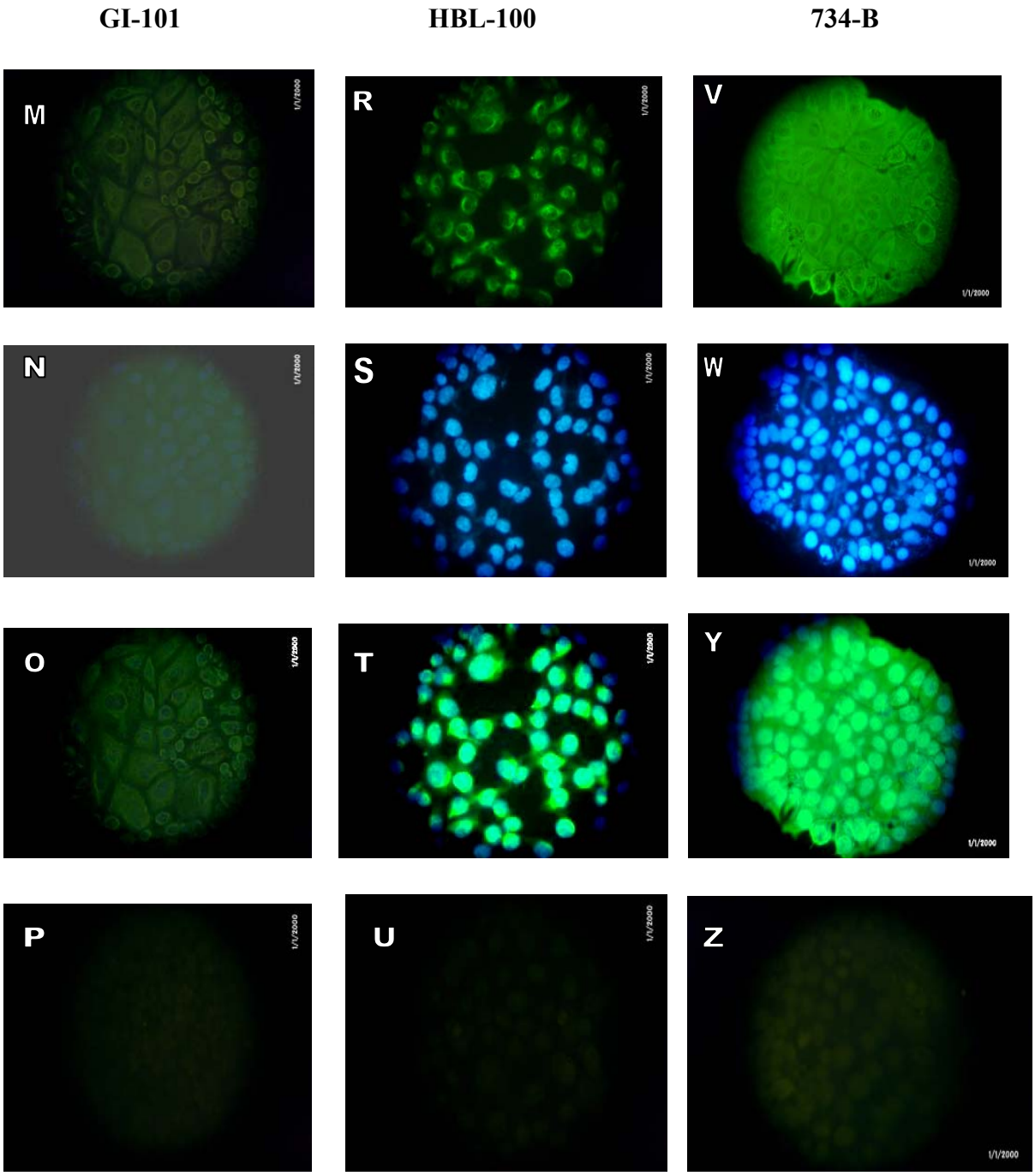
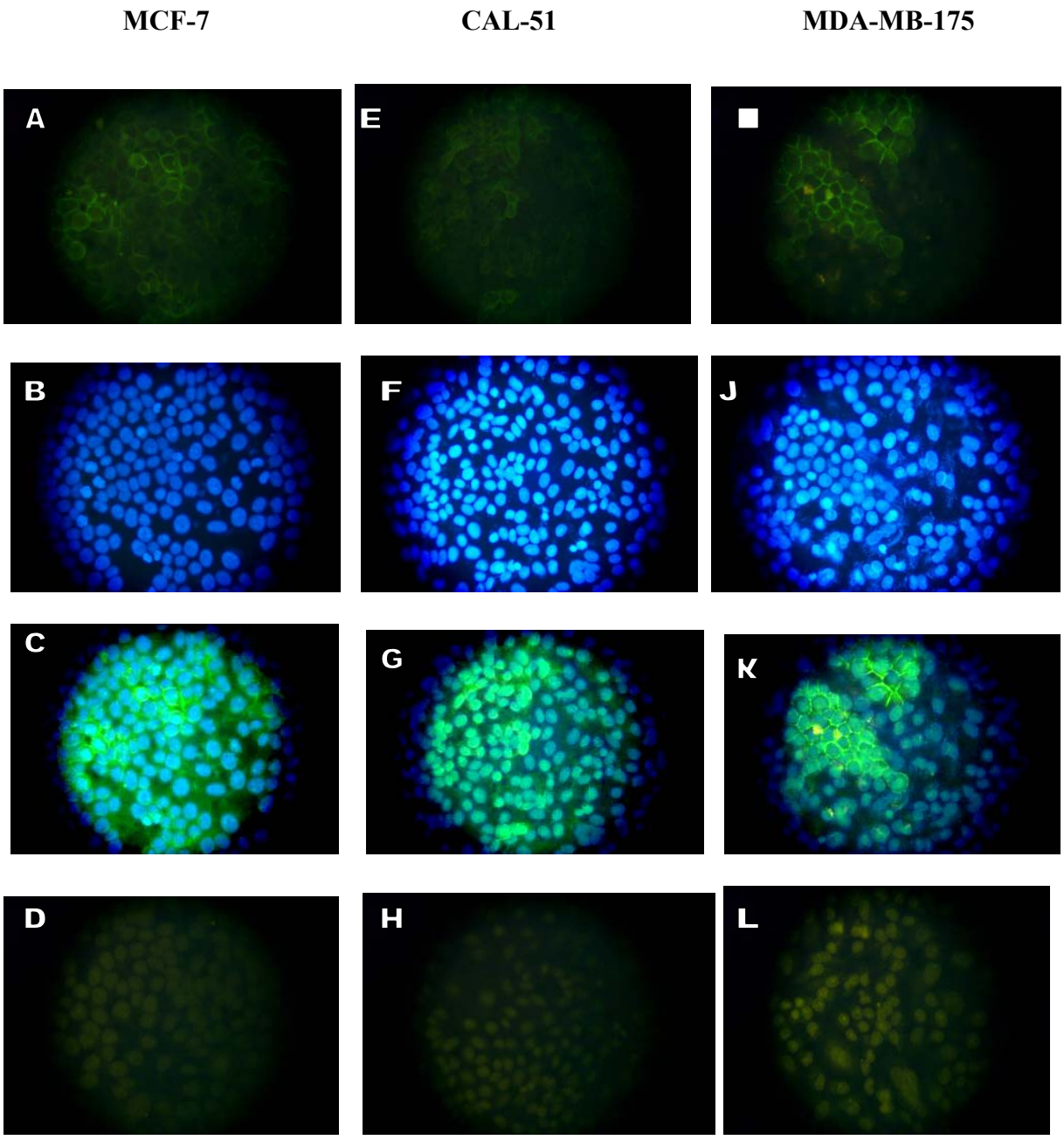


Figure 4.2 Immunofluorescence staining of breast cancer cell lines with anti-CK18 antibody. (CK18 staining: MCF-7 (A), CAL-51 (E), MDA-MB-175 (I), GI-101 (M), HBL-100 (R), 734-B (V). DAPI staining: MCF-7 (B), CAL-51 (F), MDA-MB-175 (J), GI-101 (N), HBL-100 (S), 734-B (W). Merged images: MCF-7 (C), CAL-51 (G), MDA-MB-175 (K), GI-101 (O), HBL-100 (T), 734-B (Y). Negative control (with no primary antibody): MCF-7 (D), CAL-51 (H), MDA-MB-175 (L), GI-101 (P), HBL-100 (U), 734-B (Z).)

4.1.1.3 Staining of Cell Lines with anti-ESA antibody

6 breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown on coverslips and immunofluorescence of these cell lines was performed by using anti-ESA antibody. Counterstaining with DAPI displayed that 734-B cell line was positive; but with varying intensity, HBL-100 cell line was negative; all the other cell lines displayed heterogeneity for peripherally expressed ESA antigen (with approximately 80% positivity) (Figure 4.3).



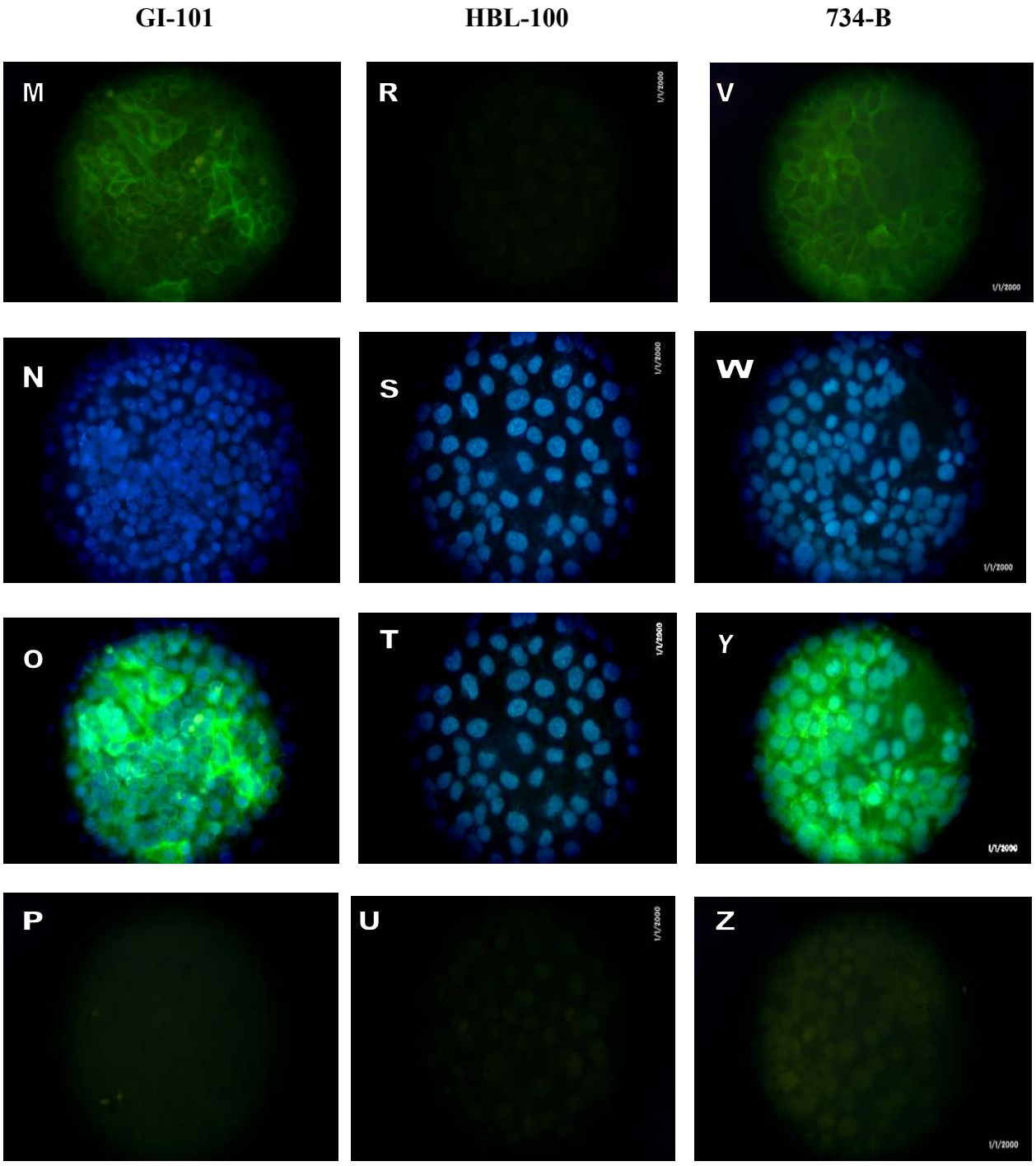
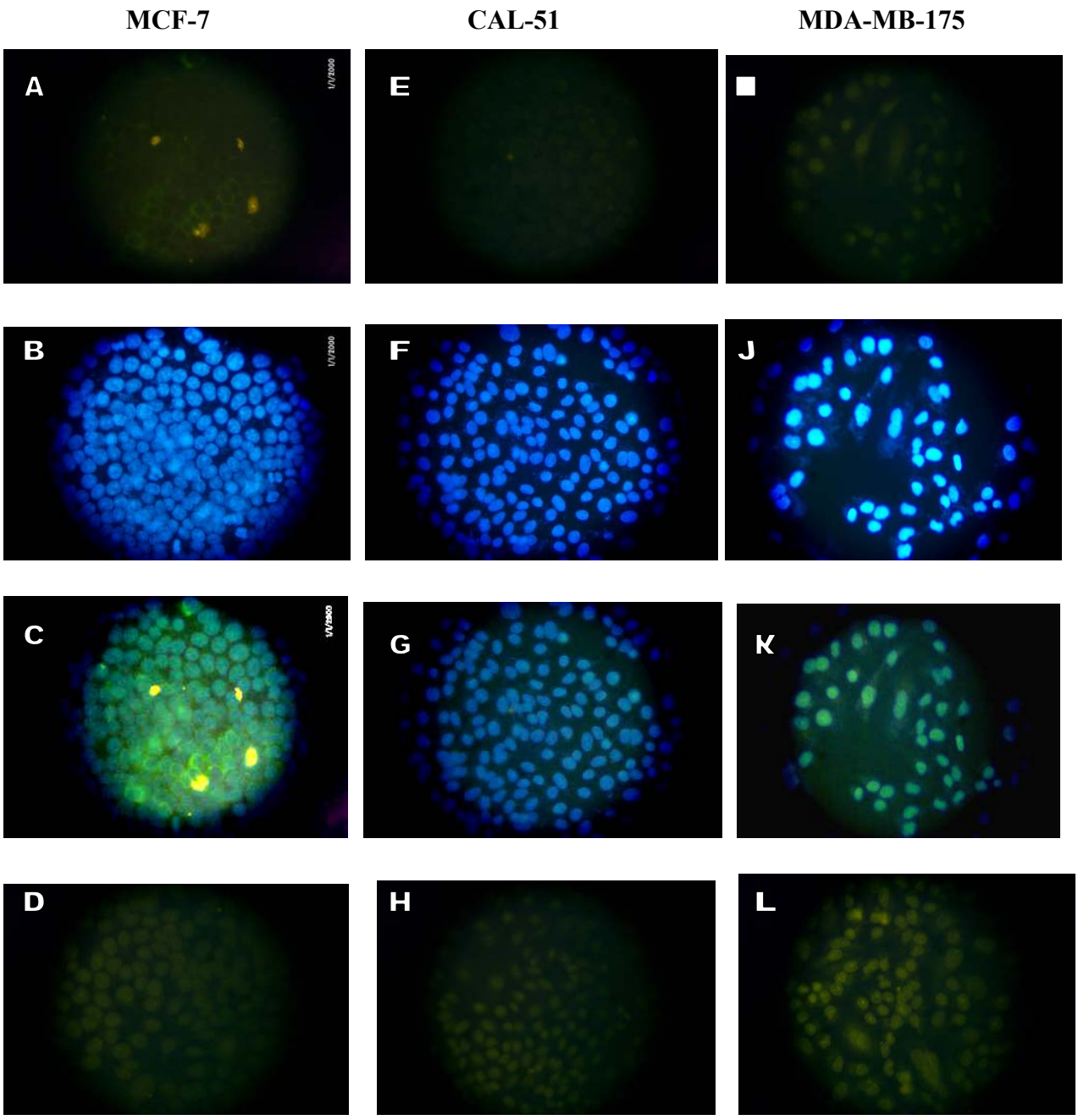


Figure 4.3 Immunofluorescence staining of breast cancer cell lines with anti-ESA antibody. (ESA staining: MCF-7 (A), CAL-51 (E), MDA-MB-175 (I), GI-101 (M), HBL-100 (R), 734-B (V). DAPI staining: MCF-7 (B), CAL-51 (F), MDA-MB-175 (J), GI-101 (N), HBL-100 (S), 734-B (W). Merged images: MCF-7 (C), CAL-51 (G), MDA-MB-175 (K), GI-101 (O), HBL-100 (T), 734-B (Y). Negative control (with no primary antibody): MCF-7 (D), CAL-51 (H), MDA-MB-175 (L), GI-101 (P), HBL-100 (U), 734-B (Z).)

4.1.1.4 Staining of Cell Lines with anti-MUC1 antibody

6 breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown on coverslips and immunofluorescence of these cell lines was performed by using anti-MUC1 antibody. Counterstaining with DAPI or Hoechst displayed that 734-B (displaying 20% positivity) and MCF-7 were heterogenous (displaying 5% positivity); all the other cell lines were negative for peripherally expressed MUC1 antigen (Figure 4.4).



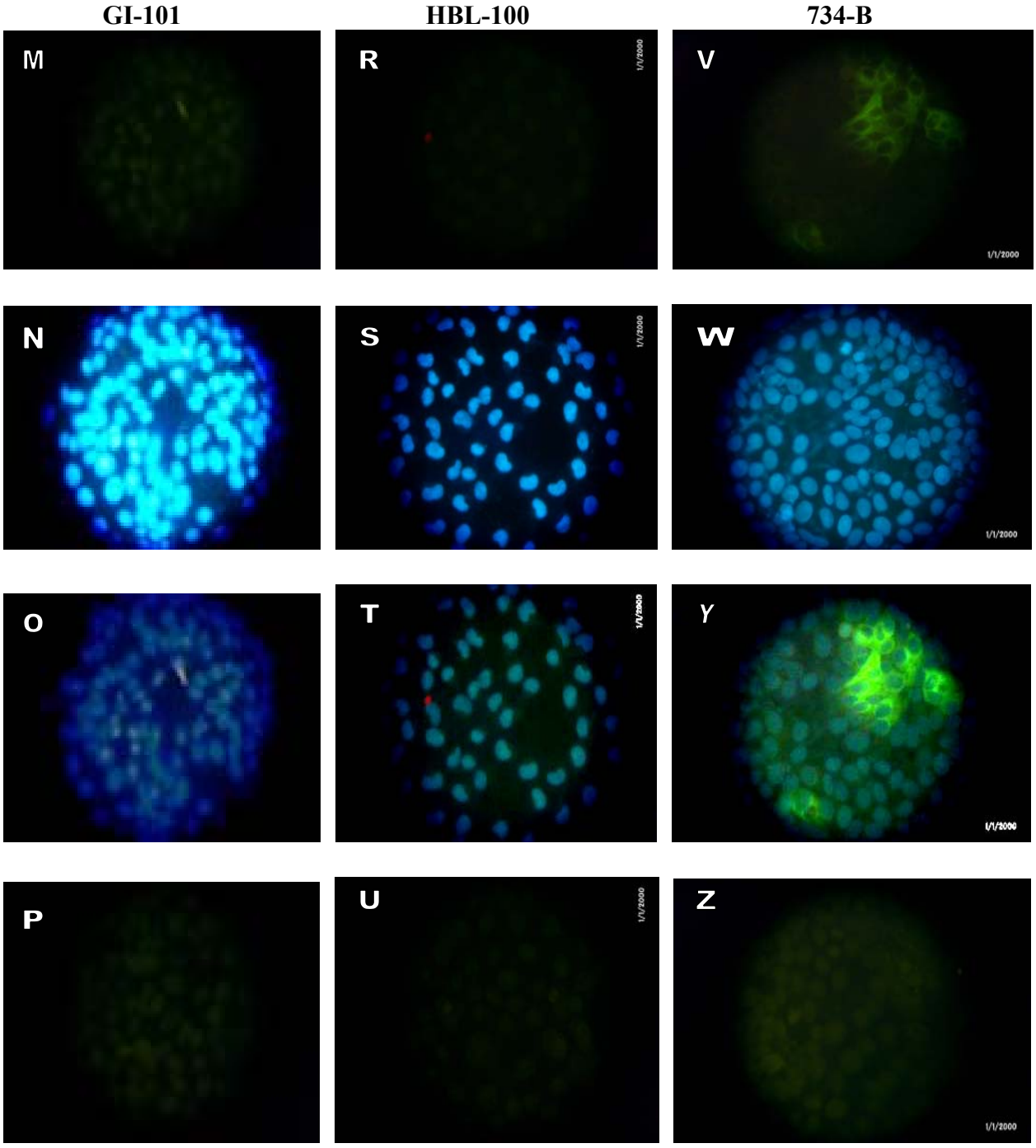
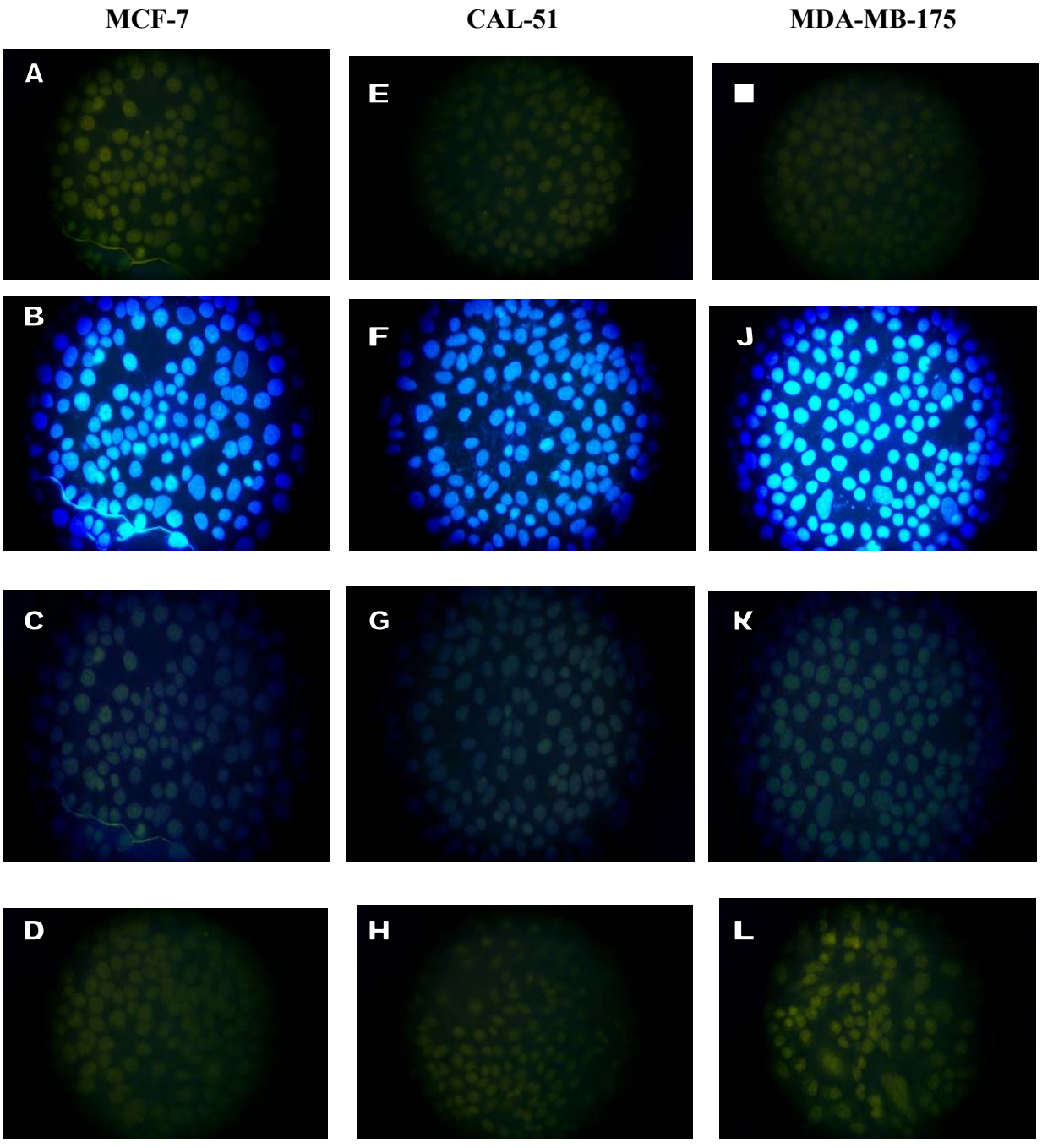


Figure 4.4 Immunofluorescence staining of breast cancer cell lines with anti-MUC1 antibody. (MUC staining: MCF-7 (A), CAL-51 (E), MDA-MB-175 (I), GI-101 (M), HBL-100 (R), 734-B (V). Counterstaining (DAPI unless otherwise stated): MCF-7 (B), CAL-51 (F), MDA-MB-175 (J), GI-101 (Hoechst) (N), HBL-100 (S), 734-B (W). Merged images: MCF-7 (C), CAL-51 (G), MDA-MB-175 (K), GI-101 (O), HBL-100 (T), 734-B (Y). Negative control (with no primary antibody): MCF-7 (D), CAL-51 (H), MDA-MB-175 (L), GI-101 (P), HBL-100 (U), 734-B (Z).)

4.1.1.5 Staining of Cell Lines with anti-ASMA antibody

6 breast cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown on coverslips and immunofluorescence of these cell lines was performed by using anti-ASMA antibody. Counterstaining with DAPI displayed that only GI-101 (displaying 30% positivity) was heterogenous and all the other cell lines were negative for cytoplasmically expressed ASMA antigen (Figure 4.5).



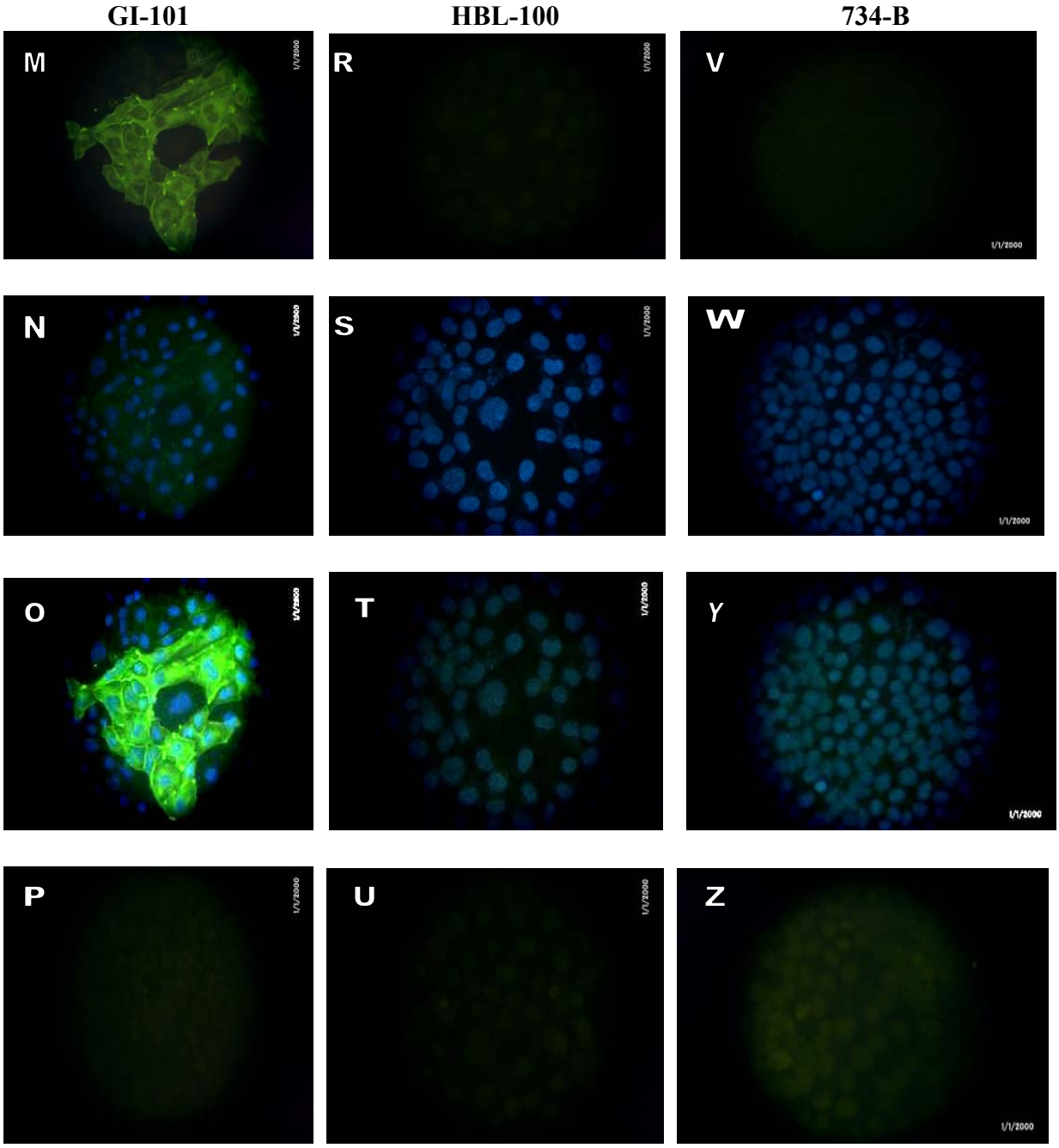


Figure 4.5 Immunofluorescence staining of breast cancer cell lines with anti-ASMA antibody. (ASMA staining: MCF-7 (A), CAL-51 (E), MDA-MB-175 (I), GI-101 (M), HBL-100 (R), 734-B (V). DAPI staining: MCF-7 (B), CAL-51 (F), MDA-MB-175 (J), GI-101 (N), HBL-100 (S), 734-B (W). Merged images: MCF-7 (C), CAL-51 (G), MDA-MB-175 (K), GI-101 (O), HBL-100 (T), 734-B (Y). Negative control (with no primary antibody): MCF-7 (D), CAL-51 (H), MDA-MB-175 (L), GI-101 (P), HBL-100 (U), 734-B (Z).)

4.1.2 Western blotting of Breast Cancer Cell Lines

6 cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were grown up to desired confluency in 100-mm and 150-mm dishes. Then, cell pellets were collected by a scraper. From the cell pellets, cell lysates were prepared by using NP-40 lysis buffer. This total cell lysate was then loaded to 10% SDS-PAGE. Afterwards, proteins were transferred to PVDF membranes and then these membranes were subjected to Western blotting as described in "Materials and Methods" section by using the antibodies of marker proteins.

4.1.2.1 Western Blotting by anti-CK19 antibody

Total cell lysates of 6 cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were subjected to Western blotting by using anti-CK19 antibody. HBL-100 displayed no CK19 band, on the other hand, GI-101 and CAL-51 displayed weaker CK19 bands than the strong CK19 bands of MCF-7, MDA-MB-175 and 734-B cell lines. Anti-calnexin antibody was used as loading control (Figure 4.6).

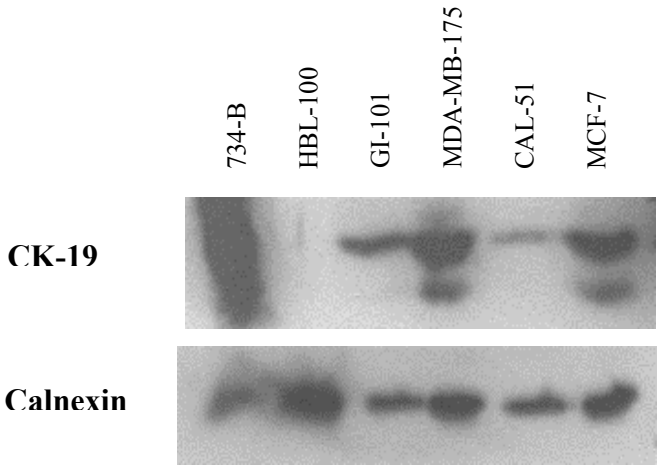


Figure 4.6 Western Blotting patterns of breast cancer cell lines by anti-CK19 antibody

4.1.2.2 Western Blotting by anti-CK18 antibody

Total cell lysates of 6 cancer cell lines (MCF-7, CAL-51, MDA-MB-175, GI-101, HBL-100, 734-B) were subjected to Western blotting by using anti-CK18 antibody. All cell lines displayed intense CK18 bands, therefore anti-calnexin staining was not necessitated as loading control (Figure 4.7).

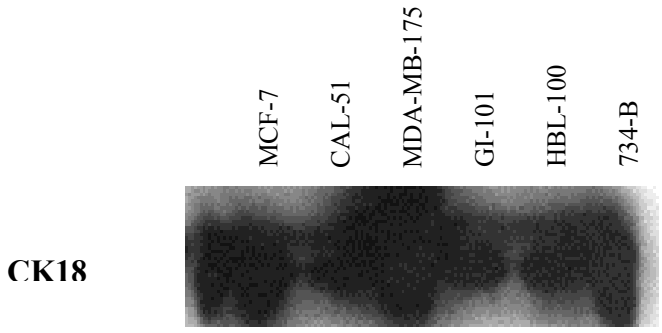


Figure 4.7 Western Blotting patterns of breast cancer cell lines by anti-CK18 antibody

4.1.2.3 Western Blotting by anti-ESA antibody

Total cell lysates of 3 cancer cell lines (MCF-7, CAL-51, MDA-MB-175) were subjected to Western blotting by using anti-ESA antibody. All three cell lines displayed ESA bands, while the ESA band of CAL-51 was very weak. Anti-calnexin antibody was used as loading control (Figure 4.8).

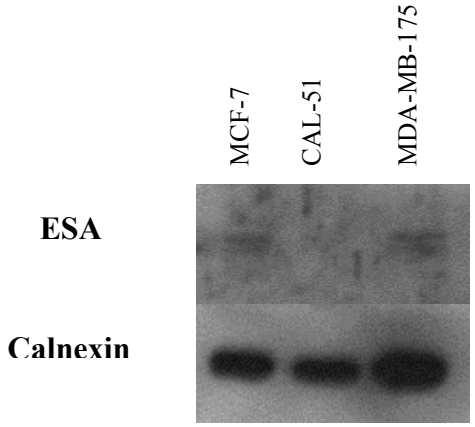


Figure 4.8 Western Blotting patterns of breast cancer cell lines by anti-ESA antibody

4.1.3 Classification of Breast Cancer Cell Lines according to Marker Protein Expression

Based on these results, breast cancer cell lines have been classified according to marker protein expression in terms of mammary epithelium differentiation status. This classification is indicated in Table 4.2. The numbers in parenthesis display the percentage of positivity for marker protein expression in heterogenous cell populations.

Table 4.2 Classification of Breast Cancer Cell Lines according to Marker Protein Expression in terms of Mammary Epithelium Differentiation Status

Marker Protein	**Cell Lines**					
	MCF-7	CAL-51	MDA-MB-175	GI-101	HBL-100	734-B
CK18	+	Het (95%)	+	+	+	+
CK19	+	Het (50%)	Het (50%)	Het (50%)	-	+
ESA	Het (80%)	Het (80%)	Het (80%)	Het (80%)	-	+
MUC1	Het (5%)	-	-	-	-	Het (20%)
ASMA	-	-	-	Het (30%)	-	-

Het: Heterogenous

4.2 Single cell cloning of GI-101 cell line and Immunofluorescence Analysis

Our immunofluorescence analysis of cell lines have revealed that GI-101 cell line displayed that this cell line contains heterogenous cell populations in terms of marker protein expression associated with mammary epithelium differentiation status. To investigate whether these heterogenous populations are clonal in origin, i.e. one cell gives rise to different types of cell populations which means that this cell line has stem/progenitor cell property, single-cell cloning of GI-101 cell line was performed as described in "Materials and Methods" section. A single cell is displayed on a well of 96-well plate in Figure 4.9. After growth of colonies derived from a single clone in 24-well, 6-well and 60-mm plates until the desired confluency, immunofluorescence of the colonies was performed as described in "Materials and Methods" section by using anti-CK18, anti-CK19, anti-ESA, anti-ASMA marker antibodies. The results have displayed that the phenotypic CK-19-, ESA-, ASMA-heterogenous populations (ASMA-positivity is low) are clonal in origin, i.e. they were derived from a single cell, which means that GI-101 cell line has stem/progenitor cell property. The colonies also displayed CK-18-positive pattern. The immunofluorescence results are displayed in Figure 4.10.

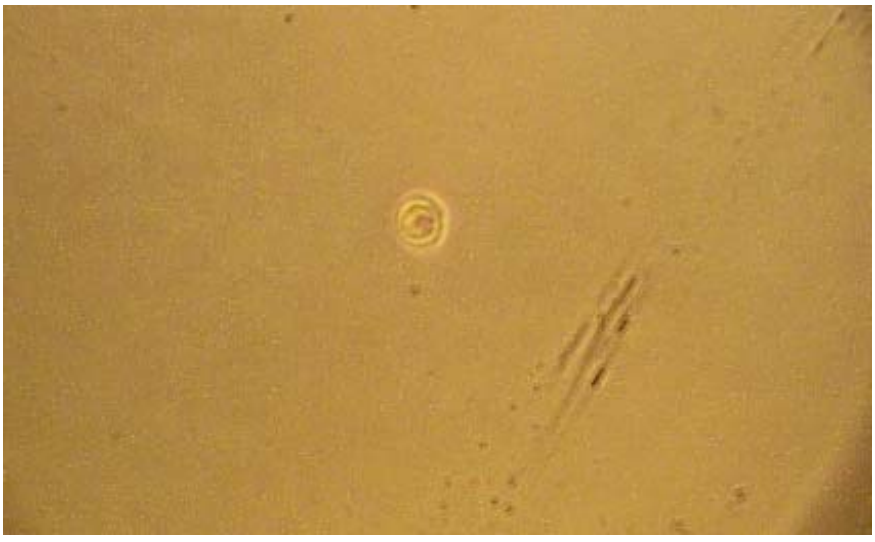


Figure 4.9 Single cell in 1 well of 96-well plate

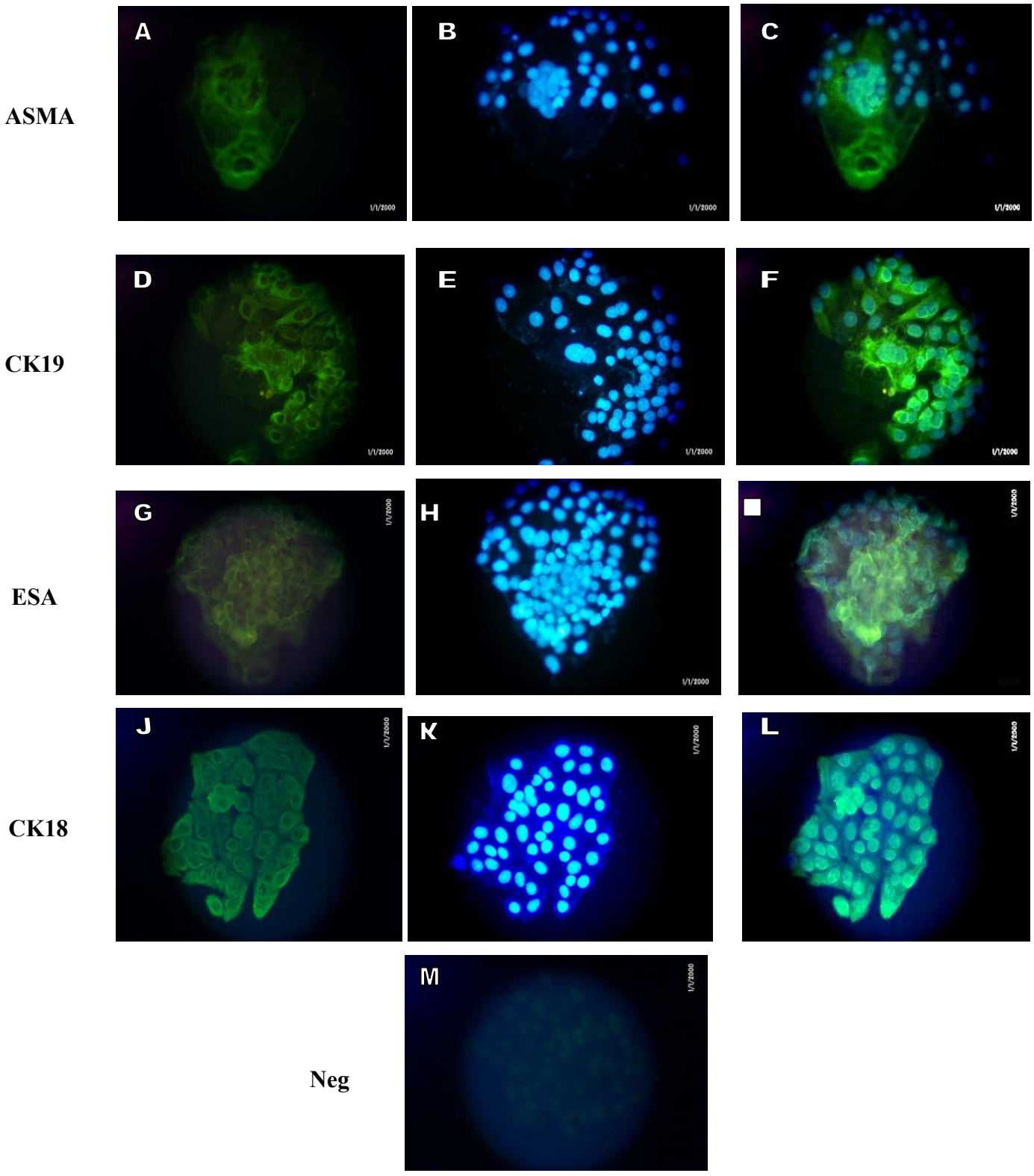


Figure 4.10 Immunofluorescence staining patterns of GI-101 colonies derived from a single clone. (anti-ASMA & DAPI stainings & merged image (A,B,C); anti-CK19 & DAPI stainings & merged image (D,E,F); anti-ESA & DAPI stainings & merged image (G,H,I); anti-CK18 & DAPI stainings & merged image (J,K,L); Negative control (no primary antibody) (M))

CHAPTER V DISCUSSION AND FUTURE PERSPECTIVES

In this study, breast cancer cell lines were classified according to marker protein expression in terms of mammary epithelium differentiation status. Immunofluorescence and Western blotting methods were used for this characterization. The marker proteins used in this study were CK18, CK19, ESA, MUC1 and ASMA. Therefore, whether these cell lines have stem/progenitor cell property was investigated by examining from which differentiation status the cell lines may have originated. The classification was displayed in Table 4.2.

Almost all the cell lines (CAL-51 with 95% positivity) displayed CK-18-(+) phenotypic profile, notifying that the cell lines originated from luminal or at least, basoluminal part of the epithelium, not from the basomyoepithelial part of the epithelium. %5 CAL-51 CK-18-(-) population would be a candidate for basomyoepithelial origin if CAL-51 expressed ASMA marker protein.

The CK-19-(+), ESA-Het (with 80% positivity) phenotypic profile makes MCF-7 cell line a strong candidate for stem/progenitor cell property. MUC1-Het (with 5% positivity) and ASMA-(-) phenotype describes that MCF-7 cell line most probably has originated from an intermediate progenitor cell of luminal differentiation. Therefore, it seems likely that this cell line represents a more differentiated status than CAL-51 and MDA-MB-175 cell lines.

734-B almost has the same phenotypic profile as MCF-7 cell line. On the other hand, ESA and MUC1 positivity is higher than MCF-7 cell line, displaying that 734-B most probably has originated from an intermediate, but more differentiated than MCF-7 cell line, progenitor cell of luminal differentiation.

HBL-100, as a breast cell line derived from milk of a nursing woman, displayed a very interesting profile of marker protein expression. This cell line is positive for only CK-18 marker protein, the other marker proteins were not expressed. Therefore, any precise conclusion of the origin of this cell line cannot be drawn, only it can be considered that this cell line is luminal in origin.

GI-101 displayed a remarkable phenotypic profile. The heterogenous expression patterns of CK-19, ESA and ASMA demonstrate that this cell line represent progenitor cell populations in different stages of differentiation. ASMA-heterogeneity describes that these stages are most probably intermediates of myoepithelial differentiation. Double immunofluorescence analysis of CK19, ESA and ASMA marker proteins will display a more precise description of origin of this cell line. For instance, the presence of any CK19(-)-ASMA-(+) cell population would represent a differentiated myoepithelial origin, CK-19-(+)-ASMA-(+) cell population would represent an intermediate step of myoepithelial differentiation, whereas CK-19-(+)-ASMA-(-) would represent a progenitor cell population in a less differentiated state. Single cell cloning of this cell line also further supported that this cell line has stem/progenitor cell property, which is described in detail below. Also, low density growth of this cell line was performed on Matrigel as a basement membrane, after cultivation for approximately for 20 days, the cells gave rise to three-dimensional structures, though not defined precisely but examined under confocal microscope, has revealed that this cell line is a strong candidate for stem/progenitor cell-like property (data not shown). The same structures were not observed for MCF-7 cell line (data not shown).

CAL-51 and MDA-MB-175 displayed almost the same phenotypic profile of marker protein expression. The absence of MUC and ASMA protein expression, which represent terminally differentiation status, and the presence of CK-19 and ESA-heterogenous cell populations make these cell lines strong candidates for stem/progenitor cell-like property. These cell lines most probably have originated from progenitor cells in much less differentiated states than MCF-7, GI-101 and 734-B cell lines, therefore their origin is much closer to a stem cell in terms of mammary epithelial differentiation.

In conclusion, it can be considered that all cell lines examined in this study display, in varying degrees, stem/progenitor cell-like protein expression, with the possibility of dedifferentiation of the cell lines in in vitro conditions and the possibility of any phenotypic instability related with genomic instability is not excluded. All cell lines do not express MUC1 or ASMA, which are representatives of terminally epithelial differentiation status, showing that the cell lines have most probably originated, at least, from progenitor cells of intermediate steps of terminal mammary gland differentiation.

To further support the hypothesis that breast cancer cell lines have stem/progenitor cell property, single cell cloning of GI-101 cell line was performed to display that the heterogenous populations observed with indirect immunofluorescence with marker protein antibodies were clonal in origin. The colonies derived from a single clone were subjected to indirect immunofluorescence with marker protein antibodies of CK19, ASMA, ESA and CK18. The colonies displayed the same pattern of marker protein expression (CK-19-Het, ASMA-Het, ESA-Het, CK-18-(+)) as their ancestral cells, demonstrating that the heterogenous cell lines are clonal in origin, i.e. a single cell has given rise to different types of cells. That is, GI-101 has the properties of differentiation into new cell types and self-renewal, therefore, it can be concluded that, with some precautions, all the cell lines display stem/progenitor cell-like property.

To draw more precise conclusions about from which differentiation status the breast cell lines may have originated, more markers can be used for analysis such as Cytokeratin 5, vimentin and CALLA.

The cell lines can also be cultivated in three-dimensional contexts such as collagen, laminin and Matrigel, by performing single cell cloning. The capability of the cell lines to form structures resembling ducts or alveoli can be tested by this approach. Therefore, the differentiation potential of the cell lines is to be examined. Our preliminary studies with GI-101 cell line on Matrigel have revealed that this cell line may have differentiation potential to form ductal/alveolar structures.

Cancer, together with deregulated cellular proliferation, also originates due to blockage of differentiation of a cell. Therefore, it can be considered that the established breast cancer cell lines have defects in pathways which determine cell fates, that is, blockage of luminal and myoepithelial cellular differentiation results in breast carcinoma. The roles of Erb-B (Troyer and Lee, 2001), Wnt (Smalley and Dale, 2001), Notch (Callahan and Rafaet, 2001), Hedgehog (Lewis, 2001), TGF-β (Wakefield et al., 2001) signaling pathways and STAT (Watson, 2001) and ETS (Shepherd and Hassell, 2001) transcription factors in mammary gland development are currently being studied. The breast cancer cell lines can be studied in terms of these pathways and transcription factors so that more precise description of the origin of the breast cancer cell lines can be made.

Elucidation of the role of stem/progenitor cells in breast cancer and also their isolation would lead to the development of much more effective therapeutic approaches which target directly these cells, so that the breast cancer can be cured effectively and the relapse of the disease can be avoided.

CHAPTER VI REFERENCES

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Appendix: Referred Web Sites

http://www.imaginis.com/breasthealth/benign.asp

http://www.imaginis.com/breasthealth/breast_health.asp

http://www.imaginis.com/breasthealth/dcis.asp

http://www.imaginis.com/breasthealth/lcis.asp

http://www.imaginis.com/breasthealth/statistics.asp