THE BIOLOGICAL ROLE OF PSORIASIN IN BREAST TUMORIGENESIS

by Tamara Hitchcock

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the Degree of

Master of Science

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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For my children.

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ABSTRACT

Alteration of psoriasin (S100A7) has previously been identified in association with the transition from preinvasive to invasive breast cancer. In this thesis we examined the *in vitro* and *in vivo* effects of psoriasin in two cell line models: MCF10AT3B, a "normal" breast cell line derived from non-neoplastic fibrocystic breast tissue and MDA-MB-231, an invasive breast cancer cell line. We first developed stable transfectants, along with appropriate vector alone controls and studied them in *in vitro* cell growth and invasion assays, as well assessed them *in vivo* with respect to tumor growth, mitotic abundance and necrotic content. We found that psoriasin has no effect on in vitro cell growth and invasive behavior in "normal" MCF10AT3B and invasive MDA-MB-231 breast cancer cells. Although we could not establish the tumorigenic "normal" breast cell line MCF10AT3B into a Balb C nu/nu mouse model, we were successful in our attempts using the invasive breast cancer cell line MDA-MB-231. Data from these experiments confirmed that psoriasin does not alter tumor cell growth *in vivo*. As well, we observed that mitotic abundance and the amount of necrosis is independent of the presence of psoriasin in invasive MDA-MB-231 breast cancer cells. These results propose that psoriasin behaves similarly in "normal" and breast cancer models, nevertheless, our previous data suggests a role in the events that govern breast cancer. Specifically, although there is no evidence suggesting that psoriasin alters cell growth and invasion directly, psoriasin may govern the progression of early breast cancer due to changes in adhesion or angiogenesis.

LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
α	alpha
β	beta
μg	microgram
μl	microgram
ALS	amyotrophic lateral sclerosis
ANOVA	analyses of variance
APC	adenamatous polyposis coli
bg	beige
bp	base pair
BRCA1	breast cancer 1
BRCA2	breast cancer 2
Ca^{2}+	calcium
CIP	calf intestinal phosphatase
CO_2	carbon dioxide
DCIS	ductal carcinoma in situ
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	epithelial
ECM	extracellular matrix
EDTA	ethylenedinitroltetracetic acid
ETOH	ethanol
FAP	familial adenamatous polyposis
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
H	heart
H&E	hematoxylin and eosin

ICAM	intercellular cell adhesion molecule
LFA1	lymphocyte functioning antigen 1
LOH	loss of heterozygosity
mA	milliamp
mets1	metastasin 1
ml	milliliter
mm	millimeter
MMP	matrix and membrane type metalloproteinases
mRNA	messenger ribonucleic acid
N	normal
NCAM	neural cell adhesion molecule
nu	nude
°C	degrees celcius
P	placental
PBS	phosphate buffered saline
RPM	revolution per minute
RT-PCR	reverse transcription - polymerase chain reaction
S100A7	psoriasin
SCID	severe combined immunodeficient
SDS	sodium didoecyl sulphate
SSC	standard saline citrate
TEMED	N,N,N',N' - tetramethylethylenediamine
TGFβ	transforming growth factor beta
TIMP	tissue inhibitors of MMPs
tPA	tissue type plasminogen activator
uPA	urokinase plasminogen activator
VCAM	vascular cell adhesion molecule
w/v	weight per volume
X	times
xid	x linked immune deficient
Zeo	zeocin

Acknowledgements

To Dr. Peter Watson, thank you very much for exposing me to the exciting world of science. You have always been an astonishing teacher and I will never forget the time I have spent in your lab.

To Dr. Leigh Murphy and Dr. Bill Orr, thank you for the scientific feedback you have given me over the years. You have taught me so very much.

To Dr. Yvonne Myal, I adore and respect you immensely. I have never met a more honest, balanced female scientist than you. I admire you for that.

To Dr. Nicole Ward, my soul sister. This thesis is as much yours as it is mine. This piece of work would have never been completed if it hadn't been for your pep talks, editing and babysitting sessions weaved in between doing my laundry.

To all of the members of my laboratory, it has been an amazing experience. Not only have I learned from your scientific expertise, I have also gained life friends.

To my family. I am so privileged to have been born into a family like ours. Your unconditional love and support have made me into the woman I am today. Thank you.

To my husband and the love of my life, Gordon. You make waking up each and every day the best day of my life. Thank you again for sharing your life and dreams with me. I love you.

To my angels, my children, Bethany and Zachary. You have given my life pure joy. Our path together is just beginning, thank you for taking it with me.

1 Introduction

1.1 Breast Cancer

Female breast cancer is a major medical problem with critical public health and societal ramifications. In 2000, The National Cancer Institute of Canada published their "Canada Cancer Statistics" and proposed that 29.9% of all cancers diagnosed in females would be breast cancer (Table 1). This translates into the leading cause of cancer in Canadian women with 19226 cases of newly diagnosed breast cancer alongside 5472 women dying of the disease in that single year. (National Cancer Institute of Canada, 2000)

1.1.2 The Pathogenesis of Breast Cancer

The phenotypic appearance of breast cancer can best be described as the proliferation of malignant cells arising within the ducts or lobules of the breast. Malignant cells may eventually invade the surrounding stromal tissue and metastasize into regional lymph nodes (Saenz RB et al.,1998). A morphological model of breast cancer based on epidemiological evidence has been used in an attempt to explain the progression of the disease. This model suggests that ductal carcinoma develops from a series of increasingly abnormal cellular alterations such that epithelial tissue changes from normal breast epithelium to atypical hyperplasia, dysplasia, in situ-carcinoma, invasive carcinoma and finally into end stage metastatic carcinoma of the breast (Figure 1) (Allred DC et al.,1993).

1.1.3 Normal Breast Pathology

At birth, breast development is quite basic as ducts will elongate and branch throughout early childhood. As the influence of estrogen becomes apparent with due time, both the ducts and the supporting connective tissue will proliferate establishing a duct system that consists of approximately 20 lobes that are radially distributed around the nipple (Rubin and Farber, 2nd edition, 1994). Figure 2 shows the normal development of the breast over the lifetime of an average woman.

1.1.4 In situ Breast Cancer

Ductal carcinoma in situ (DCIS) of the breast is a complex heterogeneous pathological condition in which malignant breast epithelial cells proceed to proliferate in the milk ducts, without invading into the surrounding epithelium (Figure 3) (Fonseca R et al.,1997). If left untreated, a proportion of these tumors will evolve into invasive breast cancer. Little information is known about possible chromosomal abnormalities in DCIS, however several alterations at the molecular genetic level have been reported (Harrison M et al., 1995). For example, 46-60% of DCIS cases overexpress c-erbB2, a proto-oncogene that codes for a transmembrane protein closely resembling epidermal growth factor receptor, as compared to 20-25% seen in invasive breast carcinomas. As well, deletions in p53, a tumor suppressor gene are found to be common in the comedo necrosis type DCIS (Liu E et al., 1991; Somerville JE et al.,1992; Walker RA et al.,1991).

1.1.5 Invasive Breast Cancer

The process of invasion describes the active translocation of neoplastic cells across the basement membrane causing disruptions of the tissue boundaries between host epithelial and extracellular matrix compartments (Figure 4). Invasion is a complex, multi-step process that involves 1) loss of adhesion of cells in the primary tumor 2) increased adhesion to the extracellular matrix and basement membrane and 3) degradation of the basement membrane by proteolytic enzymes.(Rubin and Farber, 2nd edition, 1994). Frequently, a consequence of invasion is that tumor cells will eventually metastasize into distant sites by invading into blood or lymphatic vessels (intravasation), then escape from the circulation (extravasation). These cells finally implant in a foreign tissue establishing a new tumor locus (Figure 5) (Murphy GP et al., 1995).

1.1.6 Nomenclature and Frequency of Breast Cancer subtypes

Breast carcinomas are named from a combination of their histologic patterns and cytologic characteristics. For example, the invasive form of breast cancer can be described as being ductal, lobular or papillary. Table 2 depicts the relative frequency of histologic subtypes of invasive breast carcinomas as diagnosed by a pathologist (Table 2).

1.1.7 Prognostic Indicators with Respect to Breast Cancer

Many prognostic factors have been identified and are summarized in Table 3. Of those mentioned, a few indicators have been highlighted below.

At the time of diagnosis, arguably one of the most important prognostic factors in breast cancer is the stage i.e. the extent of tumor spread. Classification

of invasive breast tumors is divided into four groups ranging from stage 1 that describes a tumor with a size of 2cm or less to stage 4, a tumor that demonstrates an involvement with the chest wall or that has clearly metastasized (Lippincott Williams & Wilkins, 5th edition, 1997).

Histological grade measures both degree of glandular differentiation, mitosis and degree of nuclear atypia in terms of size, shape and number. For example, a tumor that is described as having normal nuclei with nonconspicuous nucleoli with low mitotic abundance suggests a good prognosis (Lippincott Williams & Wilkins, 5th edition, 1997).

The presence of lymphatic and vascular invasion is also an indicator of patient outcome. Tumors that show lymph node metastasis are characterized as having a poor prognosis.

The evidence of estrogen and progesterone receptors are used as biomarkers to predict a patient's response to hormonal therapy. Moreover, patients whose tumors exhibit these receptors have higher overall survival rates than women whose tumors are estrogen and progesterone receptor negative. (Table 3) (Rubin and Farber, 2nd edition, 1994)

1.1.8 Investigating Invasive Breast Cancer

Due to the concern that invasive breast cancer metastasizes when left untreated, ultimately resulting in patient death, many research efforts are presently focused at targeting invasion specific genes. Several approaches have been used including a) investigating genes that are known to be involved in other cancers and b) dissecting out the genetic differences between invasive cancer and normal/ in

situ/ metastatic tissues by using techniques such as differential display, Loss of Heterozygosity, Subtractive Hybridization, Comparative Genomic Hybridization, and microarray.

Liang and Pardee described a technique called differential display in their 1992 paper utilizing a modified polymerize chain reaction (PCR) technique. Primers are designed to allow reverse transcription, in theory of one quarter the template. Random ten mers will then be used to PCR cDNA that binds upon recognition of that specific sequence. Samples can then be loaded on a denaturing polyacrylamide gel and the results analyzed by viewing the autoradiograph. Scientists look for the presence or absence of specific bands in their control and test samples. Bands can be isolated and sequenced in order to determine the genes that are differentially expressed (Liang et al., 1992).

Loss of heterozygosity (LOH) is another approach that enables insights to be gained by studying microsatellite markers on chromosomes by PCR. Polymorphisms can be detected and chromosomal regions of control and test sample can be compared. LOH has been described at chromosome 6q in pre-invasive and early breast carcinomas, while 11q13 demonstrates LOH in microdissected *in situ* and invasive breast cancers (Chappell SA et al., 1997; Zhuang Z et al., 1995).

Another approach is subtractive hybridization. Subtractive hybridization permits two populations (tester and driver) to be compared against one another and allows the subsequent removal of all mutually expressed genes. Mammaglobin, a breast specific member of the uteroglobin family, has been shown to be

overexpressed in the *in situ* component as compared to invasive breast carcinoma by this method (Leygue E et al., 1999). Psoriasin, a member of the S100 family, is also differentially expressed in breast cancer and was discovered by this technique (Leygue E et al., 1996). This gene will be discussed at length in following sections.

Comparative Genomic Hybridization (CGH) is capable of detecting and mapping genome wide amplifications and deletions using an equimolar mixture of two populations of tester and driver labeled genomic DNA's. Material from formalin-fixed paraffin embedded tissue blocks are most frequently used, however, most recently cells from fine needle aspirates are also successful when using this technique. The most frequently observed chromosomal changes when comparing normal and tumor cell DNA are 1q+,8q+,14q-,16p+,16q-, 17p-, 17q+, 19q+, 20q+, 21q- and 22q- (Burki NG et al., 2000 and Aubele et al., 2000)

DNA chip technology, also known as DNA microarray is the newest and arguably the most exciting method of discovering differentially expressed genes. The concept behind microarray technology is the hybridization of a "probe" to thousands of well-defined cDNAs or EST's on a solid phase chip (Scena et al., 1995). An argon-ion laser evaluates the fluorescence labeled probe fragments after binding to their appropriate partners. Using this method, entire gene expression patterns between two populations are possible, dependent only upon the amount of genes supplied on each chip. An example of this system used in cancer research is demonstrated by the introduction of normal chromosome 6 into a human melanoma cell line and then applied to a DNA chip. Out of 900 genes

examined, 1.7% were down regulated and 7% were upregulated as a result of the tumorigenic phenotype (DeRisi JL et al., 1996).

1.2 Molecular Aspects of Breast Cancer

The transition of a normal breast epithelial cell into a neoplastic cell requires several intracellular molecular events to occur. Molecular alterations pertaining to tumorigenesis can be divided into three broad categories:

1. Mutations to proto-oncogenes
2. Mutations to "gate-keeper" genes
3. Mutations to "care taker" genes

1.2.1 Mutations to Proto-oncogenes

Oncogenes are defined as naturally occurring stimulatory genes that become hyperactive due to mutational changes that include deletions, duplications and amplifications. The result of this mutation is that a dominant effect occurs in the cell. In this situation, only one gene copy needs to undergo a change - the altered gene is called an oncogene, the normal allele being a proto-oncogene (Alberts B et al., 1989). Transcription factors, signal transduction molecules, growth factors and their associated receptors, and protein kinases are included in the proto-oncogene family.

In breast cancer, ErbB, ErbB2 and C-myc are examples of mutated proto-oncogenes. Breast carcinomas that express high levels of ErbB receptors and their ligands have been associated with an aggressive clinical behavior. Therapies are being directed at these receptors and have the potential to be useful anti-cancer

treatments. Specifically, a series of monoclonal antibodies directed against the Epidermal Growth Factor (EGF) ErbB receptor and the closely related HER2/Neu (ErbB2) receptor are currently under investigation (Albanell J et al., 1999).

C-myc a is nuclear DNA binding protein that is amplified in 20-30% of human breast cancers (Berns et al.,1992; Escot C et al 1986). This nuclear DNA binding protein, found on chromosome 8q24, binds sequences that control cell proliferation (Watt RA et al.,1985). Expression of c-myc amplification is a potential marker for clinical diagnosis as it may be predictive of short-term relapse free and overall survival in patients with negative nodes and/or steroid receptor positive tumors (Klijn JGM et al.,1993).

1.2.2 Mutations to "Gatekeeper" Genes

Another genetic event responsible for the changes seen in the neoplastic cell is due to alterations of tumor suppressor genes. Tumor suppressor genes are inhibitory genes that become inactivated by a mutation. In this case, both alleles must be modified to free the cell from inhibition (Alberts B et al., 1989). Frequently, loss of one of the gene alleles, known as loss of heterozygosity (LOH), begins this process. LOH is the most abundantly found mutation in primary human breast cancers (Callahan R et al., 1992). Chromosomal regions such as 11q, 13q, 16q, 17p are frequently found to show areas of LOH in breast cancer specimens (Sato H et al., 1994; Zhuang Z et al., 1995). Many other types of malignancies also display LOH and occur because of interstitial deletions, chromosome loss or faulty mitotic events occurring during recombination (Seemayer TA et al., 1989; Callahan R et al.,1993). A recent study looked at LOH on chromosome 13q using polymorphic

microsatellite markers and 139 breast tumor cases. The authors found that LOH at 13q12-q13 was associated with low progesterone receptor value, a high S phase fraction and aneuploidy. In addition, they reported a 3-4 times increase in the risk of tumor recurrence and death. Therefore, 13q12-q13 could act as a good prognostic indicator as loss of genetic material at this site appears to correlate with poor prognosis in breast cancer patients (Eiriksdottir G et al., 1998).

Some tumor suppressor genes act as gatekeeper genes (Kinzler KW et al., 1997). These genes normally control tumor growth by inhibiting cell proliferation or promoting cell death. These genes are altered when either both alleles are affected by a) two sporadic events, or b) one inherited mutation followed by a sporadic event. Classical examples of gatekeeper genes in cancer are Retinoblastoma (Rb), p53, and the Adenomatous Polyposis Coli gene (APC). The APC gene, found at 5q21, is responsible for Familial Adenomatous Polyposis (FAP). Briefly, FAP is an inherited autosomal dominant trait characterized by the progressive accumulation of thousands of polyps found particularly in the rectosigmoid region of the colon (Rubin E et al., 1994). Patients diagnosed with FAP inevitably are diagnosed with cancer before the age of 40 unless a total colectomy is performed (Rubin E et al., 1994). Vogelstein and others looked at normal colon, premalignant lesions and colon carcinoma and developed a paradigm that encompasses the clinical, genetic and biochemical events that occur in the progression of normal colon epithelial cells towards a malignant phenotype (Vogelstein B et al., 1988; Bodmer WF et al 1987; Weinberg RA et al., 1991). Briefly, as the normal colon epithelial cell becomes neoplastic, genes such as APC

and p53 are lost and the ras oncogene is activated. This results in radical alterations of both regulatory and biochemical cell functions.

1.2.3 Mutations to "Caretaker" Genes

Genes that are required to maintain genomic stability are called caretaker genes and mutations to them can eventually lead to genetic instability in gatekeeper genes. This phenomenon often results in a neoplastic cellular phenotype (Kinzler KW et al., 1997). The mismatch repair genes MLH1 and MSH2, both of which are mutated in colon cancer as well as BRCA1 and BRCA2 in breast cancer, are all examples of caretaker genes.

Although the great majority of all breast cancers are acquired by somatic mutations, approximately 5-10% of all breast cancers are hereditary (Lynch HT et al., 1984). Recent reports suggest that BRCA1 and BRCA2 mutations account for the majority of heritable breast cancers in the United States and Europe (Rebbeck TR et al., 1996). BRCA1, the first gene associated with inherited early onset breast cancer, was mapped to chromosome 17q21 by linkage analysis in 1990 (Hall JM et al., 1990). Suggested to be a tumor suppressor gene, BRCA1 spreads over 100 kb of genomic DNA and is composed of 24 exons - 22 which encode 7.8 kb of mRNA. It is estimated that over half of all inherited breast cancers have germline mutations in BRCA1 which coincidentally also increases the risk of developing ovarian cancer (Futreal PA et al.,1994). BRCA1 tumors have been found to grow more rapidly than their sporadic counterparts and BRCA1 mRNA levels are reported to decrease during the transformation from carcinoma in situ to invasive breast cancer. This suggests a role in negative growth regulation (Marcus JN et al., 1994; Thompson

ME et al., 1995). Further evidence substantiating its role in growth is that transfected fibroblasts are able to induce apoptosis (Shao N et al., 1996). It is plausable that BRCA1 gene products may be able to activate death inducing genes, repress death inhibiting genes, activate apoptosis induced proteins or target apoptosis inhibiting proteins through direct protein-protein interactions.

A second locus, BRCA2 was mapped to chromosome 13q12-q13 in 1994 (Wooster R et al., 1994). The BRCA2 gene is composed of 27 exons distributed over roughly 70 kb of genomic DNA. This gene also confers a high risk of early onset breast cancer in females, however represents only a moderately increased risk of ovarian cancer as compared to BRCA1 carriers (Yang X et al., 1999). BRCA2 also offers some insight into male breast cancers, as male carriers have a 6% lifetime risk for developing breast cancer - an astonishing 100-200 fold increased risk as compared to the general population (Weber BL et al., 1996).

1.3 Cell Growth

The ability for a breast tumor cell to divide and differentiate is crucial for its overall survival. Historically, cancer was thought to evolve from cells that exhibited features of unregulated cell growth i.e. neoplastic cells proliferate at a faster rate than normal cells. We now know, through growth kinetic experiments using tritiated thymidine, that the major fundamental component of tumor growth is that more cells are produced than die in any given time frame (Rubin and Farber, 1994)

1.4 Invasion

Invasion is thought to result from an imbalance and deregulation of what are normally stimulatory and inhibitory events (Liotta LA et al., 1991). The molecular characterization of invasion encompasses a series of molecules that include both cell surface and secreted proteins. Molecules involved include: adhesion molecules, degradative enzymes and their inhibitors, and motility stimulating cytokines (Kohn EC et al., 1995).

1.4.1 Adhesion Molecules

A course of events that include alterations of cellular adhesion to adjacent tumor cells, the extra cellular matrix (ECM) and the basement membrane initiate the invasive process. Integrins and cadherins are intimately involved in this process (Jiang WG et al., 1994). As well, the immunoglobulin super-family contains other groups of adhesion molecules involved in tumor invasion. (Maemura M et al., 1994).

Integrins are heterodimers that are covalently linked by α and β chains. Most seem to recognize an arginine, glycine, aspartate (RGD) repeat of amino acids in the matrix they bind. There are three main families within the large superfamily of integrins: 1) members that share a common β chain but differ in their α chains, 2) receptors found on blood platelets that bind matrix components and 3) receptors that are found on white cells involved with cell-cell and cell-matrix adhesion (Alberts B et al., 1989). Integrins play an important role in cell/matrix and cell/cell attachment. Integrins allow constituents of the ECM to interact with

cytoskeletal elements such as actin, talin and vinculin in order to form a link between the cell and matrix.

Alterations in integrin expression levels have been implicated with increased basement membrane adhesion in the invasive process. For example, increased expression of β1 integrins has been associated with high proteolytic activity in invasive carcinomas (Demeure MJ et al., 1992). Recently, the interaction of α5β1 integrin and oncogenic SHC proteins - which are signal substrates for most receptors and cytoplasmic tyrosine kinases involved with cell growth, transformation and differentiation have been reported to increase cell adhesion and motility on fibronectin in breast cancer cells (Mauro L et al., 1999).

Cadherins are transmembrane calcium dependent molecules that are involved in the disruption of the normally tight associations seen between neoplastic cells and their adjacent cells. Alterations in cadherins result in the ability of a cell to leave its primary site (Liotta LA et al., 1994). Cell - cell interactions are formed because cadherins link cells together at the junction of the actin component in the cytoskeleton via α and β catenins and α actinin (Cowin P et al., 1994).

Cadherin subtypes have been identified and are named for their specific tissue distribution and binding specificities: epithelial (E), placental (P), heart (H).

E-Cadherin has been shown to be an invasion suppressor gene. Decreased expression of E-cadherin has been correlated with increased invasive phenotype in the breast (Nieman MT et al., 1999). As well, expression has been shown to alter cellular morphology and increase motility in MDA-MB-435S breast cancer cells (Handschuh G et al., 1999).

P-cadherin has recently been shown to be a prognostic indicator of survival in women who are diagnosed with invasive breast cancer. Immunohistochemistry was performed on 183 paraffin embedded breast tumors and results showed that 90% of P-cadherin negative women were alive 5 years post surgery as compared to 60% of P-cadherin positive expressing tumors (Peralta Soler A et al., 1999).

H-cadherin, localized to chromosome 16q24, is a novel cadherin that is expressed highly in the heart. A link between H-cadherin expression and breast cancer has recently been shown in the literature. Matrigel outgrowth assays demonstrate that H- cadherin expressing invasive breast cells become more normal in morphology and that H-cadherin inhibits tumor formation *in vivo* (Lee SW et al., 1998). This down regulation of malignant progression may lead scientists towards a potential therapy option in the years to come.

The immunoglobulin super-family is another group of adhesion molecules involved in tumor invasion. This family can form both homo and heterophilic cell-cell interactions. Members of this family include neural cell adhesion molecule (NCAM), vascular cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM) (Maemura M et al., 1994).

NCAM's are found mainly on neural cells and function mostly as a homophilic adhesion molecule. When transfected into breast cancer cells, NCAM has been shown to promote the invasive phenotype and increase cellular motility regardless of a tumor cells E-cadherin's expression pattern (Nieman MT et al., 1999).

VCAM-1 is an endothelial cell adhesion molecule which binds the $\alpha 4\beta 1$ integrin VLA-4 on the surface of lymphocytes (Elices MJ et al., 1990). Various cytokines are able to induce VCAM expression and levels of VCAM have been seen in high amounts in breast cancer patients (Osborn L et al., 1989; Banks RE et al., 1993).

ICAM-1 is a cytokine inducible endothelial adhesion molecule that functions as an endothelial ligand for lymphocyte-functioning antigen 1 (LFA1) but is also expressed in many different cancers (Banks RE et al., 1993; Rothlein R et al., 1986). ICAM-1 might serve a function as a tumor suppressor molecule under the host immune surveillance system in cancer cells. Therefore, expression of ICAM-1 may indicate limited cellular growth, negative node involvement and potentially a good prognosis in cases of invasive breast cancer when analyzed by immunohistochemistry (Ogawa Y et al., 1998).

1.4.2 Matrix Degrading Proteases and Their Inhibitors

The single event that defines the transition of an in situ tumor to an invasive tumor is the ability for the neoplastic cells to degrade the basement membrane and neighboring stroma. Following this event, the tumor cells are then able to migrate through the surrounding tissue and establish secondary tumor sites.

Penetration though the basement membrane, extracellular matrix and stroma is facilitated by the ability of the invasive cells to produce a variety of enzymes that can degrade collagen, laminin, hyaluronan, fibronectin and other proteoglycans. The overall balance between these degrative enzymes and their inhibitors will ultimately decide the fate of the stroma.

Three broad classes of matrix degrading enzymes have been described. These include matrix and membrane type metalloproteinases (MMP's), urokinase plasminogen activators (uPA) and the cathepsins.

Matrix degrading enzymes can be categorized based on the components of the extracellular matrix that they degrade. These include stromeolysins, collagenases, gelatinases, membrane- type matrix metalloproteases and enzymes such as macrophage metalloelastase and stromeolysin-3 (Puente XS et al., 1996).

Members of the matrix metalloprotease (MMP) family share protein domains that are conserved amongst them. The conserved "pre" domain is removed either by an autocatalytic approach or by mechanical separation preceding the MMP being secreted. Secretion of the mature enzyme is followed by the binding of zinc at the active site. This results in a conformational change that catalytically releases the "pre" domain from the zinc ion (Powell WC et al., 1996).

MMP 2, 3 and 9 are type IV collagenases that shows to be expressed and localized to the breast using both immunohistochemistry and in situ hybridization. Low amounts of MMP's are expressed in both normal and invasive breast samples at similar levels indicating that other mechanisms, such as enzyme activation and/or differences in the levels of proteinase inhibitors may be biologically essential factors (Lebeau A et al., 1999; Jones JL et al., 1999).

Tissue inhibitors of MMPs (TIMPs) regulate MMPs. Therefore, the overall balance between these two families of enzymes determines the amount of matrix degradation at any tissue site (Powell WC et al., 1996). Four TIMPs have been described, each having a preference for a specific MMP while exhibiting some

overlapping inhibitory qualities (Wilhelm SM et al., 1989; Howard EW et al., 1991; Leco KJ et al., 1994; Greene J et al., 1996).

TIMP-1, suggested as an inhibitory factor in apoptosis and TIMP-2 have been associated with tumor invasion and metastatic potential in several experimental models (Li G et al., 1999; Jones JL et al., 1999). In the eye, TIMP-3 is normally synthesized by the retinal pigment epithelium and is deposited in Bruch's membrane TIMP-4, localized to chromosome 3p25 in humans, has been shown to regulate both *in vivo* and *in vivo* growth of MDA-MB-435 invasive breast cancer cells (Olson TM et al., 1998). Cells transfected with TIMP-4 appear to have a decreased invasive index using the Boyden Chamber type invasion assays. Moreover, tumor growth was reduced 4-10 fold athymic nude mice (Wang M et al., 1997).

Another class of proteases important to invasion is the plasminogen activators and their inhibitors. Plasminogen activators are serine specific proteases that convert the inactive proenzyme plasminogen to plasmin (Jiang WG et al., 1994). Urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) are two examples of plasminogen activators. Tumor cells, epithelial cells and fibroblasts are all able to produce uPA. Available uPA converts plasminogen to plasmin and may alter other components of the ECM causing tumor cells degradation of laminin and fibronectin (Gandolfo GM et al., 1996). Expression of uPA is used in the clinical setting as a prognostic marker in cancers such as gastric, colorectal, renal, ovarian and breast, such that uPA levels result in a poorer prognostic outcome for the pateint (Duffy MT et al., 1999).

Inhibitors of uPA also must also be considered when looking at the invasive potential of neoplastic cells. Plasminogen activator inhibitors bind soluble uPA and block the catalytic site. Presently, clinical trials are underway to determine whether uPA and its inhibitor PAI-1 can differentiate between node negative cancers that are treated either surgically or by adjuvant therapy. The results may lead to future case management strategies in node negative cancers (Duffy MT et al., 1999).

Cathepsins are another group of proteases that degrade the ECM. Cathepsin D is an aspartyl protease that originates from the lysosome and is suspected to facilitate the invasion process in breast cancer by degrading ECM components (Rawlings ND et al., 1994; Rubin E et al., 1994). Results of several studies argue that high levels of cathepsin D show a negative prognostic indication for breast cancer patients (Foekens JA et al., 1999).

Increased expression of Cathepsin B has also been implicated in other human cancers. For example, human gliomas have been shown to express high amounts of Cathepsin B mRNA in their tumors. Moreover, *in vivo* and *in vitro* assays have confirmed a positive correlation to protein, activity, and secretion, paralleling the process of malignancy (Demchik LL et al., 1999).

1.5 Inflammatory Response and Cancer

It has been observed that malignant tumors evoke a chronic inflammatory response that is independent of necrosis and infection of the tumor. Assessment of the role of the immune response in both the development and control of breast cancer is extremely complex. To address this, studies have been performed and

have shown that in breast cancer the immune response is not a host defence reaction and perhaps can even serve to expedite the development of cancer (Stewart TH et al., 1997). The evidence supporting this claim comes from several clinical-pathological studies that correlate the concentration of lymphocytic infiltration into the tumor mass with poor prognosis. As well, breast cancer patients demonstrate similar correlations between in vitro assays of immune reactivity to tumor membranes and non-specific antigens to poor prognosis (Stewart TH et al., 1997).

Proposed mechanisms for this effect suggest inflammatory cell infiltrate production of direct and/or indirect modulators of breast cell growth. These include cytokines, peptide or steroid hormones, enzymes that are involved in steroid metabolism, as well as antibodies to growth factors or their receptors (Stewart TH et al., 1997)

An immunotherapeutic approach for the treatment of breast cancer is a relatively new endeavor. Although cytokine infusions, cancer vaccines and T cell therapy have been studied extensively in renal cell carcinoma and melanoma, the therapeutic efficacy is just beginning to be studied in breast cancer. With the recent detection of tumor specific immunity in breast cancer patients and the identification of several breast cancer antigens, such as ErbB and ErbB2, the application of immune-based therapies targeted towards the treatment of breast cancer maybe of clinical benefit (Knutson KI et al., 1999).

1.6 Chemotaxis as a Mode of Cell Motility

A critical step in the dynamic process of invasion is that tumor cells must be able to migrate across biological barriers. This movement is driven by a number of factors including tumor derived chemotactic factors, host -derived chemotactic factors, or a combination of the two (Rubin E et al., 1994; Grotendorst GR, 1984). These chemotactic factors stimulate the initiation, direction and speed of the tumor cells. For example, cells respond unmistakably to adhesion proteins, collagen peptides, growth factors, and proteolytic fragments in the laboratory (Liotta LA et al., 1986; McCarthy JB et al., 1985). Growth factor receptor mediated motility is one of the most common characteristics in tumor cells with an invasive phenotype (Wells A, 2000).

Studies with invasive tumor cells have confirmed the phenomenon of dysregulated cell motility in response to extracellular signals from growth factors and cytokines. This is a very common event seen in cell motility experiments and can be explained by the cellular environment consisting of both chemoattractants and chemorepellents.

Chemokines are a new family of cytokines that lead leukocyte migration to the tissues. Chemokines have a conserved motif of 4 cysteines that form pairs and consequently allow them to be classified into two groups - the alpha subfamily which preferentially attracts granulocytes and the beta subfamily that attracts mainly lymphocytes and macrophages. Chemokines are not recognized only for their chemotactic activity but also are involved in other biological processes such as hematopoesis, angiogenesis and anti-tumoral behavior (Martinez CM et al., 1999).

Chemotactic gradients have been artificially created in the laboratory to show that cells can move preferentially toward a certain molecule. Autotaxin, the first autocrine motility-stimulating factor to be isolated, purified and cloned is a potent motility-stimulating glycoprotein. Cells will move toward autotaxin when a specific concentration is reached (Liotta LA et al., 1994).

In breast tissue, prolactin serves a role in both cellular growth and differentiation. Currently, prolactin is thought to be a potential chemoattractant of T47, MCF-7 and MDA-MB-231 breast cancer cell lines. Mediated by the prolactin receptor, binding of prolactin initiates signal transduction pathways including GTP binding proteins such as ras and rac. Ras and rac's signal pathways are associated with alterations in the cytoskeleton and increased cell motility in tumor cells (Mans MV et al., 1999).

Osteonectin is an example of a chemotactic molecule that enhances MMP activity in both prostate and breast tissues. It has been postulated that prostate metastasis to the bone is mediated by the ability of osteonectin to promote tumor increased protease activity, cell migration and invasion (Jacob K et al., 1999).

1.7 Necrosis as a Primary Mechanism of Cell Death

Mechanisms of tumor cell death include necrosis, programmed cell death, inadequate blood supply, lack of access to vital nutrients or susceptibility to host defenses. Tissues that are examined by morphological features for evidence of irreversible cell injury are measured by the appearance of coagulative necrosis. Coagulative necrosis, seen easily in hematoxylin and eosin (H&E) stained tumor

sections, include changes in both the cytoplasm and the nucleus. When stained with H&E, the cytoplasm is more eosinophilic than usual, show small or absent nuclei, have increased intracellular volume, display dilated vesicular endoplasmic reticulum, present mitochondrial swelling and calcification and exhibit plasma membrane blebbing (Rubin and Farber, 1994).

1.8 Calcium Signaling and Intracellular Calcium Proteins

The concept of calcium (Ca2+) signaling was first introduced in studies attempting to explain skeletal muscle contraction. Diseases such as Alzheimer's and cancer have since then been linked to altered cellular Ca2+ levels. It is now well understood that Ca2+ plays a strategic role as an intracellular second messenger in many cell functions (Niki I et al., 1996).

Calcium coupled responses entail three major steps. First, a ligand must bind to a membrane receptor in order to raise intracellular Ca2+ levels. Secondly, Ca2+ binds to intracellular mediator proteins such as EF-hand proteins, which convey the message by modifying specific target proteins. Thirdly, these modified target proteins coordinate the stimulus with the correct cellular responses (Schafer BW et al., 1996).

Intracellular calcium binding proteins can be classified into three broad groups: EF hand proteins, Ca2+/phospholipid binding proteins and Ca2+ storage proteins.

1.8.1 EF Hand Proteins

EF hand proteins, first identified in carp muscle parvalbumin, are intracellular Ca2+ binding proteins involved in calcium signaling (Kretsinger RH, 1980). The EF hand domain is composed of 40 amino acids, 12 coding for the binding loop which is localized between α helix domains. Members of the EF hand family are described as being 4 handed, 3 handed or 2 handed i.e. one EF hand binds one ion of Ca2+. However, cases do exist where the numbers of Ca2+ binding ions are less than that of available EF hands. For example, yeast calmodulin, a 4 EF hand protein only binds three Ca2+ ions per molecule (Luan Y et al., 1987). Members of the 2 EF hand Ca2+ binding protein family are called S100 proteins.

1.8.2 S100 Proteins

S100 proteins are low molecular weight members of the 2 EF hand Ca2+ binding protein family found on human chromosome 1q21 (Niki I et al., 1996). Originally they were named S100 because constituents of a subcellular fraction of bovine brain thought to contain nervous system specific proteins were soluble in 100% saturated ammonium sulfate at neutral pH (Moore BW et al., 1965). S100 proteins are implicated in a variety of cell processes including growth and differentiation, metabolism, chemotaxis and regulation of kinase activity (Figure 6; Zimmer DB et al., 1995).

Of the previously described 2, 3 and 4 EF handed family members, the 2 EF hands are the most abundant. S100's were originally characterized as a group of abundant low molecular weight (10-12 kDa) acidic proteins found in highly enriched nervous tissues. Approximately seventeen different S100 proteins have been

identified to date and show homologies ranging from 25-65% at the amino acid level (Schafer BW et al., 1996).

1.8.3 Regulation of S100 Expression

Determining the mechanisms that regulate S100 gene expression in both normal and disease states is of great interest as S100 proteins have been implicated in diseases such as cancer, Alzheimer's, and Down's syndrome (Zimmer DB et al., 1995).

Organization of S100 genes at the intron/exon borders is highly conserved. The first exon is not translated in any of the S100 genes and the following two exons code for amino and carboxy terminal EF hand calcium binding domains. The only exception to this phenomenon is the S100A5 gene where it is organized into four introns and three exons (Polans AS et al., 1994).

S100 nucleotide sequences from coding regions and their subsequent amino acid sequences are highly conserved between species. Human and rat S100B share 85% nucleic acid sequence in the protein-coding region with variation in only two amino acids (Allore RJ et al., 1990; Jiang H et al., 1993). However, promoter regions of S100 genes and their 5' and 3' untranslated regions of their respective mRNAs can be quite different. This may explain possible differences in S100 gene expression with respect to distinct regulatory mechanisms they possess (Zimmer DB et al., 1995).

1.8.4 Tissues Expressing S100 Proteins

S100 proteins are expressed in many tissues including those of the reproductive system, nervous system, gastrointestinal system, respiratory system,

urinary system as well as muscle, skin and adipose tissues (Schafer BW et al., 1996). Nearly all S100's are confined to their own distinct cell type. For example S100A1 is expressed in neurons, heart and muscle, whereas S100A8 is expressed in granulocytes and monocytes (Zimmer DB et al., 1995). Occasionally, several different S100s can be expressed in one cell type but at different levels. For instance S100A4, S100A6 and S100A7 have all shown to be expressed in human breast cancer (Leygue E et al., 1996; Ilg EC et al., 1996).

1.8.5 Functions of S100 Proteins in Disease

As previously stated, S100 proteins are implicated in a number of diverse cellular processes and disease states. Briefly, the functions of some more notable S100 proteins are highlighted below.

S100A1 proteins are expressed in neurons, skeletal, heart and kidney cells and involved in the release of Ca2+ from the sarcoplasmic reticulum, inhibition of phosphokinase C mediated phosphorylation and inhibition of microtubule assembly.

S100 proteins A2-A7 have been implicated in cancer and metastasis. Expressions of these genes are seen mostly on epithelial cells and fibroblasts. S100A7-A9 has also been found to be chemotactic (Shaefer BW et al., 1996).

Sites of inflammation and patients diagnosed with cystic fibrosis will express S100A8 and S100A9 on their granulocytes and monocytes. These two genes can form heterodimers with one another and inhibit casein kinase (Shaefer BW et al., 1996).

S100A10, expressed in fibroblasts, is involved with the process of exocytosis and endocytosis.

S100B can homodimerize with itself or heterodimerize with S100A1. This gene is expressed in adipocytes, melanocytes, chondrocytes, glial cells and schwann cells. Diseases such as Alzheimer's, Down's syndrome, Amyotrophic Lateral Sclerosis (ALS) and epilepsy all exhibit S100B in their genotypes.

Other S100s such as calbindin 3 is associated with transport function, and profilaggrin and trichohyalin are involved with cell structure regulation (Schafer BW et al., 1996).

1.8.6 S100 Proteins in Cancer

Several members of the S100 gene family have been implicated in the progression of cancer, including S100A4, S100A6 and S100A7.

S100A4, also known as mts1 or metastasin, has been studied extensively and has been shown to regulate cell cycle progression by isolating and disabling the p53 suppressor protein that controls the G1-S transition of the cell. In addition, S100A4 controls both the invasive and metastatic properties of the cell, possibly through remodeling the ECM. Moreover, S100A4 also appears to play a role in extracellular signal transduction such that cell growth pathways are involved. In carcinoma of the breast, S100A4 correlates with the metastatic spread of cancer to the regional lymph nodes (Sherbet GV et al., 1998).

S100A4 (MTS1) calcium binding protein has specifically been implicated in motility as well as cancer growth, invasion and metastasis (Sherbert GV et al., 1998). Cell motility experiments have shown that S100A4 has the ability to interact with non-muscle myosin. Specifically, S100A4 is highly expressed during embryonic development in the highly invasive mesenchymal elements (Klingelhofer

J et al, 1997). Cell adhesion and the invasive potential of cells are also regulated by S100A4. CD44, a membrane associated adhesive glycoprotein which mediates adhesion by virtue of its function as a hyaluronate receptor, has been attributed with the ability to enhance invasive ability (Merzak A et al., 1994). In a recent study of B16 melanoma cells, S100A4 expression was upregulated and CD44 expression was assessed (Lakshmi MS, 1997). Instead of finding an increased CD44 expression, the authors found that CD44 was redistributed into a "patchy focal pattern". They proposed that this could be a result of cytoskeletal depolymerization that provides tumor cells with discrete and much stronger adhesive foci that promotes invasive behavior by increasing and strengthening anchorage and intercellular interaction (Lakshmi MS, 1997). S100A4 has been recorded in high levels in human as well as murine tumors of high metastatic potential (Ebralidze A et al., 1990). In the B16 murine melanoma, S100A4 expression can be modulated altering metastic behavior (Parker C et al., 1991). Conversely, transfection of antisense constructs has resulted in the reduction of metastatic potential (Grigorian MS et al., 1994). This has been confirmed in further experiments of highly metastic Lewis Lung carcinoma cell lines in which introduction of antisense S100A4 RNA has proved to confer very low metastatic potential (Takenaga K et al., 1997).

In breast cancer, increased levels of S100A4 gene expression correlate strongly with the potential to metastasize to axillary lymph nodes. As well, S100A4 expression is inversely correlative with estrogen and progesterone receptor status, suggesting that expression of S100A4 might also reflect the degree of aggression (Takenaga K, et al., 1997 and Albertazzi E et al., 1998).

Melanoma cells commonly overexpress S100A6. Two hybrid analyses have recently shown that S100A6 proteins isolated from melanoma cell lysates can either homo-dimerize with themselves or heterodimerize to S100B proteins. Further study is necessary in order to understand the implications of this dimerization (Yang Q et al., 1999). S100A6 also shows a potential value as a marker in the clinical management of melanoma as it correlates to the overall survival time and thickness of the primary tumor (Maelandsmo GM et al., 1997).

1.9 Psoriasin

Psoriasin (S100A7) is the most abundant protein found in psoriatic skin and is located on human chromosome 1q21 (Borglum AD et al., 1995). Isolated from noncultured unfractionated keratinocytes, psoriasin belongs to the S100 family and is composed of 101 amino acids with a molecular mass of 11,457 daltons and a pI of 6.77 (Celis JE et al., 1990; Madsen P et al., 1991; Hoffmann HJ et al., 1994). Interestingly, the peptide sequence of psoriasin shows no significant homology to any other protein (Borglum AD et al., 1995). Unlike other members of the 2 EF hand family, the structure of psoriasin suggests that the N-terminal EF hand motif contains a distorted loop. The consequence of this deformity yields the elimination of a calcium-binding residue (Brodersen DE et al., 1998). A zinc-binding site has also been discovered, formed by three histidines and an aspartate residue. These residues coordinate zinc similarly to those shown by certain metalloproteases, in particular the collagenases (Brodersen DE et al., 1999).

Psoriasin is also evident in bladder squamous cell carcinomas. Psoriasins presence may be a useful marker for early detection and/or to identify tumors that exhibit differing degrees of differentiation (Celis JE et al., 1996 and Ostergaard M et al., 1997, 1999).

Psoriasin appears to be a potent and selective chemotactic inflammatory protein for neutrophils and CD4+ T lymphocytes at concentrations at $1x10^{-11}$ M. Interestingly, psoriasin is not structurally related to either the alpha or beta chemokine subfamilies, neither to lymphotactin, a member of a newer class of chemokines. This suggests that psoriasin may be a chemotactic protein that lies outside the traditional chemokine subfamilies and may be an important new inflammatory mediator (Jinquan T et al., 1996).

Current information suggests a role for psoriasin in the pathogenesis of psoriasis, hematopoietic cell chemotaxis and in the early progression of breast cancer (Watson PH et al., 1998).

1.9.1 Psoriasin Expression in Human Tissues

Expression of psoriasin in tissues other than skin and human breast tumor has been discovered. Expression of psoriasin mRNA has also been reported in brain, heart, kidney, lung, skeletal muscle, stomach, thymus, fibroblasts and neuroblasts (Zimmer et al., 1995)

1.9.2 Psoriasin is Differentially Expressed in Breast Cancer

Moog-Lutz reported in 1995 that psoriasin was found to be expressed in breast cancer cell lines and in cancer cells of some breast carcinomas (moog-Lutz et al, 1995) Using a modified subtractive hybridization technique, Leygue et al

discovered that psoriasin was differentially expressed in human breast cancer (Leygue E et al., 1996). Later investigations demonstrated that psoriasin was constitutively more highly expressed in the in situ component of breast cancer than the invasive component of the same breast tumors by in situ hybridization (Leygue E et al., 1996).

1.9.3 Psoriasin Expression in Invasive Breast Carcinoma

Preliminary data from our laboratory suggested that psoriasin is differentially expressed within the invasive carcinoma group of breast lesions. To further investigate this phenomenon, a cohort of fifty-seven invasive ductal carcinomas and twenty-three other invasive tumor types (lobular, mucinous, medullary, tubular) were analyzed by RT-PCR and western blot. Results proved that higher levels of psoriasin were significantly associated with markers of poor prognosis, such as negative estrogen and progesterone status alongside nodal metastasis. Psoriasin expression was also correlated to the degree of host inflammatory cell response, which implicates this gene as a chemotactic factor (Al-Haddad S et al., 1999).

1.10 Animal Models of Human Breast Cancer

Several rodent breast cancer models have been recently developed although limitations still exist (Clarke R, 1996). For instance, humans are not mice and although we share considerable homology to one another, there is enough variation between our species that differences are visible in terms of pathology and genetic presentation. It is because of this that a working model of human breast cancer is very important.

In order to study human breast cancer, it is essential to find a model that will allow the introduction of human breast cells without the concern of graft rejection. The most widely used is the use of the immune-deficient athymic nude (nu) mouse. Nonetheless, other immunocompromised models also exist including the use of the beige (bg) mutation, a combined bg/nu mutations, an X-linked immune-deficient (xid) mutation, a combined bg/nu/xid mutations and lastly the severe combined immune deficiency (SCID) mutation.

Pantelouris first described the nude phenotype, named as such due to their lack of hair, in 1962 (Pantelouris EM, 1962). Mutations at the nu locus on mouse chromosome 11 resulted in a nonfunctional thymus, subsequent T cell depletion, and defective B-cell maturation. Interestingly, natural killer cell activity remains high in the nude mouse even when compared to a normal or heterozygous nu/+ mice of the same background (Clarke R, 1996). These mice are popular with scientists as they support engraftment and growth of many tumor types, have unequaled visualization of inoculation site and demonstrate a docile nature. Unfortunately, this mouse is considerably expensive and can be difficult to acquire, as breeding is more difficult.

The bg mutation in a mouse produces a block in natural killer cell activity as well as functional defects in T-cells, macrophages and granulocytes (Roder JC et al., 1979; Schultz LD, 1989). Located on mouse chromosome 13, a phenotype comparable to Chediak-Higashi syndrome in humans is present. These mice also have problems with blood clotting due to dysfunctional platelets(Clarke R, 1996).

The phenotype of the combined bg/nu mutation corresponds to the predicted phenotype one might assume. In this mouse, the bg mutation eliminates natural killer cell activity while the nu mutation depletes the T and B cell populations. While more immune-deficient as a combined mutated mouse, bg/nu mice retain the clotting disorder produced by the bg mutation (Clarke R, 1996).

B cell impairment is the major effect of the xid mutation (Kincade PW, 1977). Both males and heterozygous females are affected yet respond to thymus-independent type 1 and 2 antigens. Hence these mice are regarded as partially immune-compromised.

Combined bg/nu/xid mutations became possible upon the availability of a homozygous bg/nu mouse. Subsequent mating with a xid mouse eventually generated the NIH III mouse that is homozygous for all three mutations (Andriole GL et al., 1985). These mice are natural killer cell, T-Cell, B-cell and macrophage depleted however the clotting disorder from the bg mutation remains.

SCID mice are the most recent additions to the list of immune deficient rodent models (Bosma GC et al., 1983). The mutation occurs on mouse chromosome 16 and is equivalent to the human mutation occurring on chromosome 8q11 (Komatsu K et al., 1995). These mice are B and T cell depleted, however by 10-14 months of age small numbers of functional B and T cells are present deeming the model "leaky" at that time (Mueller BM et al., 1991). Macrophage and natural killer cell levels are essentially normal in SCID mice. Levels of natural killer cells can inhibit the engraftment of human tumors, however pre-treatment with gamma irradiation or etoposide is commonly used to solve the

problem (Williams SS et al., 1993; Visonneau S et al., 1998). These mice can also accept grafts such as osteogenic sarcoma and T cell lymphoma that nude mice cannot (Williams SS et al., 1993). In general, SCID mice are the most severely immune compromised compared to any other single gene mutated model. These mice are readily available and relatively inexpensive, resulting in their widespread use.

1.11 Breast Cancer Cell Lines

Established breast cancer cell lines offer scientists the tools to gain insights about a disease in an *in vivo* setting where the manipulation of the environment is simple and straightforward. However, care must be taken when in interpreting results, as the *in vitro* system is artificially contrived as compared to an *in vivo* setting. A more appropriate method of studying human breast cell lines is one where they are injected (xenotransplanted) into immunocompromised mice. This approach more closely reflects actual biological events (Clarke R, 1996).

Cell lines can be chosen that best imitate characteristics of the type of breast cancer to be studied. For the course of this thesis the MDA-MB-231 and MCF10AT3B cell lines will be used.

1.11.1 MDA-MB-231 Invasive Breast Cancer Cell Line

The MDA-MB-231 cell line originated from a 51-year-old woman that presented with an adenocarcinoma of the breast. Breast cancer epithelial cells were recovered from a pleural effusion and expressed epidermal growth factor (EGF) and transforming growth factor (TGF). These cells were estrogen receptor

negative, and highly invasive both in *in vitro* and *in vivo* settings (http://www.atcc.org). Following intrarvenous injection, MDA-MB-231 cells were observed in the lungs and bones (Fraker LD et al., 1984).

MDA-MB-231 cells have been demonstrated to show an increased expression of α6 integrin, following interaction between these cells and the ECM. Animal experiments have confirmed this relationship - as α6 integrin expression increases in MDA-MB-231 tumors so do their tumorigenicity and metastatic potentials (Mukhopadhyay R et al., 1999).

Suppression of tumorigenicity and metastasis has also been illustrated using MDA-MB-231 cells. A soluble truncated transforming growth factor beta (TGFβ) receptor has been reported to substantially lower tumor growth rates and eliminated spontaneous metastases as compared to control groups (Bandyopadhyay A et al., 1999).

1.11.2 MCF10 series of Normal Breast Cell Lines

The development of a cell line that imitates *in vivo* breast cancer progression from that of a normal phenotype to that of an invasive would be the finest tool available to scientists. The MCF10 breast cell line series is perhaps the closest we have achieved to finding a useful model to study the progression of early breast cancer.

Soule and colleagues characterized a spontaneously immortalized human breast cell line taken from a 36-year-old female fibrocystic breast disease patient (Soule HD et al., 1990). These MCF-10 cells maintained the phenotype of normal breast epithelial cells such that they a) lack tumorigenecity in immune-deficient

mice, b) lack three dimensional growth in collagen, c) lack a biological response to hormone and growth factors, and lastly d) they lack of anchorage dependent growth and dome formation in confluent cultures (Soule HD et al., 1990).

MCF-10 cells were later stably transfected with a point mutated T24 c-Ha-ras expression vector, renamed MCF10AT, and were found to exhibit an altered pattern of growth and morphology in 3-D collagen gels. As well, these cells became tumorigenic over time following transplantation in irradiated nude mice, resulting in tumors that exhibited an invasive phenotype (Basolo F et al., 1991). MCF10AT cells now routinely produce lesions in immune deficient mice, imitating the morphology of human proliferative breast disease, and in situ and invasive breast cancer (Dawson PJ et al., 1996). Later it was found that the activated c-Ha-ras was not solely responsible for the presentation of the preneoplastic phenotype (Miller FR et al., 1989).

Further work on the MCF10AT cells involved their injection into immune deficient mice with the developing lesions being subsequently transplanted into Balb C nu/nu mice. Multiple transplant generations were performed with the resulting lesions including mild hyperplasia, moderate hyperplasia, atypical hyperplasia, in situ carcinoma and invasive carcinoma (Dawson PJ et al., 1996). The last transplant generation, named MCF10AT3B was the most successful and the cell line that we chose in our study.

2 Rationale and Hypothesis

As previously discussed, invasion is an extremely complex process that requires a number of genes, including those that code for adhesion molecules, proteases and motility factors. Invasion necessitates the neoplastic cell to display altered adhesive properties towards the extracellular matrix and basement membrane, as well as exhibit proteolytic activity.

Psoriasin is a member of the S100 family of genes, the proteins which are Ca^{2+} binding molecules with 2 EF hand motifs and has been implicated in the pathogenesis of breast cancer. In our laboratory, initial studies of normal, in situ and invasive carcinomas demonstrated that loss of psoriasin expression was associated with poor prognosis tumors. This result suggested that altered expression of psoriasin may be functionally involved in the process of invasion. Our initial hypothesis was that loss of psoriasin facilitated the process of invasion. In order to elucidate this further we proposed to select a highly invasive cell line that did not express psoriasin. MDA -MB-231 was chosen to be transfected with psoriasin and in vitro and in vivo growth and invasion studies were performed. We anticipated that the introduction of psoriasin into MDA-MB-231 would decrease cell growth and decrease in invasion.

Our initial psoriasin transfection experiments failed to find an effect on in vitro growth and invasion. At the same time, subsequent RT-PCR studies in our laboratory were being performed that included ductal carcinoma in situ (DCIS) as well as a diverse range of invasive tumors. Results from these studies indicated

that psoriasin can be highly expressed in DCIS tumors that are associated with invasive tumors as well as those tumors who are classified as having a poor prognosis (Al- Haddad et al., 1999). This led us to change our hypothesis. We believed now that high psoriasin expression would be associated with a highly invasive phenotype because the presence of psoriasin in a DCIS tumor suggests it may be a positive factor in the invasive process. To test this hypothesis we stably transfected a noninvasive "normal" cell line, MCF10AT3B, with psoriasin. We wanted to determine if these cells could show increased characteristics of invasiveness in vitro and in vivo as well as an increased progression towards the invasive phenotype in vivo.

3 Methods

3.1 Tissue Cell Culture

The MDA-MB-231 (ER- human breast cancer cell line, adenocarcinoma, Mundy G laboratory) was grown and maintained as a monolayer culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 units/ml penicillin, 10 units/ml streptomycin, 30% (weight/volume) glucose and 2mM final concentration L- Glutamine. MCF10A1 (immortalized normal breast cell line, fibrocystic breast) and MCF10AT3B (immortalized mammary cell line; generously provided by Fred Miller and Gloria Heppner, Michigan Cancer Foundation, Detroit, Michigan, USA) were grown and maintained as monolayer cultures in DMEM supplemented with 10% equine serum, 0.1μg/ml cholera enterotoxin (Sigma Chemical Co.), 10μg/ml insulin, 0.5 μg/ml hydrocortisone (Sigma Chemical Co), 0.02μg/ml Epidermal growth factor (UBI), 10 units/ml penicillin and 10 units/ml streptomycin. With the exceptions noted, all of the above reagents were purchased from GibcoBRL. All cells were grown at 37^0C, in 5% carbon dioxide and 100% humidity.

3.1.1 Passaging Cell Lines

Cell lines were grown to ~80% confluence in a T_{150} flask and harvested in the following manner: medium was aspirated off, cells were rinsed with 5mls of 1X phosphate buffered saline (PBS; Amresco), PBS was aspirated off and 2mls of 1X trypsin/EDTA (ethylenedinitroltetracetic acid; TE) (GibcoBRL) was added and incubated at 37^0C, 5% $C0_2$, 100% humidity for approximately 3 minutes. Cells harvesting was completed by adding 8 ml of supplemented DMEM to the

cells-TE suspension, and drawn up and down through a 10ml glass pipet in order to suspend the cells evenly and completely. A 1:10 volume of the cell suspension was used to maintain the culture in a new T_{150} flask.

3.1.2 Freezing Cell Lines

Cells were frozen down for storage in liquid nitrogen by using a cell-freezing medium consisting of 10% dimethylsofoxide (DMSO), 10% serum and 80% supplemented DMEM. Cells were split as described above, spun down at room temperature and pelleted at 500 rpm for 5 minutes. Cells were resuspended in a solution of 1.5 ml of freezing medium and 1.5 ml of supplemented DMEM and aliquoted into 3 cryovials of 1 ml each final volume. Cells were placed in a styrofoam insulated box over night at -70^{0}C and placed into liquid nitrogen the following day.

3.2 Cloning

3.2.1 Restriction enzyme digest

A 373 bp fragment of Psoriasin was cut out of a PCR II TA vector, previously cloned in Dr. Leigh Murphy's laboratory, University of Manitoba, using a restriction enzyme digest. Briefly, 10 μg of plasmid DNA was cut using 20 units EcoR1, 2mg/ml RNAse A, 10X React 3 buffer in a final volume of 50 μl. The DNA was cut for one hour using a temperature of 37^{0}C. DNA was precipitated using 1:10 volume 3M Sodium Acetate, pH 5.2 and 2X volume cold 100% ethanol (ETOH) and placed in the -20^{0}C freezer overnight. The sample was pelleted at 4^{0}C, 12500 rpms for 15 minutes, liquid removed, rinsed with 70%

ETOH, dried for 10 minutes and finally resuspended in 38.5μl sterile water. To prevent the fragment from re-ligating onto itself, 5 μl 10X Calf Intestinal Phosphatase (CIP) buffer, 2 μl CIP was added to the resuspended DNA to a final volume of 50μl and incubated for 30 minutes at 37^0C. The mixture was then washed with a 1:1 volume of phenol chloroform, mixed thoroughly, and spun down at 12500 rpm for 90 seconds. The top layer was precipitated using sodium acetate and ethanol as mentioned above and resuspended in 10 μl of sterile water.

3.2.2 Excision of Psoriasin from Agarose Gel

Psoriasin was isolated from a 0.7% agarose gel by separating the DNA fragment of interest from the vector (~120 V for 30 minutes). An incision in the gel was made below the fragment and a 3MM Whatman paper was placed in front of a piece of dialysis tubing and was inserted into the cut gel. The gel was run for an additional 15 minutes to allow for the DNA fragment to run into the paper. DNA was eluted from the paper by adding 100μl elution buffer (200 mM NaCl, 50mM Tris pH 7.5, 1mM EDTA pH 8, 0.1% 10%SDS) three times into a 0.5ml tube containing the paper and swirling the paper around the elution buffer. A hole was made in the bottom of the tube and was inserted into a second larger 1.5 ml tube to collect the DNA containing elution buffer. These tubes were spun down at 2000 rpm for 10 seconds. The solution was then precipitated using sodium acetate/ethanol and resuspended in 10μl TE.

3.2.3 Ligation of Psoriasin into pcDNA3.1/Zeo

pcDNA3.1/Zeo (Invitrogen) was linearized, precipitated, and verified using the same methods as above. The isolated Psoriasin fragment was then cloned into the vector in the following manner: 1μl vector , 1μl Psoriasin fragment (25 ng DNA), 2μl 5Xligation buffer, 0.25μl T4 ligase in a total volume of 20 μl and incubated overnight at 12^{0}C. The following morning, the ligation was precipitated using sodium acetate/ethanol and was resuspended in 10μl TE.

3.2.4 Electroporation into DH5α competent cells

DH5α competent cells (Gibco) were used to accept DNA from the ligations. Briefly, 1μl of purified ligation product was added to 10μl of competent cells and placed into a 0.2μm electroporation vial. This vial was placed into the electroporation apparatus and charged to 1.25 V, 25 Farads and 400 ohms. A volume of 1ml SOC was added to the electroporation vial and cells were transferred to a 1.5ml tube and incubated at 37^{0}C for 30 minutes. Cells were then plated on LB plates with selection for Carbenicillan and incubated overnight at 37^{0}C. Colonies were picked for miniprep analysis after ~24 hours.

3.2.5 Plasmid DNA preparation - Miniprep analysis

Colonies were randomly chosen and placed into 2ml cultures of LB/carbenicillin (20mg/ml) and grown overnight at 37^{0}C with shaking. Cultures were transferred into a 1.5ml microfuge tubes and spun at 12500 rpm for 45 seconds to pellet the bacteria. The remaining 0.5ml culture was placed into the fridge for future use. Media was poured off the pellet and bacteria was suspended in 100μl of solution 1 (50mM glucose, 50mM Tris-HCl pH8, 10mM

EDTA), vortexed and placed at room temperature for 5 minutes. To this, 200 μl of solution 2 (1% SDS in 0.2M NaOH) was added and quickly mixed by inverting the tube twice, followed by adding 150μl of solution 3 (3M KOAc, 11.5 v/v glacial acetic acid) Once mixed well, the solution was incubated on ice for 10 minutes, followed by 5 minutes centrifugation at 12500rpm at 4^0C. The supernatant was then placed into a fresh microfuge tube and an equal volume of phenol/chloroform was added. The contents were well mixed and centrifuged for 90 seconds at 12500rpm at room temperature. The aqueous layer was transferred to a new tube and DNA was precipitated by adding 2 volumes of 100% absolute ethanol and placed in the -20^0C overnight. The following morning, tubes were centrifuged for 15 minutes at 12500rpm at 4^0C to pellet the DNA. The supernatant was poured off and the pellet was rinsed with 70% ETOH, air dried for 10 minutes and resuspended in 50μl TE. Both restriction enzyme digests and DNA sequencing were performed on miniprepped DNA, confirming the effective ligation of pcDNA3.1/Zeo/Psoriasin (pcDNA3.1/Zeo/Psor). One bacterial clone consisting of pcDNA3.1/Zeo/Psor was used to generate larger amounts of plasmid using the Qiagen Mega prep kit (Qiagen). Clones were sequenced to determine forward psoriasin orientation.

3.2.6 Large scale plasmid preparation

Plasmid DNA for use in future stable transfections was generated using the Mega Plasmid kit (Qiagen). Using this method, a bacterial clone confirmed to contain pcDNA3.1/Zeo/Psor was grown overnight at 37^0C in 5ml LB containing 20 mg/ml carbenicillan. This culture was used to inoculate a 500ml

LB/carbenicillan flask and left to grow overnight, shaking at 37^{0}C. The bacteria were pelleted and resuspended in 50ml Buffer P1. To the suspension, 50ml Buffer P2 was added and incubated at room temperature for 5 minutes. Next, 50ml of chilled Buffer P3 was added, and solutions were mixed and left to incubate on ice for 30 minutes. The supernatant was recovered by centrifuging the solution at 12000 rpm for 30 minutes at 4^{0}C. This was centrifuged a second time at 12000 rpm for 15 minutes at 4^{0}C. The supernatant was loaded onto the Qiagen-tip 2500 column, washed with 200ml Buffer QC and eluted with 40ml Buffer QF. DNA was precipitated with 0.7 volume of room temperature isopropanol and pelleted by centrifuging at 11250 rpm for 30 minutes at 4^{0}C. The pellet was washed with 7ml 70% ethanol, air dried and redissolved in 200µl TE.

3.3 Stable Transfection

3.3.1 Stable Transfection method

MDA-MB-231 stable transfectants were generated in Dr. Leigh Murphy's laboratory using the calcium phosphate transfection method and were generously provided to our laboratory. MCF10AT3B stable transfectants were created using the Qiagen Superfect kit according to the manufacturers instructions. The day before transfection, cells were seeded to approximately 4x10^5 and grown in the absence of antibiotic in a 60mm dish. Cells were incubated at 37^{0}C, 5% $C0_2$ overnight. The following day the dishes were 20% - 40% confluent. Cells adherent to 60mm dishes were washed with 4mls of media

in the absence of serum and antibiotics. Five micrograms of plasmid DNA were dissolved in TE (pH 7.4) with cell growth media containing no serum or antibiotics to a total volume of 150μl. To the suspension, 30μl of Superfect transfection reagent was added and mixed by pipetting up and down 5 times. The solution was then incubated for 5-10 minutes at room temperature, allowing for complex formation to occur. Next, 1ml of cell growth media (containing serum and antibiotics - 400μg/ml Zeocin, 10 U/ml PenStrep (GibcoBRL) was added to the reaction tube that contained the transfection complexes. Pipetting up and down twice mixed the contents in the tube, and the entire volume was immediately transferred to the 60mm dish. The dish was incubated for 2-3 hours at 37^0C, 5% $C0_2$ and the media containing the remaining complexes was removed from the cells with gentle aspiration. Cells were washed once with 4ml of PBS and new cell growth media containing antibiotics and serum was added to the dish. Media containing serum and antibiotics was used until non-transfected cells died and colonies of transfected cells could be visualized.

3.3.2 Cloning the transfectants

After removing the media, Whatman paper disks of approximately 0.5 mm diameter were soaked in 1X Trypsin/EDTA (GibcoBRL) and applied to the colonies on the 100mm for about 60 seconds. Using forceps, the disk and colony were placed into a well on a 24-well dish containing 500μl of supplemented DMEM containing 400μg/ml Zeocin. The clones were systematically expanded in order to freeze down cell stocks and pellet for RNA/protein analyses.

3.4 RNA Extraction

RNA was extracted using the Tri-Reagent protocol (Molecular Research Centre Inc, OH), is based on the original protocol of Chomczynski and Sacchi (1987). Normally, 1ml of Tri-Reagent was used per 20-100mg of cells or per 100mm^2 culture dish. After addition of Tri-Reagent, pellets were homogenized on ice for 15-30 seconds and incubated at room temperature for 5 minutes. Then 200µl of chloroform was added to the samples, which were shaken and left at room temperature for an additional 5 minutes. The samples were centrifuged at 12000 rpm for 15 minutes at 4^{0}C, and the clear, aqueous phase containing the RNA was transferred to a new tube. Precipitation of the RNA occurred by adding 500µl of isopropanol and incubating the samples at 4^{0}C for 30 minutes, then centrifuging at 12000 rpm for 10 minutes at 4^{0}C. The subsequent pellet was washed with 75% ethanol and centrifuged at 7500 rpm for 5 minutes at 4^{0}C. After briefly air-drying the pellet for 5 minutes on ice, the RNA was dissolved in 30µl diethylpyrocarbonate (DEPC) treated; (Sigma) sterile water.

3.5 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RNA (200ng) was reverse transcribed using 5X RT buffer, 2.5mM of each nucleotide, 0.1% bovine serum albumin, 0.1M dithiothreitol (DTT), 10% DMSO, and 200 units/µl MMLV reverse transcriptase (Gibco BRL) in a final volume of 20µl.

For PCR using psoriasin, estrogen receptor, or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers, 2µl of the above cDNA was used in the

PCR mixture, which consisted of: 10X PCR buffer, 25mM magnesium chloride, 2.5mM of each nucleotide, 10% DMSO, 5units Taq polymerase (Promega), and 50mM of each primer, in a final volume of 50μl. DNA was amplified for 35-45 cycles in a Perkin Elmer Thermal cycler after an initial 10 minute denaturing step at 94^0C using the following program: 45 seconds at 94^0C, 45 seconds at 55^0C, then 90 seconds at 72^0C. The PCR was completed by a 15-minute primer extension step, before cooling down to 4^0C permanently.

For studies using RT-PCR, 35 cycles were used for GAPDH, 40 cycles for estrogen receptor and 45 cycles for psoriasin in order to keep expression in the linear range of PCR amplification. Cycle number was derived by cycle saturation experiments done by previous members of our laboratory.

3.6 Northern Analysis

3.6.1 Sample Preparation

40 μg of total RNA was diluted in a volume of 10μl using DEPC-treated distilled water. To this dilution, 10μl of formamide (Sigma), 2.73μl of gel running buffer (5X GRB: 0.2M MOPS pH 7.0, 50mM sodium acetate, 5mM EDTA pH 8.0) and 4.73μl of formaldehyde was added (all done on ice), the samples heated at 65^0C for ten minutes, then cooled on ice.

3.6.2 Gel preparation and running of samples

A Northern gel was comprised of a 1% (w/v) agarose gel containing 18% formaldehyde and 20% GRB. The samples were loaded using 3μl of a loading

buffer (0.25% bromophenol blue, 0.25 xylene cyanol, 30% glycerol) and run overnight at 40 volts. The following morning, the gel was rinsed three times: 30 minutes in1% (w/v) glycine, 20 minutes in 0.05N sodium hydroxide, and 40 minutes in 20X standard saline citrate (SSC). The gel was transferred overnight onto a Zeta probe membrane (BioRad) using 10X SSC. The membrane was then baked at 80^0C for 30 minutes and ready to probe.

3.6.3 Probe Synthesis

The radiolabelled probe used for the Northern blots was synthesized from a 373bp genomic DNA template using PCR techniques by the RadPrime method (GibcoBRL) for psoriasin specifically. Briefly, 25ng of DNA was dissolved in 20 μl of sterile TE, boiled for 5 minutes and cooled on ice. 1μl of each nucleotide (excluding the labeled one used), 20μl of 2.5x Random Primer solution and 5μl of dCTP ^{32}P were added to the mixture. Lastly, 1μl of Klenow was added and the solution was incubated at 37^0C for 10 minutes, following which 5μl of stop solution (0.5M EDTA, pH 8.0) was added. The now-labelled probe was eluted using two 400μl volumes of TE buffer. The probe was denatured by boiling for 5 minutes, cooled on ice immediately, and then applied to the membrane in hybridization buffer overnight after blocking the membrane in a prehybridization solution overnight.

3.6.4 Washing of blot

The blot was washed with 1x SSC, 0.2% SDS for 20 minutes followed by

A 10 minute wash of 0.2x SSC, 0.1% SDS. The membrane was then placed in a sealed bag and x-ray film was placed on top and developed after being exposed for 7-10 days at -70^{0}C.

3.7 Western Blot analysis

3.7.1 Preparation of samples

Protein was extracted from either cell pellets or tumors using 200μl SDS-isolation buffer (50mM Tris pH 8.0, 20 mM EDTA, 5% SDS, 5mM b-glycerophosphate, 1mM AEBSF, 5mg/ml aprotenin). Cell lysates were passed through a 23-gauge needle to shear DNA and centrifuged at 13000 rpms for 20 minutes at room temperature followed by the addition of 5μl glycerol and 0.5μl of 100mM DTT. Protein concentrations were determined using a Pierce's protein assay. Protein standards were created using BSA protein diluted in ddH_20 to final concentrations of 0, 2, 5, 10, 15, 20, 25, 30, 40 and 50μg respectively. The experimental protein was diluted by adding 1μl of protein to 500μl ddH_20. 1000 μl of a two part assay reagent solution was added to each sample consisting of a 98:2 ratio of solution A (sodium carbonate, sodium bicarbonate, tartrate in 0.2N sodium hydroxide) and solution B (4% cupric sulfate pentahydrate). Samples were incubated for 90 minutes at 60^{0}C and optical densities were read at 562nm on a Milton Roy Spectronic spectrophotometer. Samples were stored at –20^{0}C until needed. Prior to loading, 50μg of protein were warmed at 65^{0}C to dissolve any SDS precipitate and was added to an equal volume of 2X SDS Tris sample

buffer (2.5 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.2% (w/v) bromophenol blue) and boiled for 5 minutes.

3.7.2. Preparation of Western Gel

To recover proteins of interest less than 20 kDa in size, a Tricine-SDS PAGE gel running and blotting technique was used visualize psoriasin. Briefly, the plates were set up according to manufacturer's instructions. The 16.5% separating and 4% stacking gels contained 3x separating buffer (3.0 M Tris, pH 8.45, 0.3%SDS), 30% polyacylamide (29:1, 3.3%C), 10% ammonia persulfate (Biorad) and TEMED (N,N,N',N' – tetramethylethylenendiamine, Sigma). A Tricine-SDS running buffer (100 mM Tris, 100mM Tricine, 0.1% SDS, pH 8.25) was added to the inner chamber of the BioRad running apparatus while the outer chamber contained a 0.2M Tris, pH 8.9 solution. The gel was run at 20mA for one hour, followed by 35mA for an additional three hours. Transfer of the Western gel was completed wet onto a 0.2 μm nitrocellulose (Zeta probe) blotting membrane. The transfer assembly components (membrane, Whatman paper, scrub pads) were presoaked in chilled 1x Tris glycine transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.0). The transfer took place at 100 volts for 1 hour while the entire apparatus was cooled on ice. The membrane was blocked in 5% dried skim milk powder in TBST (20 mM Tris, 137 mM NaCl, 0.5% Tween 20, pH 7.6) shaking for one hour at room temperature or at 4^0C overnight. The blocking solution was then removed and the anti-rabbit psoriasin primary antibody was added to a new aliquot of 1% blocking solution (1:500) and left to

incubate overnight at 4^0C with shaking. Psoriasin antibody was generously provided by Dr. JE Celis, Denmark) The blot was washed six times with 1x TBST at room temperature (five 5-minute rinses, one 15-minute rinse). The secondary antibody goat anti-rabbit IgG in 1% blocking solution (1:5000) was added along with a 1:10 dilution of horse radish peroxidase conjugated anti-biotin antibody in order to visualize the protein. The blot was washed again six times. The Pierce "SuperSignal" method was used to develop all western blots that were performed. Briefly, equal volumes of two solutions (a luminol/enhancer solution and a stable peroxide solution) were pipetted onto the blot and left to incubate 10 minutes at room temperature. The solution was removed and x-ray film was placed onto the blot and developed after various time points (1, 5, 10, 30, 60 seconds).

During the course of these experiments, our laboratory created two primary psoriasin antibodies of our own. A psoriasin-specific chicken IgYand sheep IgG polyclonal anitibody was generated by immunizing chicken and sheep with a 14 amino acid peptide corresponding to the carboxy terminus of psoriasin (KQSHGAAPCSGGSQ; Bionostics, Toronto and Aves labs). Greater than 90% pure IgY fractions were obtained in phosphate buffered saline and then further purified by passing it over a psoriasin peptide affinity column made by binding the synthetic pepti9de to N-hydroxy-succunimide-activated Sepharose 4B (Pharmacia Biotech), according to the manufacturers instructions. The bound IgY was then eluted with 5.0 mol/L sodium thiocyanate, followed by the dialysis against phosphate buffered saline (Al-Haddad et al., 1999)

We chose to use the Danish antibody for the course of the experiments, a gift from Dr. Celis, Denmark, as we had it in our possession at the beginning of this thesis. Briefly, this psoriasin polyclonal antibody was raised in rabbit against recombinant human psoriasin. This antibody was precipitated with ammonium sulfate, resuspended in 1/5 of the original volume of serum with Hanks buffered saline and dialysed extensively against three changes of Hank's buffered saline (Celis et al., 1996)

3.8 Growth assays

MDA-MB-231 and MCF10AT3B and their transfected clones ($CL7FD3^{psor}$, $CL7FB2^{psor}$, $CL6FA1^{vector}$, $CL6VA1^{vector}$, $CL71A1^{vector}$, $ZPB13^{psor}$, $ZP8E12^{psor}$, $ZV1C1^{vector}$, $ZV2B4^{vector}$), were grown as previously described in $35mm^2$ dishes at a concentration of 10000 cells per well. MDA-MB-231, $CL7FD3^{psor}$, $CL7FB2^{psor}$, $CL6FA1^{vector}$, $CL6VA1^{vector}$, $CL71A1^{vector}$ were trypsinized and counted in triplicate on days 1, 3, and 7 and the growth assay was repeated four times by Caroline Leygue in Dr. L.C Murphy's laboratory. This was done in triplicate, and the growth assay was repeated three times. MCF10AT3B, $ZPB13^{psor}$, $ZP8E12^{psor}$, $ZV1C1^{vector}$, $ZV2B4^{vector}$ cells were trypsinized and counted at days 1, 4, 6, 8, 11, 13 using a Coulter Counter in order to determine growth curves. This was done in triplicate and the growth assay was repeated three times.

3.9 Invasion assays

The invasion assays were performed as per the manufacturer's instructions. Briefly, the Biocoat Matrigel and control insert invasion chambers (Collaborative Biomed) were rehydrated with 37^0C DMEM for 90 minutes. Cells were counted to a concentration of $1x10^5$ cells per ml, in DMEM supplemented with 0.1% BSA. 750µl of conditioned media (0.1% BSA, ascorbic acid) was added to the wells below the invasion chamber, and 5000 cells were added to the invasion chamber. The cells were grown at 37^0C, 5% $C0_2$ for 18 hours. Any non-invading cells were carefully removed from the upper surface of the membrane with a Q-tip moistened with DMEM. The membrane was fixed with methanol, stained with hematoxylin and eosin and finally removed from the invasion chamber to be placed on a slide for cell counts. Three fields of view of each slide were photographed, and cells were counted. Percent invasion was calculated by dividing the mean number of cells invading the Matrigel coated membrane by the mean number of cells migrating through the uncoated control insert membrane multiplied by 100.

3.10 Animals

Adult (at least 6 weeks old), female, Balb/C *nu/nu* mice were obtained from Charles River Laboratories (Quebec) and were cared for at the University of Manitoba's animal care facility.

3.11 Injections

Cells (MCF10AT3B, MDA-MB231, CL71A1^{vector}, CL7FD3^{psor}, CL7FB2^{psor}) were grown in tissue culture and were harvested at 70% confluence. Animals (12 in each group) received two subcutaneous injections of $1x10^7$ cells suspended in DMEM bilaterally in the mammary fat pad region, one on each side of their bodies.

3.12 Assessment of tumor growth

Growth of tumors and health of the animals were measured. Animals were anesthetized according to CCAC guidelines, using isoflurane, and tumor growth was measured in mm using vernier calipers. Length, width, and depth of the tumor were measured in order to find the volume of the tumor every three to five days . Animals were humanely euthanized using an isoflurane overdose under two conditions: once a sufficient tumor growth curve could be plotted(generally 30-60 days); or due to an animal's ill health as a result of tumour growth and the related side-effects.

3.13 Autopsy and recovery of tissue

Animals were autopsied immediately after euthanization. A brief examination of internal organs was performed and lung, liver, spleen, femur and tumor were harvested for further investigation. Lung, liver, spleen and tumors were dissected bilaterally allowing one piece for formalin fixation/paraffin embedding with the remainder of the tissue being stored for later use at -70^0C.

Femurs were placed overnight in 10% formic acid (Sigma) in order for decalcification to take place and then formalin fixed and paraffin embedded. Tumor, liver, lung, and bone were sectioned at 5μm thickness using a microtome (Leica) and placed onto slides. Slides were deparaffinized using Xylene (Fisher) and stained with Hematoxylin and Eosin.

3.14 Assessment of Necrosis

The amount of necrosis within each tumor was measured using computer-imaging software (MCID). A picture from each 5 μm slide was visualized on a computer monitor, captured, and saved. The image analysis software was able to distinguish between tumor and necrotic tissue as these tissue types stain differently using hematoxylin and eosin. Briefly, necrotic tissue stains pink while tumor tissue will stain blue. Surface area was analyzed using an interactive methodology such that one can highlight a pixilated area. Percent necrosis was found by dividing the area of necrosis over the total area of the tumor and multiplying by 100. Since there was a vast difference in final tumor size, a subset of tumors with tumor volumes of 10000 +/- 10% were also analyzed in order to see if equal volume tumors behaved similarly to entire cohort.

3.15 Assessment of mitotic events

The abundance of mitotic events was determined using a Merz grid. Cells that were actively undergoing mitosis i.e. ones that prophase, metaphase, anaphase or telephase could be visualized under the microscope could be

visualized under 100X magnification using a microscope. Mitotic cells that were touching the crosshatch were counted in the data set. Slides were analyzed in triplicate and % of mitotic events was determined by the number of mitosis lying overtop a crosshatch divided by 36 and multiplied by 100. Since there was a vast difference in final tumor size, a subset of tumors with tumor volumes of 10000 +/- 10% were also analyzed in order to see if equal volume tumors behaved similarly to the entire cohort.

4 RESULTS

4.1 Psoriasin is not expressed in MCF10AT3B or MDA-MB-231 cell lines

RT-PCR analyses were completed on MCF10AT3B cell line, an immortalized T-24 ras transfected fibrocystic human breast epithelial cell line, and MDA-MB-231, a highly invasive human breast cancer cell line, to determine the constitutive expression of psoriasin. No constitutive expression of psoriasin was found (Figure 7). Therefore, these two cell lines offered a potential model to study the effects of introducing psoriasin into both normal and cancerous breast cells. The originating MCF10A1 fibrocystic cell line did show evidence of some psoriasin expression, thus we chose not to use these as our parental line. Also, we were able to show that psoriasin RNA expression was induced following 48 hours β-estradiol exposure in the MCF10AT3B cells, therefore, ovariectomized female mice were chosen for all *in vivo* experiments to avoid potential interactions between endogenous β-estradiol-induced psoriasin expression and exogenous (experimentally) -introduced psoriasin (Figure 7).

4.2 Psoriasin is stably transfected into the normal breast (MCF10AT3B) and invasive breast cancer (MDA-MB-231) cell lines

Various stable transfection methodologies and techniques achieved the introduction of psoriasin into the "normal" breast cell line MCF10AT3B and the invasive breast cancer cell line MDA-MB-231. The calcium phosphate methodology developed by Chen *et al* (1987) facilitated the uptake of plasmid DNA containing psoriasin into MDA-MB-231. This technique resulted in one high

expressing positive clone labelled CL7FD3^{psor}, two low expressing clones, CL7FA1^{psor} and CL7FB2^{psor}, and two vector alone - CL6VA1^{vector} and CL71A1^{vector} controls (Table 4). All of the above clones were generously supplied by Leigh Murphy's laboratory, University of Manitoba). Previous characterisation of these clones, along with proper loading controls, was completed by Dr. E. Leygue (Dr. L. Murphy's lab, U of M, Winnipeg, Manitoba). Western blot analysis completed in Dr. P Watson's laboratory confirmed these psoriasin protein expression levels in the CL7FB2^{psor} (low expressing) clone and the CL7FD3^{psor} (high expressing) clone (Figure 8; Table 4). Normal breast cell MCF10AT3B was transfected with psoriasin cDNA using lipofection techniques, and two similarly high psoriasin-expressing clones were generated: ZP1B3^{psor} and ZP5E12^{psor} (Table 4). Vector alone controls were also constructed and were labelled ZV1C1^{vector} and ZV2B4^{vector} (Table 4). In order to achieve these stably transfected MCF10AT3B^{psor} clones five separate attempts were made. Approximately 50 - 75 clones were established from each original transfection plate, however less than 10 could be established to the T150 size flask. All clones were subjected to Northern analysis and indicated psoriasin RNA was present in only two clones and absent in all others . Psoriasin containing clones ZP1B3^{psor} and ZP5E12^{psor} clones as well as vector alone controls ZV1C1^{vector} and ZV2B4^{vector} were harvested from separate transfection attempts . Western blot analysis confirmed psoriasin protein expression was much higher in ZP1B3^{psor} than in the ZP5E12^{psor} clone, and the absence of psoriasin protein in the vector alone controls (ZV1C1^{vector} and ZV2B4^{vector}) (Figure 9). This indicates that RNA

was being translated into protein. Gels were stained with Coomassie Blue prior to transfer as a loading control.

4.3 *In vitro* results

4.3.1 Psoriasin has no effect on growth in "normal" breast cells (MCF10AT3B)

To evaluate whether psoriasin had an effect on the rate of cell division, a cell growth assay was performed on the "normal" breast cell line (MCF10AT3B) parental cell line, ZV2B4^{vector} and ZV1C1^{vector} vector alone controls, and the psoriasin expressing ZP1B3^{psor} and ZP5E12^{psor} clones (Figure 10). ANOVA analysis of the results indicated that there was no difference in growth in terms of doubling time when comparing cell lines. ($p>0.05$; Figure 11).

4.3.2 Psoriasin may decrease *in vitro* invasive behavior in "normal" fibrocystic breast cells (MCF10AT3B)

To assess whether psoriasin had an effect on the invasive potential of cells, a Boyden Chamber invasion assay was performed using the "normal" MCF10AT3B parental cell line, ZV2B4^{vector} and ZV1C1^{vector} vector alone controls, and ZP1B3^{psor} and ZP8E12^{psor} high psoriasin-expressing clones. Invasion, as measured by percent, was analysed using ANOVA. An overall main effect of cell line [$F(5,12) = 19.31$, $p<0.001$] was found. However, post-hoc Students Newman Keuls analyses revealed no differences between the parental control (MCF10AT3B), the vector alone (ZV2B4^{vector}), and the psoriasin-expressing ZP1B3^{psor} clone ($p > 0.05$). Specifically, ZP1B3^{psor} shows slightly higher invasion and ZP5E12^{psor} shows decreased invasion than vector and parental controls

however not statistically significant. There was a difference observed between the two psoriasin-expressing clones ($ZP1B3^{psor}$ > $ZP5E12^{psor}$; p = 0.028) (Figure 12).

4.3.3 Psoriasin has no effect in *in vitro* cell growth in invasive breast cancer cells (MDA-MB-231)

To evaluate whether psoriasin had an effect on the rate of cell division, a cell growth assay was performed on the invasive breast cancer (MDA-MB-231) parental cell line, $CL6VA1^{vector}$ and $CL71A1^{vector}$ vector alone controls, $CL7FA1^{psor}$ and $CL7FB2^{psor}$ (low expressing) clones and the $CL7FD3^{psor}$ (high expressing) psoriasin clone (Figure 13). Doubling time, as measured in days, did not differ (p>0.05) between psoriasin expressing clones – $CL6FA1^{psor}$ (1.04±0.02), $CL7FB2^{psor}$ (1.27±0.06), $CL7FD3^{psor}$ (1.13±0.07), the parental control, MDA-MB-231 (1.03±0.05), and the vector alone controls $CL6VA1^{vector}$ (1.32±0.13), $CL7VA1^{vector}$ (1.26±0.04) (Figure 14).

4.3.4 Psoriasin has no effect on *in vitro* invasive behavior in invasive breast cancer cells (MDA-MB-231)

To assess whether psoriasin had an effect on the invasive potential of cells, a Boyden Chamber invasion assay was performed using the MDA-MB-231 parental cell line, $CL6VA1^{vector}$ and $CL71A1^{vector}$ vector alone controls, $CL7FA1^{psor}$ and $CL7FB2^{psor}$ (low expressing) and $CL7FD3^{psor}$ (high expressing) psoriasin expressing clones (Figure 15). Invasion, as measured by percent, did not differ (p>0.05) between psoriasin expressing clones – $CL6FA1^{psor}$ (34.5±6.0), $CL7FB2^{psor}$ (30.8±1.9), $CL7FD3^{psor}$ (21.4±4.7), the parental cell line, MDA-MB-

231 (23.4±6.8), and vector alone controls CL6VA1^{vector} (22.9±3.3), CL7VA1^{vector} (32.9±5.7)

4.4 *In vivo* results

4.4.1 "Normal" breast cell line (MCF10AT3B) was not established into Balb C nu/nu mice

In an attempt to grow a cell line that mimics breast cancer progression in an animal model, MCF10AT3B was injected into a Balb C *nu/nu* mouse according to the recommendations of Dr. Fred Miller (Dawson PJ et al, 1996). After 200 days, twelve mice were euthanized and an autopsy was performed. No evidence of any tumors were found, however an enlarged salivary gland was observed in each mouse.

During the course of this experiment, new evidence suggested that implanting a time release estradiol pellet into an ovariectomized Balb C *nu/nu* when injecting "normal" breast cell line MCF10A1, decreased the length of the experimental paradigm significantly, such that tumors were formed within 60 days. Our attempts at replicating this experimental protocol were unsuccessful (n=6).

4.4.2 Psoriasin does not affect the growth rate of tumors *in vivo* following injection of invasive breast cancer cells (MDA-MB-231)

The parental cell line, MDA-MB-231, vector alone CL71A1^{vector}, and psoriasin transfected CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cell lines were injected bilaterally into Balb C nu/nu mice in an

attempt to determine the effect of psoriasin in *in vivo* cell growth, as measured by tumor volume. Mice were injected on three separate occasions. Set 1 represents n=6 per cell line, Set 2 represents n=3 per cell line and Set 3 represents n=3 per cell line injected (Table 5). Figures 17-19 show individual mouse set growth summaries whereas Figure 20 depicts the mean cell line growth trend. (Figures 17-20).

To analyse the data we chose to look at the slope of the growth trend. Because all cell lines in the three experiments performed followed the same growth trend, we analysed the slopes of the curves together as one data set i.e combined all three sets together (Figure 21). The ANOVA showed no main effect of side confirming that there was no differences between injecting on either side of the animal ($F(1,88) = 0.15$; $p > 0.05$], therefore the data from both sides of injection were grouped together for all further data analyses. Cell lines transfected with psoriasin or with vector alone appeared to have an effect on tumor volume ($p < 0.05$). Specifically, tumors containing $CL71A1^{vector}$ (vector alone) and $CL7FD3^{psor}$ grew much larger than parental MDA-MB-231 cells ($p < 0.05$). Interestingly $CL7FB2^{psor}$ (low expressing) grew larger than parental MDA-MB-231 but was not of any statistical significance ($p > 0.05$). These data present evidence that psoriasin might not be involved directly in in vivo growth of invasive carcinoma, as a vector effect is suggested.

To confirm the presence and absence of psoriasin in these tumors, tumor tissue was harvested post mortem and western blot analysis completed. Western blot results confirmed that the post-mortem psoriasin protein expression

in vivo was still present in psoriasin transfected CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) clones and not in the in the CL71A1^{vector} (vector alone) and MDA-MB-231 parental controls (Figure 22).

4.4.3 Amount of necrosis is independent of the presence of psoriasin in invasive breast cancer cells (MDA-MB-231)

To assess whether psoriasin alters cell death *in vivo*, histological sections examining the area of a tumor that necrotic cells occupy was analysed using morphometry and image analysis software (Figure 23). ANOVA revealed no main effect of cell line [$F(3,44) = 1.20$; $p>0.05$]. No differences relating to the amount of necrosis were observed between tissues expressing high levels (CL7FD3^{psor}), low levels (CL7FB2^{psor}), or no psoriasin (CL71A1^{vector} vector alone and MDA-MB-231 parent) ($p>0.05$) (Figure 24). Because we did not sacrifice animals at a set volume but rather until their health was at issue, we chose to take a subset of animals to investigate the relationship between necrotic abundance and constant tumor volume as well. Tumors that reached a volume of 10 000 +/- 10% were included. Six animals from MDA-MB-231, four animals from CL71A1^{vector}, four animals from CL7FB2^{psor}, and three animals from CL7FD3^{psor} were examined. ANOVA was performed and no difference was seen between the amount of necrosis and constant tumor volume in the above mentioned cell lines ($p>0.05$) (Figure 25).

4.4.4 Amount of mitotic events in tumors is independent of the presence of psoriasin in invasive breast cancer cells (MDA-MB-231)

To assess whether psoriasin alters the amount of cell growth as measured by evidence of mitosis *in vivo*, histological sections were examined using a Merz grid (Fig 26). The number of cells classified as actively mitotic in the H&E stained histological sections of tumours was not significantly different between tissues expressing high levels ($CL7FD3^{psor}$), low levels ($CL7FB2^{psor}$), or no psoriasin ($CL71A1^{vector}$ vector alone and MDA-MB-231 parent) ($p>0.05$) when analysing the data by ANOVA. (Figure 27). Because we did not sacrifice animals at a set volume but rather until their health was at issue, we chose to take a subset of animals to investigate the relationship between mitotic abundance and constant tumor volume as well. Tumors that reached a volume of 10 000 +/- 10% were included. Six animals from MDA-MB-231, four animals from $CL71A1^{vector}$, four animals from $CL7FB2^{psor}$, and three animals from $CL7FD3^{psor}$ were examined. ANOVA was performed and no difference was seen between the mitotic abundance and constant tumor volume ($p>0.05$) (Figure 28).

5 SUMMARY OF RESULTS

1. Psoriasin was successfully transfected into the normal breast cell line MCF10AT3B resulting in ZP1B3^{psor} and ZP5E12^{psor} clones. As well, ZV1C1^{vector} and ZV2B4^{vector} vector alone transfected clones were generated.
2. Psoriasin was successfully transfected into the highly invasive MDA-MB-231 cell line resulting in CL6FA1^{psor} (low expressing), CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) clones. As well, CL6VA1^{vector} and CL71A1^{vector} vector alone transfected clones were generated.

***In vitro* experiments:**

1. Psoriasin has no effect on *in vitro* cell growth in normal fibrocystic breast cells (MCF10AT3B)
2. Psoriasin may increase *in vitro* invasive behavior in normal fibrocystic breast cells (MCF10AT3B)
3. Psoriasin has no effect on *in vitro* cell growth in invasive breast cancer cells (MDA-MB-231)
4. Psoriasin has no effect on *in vitro* invasive behavior in invasive breast cancer cells (MDA-MB-231)

***In vivo* experiments:**

1. Normal breast cell line (MCF10AT3B) was not established into Balb C nu/nu mice, therefore psoriasin effects *in vivo* could not be assessed
2. Psoriasin had no affect on the growth rate of tumors *in vivo* following injection of invasive breast cancer cells (MDA-MB-231)
3. Amount of necrosis is independent of the presence of psoriasin in invasive breast cancer cells (MDA-MB-231)
4. Mitotic abundance is independent of the presence of psoriasin in invasive breast cancer cells (MDA-MB-231)

6 DISCUSSION

6.1 Psoriasin

Psoriasin (S100A7) is a member of the S100 gene family of calcium binding proteins of which the precise function is unknown (Hoffmann HJ et al., 1994; Madsen P et al., 1991). The expression of psoriasin was first identified in psoriatic skin and other skin diseases, and more recently in other human tissues (Madsen P et al., 1991; Sitzmann J et al., 1994; Zimmer et al., 1996). Studies show that psoriasin gene expression is altered as normal skin epithelial cells differentiate towards a psoriatic phenotype. Specifically, psoriasin is highly expressed in psoriatic skin lesions as compared to normal epithelial skin cells. This suggests that psoriasin is differentially expressed and can potentially be used as a molecular marker for diagnosing psoriasis, a relatively common skin disease.

Using a modified subtractive hybridization technique, Leygue discovered that psoriasin was also differentially expressed in human breast cancer (Leygue et al., 1996). Later investigations demonstrated that psoriasin was more highly expressed in the *in situ* component than the invasive component of the same breast tumors by *in situ* hybridization (Leygue et al., 1996). This observation supports psoriasin's involvement in the molecular events that surround breast cancer progression.

In our laboratory, we studied psoriasin's relationship further comparing *in situ* and invasive breast tumors and observed that psoriasin could also be expressed in invasive breast tumors. A small cohort of invasive breast tumors was selected and we concluded that psoriasin is expressed by RT-PCR analyses

in tumors that are characterized as having a poor prognostic outcome i.e. estrogen and progesterone receptor negative by ligand binding (data not shown).

Al-Haddad confirmed this observation and expanded her study using a cohort of fifty-seven (57) invasive tumors of different histological sub-types and ranges of differentiation as determined by tumor grade and estrogen receptor status. RT-PCR and western blot analysis established that psoriasin expression levels were similar between mRNA levels and protein expression in invasive tumors. As well, high psoriasin expression is significantly associated with poor prognostic markers that include estrogen and progesterone receptor negative status, alongside positive lymph node involvement. Psoriasin expression within breast tumor cells was also associated with evidence of inflammatory infiltrate (Al-Haddad et al., 1999). Evidence that psoriasin is a chemotactic protein for inflammatory cells is also supported in the literature as psoriasin attracts CD^4+ lymphocytes and neutrophils (Jinquan T et al., 1996).

Other methods that could be used to elucidate psoriasins role in breast tumorigenesis include a) looking for protein - protein interactions, b) looking for the biological effects of over expression and c) looking for the biological effects of underexpression.

In order to describe signal transduction pathways associated with a specific disease process it is important to elucidate the proteins that are involved. Co-immunoprecipitation studies and the yeast two hybrid system are examples of techniques that will detect those proteins that are associated and/or bind with your protein of interest. Barraclough's laboratory discovered an association

between S100A4 and S100A1 using the yeast two-hybrid system and confirming it further with affinity column chromatography and gel overlay techniques. They hypothesised that the presence of S100A1 might control the metastasis inducing capabilities of S100A4 when co-expressed in the same cultured mammary cells (Wang G et al., 2000). They suspected that the heterodimerization had some biological significance as they had found it in human breast cancer cells, rat mammary cells (RAMA 29) and smooth muscle cells. S100A1 and S100A4 were both located in the perinuclear region and on the stress fibers of the cytoskeleton, suggesting that they interact inside the cell at these same locations and may share a common intracellular target (Wang G et al., 2000). Mandinova and colleagues suggested that it is possible that the interactions of S100A4 with myosin, which might be associated with the protein's metastasis-inducing capabilities, could be modulated by interaction with cytoskeleton-associated S100A1 (Mandinova et al., 1998). For instance, S100A1 and S100A4 are both co-localized in the high S100A4-expressing cultured rat mammary cells, RAMA 29, which do not express a metastatic phenotype (Wang G et al,. 2000)

Cell lines can also offer great assistance in understanding biological questions. For example we can use breast cell lines that are similar to each other in terms of estrogen receptor status, progesterone receptor status etc... but differ in terms of the genes they endogenously express. This can be a wonderful tool to explain the specific role of the gene of interest upon completing appropriate in vitro and in vivo experiments. As well, transgenic animal models are useful. A psoriasin transgenic mouse model could be created for example

and crossed with a mouse expressing HER2/neu in the attempt to explain psoriasins role in poor prognosis breast cancer.

Alternatively, looking at underexpression studies are equally valuable. For example a psoriasin knockout mouse could be created in order to illustrate psoriasins importance in developmental processes.

Although no studies have been reported regarding psoriasin's effect on cell growth and death, other S100 proteins, similar in structure to psoriasin, have been studied. Of the S100 family members - S100A1, S100A4, S100A6, S100A8 and S100B are implicated in alterations to patterns of cell growth (Zimmer et al., 1995).

S100 proteins are also involved in the progression of cancer. For example, S100A2 and S100A4 proteins are differentially expressed between normal and neoplastic cells, whereby altered gene expression ultimately affects the overall invasive and metastatic phenotype (Wicki R, 1997; Ebralidze A, 1989).

These observations led us to the belief that psoriasin (S100A7) might be involved further in the processes of cell growth/death, invasion, and chemotaxis as members of the S100 family of proteins share similar cell functions and behaviors.

We have studied psoriasin in terms of the above mentioned characteristics through both *in vitro* and *in vivo* methods that attempt to assess psoriasins affect on the pathology and behavior in "normal" (MCF10AT3B) and invasive breast cancer (MDA-MB-231) cell line models.

6.2 *In vitro* experiments

6.2.1 *In vitro* growth and invasion in a normal breast (MCF10AT3B) cell line

Our results show that forced expression of psoriasin into a normal breast (MCF10AT3B) cell line does not alter growth and invasive behavior.

Although "cancer associated" S100's have been introduced into breast cancer cell lines (Onishenko A et al., 1996) there are no published studies reporting the introduction of them into "normal" breast cells.

Other results are reported in the literature with respect to high S100 protein expression levels and decreased rates of cell growth in normal cells. As one of the first S100s identified, S100B is primarily expressed in neural cells (Zimmer et al., 1995). Decreased expression of S100B in C6 neural cells resulted in a decreased rate of cell growth suggesting a reversal of the transformed phenotype (Selinfreund RH et al., 1990). Like wise, when introducing S100B into neonatal rat cardiac myocyte cultures, growth of these cells is greatly increased. This suggests that S100B is capable of serving as a molecule in a negative feedback mechanism that limits cellular growth due to altered gene expression (Parker TG, 1998)

Introduction of psoriasin into a "normal" breast cell line MCF10AT3B also appears to decrease *in vitro* invasive behavior. Of the two similarly expressing psoriasin transfected clones ($ZP1B3^{psor}$ and $ZP5E12^{psor}$), only one clone ($ZP5E12^{psor}$) significantly decreased its ability to invade compared to vector alone ($ZV2B4^{vector}$) and parent (MCF10AT3B) controls. This suggests that a)

introduction of psoriasin into normal breast cells further decreases any invasive capability or b) due to the transfection technique, integration of psoriasin into the chromosomal DNA of MCF10AT3B normal breast cells differed between the two clones which may in turn alter the invasive phenotype i.e. ZP5E12^{psor} is an aberrant clone and psoriasin does not have any affect on the invasive ability of normal breast cells. Studies suggest that introduction of antisense S100A4 RNA into Lewis lung carcinoma cells decrease *in vitro* invasiveness, whereas other studies introducing S100A4 into cells show other effects (Takenaga K, 1997).

In order to confirm psoriasin's affect on the invasive behavior of normal breast cells (MCF10AT3B), additional clones would need to be generated and tested using the Boyden Chamber invasion assay.

6.2.2 *In vitro* growth and invasion in an invasive breast cancer cell line (MDA-MB-231)

In the attempt to explain the relationship between the regulation of cell growth and the expression of S100 proteins, it is important to correlate the presence of S100 proteins with the proliferative behaviors of normal and tumor cells in culture.

Introduction of psoriasin (S100A7) into MDA-MB 231 invasive breast cancer cells produced no difference in behavior related to cell growth and invasion in our experiments.

Similar studies have been performed introducing S100A4 into breast cancer cell lines, observing both cell growth and invasive responses.

In the literature, positive proliferative responses such as those imparted by growth factors have been reported to increase the doubling time of cells. Sherbet et al asserted when overexpressing S100A4 in human tumor cells, a high level of epidermal growth factor receptor was elicited and in turn cell proliferation increased (Sherbet GV, 1995). Interestingly, when Onischenko and colleagues introduced S100A4 into breast cancer cell line MCF-7 they found that *in vitro* S100A4 expression did not modify proliferative or invasive properties (Onishenko A et al., 1996). In order to elicit their findings, their group performed gene transfer experiments using a human sense S100A4 expression vector and human MCF7 malignant epithelial cells that do not express endogenous S100A4. In vitro, S100A4 expression did not modify proliferative or invasive properties of transfected MCF7 cells.

Similarly, Zain's laboratory reported that S100A4 did not alter any invasive properties when introducing its forced expression into nonmetastatic mouse mammary adenocarcinoma cell line CSML0 using Boyden chamber experiments (Ford HL et al., 1995). However, what was found was the presence of S100A4 did correlate with an increase in cellular motility. The authors also raise an important point that alongside the presence or absence of S100A4 in their metastatic and nonmetastatic cell lines, these cell lines differed also with respect to another known marker of metastasis and tumor progression, being MMP expression. They argue that due to the low levels of MMP activity in the CSML0 cell lines and their transfectants, they were not able to detect an increase in invasive behavior (HL Ford et al., 1995)

Our observations combined with those of Onischenko and Ford may support that S100A4 and S100A7 proteins which are introduced into breast cancer cell lines behave similarly in terms of not affecting *in vitro* growth and invasive behavior. On the other hand, an increase in the in vitro invasive potential of glioma cells of S100A4 has been reported in culture, demonstrating that the results can vary depending on the model used (Sherbet et al.,1998 and Merzak et al., 1994).

6.3 *In vivo* experiments

6.3.1 "Normal" breast cell line (MCF10AT3B) could not be established *in vivo*

We attempted to introduce "normal" human breast cell line MCF10AT3B into Balb C *nu/nu* mice in order to generate lesions that range from atypical hyperplasia to invasive carcinoma. It was our hope that if successful, we would next introduce psoriasin transfected MCF10AT3B clones into the Balb C *nu/nu* mouse and observe psoriasins effect on a breast cell line that will spontaneously mimic breast cancer tumorigenesis. Unfortunately, we were not successful in our endeavour (n=10). Specifically, MCF10AT3B cells that were injected into the mammary fat pad region of Balb C *nu/nu* mice (n=10) failed to establish a palpable tumor mass. At day 200, we euthanised the mice and performed an extensive autopsy and were disappointed to find no evidence of a tumor mass. Interestingly, the mice (n=10) did show grossly enlarged salivary glands. No mention of this finding was mentioned in any of the MCF10AT3B literature, neither did the animal care veterinarian have any explanation for this discovery.

During the course of our 200 day pilot experiment, new evidence suggested that implanting a time release estradiol pellet into an ovariectomized Balb C *nu/nu* mouse decreased the length of the experimental paradigm significantly, such that tumors can form within 60 days (Shekhar MP, 1998). Again, our attempts at replicating this experimental protocol were unsuccessful (n=6). At day 60 we euthanised the mice and performed an autopsy and no evidence of a tumor mass was apparent. The mice looked normal in all other respects, including the salivary gland that was previously enlarged in the 200-day pilot experiment.

Possible explanations for our failure at replicating these experiments are many. Firstly, handler error could have accounted for our inability to replicate the experiment in terms of cell handling and injection technique. This explanation may have some merit, however is highly unlikely as we were able to successfully grow tumors from the invasive breast cell line MDA-MB-231 in the same mouse type (Balb C *nu/nu*) at the same time we were attempting these experiments. The MCF10AT3B literature does mention that injections were to be placed into the "mammary fat pad" of the female mouse. When learning injection technique with the animal care veterinarian, it was suggested dissecting out and injecting cells into the mammary fat pad would be almost impossible as nulliparous 6 week old female mice only have rudimentary breast tissue. Instead, the ideal solution would be to inject into the general mammary fat pad area of the mouse. Because we were successful in establishing tumors using MDA-MB-231 cells using the same mice and the same injection technique we strongly feel that handler error was not likely. We believe that the cells

themselves or the handling of the cells was more likely the problem. For instance, the choice of using Matrigel is controversial as some claim it to be beneficial for the cells establishment into the mouse. As well, only few laboratories are capable of growing MCF10AT3B cells in the Balb C *nu/nu* mouse. It may be of some value to grow the breast cells in a different mouse, such as the Balb C *nu/bg* mouse, as did the authors that first developed this xenograft model (Miller et al., 1993).

Another possibility to consider is the source of the animals. It may be of interest to compare two breeding house companies.

6.3.2 *In vivo* tumor growth of the invasive breast cancer cell line MDA-MB-231

Introduction of psoriasin into invasive breast cancer cell line MDA-MB-231 was used to assess psoriasin's effect on *in vivo* tumor cell growth. High expressing ($CL7FD3^{psor}$), low expressing ($CL7FB2^{psor}$), vector alone ($CL71A1^{vector}$) and parental MDA-MB-231 invasive breast cancer cells were injected subcutaneously into the mammary fat pad region of Balb C *nu/nu* mice. Tumors were allowed to grow until a sufficient growth curve could be plotted or the animals ill health was at risk. We observed that psoriasin had no affect on the growth rate of tumors following the injection of the above mentioned MDA-MB 231 breast cancer cells and transfected clones.

This observation further supports the *in vitro* data generated by the same cells as mentioned previously. Again, it is plausible that psoriasin conducts its self in a similar fashion as the S100A4 gene i.e introduction of S100A4 into

breast cancer cell lines does not change patterns of tumor cell growth (Ford HL et al., 1995).

Upon looking at the tumor cells under the microscope, we noticed that the MDA-MB-231 cells that were transfected with psoriasin appeared to be loosely attached to one another. Again, S100A4 behaves in a similar fashion. There is some evidence that S100A4 expression may alter the adhesive properties of cells, possibly by remodelling the extracellular matrix and promoting a redeployment of adhesion-mediating macromolecules occurring in the extracellular matrix (Sherbet GV,et al., 1998; Onishchenko A et al., 1996). Psoriasin may in fact share the same molecular pathways that S100A4 employs with respect to cellular adhesion molecules.

6.3.3 Mitotic abundance in tumors is independent of the presence of psoriasin in invasive breast cancer cells (MDA-MB-231)

We observed that psoriasin has no effect on mitotic abundance. CL7FB2^{psor} (low expressing) invasive breast cells did not differ from the amount of active mitosis compared to high expressing (CL7FD3^{psor}), vector alone (CL71A1^{vector}) and parental (MDA-MB-231) controls using merz grid microscopic analysis.

To confirm if low psoriasin levels increases the rate of *in vivo* cell division in invasive breast cancer cells, other techniques should be considered. It is important to remember that tumor size, as a measure of cell growth, encompasses not only active cell division but also cell death. For example, bromodeoxyuridine (BrdU) staining is commonly used as it becomes

incorporated into replicating DNA. Tumors can then be stained with antibodies against BrdU in order to localize the signal to tumor cells. Injecting thymidine into an animal works similarly to BrdU, as it to will yield a signal that indicates a cell is undergoing cell division.

6.3.4 Amount of necrosis is independent of the presence of psoriasin in MDA-MB-231 invasive breast cancer cells

We showed through examining the area of a tumor that necrotic cells occupy that psoriasin has no effect on necrotic abundance, regardless of volume of tumor burden . The literature is quite limited regarding S100 protein's effects on cell death as caused by necrosis. Onsischenko's gene transfer experiments as mentioned previously were also introduced into an in vivo animal model. In vivo, MCF7 cells expressing S100A4 were associated with tumors exhibiting necrosis, and abundant fibrous and poorly arranged cellular stroma (Onischenko et al., 1995). Fascinatingly, our in vivo MDA-MB 231 cells that were transfected with psoriasin also exhibited a loose cellular arrangement. Although not shown to be significant, we also found that our in vivo tumors, irrespective of transfection manipulation exhibited a high degree of necrosis upon microscopic investigation. Future experimentation might include psoriasin being transfected into MCF7 cells and injected into a mouse model. This may help to distinguish between the actual effects that S100 exhibit and those that may be skewed due to cell line effect.

As necrosis is just one cause of cell death, other causes of cell death such as apoptosis would be interesting to investigate. S100s have been reported to

demonstrate involvement in programmed cell death as S100B expression is associated with apoptotic cell death in Alzheimer type neurodegenerative lesions associated Downs syndrome (de la Monte, 1999)

6.4 Summary

In conclusion, we have shown that expression of psoriasin does not alter the in vitro growth and invasion in "normal" MCF10AT3B cells. As well, psoriasin has no effect on *in vitro* and *in vivo* cell growth, invasion and necrosis and mitotic abundance in invasive MDA-MB-231 breast cancer cells.

6.5 Future Considerations

We attempted to explain a biological role of psoriasin in breast tumorigenesis using in vitro and in vivo means. We used in vitro growth and invasion assays as well as in vivo growth and histological studies in order to determine the relationship psoriasin has to these biological processes. As the result of an incorrect hypothesis, we found that we only had more questions at the completion of this thesis.

Forthcoming studies might include looking at properties other than growth and invasion that might be relevant in the transition of DCIS to invasive cancer where we see alterations of psoriasin expression. Experiments including looking at differences in adhesion, motility, apoptosis, and angiogensis should be considered.

In terms of our results, we must revisit the way we performed our experiments. It is important to look at the technical aspects first. We should ask ourselves if we are confident with our reagents that include tissues, cells and vectors as well as any errors that may be caused by the handler. In our case, we are certain that our reagents were acceptable. In terms of handler error, we would have liked to explore the mammary fat region further. It was our hope to inject cells into the mammary fat pad that lay between the dermis and the facia/muscle in order to assess psoriasin's invasive potential. However, upon looking at the histological sections, we did not always inject into the right space. Recent meetings between University of Manitoba's veterinarian, Dr. Kathy McCutcheon and our laboratory indicate that this issue maybe resolved in upcoming animal experiments.

Next, biological considerations may include a) our results are correct but we asked the wrong questions or used the wrong tools. Perhaps we need to manipulate a cell line by transfecting it with an inducible psoriasin gene that is under the control of an inducible promoter. By using a cell line that has the molecular machinery to accept psoriasin we can then ascertain its importance when the gene is turned on or off.

Nevertheless, MDA-MB-231 maybe suitable for our experiments, however the assays we performed did not unearth the real results. For example, we know that psoriasin relates to host immune responses and this in turn may influence invasion. We however put human cells into a Balb C nu/nu mouse that does not have an intact immune system because this is the best available model to us at

present. As well, in vitro invasion experiments are limited as they lack ECM, vessels and all of the proteins and enzymes that would normally be seen in an in vivo setting.

Providing that all of the above-proposed considerations are performed and we still see that psoriasin does not influence the process of invasion, we can explore different avenues for psoriasin's involvement. For example, it would be interesting to investigate whether psoriasin's expression in in situ carcinoma is due to a survival mechanism in place. Perhaps psoriasin is important for survival in an hypoxic duct. Alternatively psoriasin may have a major effect on immune cells and vessels and other secreted chemokines within the context of angiogenesis.

Psoriasin is a complicated protein that seems to be involved in many biologically processes as well as found in many different cell types. In due time we will elucidate its importance.

TABLES

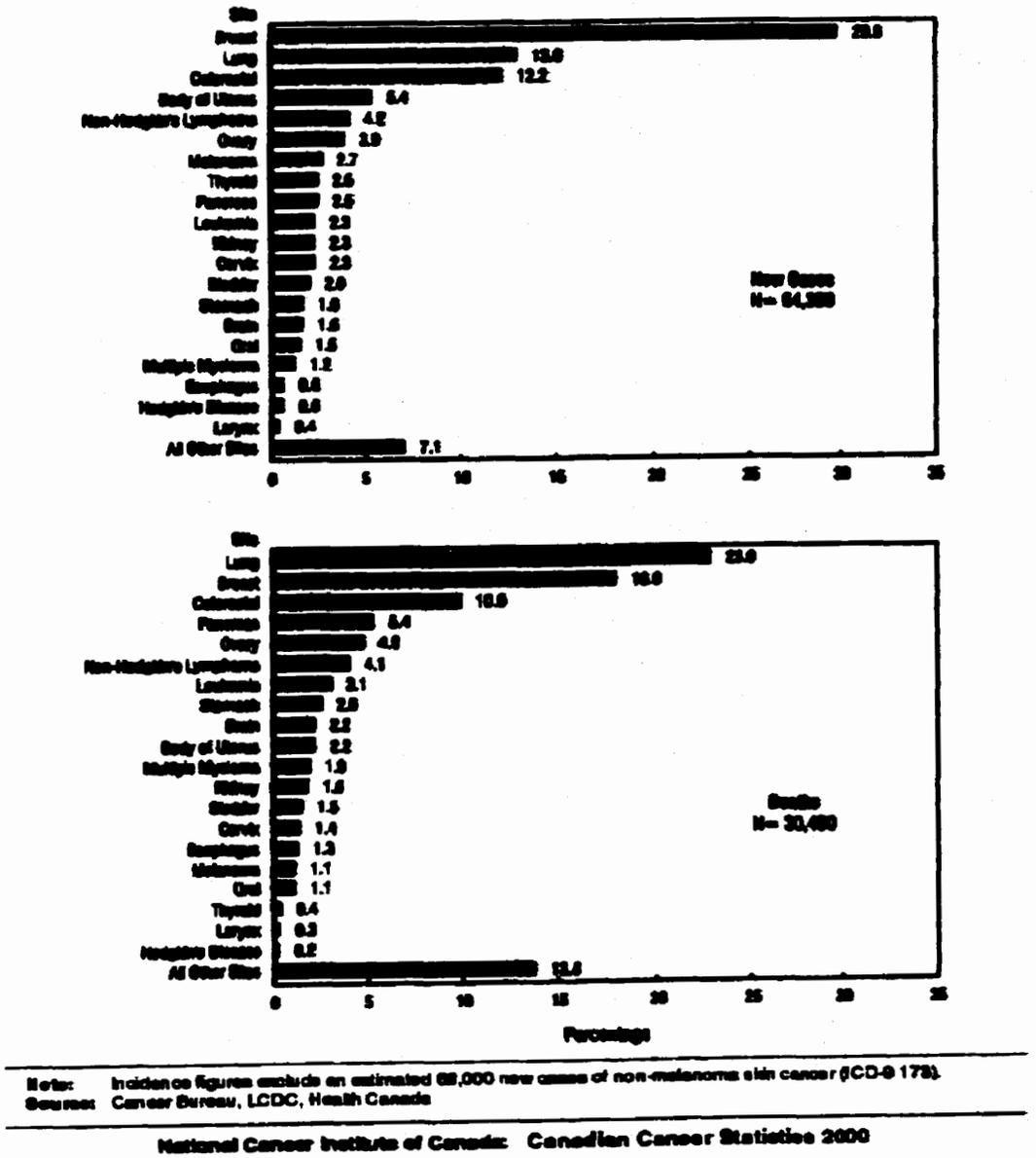


Table 1. Year 2000 Canadian Cancer Statistics – National Cancer Institute of Canada (NCIC). NCIC proposes that 29.9% of all female cancers will be that of breast cancer translating into 19226 newly diagnosed cases for the year 2000. Approximately 5472 women will also die of the disease, which depicts 23% of all deaths caused by cancer in females

Subtype	Frequency
Invasive ductal carcinoma (pure)	**50 %**
Ductal + Lobular (mixed)	**30 %**
Invasive lobular (pure)	**10 %**
Other	**10 %**

Table 2. Frequency of Histologic Subtypes of Invasive Breast Cancer. Invasive ductal carcinoma comprises the largest quantity of diagnosed invasive breast carcinomas, followed by lobular, and "other" types including medukkary, mucinous and tubular..

BREAST CANCER PROGNOSTIC FACTORS

Patient characteristics:
- • Age at menopause

Tumor differentiation:
- * Histological variables
- * Estrogen and progesterone receptors
- • DNA ploidy status
- pS2 protein
- HER2/neu, Epidermal Growth Factor receptors, mutant p53

Proliferative rate:
- * Mitotic Index
- Thymidine labelling index
- S-phase fraction

Tumor invasiveness or metastatic potential:
- • Axillary lymph node status
- * Tumor size
- Tumor related angiogenesis
- Oncogene and growth factor receptor expression
- Cathepsin D
- Urokinase plasminogen activator and its inhibitor (PAI-1)

Table 3. Breast Cancer Prognostic Factors. Summarised are prognostic factors that are implicated in breast cancer which include age, tumor differentiation, proliferative rate and tumor invasive and metastatic potential. Asterisk * denotes routine clinical use/application in patient assessment. (excerpted from Pathology, 2nd edition, Rubin and Farber, 1994)

CELL LINE	DESCRIPTION	EXPRESSION LEVEL OF PSORIASIN	
		RNA	PROTEIN
MCF10AT3B	Normal breast cell line (parent)	-	-
ZV1C1^{vector}	Normal breast cell line + vector	-	-
ZV2B4^{vector}	Normal breast cell line + vector	-	-
ZP1B3^{psor}	Normal breast cell line + vector + psoriasin	++	++
ZP5E12^{psor}	Normal breast cell line + vector + psoriasin	+++	+++
MDA-MB-231	Invasive breast cancer cell line (parent)	-	-
CL6VA1^{vector}	Invasive breast cancer cell line + vector	-	-
CL61A1^{vector}	Invasive breast cancer cell line + vector	-	-
CL6FA1^{psor}	Invasive breast cancer cell line + vector + psoriasin	+/-	+
CL7FB2^{psor}	Invasive breast cancer cell line + vector + psoriasin	++	++
CL7FD3^{psor}	Invasive breast cancer cell line + vector + psoriasin	++++	++++

Table 4. Description of Cell Lines. "Normal" breast cell line MCF10AT3B and invasive MDA-MB-231 breast cancer cell lines were used for the course of the experiments. MCF10AT3B was transfected with pcDNA 3.1 Zeo + psoriasin and generated ZV2B4^{vector} and ZV1C1^{vector} vector alone controls, as well as psoriasin + vector ZP1B3^{psor} and ZP5E12^{psor} clones. MDA-MB-231 was transfected with pcDNA 3.1 Neo + psoriasin and generated CL7VA1^{vector}, CL71A1^{vector} vector alone controls, low expressing CL7FA1^{psor} and CL7FB2^{psor} clones and a high expressing psoriasin + vector CL7FD3^{psor} clone. Expression was assessed subjectively, + low expression, ++ medium expression, +++ high expression, ++++ very high expression.

SET	CELL LINES USED	NUMBER OF ANIMALS
1	MDA-MB-231 CL71A1^{vector} CL7FB2^{psor} CL7FD3^{psor}	6 6 6 6
2	MDA-MB-231 CL71A1^{vector} CL7FB2^{psor} CL7FD3^{psor}	3 3 3 3
3	MDA-MB-231 CL71A1^{vector} CL7FB2^{psor} CL7FD3^{psor}	3 3 3 3

Table 5. Representation of animals used in in vivo experiments. The in vivo experiments were designed to allow Balb C nu/nu mice be injected bilaterally with MDA-MB-231 (parent), CL71A1^{vector}, CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cell line for a total of 12 mice per cell line.

FIGURES

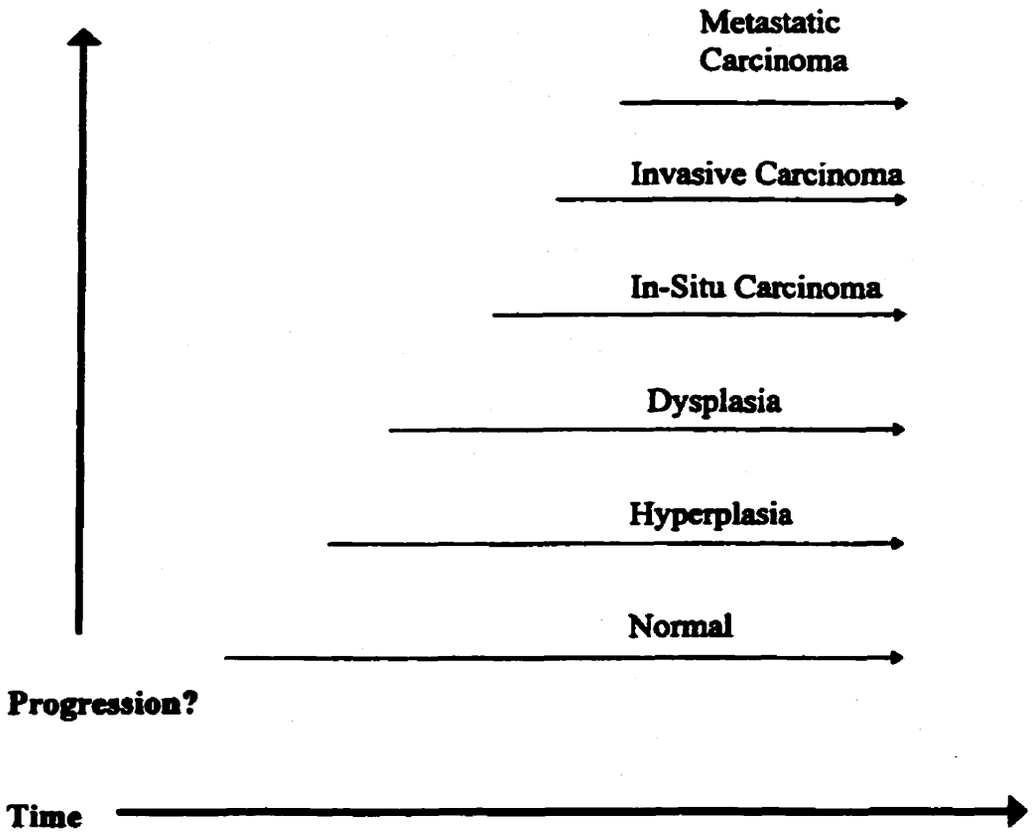


Figure 1. Morphological model of breast cancer evolution. Breast cancer evolves from normal epithelium going through a series of increasingly abnormal, but non-obligatory, cellular changes from hyperplasia, to dysplasia, to carcinoma in situ, to primary invasive carcinoma and, finally to metastatic carcinoma.

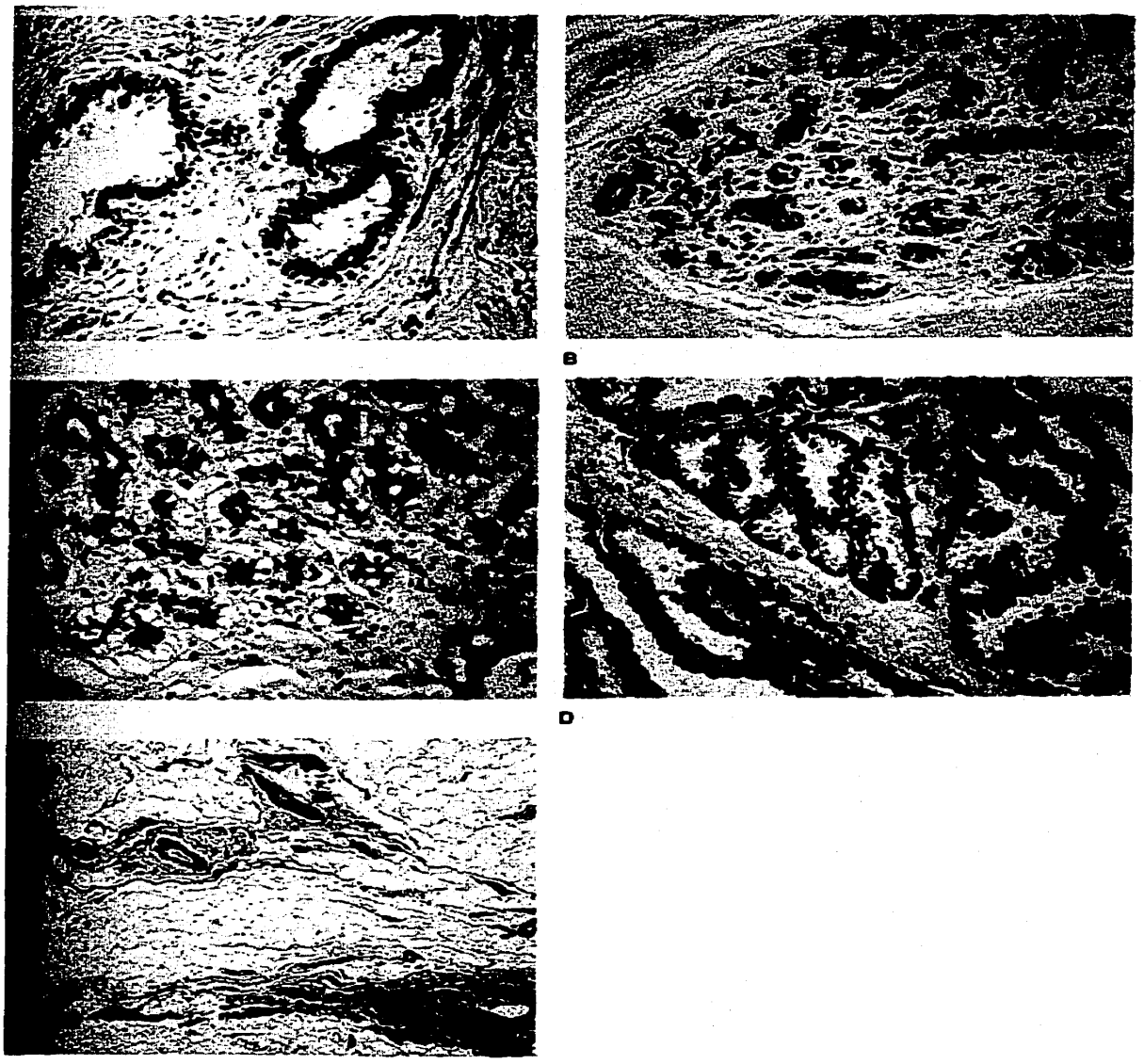


Figure 2. Normal breast architechture at various ages. (A) Adolescent breast: Large and intermediate size ducts are seen within a dense fibrous stroma. No lobular units are present. (B) Post-pubertal breast, first half of menstrual cycle:The termainal duct lobular unit consists of small ductules arrayed around intralobular duct. The two-layered epithelium shows no secretory or mitotic activity. (c) Postpubertal breast, second half of menstrual cycle: The terminal duct lobular units are enlarged, with increased numbers of terminal ducts. The basal epithelial cells are vacuolated, and mitosis is present (D) Lactating breast: Individual terminal ducts show prominent epithelial secretory activity. (E) Postmenopausal breast: The terminal duct lobular units are absent. The remaining intermediate ducts and larger ducts are commonly dilated. There is little interlobular fibrous tissue, and most of the breast is composed of fat. (excerpted from Pathology, 2nd edition,Rubin and Farber,1994)

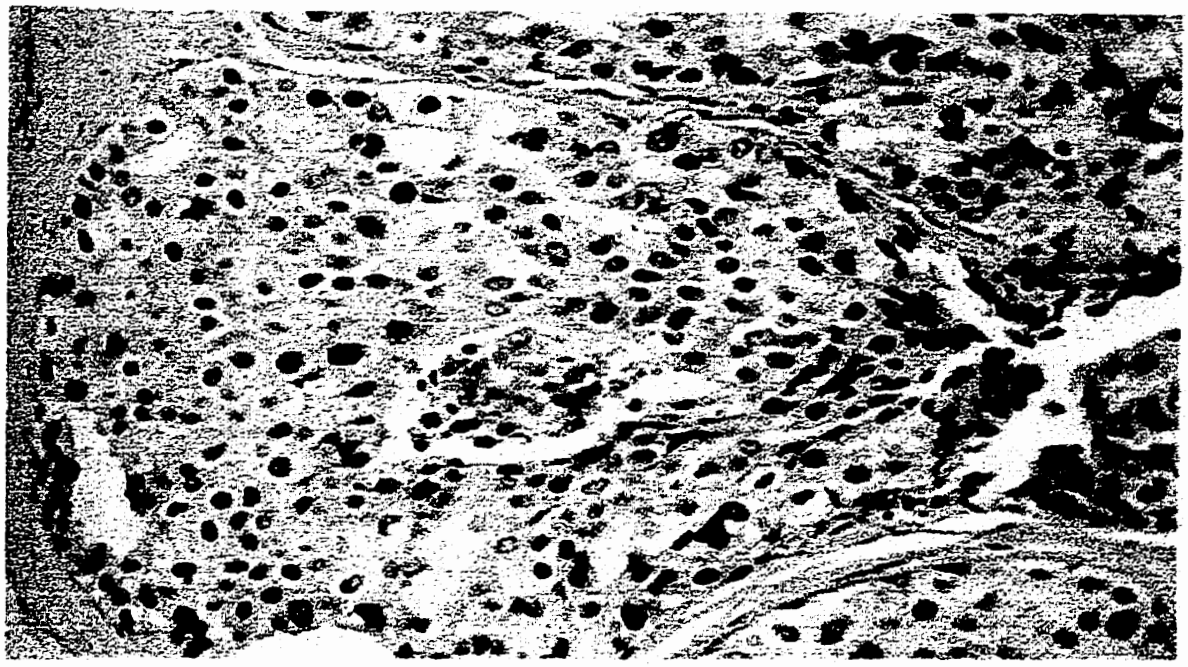


Figure 3. Ductal Carcinoma In situ. Tumor cells, encased in a basement membrane are large and have abundant cytoplasm (excerpted from Pathology, 2nd edition, Rubin and Farber, 1994)

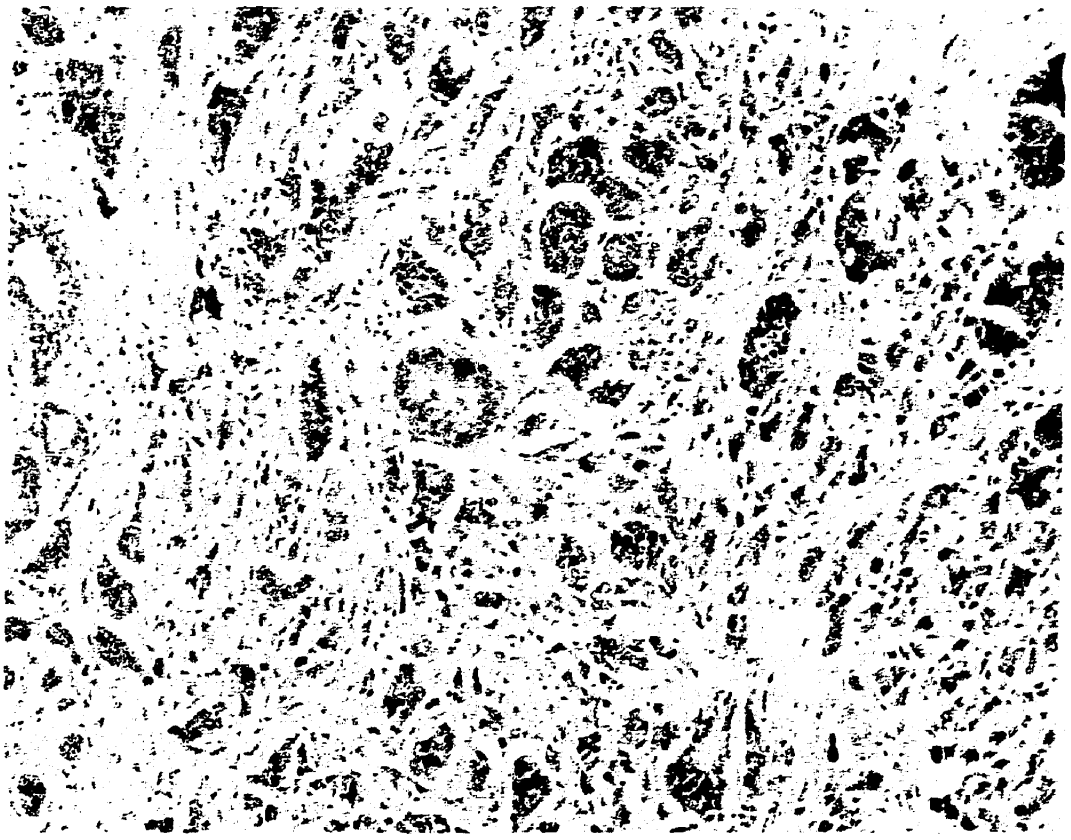


Figure 4. Invasive Ductal Carcinoma. Irregular cords and nests of tumors composing the intraductal component invade the surrounding stroma (exerpted from Pathology, 2nd edition, Rubin and Farber, 1994).

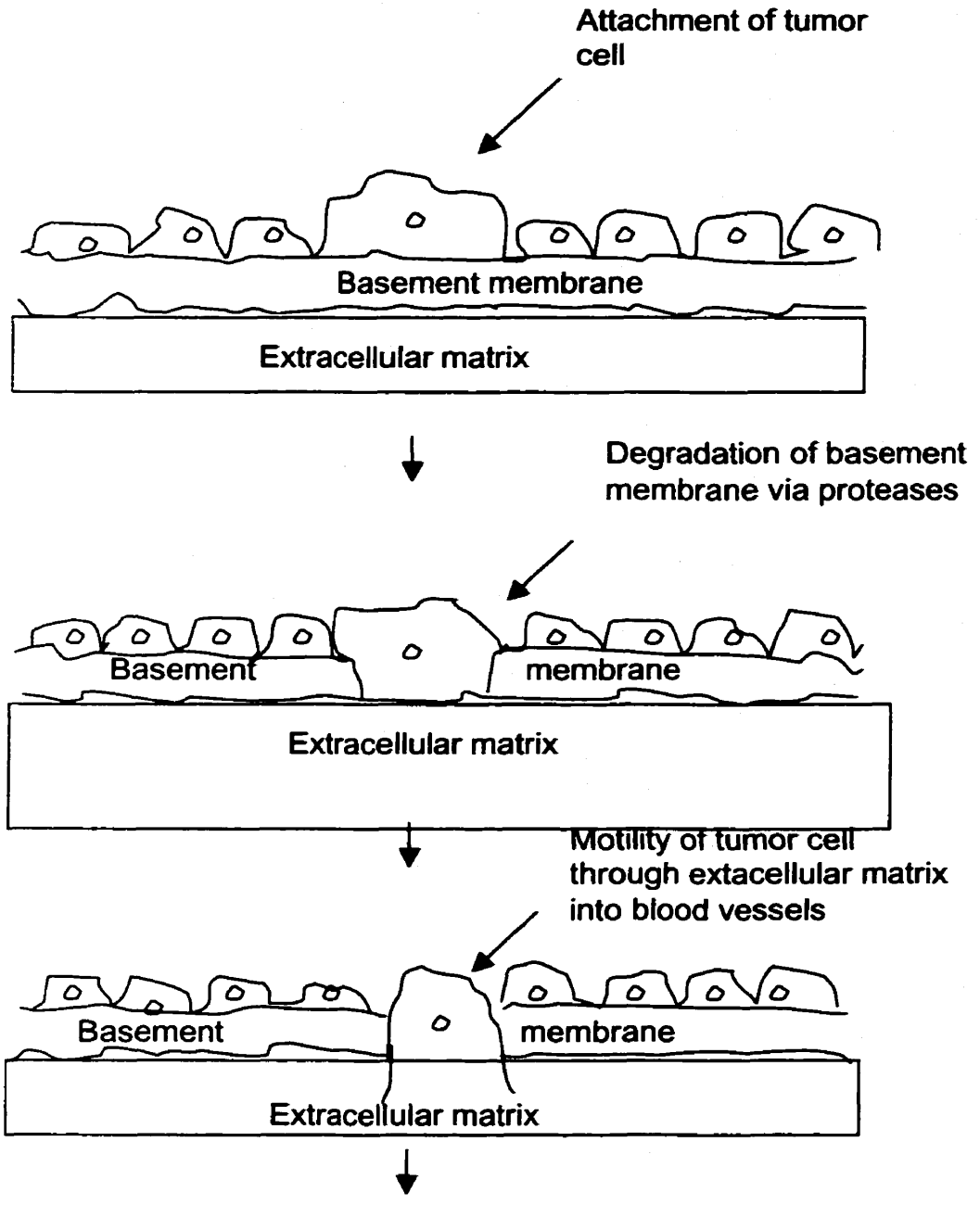
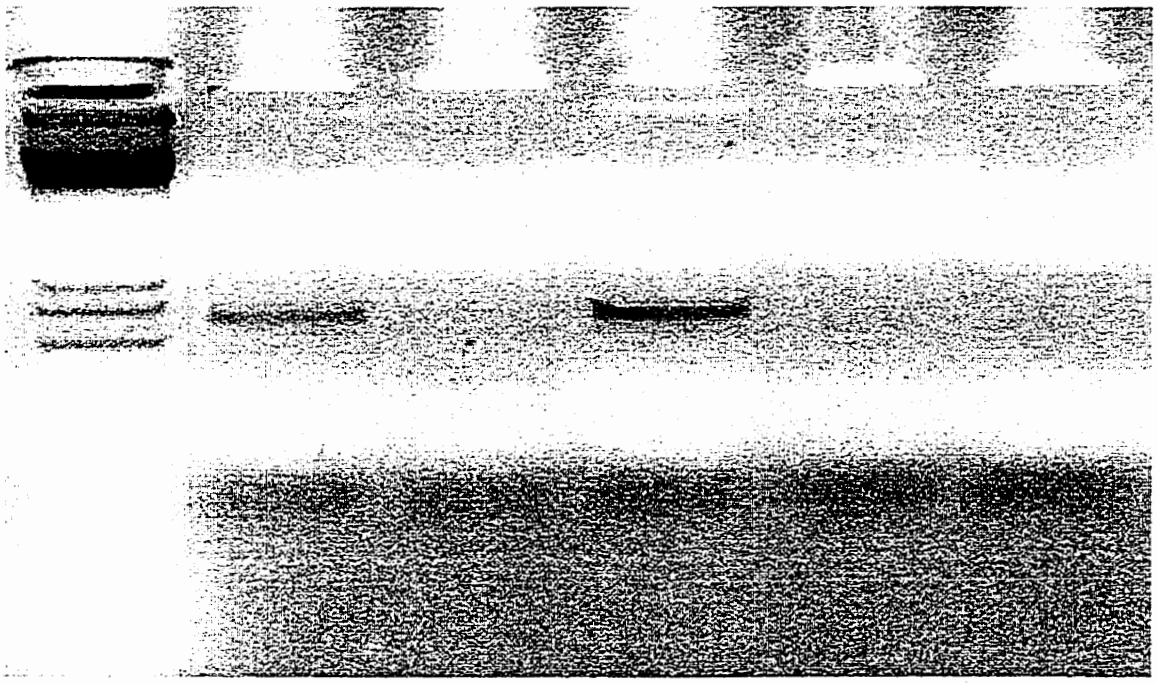


Figure 5. Process of Invasion. Invasion is a well defined, multi-step process involving loss of adhesion to the primary tumor and increased adhesion to both the extracellular matrix and the basement membrane. Proteolytic enzymes next degrade the basement membrane and invasive cells move to a nearby secondary site. Often tumors will metasasize to a distant site by invading the blood or the lymphatic system and enter other tissues by the same mechanism.

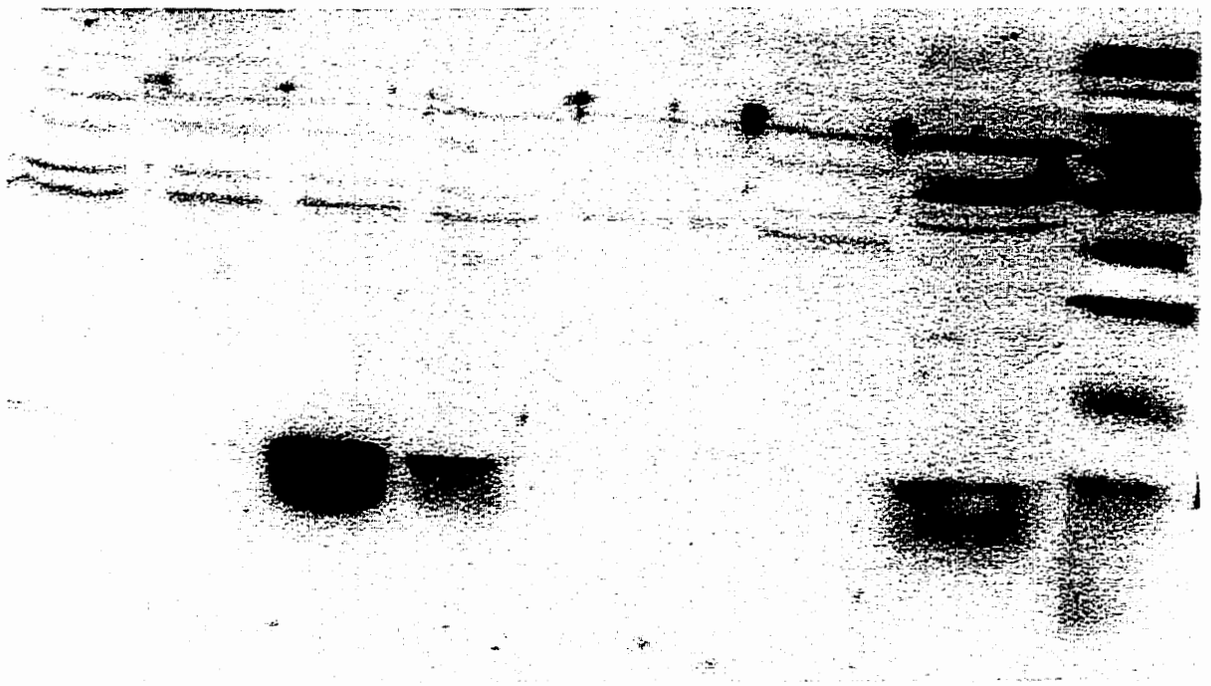
Name	Synonyms	cDNA[a]	Gene[a]	Protein[a]	Binding	Potential Function(s)
S100A1	S100α	+	+	+	+	Regulation cell growth Regulation energy metabolism Regulation contraction
S100A2	S100L, CaN19	+	+	+	+	Unknown
S100A3	S100E		+			Unknown
S100A4	CAPL, p9Ka, 42A, pEL98, mts 1, Metastasin, Calvasculin, 18A2	+	+	+	+	Regulation cell growth Cell differentiation Regulation cell structure
S100A5	S100D		+			Unknown
S100A6	CACY, 2A9, PRA, CaBP 5B10, Calcyclin	+	+	+	+	Regulation cell growth Intracellular signal transduction
S100A7	PSOR1, Psoriasin	+	+	+	+	Unknown
S100A8	CAGA, MRP-8, p8, MAC 387, B8Ag, L1Ag, CP-10, MIF, NIF	+	+			Response to chemotactic factors Regulation cell growth Regulation kinase activity Cell differentiation
S100A9	CAGB, CFAg, MRP-14, p14, MAC 387, B8Ag, L1Ag, MIF, NIF	+	+			Response to chemotactic factors Regulation cell growth Regulation kinase activity
S100A10	Ca[1], p11, p10, 42C, Calpactin light chain		+	+	-	Regulation neurotransmitter release Anti-inflammatory
S100B	NEF, S100B	+	+	+	+	Regulation cell growth Regulation energy metabolism Regulation contraction Regulation cell structure Memory and learning Intracellular signal transduction Cell differentiation
S100C	S100C, Calgizzarin	+		+	+	Unknown
S100P	S100P	+		+	+	Unknown
CALB3	ICaBP, Calbindin-D9k	+	+	+	+	Calcium buffer Regulation calcium homeostasis
S100F-P	Profilaggrin	+	+	+	+	Regulation cell structure
S100F-T	Trichohyalin	+	+	+	+	Regulation cell structure

Figure 6. The S100 Subfamily of the EF Hand Protein Family. Described are the members of the EF hand protein family with their respective number of EF hand motifs, molecular weights and proposed functions (excerpted from Zimmer et al., 1995)



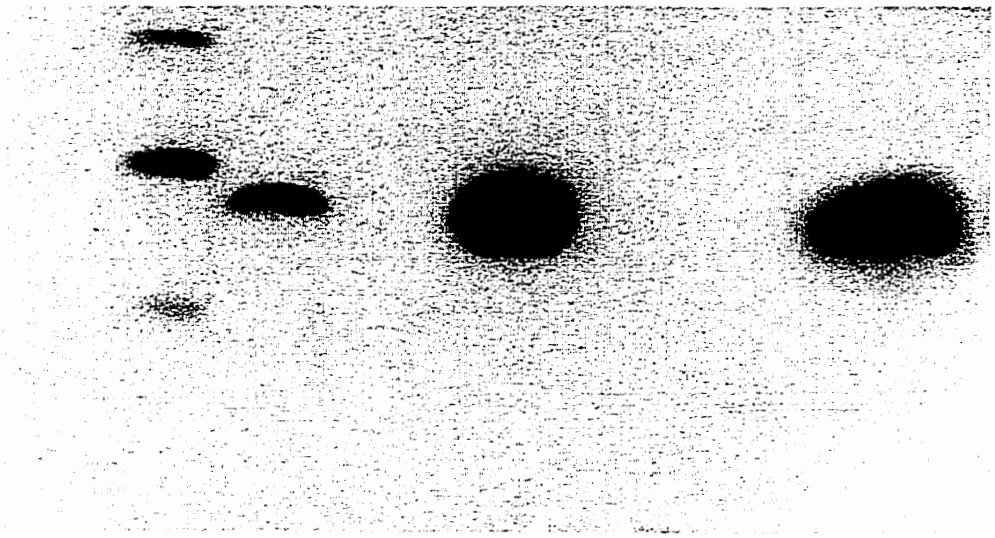
ladder MCF10A1 MCF10AT3B MCF10AT3B + E_2 MDA-MB-231 RNA-

Figure 7. RNA Expression of Psoriasin in Breast Cell Lines. RT-PCR analyses in MDA-MB-231 and MCF10AT3B cell lines show no constitutive expression of psoriasin. Immortalised MCF10A1 shows expression of psoriasin. Psoriasin can be induced by a 48-hour exposure to β-estradiol in MCF10AT3B cell lines. Psoriasin is 373 bp in length. GAPDH controls were performed (data not shown).



CL6FA1 CL71A1 CL7FD3 CL7FB2 CL6VA1 MDAMB231 MCF7+E_2 ladder

Figure 8. Protein expression of MDA-MB-231 and transfected clones. Western blot analysis demonstrates the presence of psoriasin in clones CL7FA1^{psor}, CL7FB2^{psor} and CL7FD3^{psor}. Vector alone control clones CL71A1^{vector} and CL6VA1^{vector} show an absence of psoriasin expression. Parent cell line MDA-MB-231 shows an absence of psoriasin expression. Positive control MCF7 + E^2 reveals a positive expression for psoriasin using Danish anti-rabbit antibodies. 50μg crude protein loaded to gel. Coomassie blue loading control was performed (data not shown).



ladder CL7 FD3 MDA-MB-231 ZP1B3 ZV1C1 ZV2B4 ZP5E12

Figure 9. Psoriasin Protein Expression of MCF10AT3B and Transfected Clones. Western blot analysis demonstrates the presence of high levels of psoriasin in clones ZP1B3^{psor} and ZP5E12^{psor} using Danish anti-rabbit antibody. Vector alone control clones ZV1C1^{vector} and ZV2B4^{vector} show an absence of psoriasin expression. Parent cell line MCF10AT3B shows no psoriasin expression. CL7FD3^{psor} is positive control. 50 μg crude protein loaded. Coomassie blue stain was performed as loading control (data not shown).

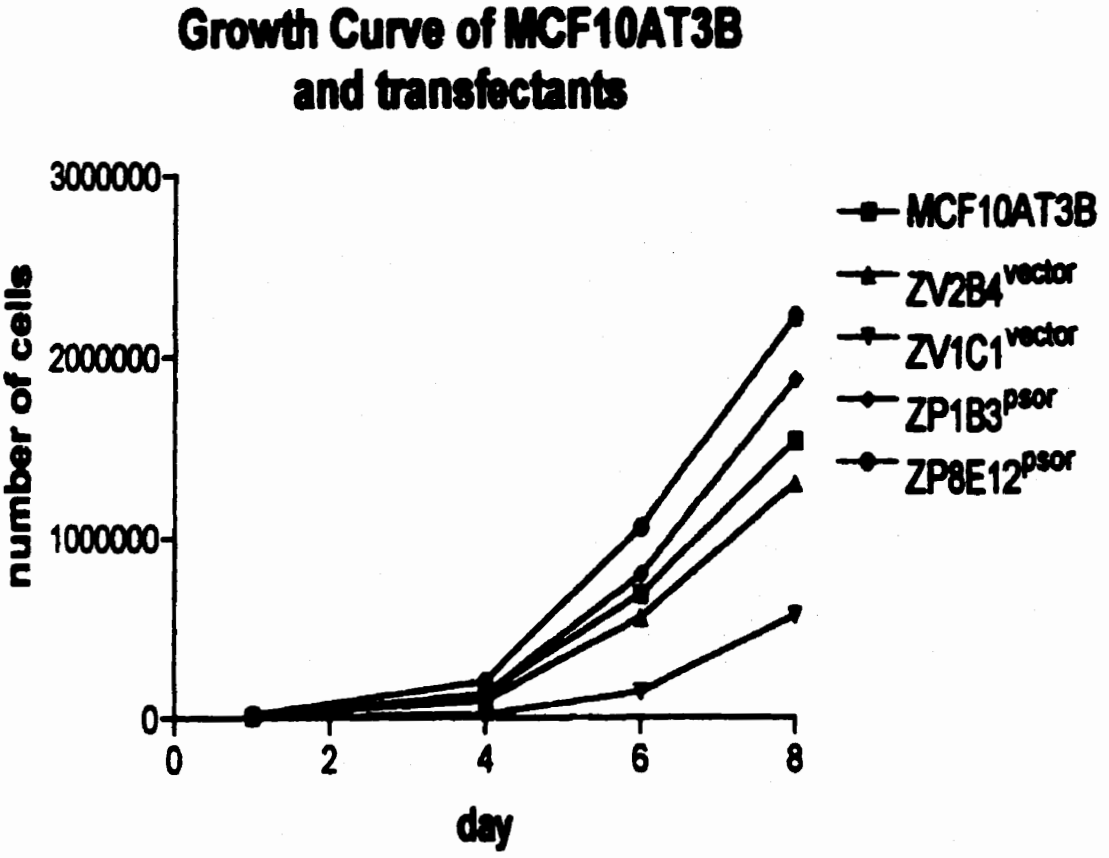


Figure 10. *In vitro* Growth of MCF10AT3B series. *In vitro* growth of parent MCF10AT3B, ZV1C1^{vector} and ZV2B4^{vector} vector control and ZP1B3^{psor} and ZP8E12^{psor} psoriasin transfected cell lines is depicted (n=3). There is no statistical difference in growth rate of these cell lines ($p>0.05$)

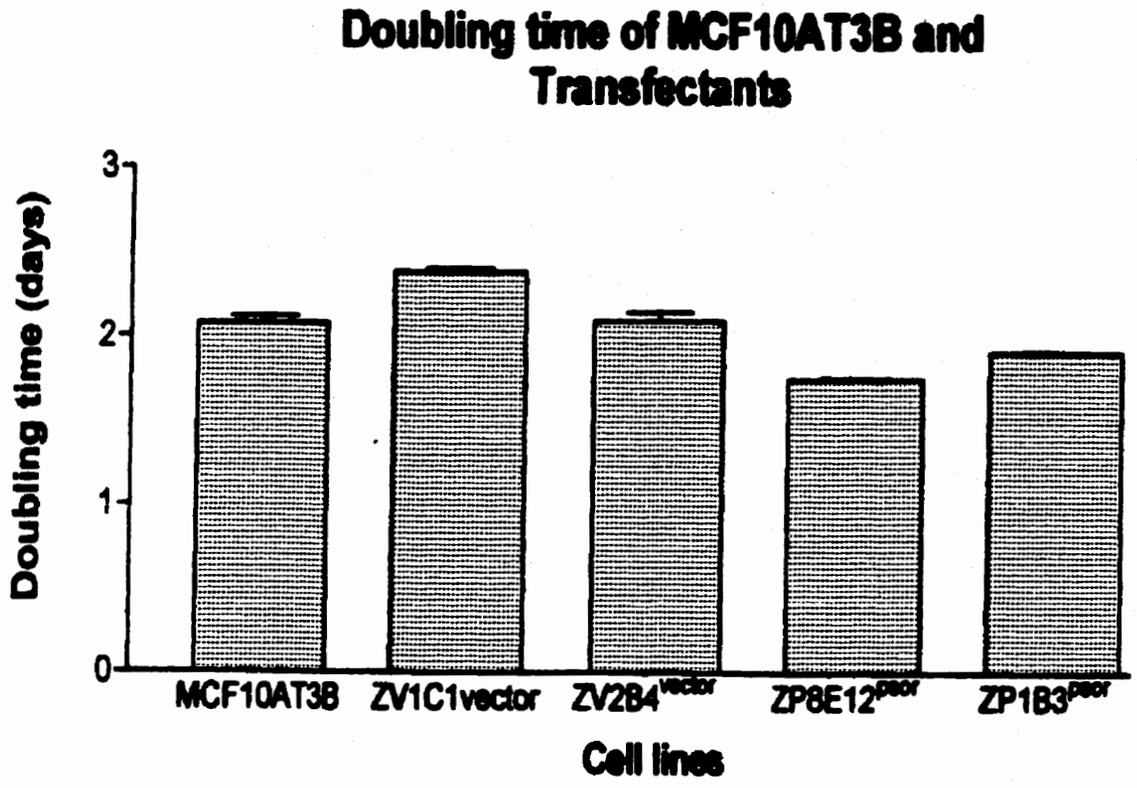


Figure 11. *In vitro* Growth of MCF10AT3B series. *In vitro* growth of parent MCF10AT3B, ZV1C1^{vector} and ZV2B4^{vector} vector control and ZP1B3^{psor} and ZP8E12^{psor} psoriasin transfected cell lines is depicted doubling time (n=3). There is no statistical difference between the growth of the above cell lines ($p>0.05$) SEM.

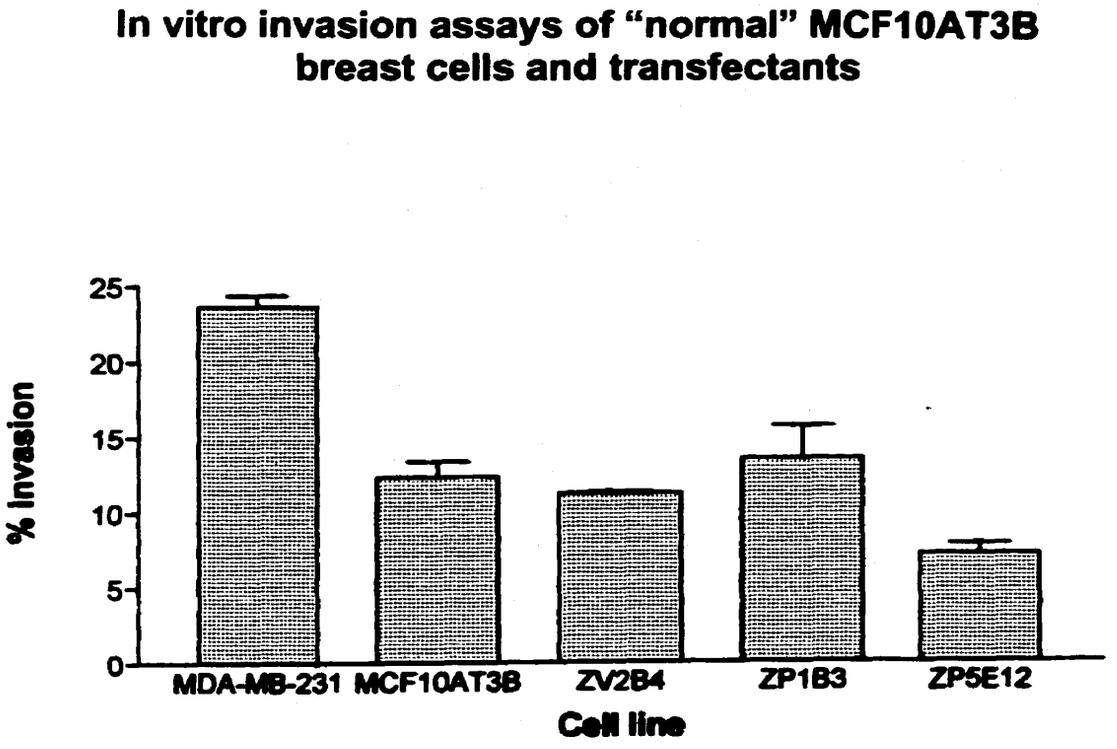


Figure 12. *In vitro* invasive response of MCF10AT3B Series. Boyden Chamber invasion assays show that of the two clones that were transfected with psoriasin, ZP5E12^{psor} exhibited a 50% reduction of *in vitro* invasive behavior compared to MCF10AT3B parent and ZV2B4^{vector} vector alone controls ($p<0.05$) ($x \pm SEM$) (n=3). MDA-MB-231 is represented as a positive control for invasive cellular behavior.

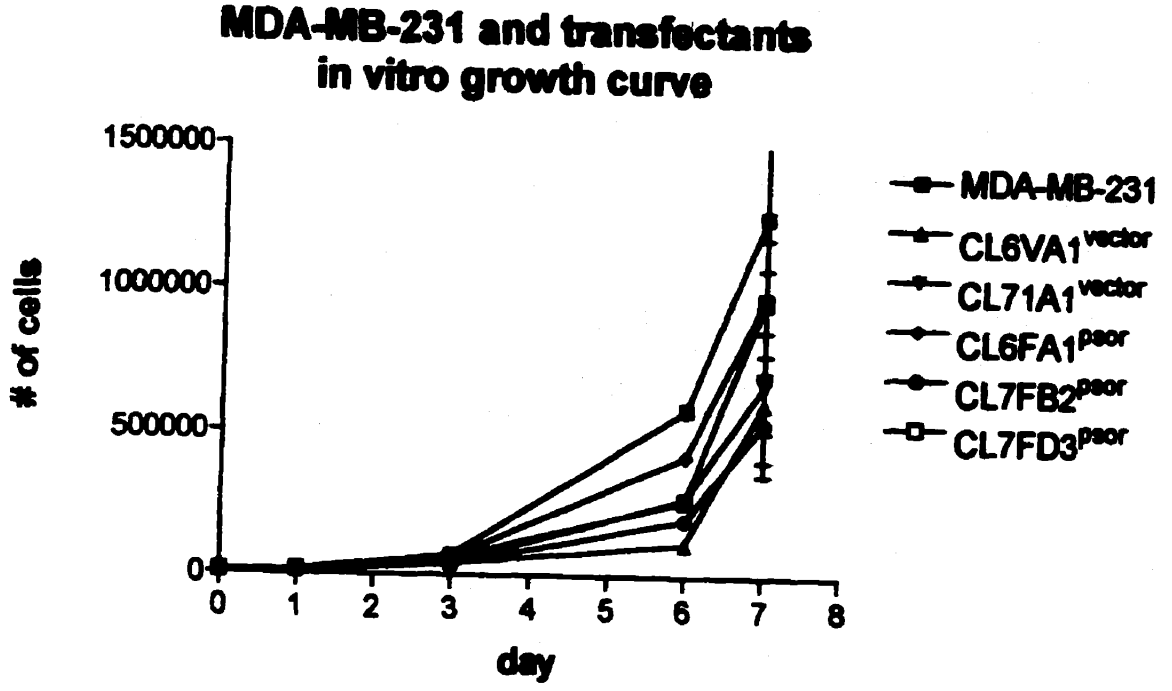


Figure 13. ***In vitro*** **Growth of MDA-MB-231 series.** *In vitro* growth of parent MDA-MB-231,CL6VA1^{vectorr},CL6VA1^{vector},CL71A1^{psor}, CL7FB2^{psor}, and CL7FD3^{psor} is depicted by a growth curve (n=3). There is no statistical difference in growth between the above mentioned cell lines ($p>0.05$) ($x \pm SEM$)

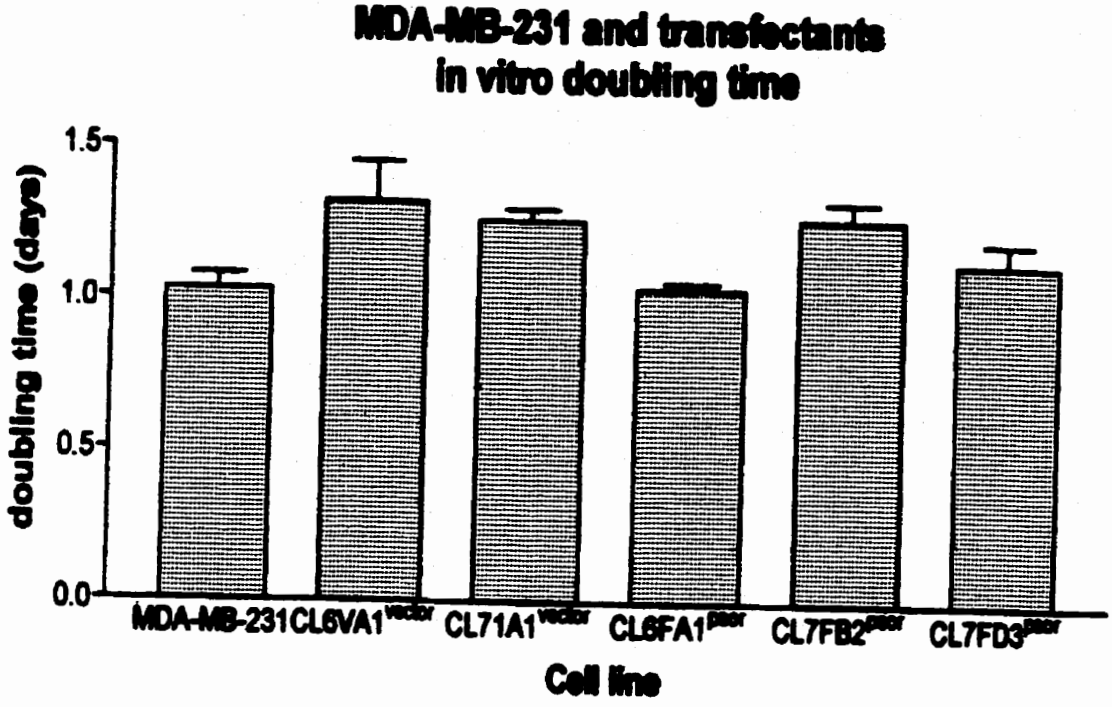


Figure 14. *In vitro* growth of MDA-MB-231 series. *In vitro* growth of parent MDA-MB-231,CL6VA1^{vectorr},CL6VA1^{vector},CL71A1^{psor}, CL7FB2^{psor}, and CL7FD3^{psor} is depicted by doubling time (n=3). There is no statistical difference in the doubling times of the above mentioned cell lines ($p>0.05$) ($x \pm SEM$)

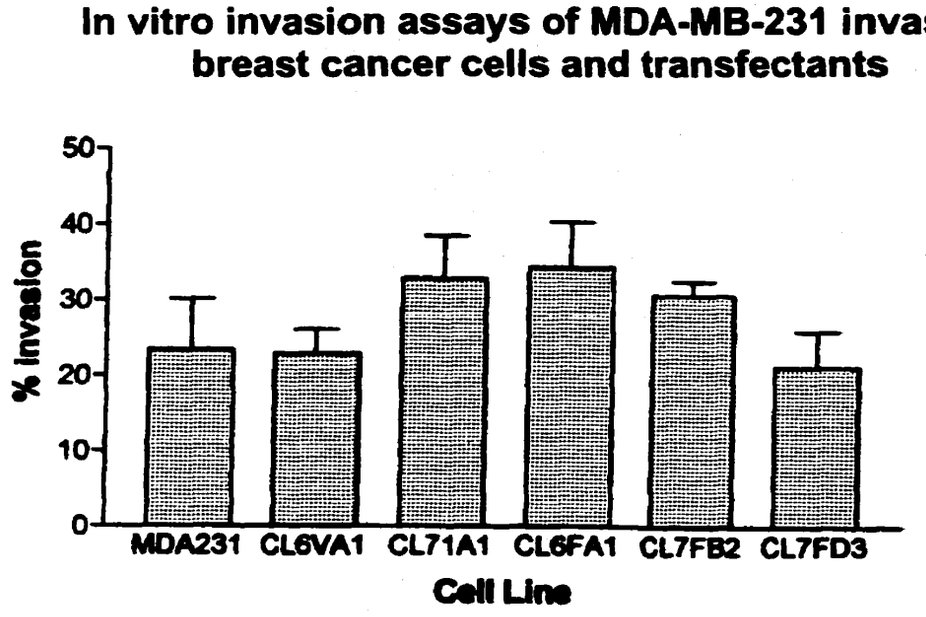


Figure 15. *In vitro* invasive response of MDA-MB-231 Series. Psoriasin does not effect the rate of invasion (n=3), as measured by percent, when comparing CL7FB2^{psor} and C67FA1^{psor} (low expressing), CL7FD3^{psor} (high expressing), CL71A1^{vector} and CL7VA1^{vector} vector alone and MDA-MB-231 parent controls ($p>0.05$) ($x \pm SEM$).

Figure 16. Representation of tumors formed from injecting breast cancer cell line MDA-MB-231 into Balb C nu/nu mice. Balb C nu/nu mouse displays the *in vivo* growth of 1 x10^7 cells 42 days post-injection. All Balb C nu/nu mice injected with MDA-MB-231 (parent), CL71A1^{vector} (vector alone), CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cells looked similar to this one pictured.

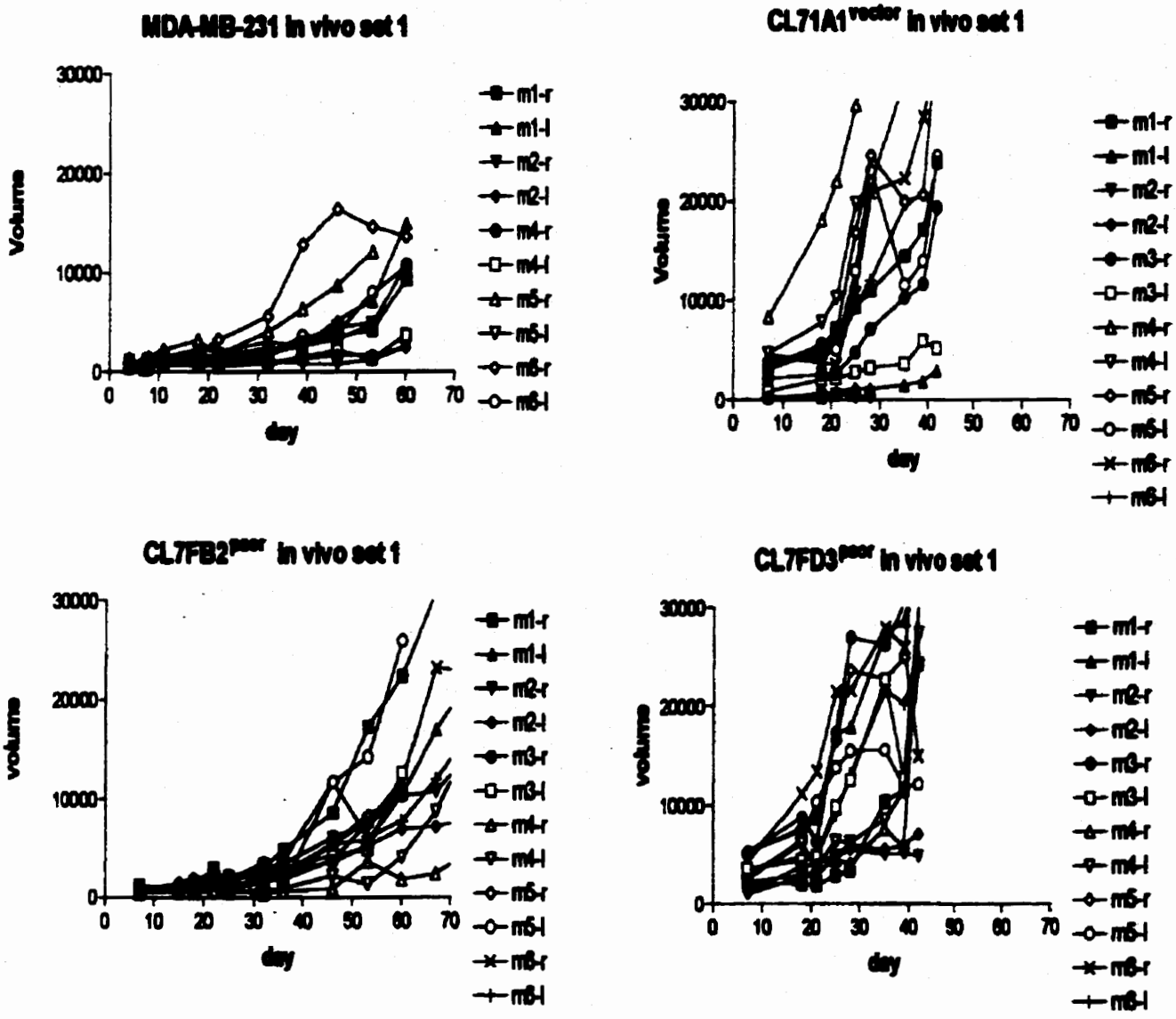


Figure 17. In vivo growth set 1 mouse experiment. Depicted are the growth curves from Balb C nu/nu individual mice injected with MDA-MB-231, CL71A1^{vector}, CL7FB2^{psor}, and CL7FD3^{psor}. Six mice were injected per cell line and tumor volume was measured and plotted onto the above graphs (m= mouse number, l= left side of mouse, r= right side of mouse). CL71A1^{vector} and CL7FD3^{psor} cell lines bearing tumors grow larger than CL7FB2^{psor} and parental MDA-MB-231cells ($p<0.05$)

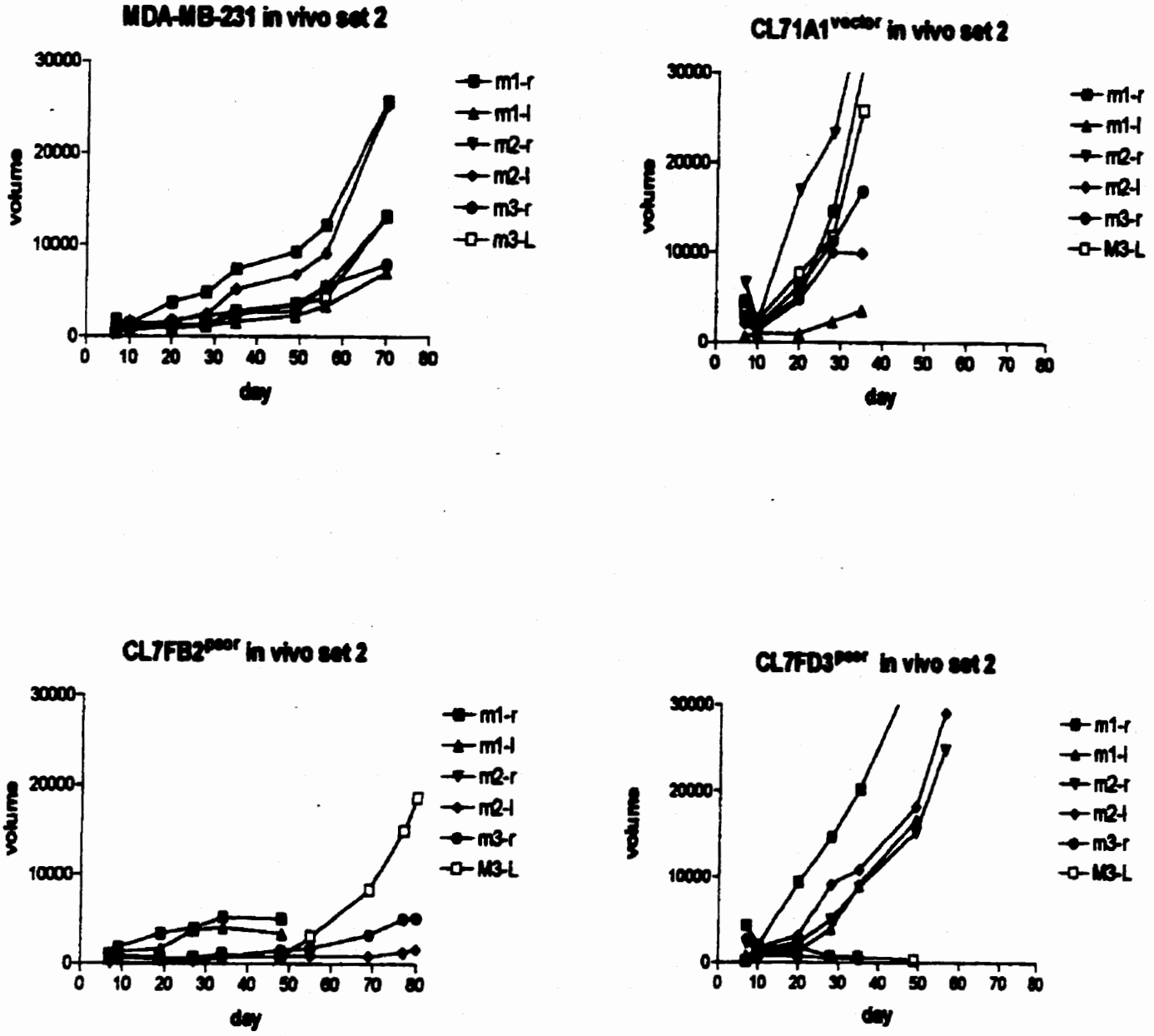


Figure 18. In vivo growth set 2 mouse experiment. Depicted are the growth curves from Balb C nu/nu individual mice injected with MDA-MB-231, CL71A1^{vector}, CL7FB2^{psor}, and CL7FD3^{psor}. Three mice were injected per cell line and tumor volume was measured and plotted onto the above graphs (m= mouse number, l= left side of mouse, r= right side of mouse). CL71A1^{vector} and CL7FD3^{psor} cell lines bearing tumors grow larger than CL7FB2^{psor} and parental MDA-MB-231cells ($p<0.05$)

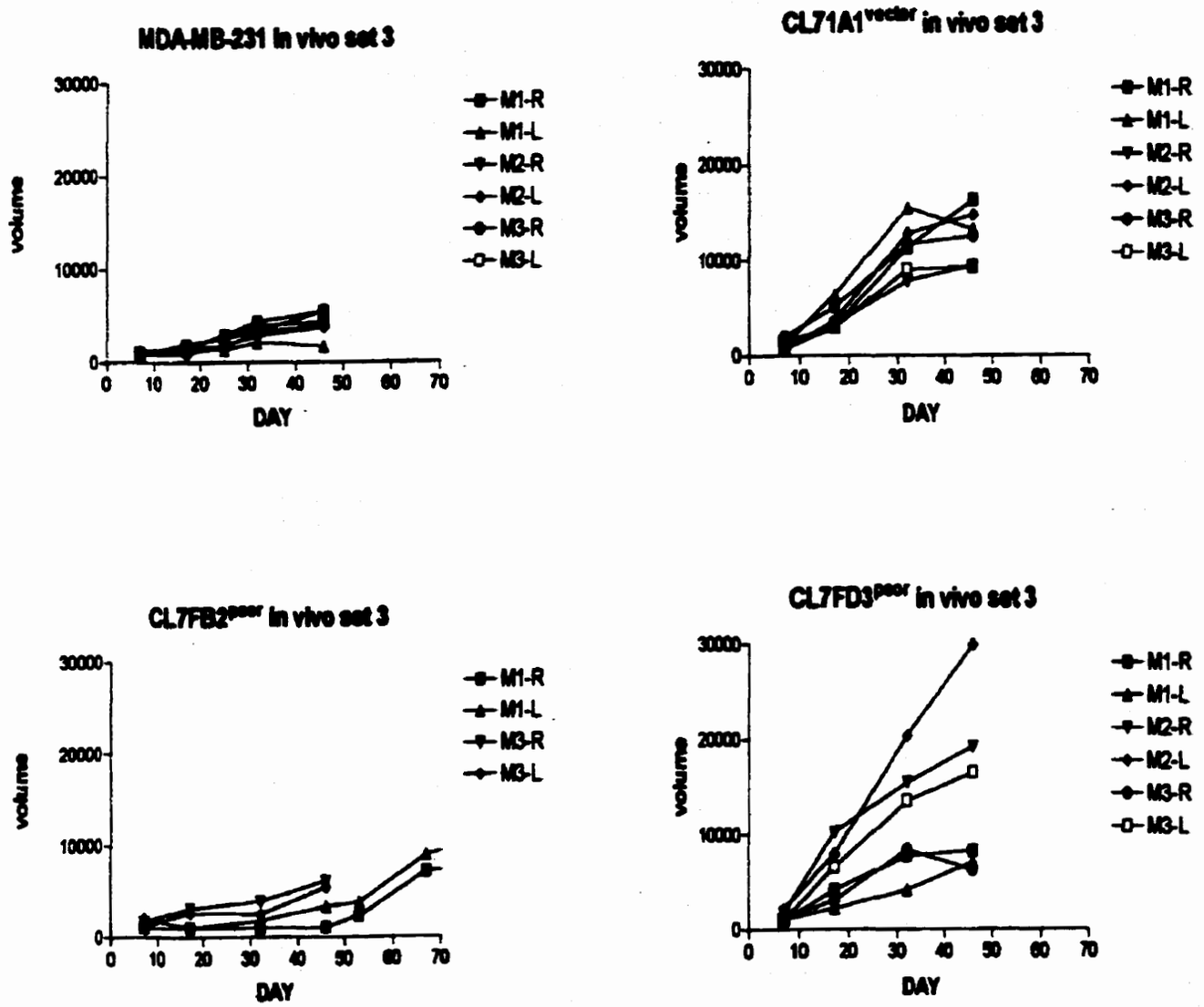


Figure 19. In vivo growth set 3 mouse experiment. Depicted are the growth curves from Balb C nu/nu individual mice injected with MDA-MB-231, CL71A1^{vector}, CL7FB2^{psor}, and CL7FD3^{psor}. Three mice were injected per cell line and tumor volume was measured and plotted onto the above graphs (m= mouse number, l= left side of mouse, r= right side of mouse). CL71A1^{vector} and CL7FD3^{psor} cell lines bearing tumors grow larger than CL7FB2^{psor} and parental MDA-MB-231cells ($p<0.05$)

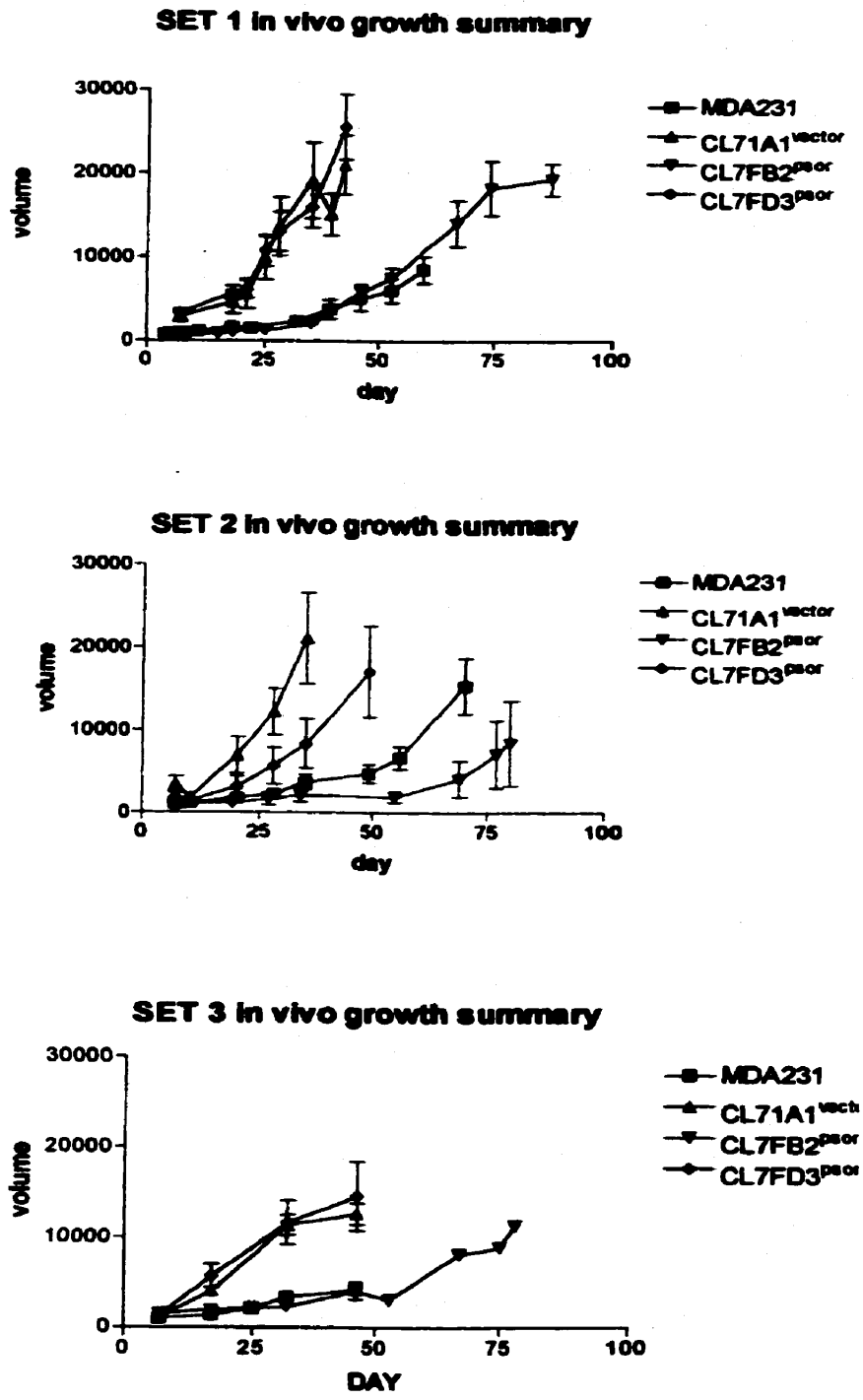


Figure 20. Summary of in vivo growth mouse experiment. Summarised are the mean growth curves from sets 1-3 Balb C nu/nu mice injected with MDA-MB-231, CL71A1^{vector}, CL7FB2^{psor}, and CL7FD3^{psor}. CL71A1^{vector} and CL7FD3^{psor} cell lines bearing tumors grow larger than CL7FB2^{psor} and parental MDA-MB-231cells ($p<0.05$)

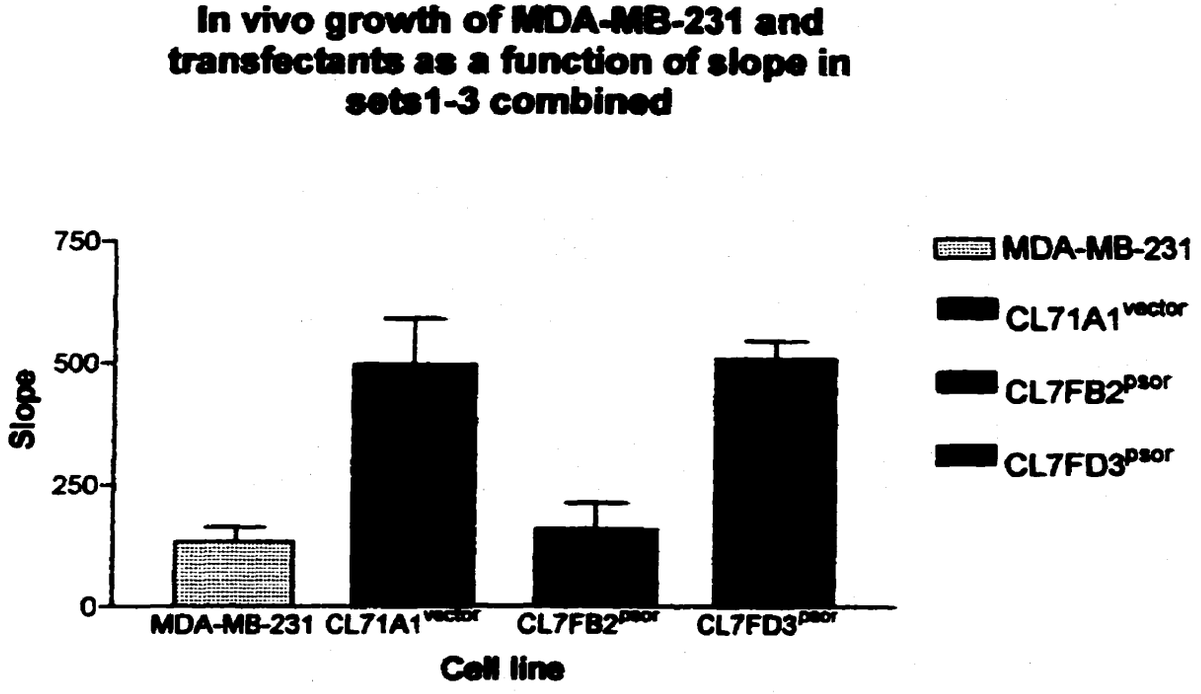


Figure 21. In vivo growth summary. Depicted is the in vivo growth of Balb C nu/nu mice injected with MDA-MB-231, CL71A1^{vector}, CL7FB2^{psor}, and CL7FD3^{psor}. CL71A1^{vector} and CL7FD3^{psor} mice grew tumors significantly larger than parental MDA-MB-231 and CL7FB2^{psor} mice ($p<0.05$)

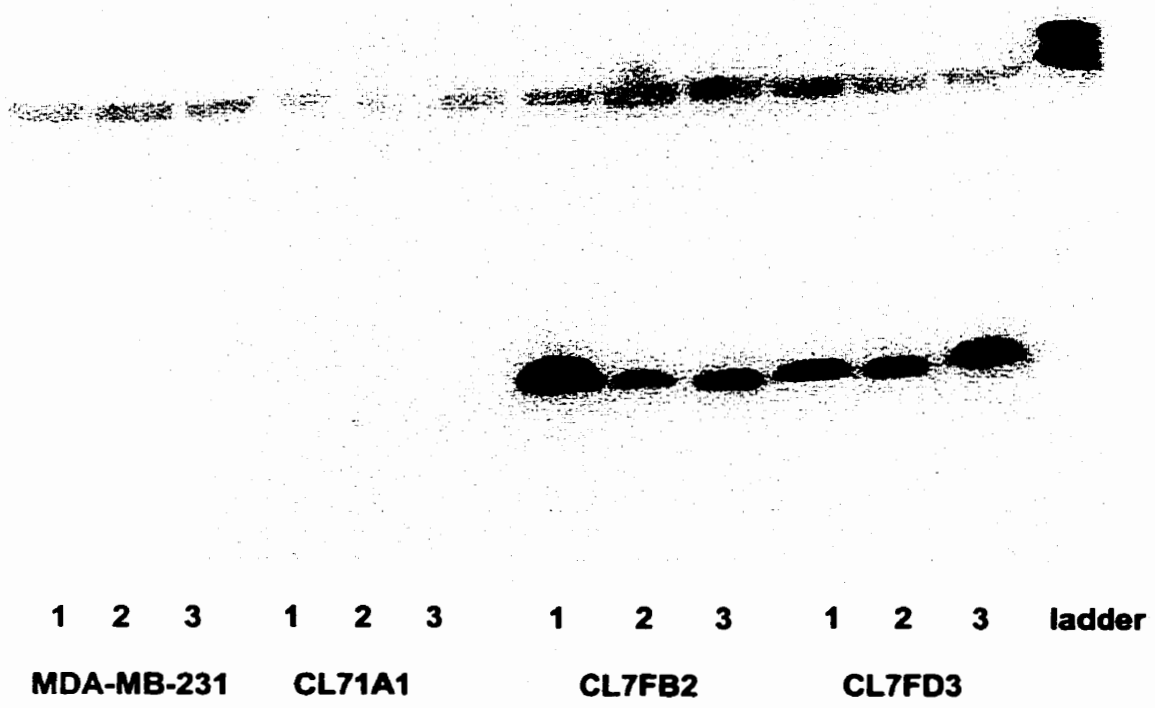


Figure 22. Protein expression of psoriasin post mortem in Balb C nu/nu mice. Western Blot analysis shows the post mortem expression of psoriasin from MDA-MB-231, CL71A1^{vector} , CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cell lines grown *in vivo* into Balb C nu/nu mice. Three tumors were selected at random from each group to perform this experiment. Parental MDA-MB-231 (parent) and CL71A1^{vector} control mice show no expression of psoriasin while psoriasin + vector transfected CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cell lines show positive expression for psoriasin. Danish anti-rabbit antibody was used in these experiments.

Figure 23. Necrotic cells. Represented is a histological section from an MDA-MB-231 tumor containing necrotic cells. Outer darker rim is tumor and center is necrosis.

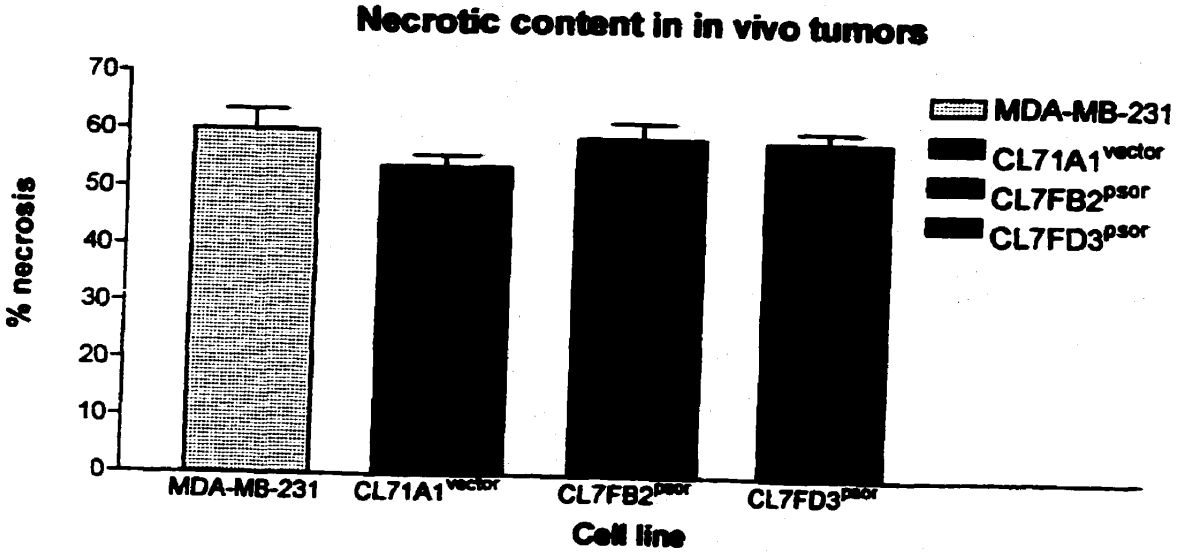
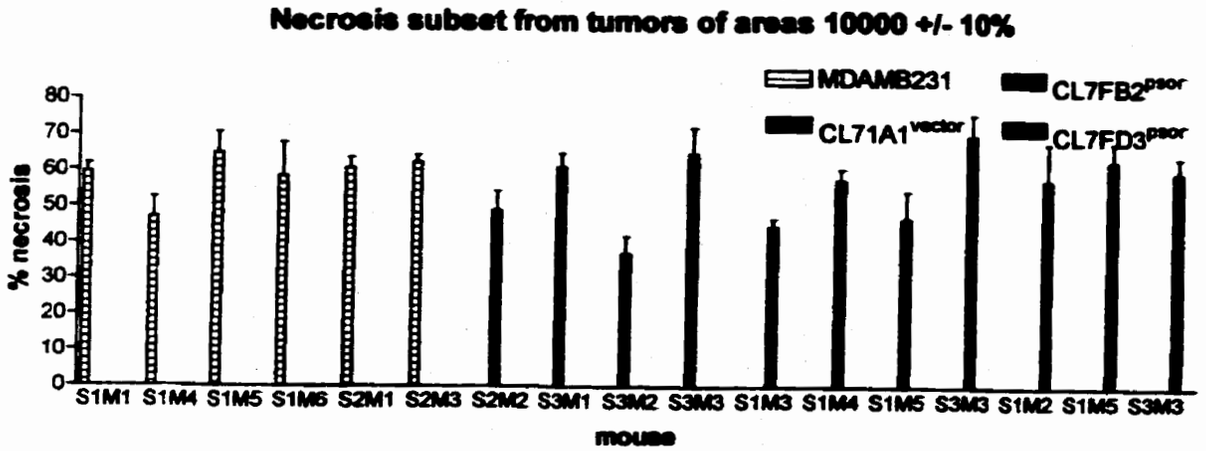
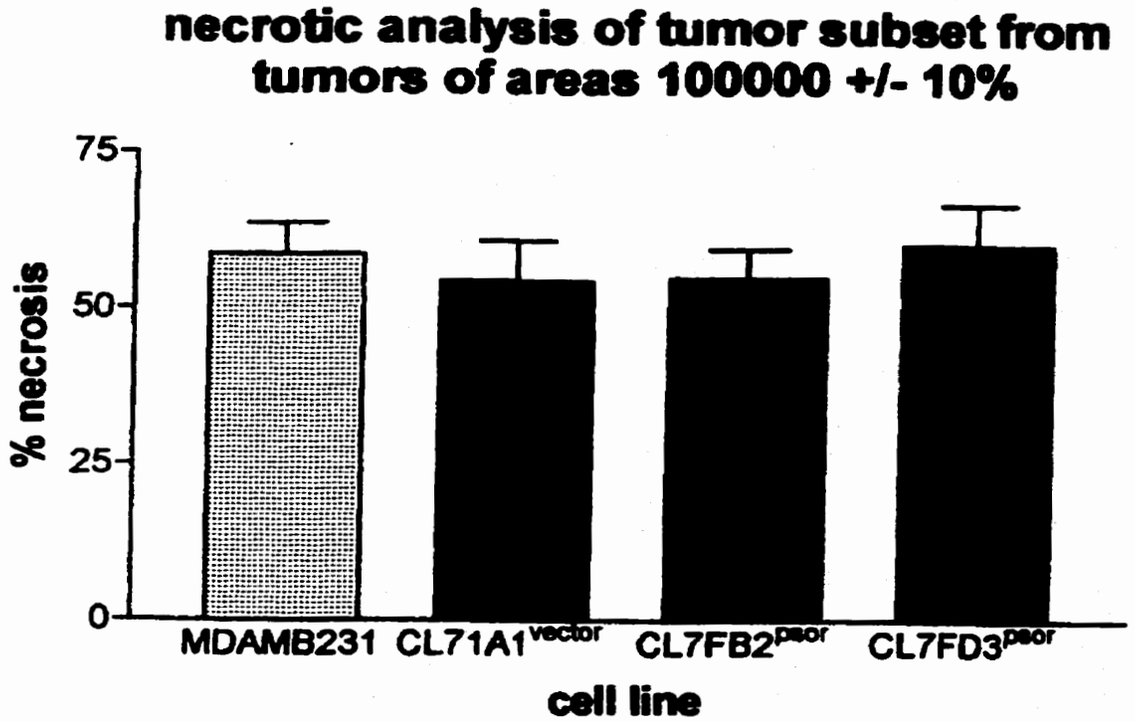


Figure 24. Necrotic content relative to size in in vivo tumors. Represented is the mean necrotic content found in MDA-MB-231, CL71A1^{vector} , CL7FB2^{psor} (low expressing) and CL7FD3^{psor} (high expressing) cell lines grown *in vivo* into Balb C nu/nu mice. There was no statistical difference in the necrotic abundance in the above mentioned cell lines ($p>0.05$)



a



b

Figure 25. Necrotic abundance subset. a) Represented are the individual amounts of necrosis found in MDA-MB-231 (n=6), CL71A1^{vector} (n=4), CL7FB2^{psor} (n=4) and CL7FD3^{psor} (n=3) cell lines grown *in vivo* into Balb C nu/nu mice with tumor volumes approaching 10000 μm^3 +/- 10%. b) Summarized is the mean necrotic abundance from the above mentioned cell lines. No statistical difference was seen in necrotic abundance when tumor volumes are held constant ($p>0.05$)

↓ **mitotic figure**

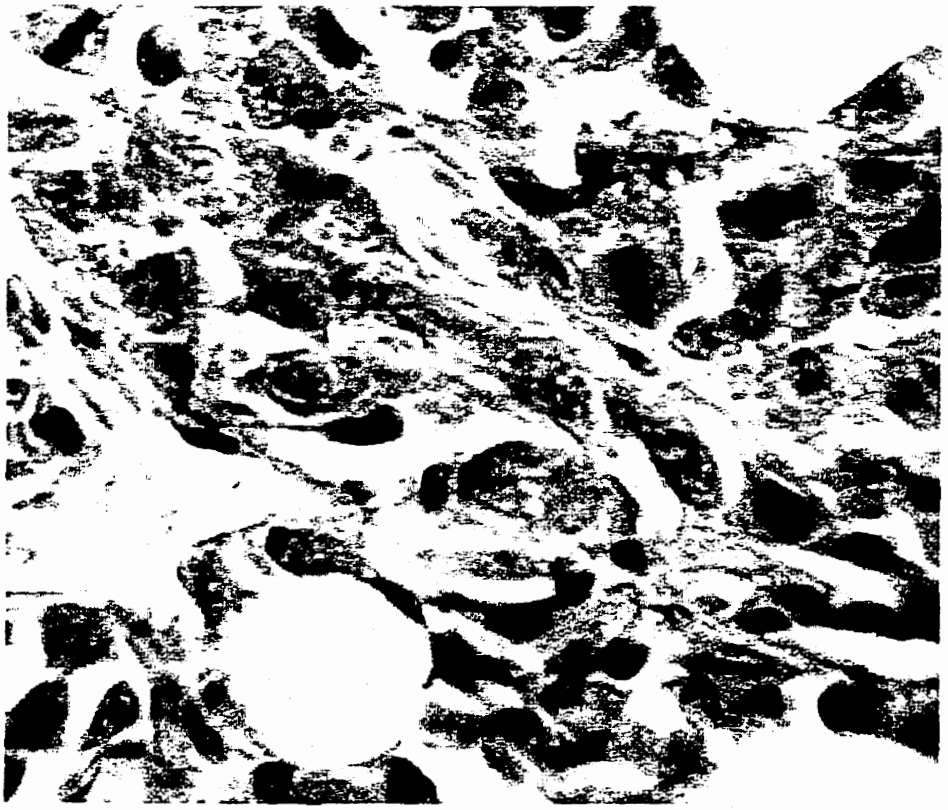


Figure 26. Mitotic Figure. Represented is a histological section from an MDA-MB-231 tumor showing mitotic figures. Arrow represents a cell that is undergoing active mitosis.

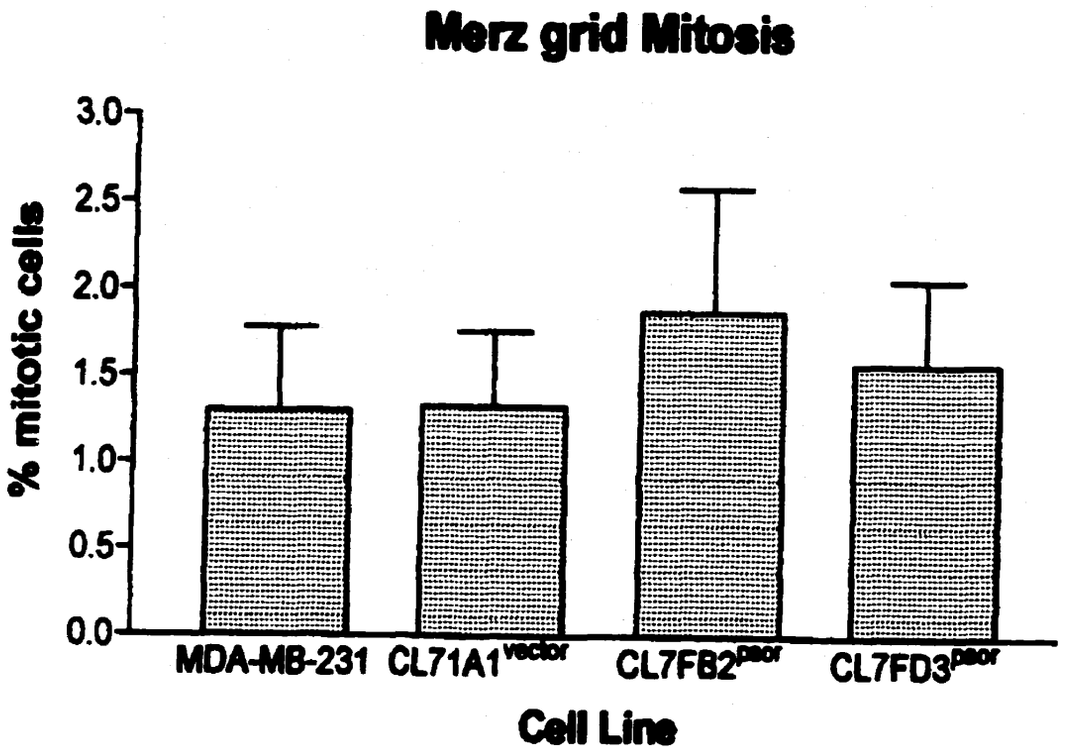
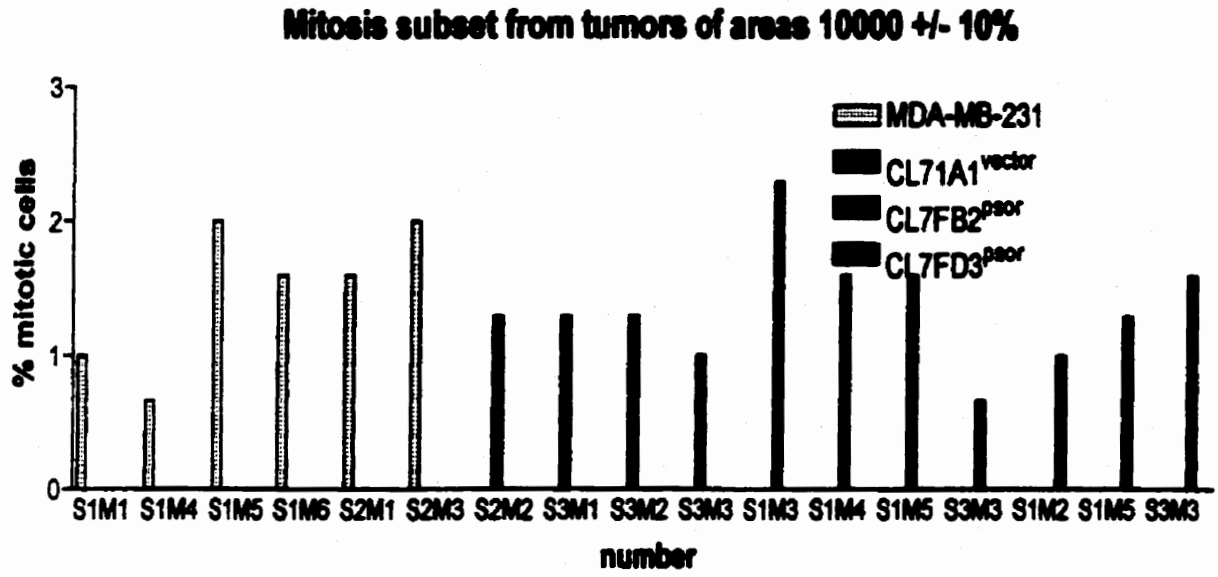
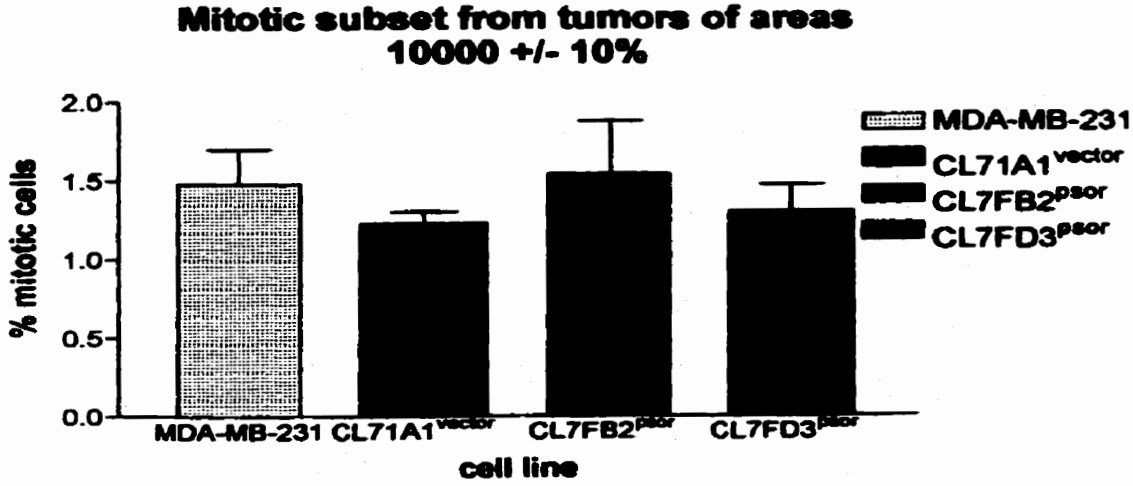


Figure 27. Mean mitotic event. Represented is the mean number of mitotic events in tissues expressing high levels ($CL7FD3^{psor}$), low levels ($CL7FB2^{psor}$), or no psoriasin -$CL71A1^{vector}$ vector alone and MDA-MB-231 parent (n=3). There is no statistical difference in the abundance of mitotic events in the above mentioned cell lines when injected into a Balb C nu/nu mouse ($p>0.05$) ($x \pm SEM$)



a



b

Figure 28. Mitotic abundance subset. . a) Represented are the individual amounts of mitotic abundance found in MDA-MB-231 (n=6), CL71A1^{vector} (n=4), CL7FB2^{psor} (n=4) and CL7FD3^{psor} (n=3) cell lines grown *in vivo* into Balb C nu/nu mice with tumor volumes approaching 10000 μm^3 +/- 10%. b) Summarized is the mean mitotic abundance from the above mentioned cell lines. No statistical difference was seen in mitotic abundance when tumor volumes are held constant ($p>0.05$) ($x \pm SEM$)

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