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EXPRESSION OF CD44 IN HUMAN BREAST CANCER

by

Janine Sidorchuk

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

MASTERS OF SCIENCE

Department of Pathology

University of Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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This thesis is dedicated to the memory of Gordon Sones Sr- who always told me he was proud of me and encouraged me in my studies.

ABSTRACT

CD44 is a cell surface glycoprotein that functions as an adhesion molecule for the extracellular matrix, and is alternatively spliced to form mRNA variants. It has been demonstrated that expression of CD44 variants may be associated with many invasiveness cancers, including breast cancer. We have shown that patterns of CD44 expression are different between ER- (highly invasive) and ER+ (less invasive) breast cancer cells. In ER- breast cancer cell lines, the CD44E (epithelial) variant, containing the alternately spliced exons v8 to v10, is reduced when compared to CD44H (standard form). As well, there is a decreased incorporation of v7 and v10 containing variants in ER- cells. This difference is partly conserved in ER- and ER+ tumours. Based on these studies, our aim is to determine if the tumor cell microenvironment influences CD44 splicing patterns, and whether the difference in CD44 expression contributes to increased invasiveness of ER- cell lines. We studied the effects of cell density and cell substrates on both ER+ and ER- cells to determine the effects of the microenvironment on CD44 expression and splicing. Cell density had no effect on levels of CD44H or CD44E expression or splicing for either cell line, nor did substrates affect overall expression of CD44H in either cell line. However, in ER+ cells, a decrease of 59% in expression of CD44E was observed in cells grown on Matrigel compared to plastic. ($p<.0303$) Since it has been suggested SR (serine-arginine rich) proteins are involved in regulating alternative splicing of CD44, we also measured the levels of SR proteins in ER+ cells plated on several substrates by Western blot. Results showed there was no change in the relative SR levels of cells grown on Matrigel, collagen, or p-HEMA when compared with plastic. To assess effects of

CD44E overexpression on invasiveness, we transfected an ER- breast cancer cell line with the CD44E gene. Integration of the transgene, mRNA and protein overexpression were assessed, and changes in invasiveness in the transfectants were determined by invasion assays. Growth assays determined that transfection with CD44E did not significantly alter the growth rates of the cells, and invasion assays performed indicated that CD44E overexpression did not alter invasiveness in the JS11-H2 clone. In summary, since patterns of CD44 expression in ER- breast cancer cells were unaffected by cell density or growth on substrates, this suggests that an intrinsic property related to the differentiation of ER- breast cancer cells regulates CD44 expression. In contrast, extracellular signal conferred by substrates regulates the expression pattern of the CD44E variant, in ER+ cells. This alteration in CD44 splicing was not due to a change in the expression pattern of SR proteins. Overexpression of CD44E did not result in any increase in invasive potential as measured by the Boyden chamber assay, but it remains to be determined whether an increase in CD44E may increase a cell's ability to invade through other tissue components beyond the basement membrane.

ABBREVIATIONS

CD44E/R1	epithelial variant of CD44
CD44H	standard form of CD44
CD44v	variant forms of CD44
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix
EDTA	ethylenedinitrolltetracetic acid
ER	estrogen receptor
FACS	fluorescence-activated cell sorting
HA	hylauronic acid
HGF	hepatocyte growth factor
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
kb	kilobase
mg	milligram
ml	millilitre
MMLV	Moloney murine leukemia virus
MMP	matrix metalloprotease
mRNA	messenger RNA
MT MMP	membrane-type matrix metalloprotease
NCAM	neural cell adhesion molecule

PAI	plasminogen activator inhibitor
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
pHEMA	polyhydroxyethylmethacrylate
Rb	retinoblastoma gene
RHAMM	receptor for hyaluronan mediated motility
RNA	ribonucleic acid
RT PCR	reverse transcription polymerase chain reaction
SR	serine-arginine
snRNP	small nuclear ribonucleic protein
TIMP	tissue inhibitor of metalloproteases
TNF	tumor necrosis factor
uPA	urokinase plasminogen activator
VCAM	vascular cell adhesion molecule

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1 Literature Review

1.1 Molecular aspects of breast cancer

The initiating molecular changes that occur when a normal breast cell becomes neoplastic can be separated basically into three main categories:

1.Genetic alterations to proto-oncogenes that may include point mutations and gene amplification which lead to oncogenic activation;

2. Mutations to "gatekeeper" genes identified in breast cancer;

3.Thirdly, mutations to genes falling under the "caretaker gene" category, including BRCA1 and BRCA2.

1.1.1 Oncogenes and breast cancer

Proto-oncogenes that are commonly involved in breast cancers fall into the categories of transcriptional factors, protein kinases, signal transducers and growth factors/receptors. (Darkee, 1996) Most often, these proto-oncogenes are amplified, leading to their activation. A typical example of this is in the oncogene c-myc. The c-myc oncogene, found on chromosome 8q24, encodes a nuclear DNA binding protein that binds sequences that modulate transcription and therefore control cell proliferation. (Watt et al., 1985) Activation of this oncogene often occurs by amplification (Spencer et al., 1991) which is correlated with 20-30% of primary breast cancers, (Berns et al., 1992 and Escot et al., 1986) as well as with more aggressive forms of breast cancer, (Garcia et al., 1989 and Varley et al., 1987) indicating a possible prognostic value of c-myc.

A second commonly mutated oncogene in breast cancer is erbB-2, or the HER/neu oncogene. ErbB-2 was one of the first consistent genetic mutations to be found in human breast cancers, as amplification of erbB-2 occurs in about 20-25% or breast tumours, leading to protein overexpression. (Gusterson et al., 1992) ErbB-2 is a receptor tyrosine kinase and a member of the ErbB/type I family, and involved in activation of signalling pathways. (Ullrich et al., 1990) ErbB-2 kinase activity is induced by first binding members of the epidermal growth factor family to their receptors (Peles and Yarden, 1993, Wen et al., 1992) dimerization of the receptor tyrosine kinases, and lastly autophosphorylation of their tyrosine residues by members of a receptor kinase family. (Sliwkowski et al., 1994) C-erbB-2 amplification has also been associated with poor prognosis in breast cancer (Borg et al., 1990) and its overexpression in invasive breast carcinomas may correlate with lymph node status (Pavelic et al., 1992), tumours of high-grade (Paik et al., 1990), and larger tumour size. (Borg et al., 1991) C-erbB-2 protein levels have also been correlated with the overexpression of the tumour suppressor gene p53, as well as a more negative prognosis. (Nakopoulou et al., 1996) Currently, breast cancer therapies involving erbB-2 targeting are being looked into. Potential strategies include: antibodies, immunotoxins, and peptide/chemical inhibitors. (reviewed in Hynes and Stern, 1994)

1.1.2 Mutations to "gatekeeper" genes

The second main group of molecular alterations causing breast cancer is to a group of genes classified as tumour suppressor genes. Often this group of genes is affected by initial loss of one of the alleles of the gene, or loss of heterozygosity (LOH). This type of mutation

represents one of the most frequent types of mutations in primary breast carcinomas. (Callahan et al., 1992) Loss of heterozygosity, the loss of one allele can occur as a result of deletions (either whole or sections of chromosomes), or inappropriate recombination (or crossing over) of chromosomes during mitosis. Loss of heterozygosity in breast cancer occurs with greater frequency on specific chromosomal regions, such as 13q, 16q, and 17p. (Sato et al., 1990) They also noted that in the transformation of normal breast cells required multiple genetic alterations and often included more than one allelic loss. Recently, a study of 1280 breast tumours looked at LOH of several areas on the p and q arm of chromosome 17. They found that several of these areas were deleted in nearly half of the breast tumours studied. These deletions were found to correlate with larger tumour size, ER- negative status, and earlier age of cancer onset. (Phelan et al., 1998) An earlier study chose a region on the long arm of chromosome 16 to be studied in 200 breast tumours. They determined that allelic loss of this segment correlated with favourable prognosis in that there was statistical association of LOH with decreased metastasis, overall and disease-free survival. (Hansen et al., 1998) They postulated that this observation could be due to the loss of a gene on 16q that could lead to tumour development, but simultaneously, a "metastasis-inducing" gene could be lost, preventing the tumour from spreading.

Kinzler and Vogelstein (1997) have defined some of these tumour suppressor genes as gatekeeper genes: ones that normally regulate tumour growth directly by promoting tumour cells death or inhibiting tumour cell growth. Mutations to both alleles of a gatekeeper gene (either by two sporadic events, or one inherited mutation and one sporadic one) will

initiate a neoplasm. Several examples of genes classed as gatekeeper genes are the APC (adenomatous polyposis coli gene) and β-catenin genes in colorectal cancer, the Rb gene in retinoblastoma and p53, commonly mutated in many cancers. The p53 tumour suppressor gene has been shown to be the most commonly altered gene in breast cancer (Osborne et al., 1991) and is lost in approximately 50% of all breast tumours. (Baker et al., 1989) p53 is normally functions to preserve the stability of the genome by monitoring DNA for damage caused by a number of factors, e.g. ultraviolet light. (Lane, 1992) The accepted model of p53 function shows us that p53 protein levels rise in DNA-damaged cells (Kastan et al., 1991) until the cells arrest in the G1 phase, where DNA repair can take place, or alternately p53 causes the cell to undergo programmed cell death. (Lane, 1992) Loss of p53 function in a cell involves two mutations: both alleles are either rendered inactive or dysfunctional by mutation or deleted altogether. (el-Ashry and Lippman, 1994) Since the function of p53 is to monitor the genome for DNA mutations, it follows that p53 loss or mutation would correlate with genomic instability. Eyfjord et. al. (1995) showed that in a panel of primary breast tumours, a loss of functional p53 correlated with loss of other chromosome 17 alleles, as well as general clonal chromosomal abnormalities.

p53 also may correlate with prognosis of breast cancer, and perhaps overall patient survival. p53 levels (as detected by immunohistochemical analysis) showed a correlation with other indicators of prognosis; oestrogen receptor status, tumour grade, and level of proliferation. (Haerslev and Jacobsen, 1995)

1.1.3 Mutations to "caretaker" genes

In addition to gatekeeper genes, there exists a class of genes known as the caretaker genes, which are required to maintain genomic stability. They differ from gatekeeper genes in that a mutation to caretaker genes does not cause neoplasia directly, but causes genetic instability which may lead to a mutation in a gatekeeper genes, which could cause cancer to arise. (Kinzler and Vogelstein, 1997) In other words, in this model it would take two sporadic mutations to both alleles of the caretaker gene, as well as two sporadic mutations to both gatekeeper alleles to cause cancer. Some examples of caretaker genes are the mismatch repair genes MSH2 and MLH1 (mutated in colon cancer), and recently added to the list of caretaker genes,- BRCA1 and BRCA2 in breast cancer.

As mentioned previously, a relatively small number of all breast cancers (about 5%) are said to be inherited, due primarily to the presence of two caretaker genes, namely BRCA1 and BRCA2. The BRCA1 gene, located on chromosome 17q21, is mutated in about half of all inherited breast cancers, and germline mutations in BRCA1 have been shown to increase the chances of getting ovarian cancer. (Futreal et al., 1994) This gene codes for a 220 kDa phosphoprotein, normally found in the nucleus, which may act as a transcription factor. This has been demonstrated in GAL4/DNA binding studies that have indicated increased transcriptional activation when BRCA1 was used. (Chapman and Verma, 1996) It was discovered that in many breast tumours (both primary and metastatic) as well as breast cancer cell lines, there was abnormal localisation of BRCA1 to the cytoplasm, showing one way BRCA1 regulates the growth of a cell. (Chen et al., 1995)

The other breast cancer susceptibility gene, BRCA2, was found as a result of analysis of families with increased incidence of breast cancer, but that were not linked to BRCA1. As in the case of BRCA1, BRCA2 also confers a higher risk of breast cancer, but not for ovarian cancer. (Wooster et al., 1994) Recently, both BRCA2, and possibly BRCA1 have been shown to bind to Rad51, a gene involved in DNA repair and recombination during mitosis and meiosis. Sharan et al (1997) postulated that since BRCA2- mutants were radiation sensitive and had many DNA repair problems, BRCA2 may be a cofactor for RAD51 and help repair double stranded DNA breaks.

1.2 Invasion and metastasis

Once the irreversible genetic mutations have converted the once normal breast cell to a neoplastic one, the in situ tumour may acquire the ability to spread to the adjacent tissue - this is known as the process of invasion. Invasion is a well-defined, multi-step process involving, in epithelial tumours, loss of adhesion to the primary tumour and increased adhesion to the extracellular matrix and the basement membrane, proteolysis of the basement membrane, and motility of the invasive cells through the basement membrane to the nearby secondary tumour site. Often, tumours go one step further and metastasise to a distant site by invading the blood of lymphatic systems and entering other tissues by the same mechanism as the initial invasion. (Aznavoorian et al., 1993)

1.2.1 Adhesion molecules

The first step in this process involves changes in tumour cell adhesion to the adjacent

tumour cells as well as to the extracellular matrix and basement membrane. There are four basic groups of adhesion molecules: integrins, cadherins, immunoglobulins, and other assorted adhesion molecules. (Jiang et al., 1994)

Integrins are formed by two subunits which covalently interact, known as the α and the β subunits, to form a heterodimer. This transmembrane glycoprotein is a specific receptor for a number of ligands in the extracellular matrix. (Hynes, 1987) There are at least 14 alpha and 8 beta subunits identified to date, and are found in every type of tissue, with at least one integrin type expressed in each tissue. (Albelda et al., 1989) At least 20 integrins have been discovered, (Hynes, 1992) and function in cell/matrix and cell/cell attachment. (Sriramarao et al., 1993) In terms of cell/matrix adhesion, integrins link components of the extracellular matrix to the cytoskeletal elements such as actin, talin, and vinculin. (Burridge et al., 1988) Specifically, the integrins bind laminin, fibronectin, and vitronectin in the extracellular matrix, and to a specific RGD repeat of amino acids: arginine, glycine, and aspartate. (Vogel et al., 1990; Cheresh et al., 1989; Yamada, 1991)

With respect to the process of invasion, changes in the levels of integrin expression have been correlated with increased adhesion to the basement membrane, which is one of the first steps in the process of invasion. For example, $\beta 1$ integrins have been shown to have increased expression in invasive carcinomas and that has been correlated with an increased activity of proteases, another step in invasion. (Demeure et al., 1992) As well, the increased expression of the $\alpha_v\beta_3$ integrin (the vitronectin receptor) in malignant melanoma cells

correlates with an increased adhesion to vitronectin and fibrinogen, as well as increased invasiveness. (Gehlsen et al., 1992) More recently, another aspect of the role of integrins in cancer was described, by Weaver et al., (1998 who illustrated that breast tumour cells treated with β_1 integrin-blocking antibodies reverted functionally and morphologically to a "normal" state, re-assembled a basement membrane, reorganised their cytoskeleton, and down regulated cyclin D1 to the point that they stopped growing. By blocking β_1 integrins in the cell lines before injection into nude mice, the researchers also showed that *in vivo* tumour size and number of tumours formed was decreased significantly.

Another major group of cell adhesion molecules involved in invasion and metastasis are the cadherins. Cadherins are calcium-dependant transmembrane cell adhesion molecules which are important in cell/cell interactions as they bind one another by homophilic interactions. (Geiger and Ayalon, 1992) As well, cadherins are linked to the cytoskeleton. Specifically, cadherins link cells together at adheren junctions to the actin component of the cytoskeleton via α- and β-catenins and α-actinin. (Cowin, 1994)

E-cadherin, also known as uvomorulin, is found on epithelial cells, and has been described as an anti-invasion molecule. E-cadherin normally helps cells to adhere to one another, and when transfected into invasive cells (ones that are less adherent to the surrounding cells) it decreased the invasiveness of cancer cells. (Vleminckx et al., 1991) Along the same lines, the use of an anti-E-cadherin antibody (or use of antisense mRNA) can induce increased tumour cell motility and invasive potential in normal cells. (Vleminckx et

al., 1991) So, the reduction of E-cadherin expression in normal cells enhances invasion and metastasis by diminishing the cell/cell adhesive contacts. (Oka et al., 1993; Doki et al., 1993) Lastly, the presence of E-cadherin has been shown in squamous cell carcinomas of the had and neck to be inversely correlated with metastasis and invasion. (Schipper et al., 1991)

The third major group of adhesion molecules involved in invasion and metastasis are the immunoglobulin super-family. This family includes ICAM-1,2,3 (intercellular adhesion molecule), NCAM (neural cell adhesion molecule), VCAM-1 (vascular cell adhesion molecule) and PECAM-1 (platelet endothelial cell adhesion molecule). This family is named for the immunoglobulin-like domain they share, and these molecules can form either homo- or heterophilic cell/cell interactions and are directly involved in invasion and metastasis. (Maemura and Dickson, 1994)

ICAM-1 is a cytokine-inducible endothelial adhesion molecule, found to function as an endothelial ligand for LFA-1 (lymphocyte-functioning antigen 1) (Rothlein et al., 1986) It has been established that higher levels of soluble ICAM-1 have been associated with several cancers (including bladder, breast, gastrointestinal, and ovarian carcinomas) when levels were compared to the normal patient's levels of ICAM-1, (Banks et al., 1993) as well as in cases of liver metastasis occurring in patients with gastric, colon, and pancreatic carcinomas. (Tsujisaki et al., 1991)

NCAM's are a group of cell adhesion molecules found mainly on neural cells, and

also functions as a homophilic adhesion molecule. (Maemura and Dickson, 1994) A decrease in expression of NCAM on glial cells was shown to correlate with increased metastatic ability in neurogenic tumours. (Andersson et al., 1991)

VCAM-1 is an example of an endothelial cell adhesion molecule which binds VLA-4 (an $\alpha_4\beta_1$integrin) on the surface of lymphocytes. (Elices et al., 1990) VCAM-1 expression can be induced by various cytokines, (Osborn et al., 1989) and has been shown to mediate some metastases; specifically ones that express VLA-4 such as melanomas. (Rice and Bevilacqua, 1989) As well, soluble VCAM-1 has also been found in higher levels in cancer patients (e.g. breast, ovarian, bladder tumours), similar to ICAM-1. (Banks et al., 1993)

Other cell adhesion molecules that affect invasion and metastasis include: selectins, and CD44. Selectins are calcium dependent proteins which bind carbohydrate ligands that contain the sialyl Lewis X or A structures. (Berg et al., 1991) Since it is known that tumours such as those in lung, colon and stomach express SLe^X, (Fukushima et al., 1984) it follows that E-selectin may be helping in binding tumour cells to the endothelium, and increasing the chance of metastasis. Another cell adhesion molecule thought to be involved directly in invasion and metastasis is CD44, which will be discussed in great detail in the following sections.

1.2.2 Matrix degrading proteases and their inhibitors

The second major event in the process of invasion and metastasis is the degradation of the basement membrane and surrounding stroma; an event which defines an invasive

tumour from an *in situ* one. (Aznavoorian et al., 1993) By degrading the basement membrane and local stroma, tumour cells are more able to migrate through the tissue and begin forming new tumours.

To penetrate the basement membrane, extracellular matrix and stroma, the production of enzymes is one mechanism that will degrade collagen, hyaluronan, fibronectin, laminin, and other proteoglycans which make up the basal lamina and the extracellular matrix. Whether or not degradation of the stroma will occur depends on the balance between levels of these enzymes and their inhibitors. (Stetler-Stevenson et al., 1993)

There are several classes of matrix degrading enzymes; the major ones include:

1. matrix and membrane type metalloproteases
2. the urokinase plasminogen activator (uPA)
3. cathepsins

The matrix-degrading metalloproteases can be further subdivided into three subclasses based on the component of the extracellular matrix which they degrade; namely into the collagenases, gelatinases, and stromelysins. A fourth and fifth subclass have been suggested to include the four membrane-type matrix metalloproteases and an "other" group that contains enzymes such as stromelysin-3 and macrophage metalloelastase. (Puente et al., 1996) Although these enzymes are grouped by substrates, there is considerable overlap between subgroups as to extracellular matrix component degradation. Generally, collagenases (interstitial, neutrophilic, collagenase-3) degrade fibrillar collagens (types I to III), the gelatinases (gelatinase A/MMP-2 and gelatinase/MMP-9)degrade denatured collagens (type IV, V, VII) as well as elastin and fibronectin. (Matrisian, 1992) The

stromelysins (both 1 and 2) as well as the other member of that group, matrilysin, have been shown to preferentially degrade proteoglycans and glycoproteins, as well as laminin and fibronectin and several collagen types. (Matrisian, 1992) The last functionally similar subgroup of MMPs, the membrane-type matrix metalloproteases (MT-MMP 1 to 4) do not seem to degrade any substrate but rather have an ability to activate other MMPs at the cell surface.(Cao et al., 1995) For example, proMMP-2 (gelatinase A) processing was increased in MT-MMP transfected cells, as was it's gelatinase-degrading activity. (Sato et al., 1994)

The MMP family share several conserved protein domains, and it is the conserved "pre" domain that is removed prior to MMP secretion, either by autocatalytic means or by a separated MMP. This same sequence binds a zinc ion in it's active site at a cysteine residue. Disruption of the zinc ion-cysteine interaction results in a conformational change that catalytically releases the "pre" domain from the zinc ion and produces the mature enzyme. (Powell and Matrisian, 1996)

MMPs as a family are regulated by a small family of endogenous inhibitors called tissue inhibitors of MMPs or TIMPs. The relative levels of MMPs in relation to TIMP levels can determine the amount of matrix degradation and therefore invasiveness. (Powell and Matrisian, 1996) There have been four TIMPs isolated and characterised so far, and they posses overlapping inhibitory effects, with some TIMPs having a preference for specific MMP's. TIMP-1 has been shown to bind activated MMP-1 (interstitial collagenase), stromelysin-1 (MMP-3), and latent or active gelatinase B (MMP-9). (Wilhelm et al., 1989) TIMP-2 has a high binding affinity for latent progelatinase A (MMP-2), but can inhibit activity of almost all activated MMP's.(Howard et al., 1991) Although TIMP-3 has

overlapping inhibitory effects with TIMP 1 and 2, TIMP-3 differs from them in that TIMP-3 is associated with the extracellular matrix, and is not a soluble protein. (Leco et al., 1994) The newest TIMP to be isolated and characterised, TIMP-4, has been found to inhibit most MMPs as well. (Greene et al., 1996)

As an example of the role of TIMPs in human cancers, Mohanam et al. have observed a correlation between a decrease in TIMP-1 and TIMP-2 expression and an increase in invasive ability in glioblastomas. The implications of TIMPs in breast cancer has also been noted, and will be discussed in further detail.

Another example of a protease important in invasion is the plasminogen activators and their inhibitors. There are two types of plasminogen activators, uPA (urokinase plasminogen activator) and tPA (tissue-type plasminogen activator). These are serine-specific proteases which convert the inactive proenzyme plasminogen to plasmin. (Jiang et al., 1994) uPA is a 55 kDa serine protease synthesised by many cell types including tumour cells, fibroblasts, epithelial cells and is produced as a proenzyme bound to the tumour cell surface by a specific receptor called the uPA receptor or uPAR. (Dano et al., 1985)

The ability of uPA to convert plasminogen to plasmin affects the breakdown of many other components of the extracellular matrix. Plasmin can bind plasminogen receptors on tumour cells to cause the degradation of laminin, fibronectin, and proteoglycans. (Gandolfo et al., 1996)

Inhibitors of uPA also factor into the invasive and metastatic potential of many cancers. PAI-1 and 2 (plasminogen activator inhibitors) are the two best characterised inhibitors of uPA, and function by binding soluble uPA and blocking the catalytic site. The

levels of uPA and it's inhibitors are crucial in regulating the invasive and metastatic ability of cancer cells. (Sumiyoshi et al., 1992)

Another family of matrix-degrading proteases are the cathepsins. Of particular interest to breast cancer are cathepsins D and B. Cathepsin B is a cysteinyl, secreted zymogen while cathepsin D is an aspartyl protease, both which are of lysosomal origin. (Rawlings and Barrett, 1994) Both cathepsins have been shown to activate receptor-bound prourokinase, and to have altered expression in human breast cancer cells. (Johnson et al., 1993) However, the relevance of cathepsin D as a prognostic indicator in breast cancer is debatable. Several other prognostic factors have been investigated to observe if there was any correlation with cathepsin D levels. These included oestrogen receptor status, (Simony et al., 1988) which showed no correlation, and nodal status, (Namer et al., 1991) which yielded conflicting results. Differences in methods of assaying for cathepsin D (ELISA, Western blot, immunohistochemistry) may have accounted for the discrepancies in these findings. (Westley and May, 1996)

Cathepsin B is also associated with invasive cancer. In microdissected colon cancer samples, increased cathepsin B levels were associated with more invasive regions of the tumour, when compared with matched normal epithelium. (Emmert-Buck et al., 1994)

1.2.3 Motility factors

In addition to adhesion and extracellular degradation, motility of a tumour cell is critical in invasion and metastasis. A great many factors have been identified as inducing tumour cells to migrate. These have been subdivided into three groups in a review by

Woodhouse et al.: (1997)

1. Factors secreted by tumour cells (autocrine motility factors)
2. Host secreted growth factors
3. Extracellular matrix proteins.

The first group includes HGF/SF (hepatocyte growth factor/scatter factor), IGF-II (insulin-like growth factor II) and autotaxin. HGF/SF and it's receptor, c-met, function as a tyrosine kinase to phosphorylate a number of components in the MAP (mitogen activated pathway) kinase pathway, (Santos et al., 1993) including phosphotidylinositol-3-phosphate, ras, src, and phospholipase C. Another member related to HGF is autotaxin, a strong chemotactic and chemokinetic cytokine with phosphodiesterase activity. (Murata et al., 1994) It has been observed that autotaxin, which belongs to the autocrine motility factor family, is able to stimulate motility in a number of cell lines including breast, bladder, and ovarian carcinoma cell lines. (Kohn et al., 1990)

Host-derived motility factors can sometimes act as homing factors for tumour cell motility to specific tissues. Examples of secreted factors that do this are IL-8 , which is known to induce melanoma tumour cell migration (Wang et al., 1990), and insulin-like growth factors. These have been postulated as potential "homing" factors for tumour cells that have invaded the blood vessels, and may "direct" the cells to a secondary site for new growth. (Aznavoorian et al., 1993)

Lastly, extracellular matrix proteins also stimulate motility, or more specifically, chemotaxis and haptotaxis (motility toward an immobilised substrate.) As mentioned before, the primary adhesion molecules to the extracellular matrix are integrins. (Vogel et al., 1990;

Cheresh et al., 1989; Yamada, 1991) Matrix proteases aid in this extracellular induced motility by cleaving the matrix proteins which stimulate motility in the tumour cells. Examples of extracellular matrix proteins that have been determined to affect motility of tumour cells are fibronectin, laminin, type IV collagen in mesotheliomas, and several melanomas cell lines. (Klominek et al., 1993; Anazvoorian et al., 1990)

1.2.4 Invasion and metastasis genes in breast cancer

Of the previously mentioned classes of genes involved in invasion and metastasis, several genes have been implicated in breast cancer. For example, adhesion molecules are frequently up or down-regulated in breast cancer. Jones et al. (1996) have found a correlation between the reduction in membrane staining for E-cadherin, and an increase in cytoplasmic E-cadherin levels, with an increase in lymph node metastasis. It was also observed that different types of breast cancers expressed E-cadherin in a different manner. Jones et al. in 1996 also observed that infiltrating lobular carcinomas were completely negative for membrane E-cadherin while most infiltrating ductal carcinomas retained some E-cadherin expression.

Integrins are another family of cell adhesion molecules that are affected in breast cancers. Since integrins are cell/matrix adhesion molecules, altered expression resulting in stronger adhesion to the extracellular matrix could result in enhanced invasive ability of the cancer cells. Studies of integrin expression at the protein (Natali et al., 1992) and mRNA levels (Zutter et al., 1993), specifically integrins $\alpha_2\beta_2$, $\alpha_6\beta_4$, and $\alpha_6\beta_1$, indicated that in breast cancer there is a reduced expression. Pignatelli et al., (1991) were able to correlate this reduction in expression with more poorly differentiated carcinomas.

Recently, the intracellular adhesion molecule ICAM-1, was studied in a panel of patients with invasive breast cancer. (Ogawa et al., 1998) In this study (which had an average length of follow-up of 98 months) patients with tumours that were ICAM-1 positive had better overall survival than patients with tumours lacking the molecule. As well, the expression of ICAM-1 also correlated negatively with tumour size, and lymph node metastasis. (Ogawa et al., 1998)

Matrix-degrading proteases like MMP's have also been implicated in the invasion and metastasis of breast cancer. (Powell and Matrisian, 1996) Stromelysi-3 (MMP-11) overproduction by stromal cells specifically has been associated with the grade/stage of breast tumours (Basset et al., 1993) as well as increased metastasis and poorer long term survival. (Engel et al., 1994)

The role of gelatinase A (MMP-2) has also been studied in human breast cancer both *in vivo* and *in vitro* experiments and it has been shown that the activated form of gelatinase A is present in breast cancer. (Brown et al., 1993) Although MMP-2 has an association with an invasive phenotype, MMP-2 levels do not necessarily correlate with metastatic progression.

Inhibitors of MMPs also have altered expression in cancers. For example, TIMP-2 overexpression in breast cancer cells has been shown to correlate with decreased bone metastases. (Leco et al., 1994) TIMP-4 involvement in breast cancer and invasion was observed in transfection experiments of a metastatic breast cancer cell line with TIMP-4. As a result of the transfection, a decrease in tumour size and lung and lymph node metastases was evident. (Wang et al., 1997)

The protease, uPA also has an important role in breast cancer as a prognostic factor,

particularly with disease-free and overall survival. Duffy et al. (1994) observed that breast cancer patients with high levels of uPA, specifically patients that were positive for lymph node involvement, had a statistically significant decrease in length of disease free survival as well as overall survival, when compared to patients with low uPA levels. The effect of combined levels of uPA and PAI-1 was observed by Janicke et.al. (Janicke et al., 1993) to enhance the prognostic value for node-negative breast cancer patients. They observed that with high levels of both uPA and PAI-1 occurred a statistical increase in the risk for relapse. High PAI-1 levels should block the effects of high uPA, but Janicke postulated that high PAI-1 may aid metastatic cells in reimplantation in another organ by blocking stromal degradation.

The prognostic effect of PAI-2 is also significant in breast cancer. Whereas increased PAI-1 levels seem to correlate with poor prognosis (Janicke et al., 1993), increased levels of PAI-2 is correlated with an increased overall favourable prognosis, especially in node-negative, post-menopausal women with breast cancer. (Bouchet et al., 1994) A study by Foekens et al. in 1995 showed that in tumours with high levels of uPA, the increased PAI-2 levels correlated with increased disease-free, metastasis free survival, indicating that in breast cancer PAI-2 may be the most important inhibitor of uPA.

A further example of the relevance of protease expression in breast cancer are the cathepsins, particularly cathepsins B and D. Using immunohistochemical techniques, Castiglioni et.al. (1994) analysed cathepsins B, D, and L levels in human breast cancer. They found that there was increased expression of all three cathepsins in a statistically significant number of tumours (when compared to normal epithelium). These enzyme levels did not

correlate with oestrogen receptor status, tumour size, or clinical stage, (Castiglioni et al., 1994) which further supports the idea that cathepsins D and B do not have good prognostic value in breast carcinomas, but have a role in invasion and metastasis.

Motility factors like HGF also have a role in the invasion and metastasis of breast cancer. Studies on the c-Met/HGF receptor in breast cancer cell lines indicated that cell lines that were moderately differentiated did not express c-met and did not respond to HGF stimulation. Less differentiated and more invasive cell lines expressed c-met in high levels and had increased invasiveness and motility in response to HGF, indicating that HGF may contribute to invasion of breast cancer cells.(Beviglia et al., 1997)

1.3 CD44

As previously discussed, adhesion molecules are integral components in invasion and metastasis, whether they function in cell/cell or cell/matrix adhesion. The focus of this project has been the extracellular matrix adhesion molecule CD44; a molecule that has been well characterised and its role in invasion and metastasis in other systems, well documented.

CD44 is a cell surface glycoprotein encoded by a single-copy gene at 11p13 (in humans) and spans approximately 60 kb. (Goodfellow et al, 1982) The organisation of the CD44 gene was described in 1992 by Screaton et al. (Screaton et al., 1992) and involved 20 exons, 10 of which could be alternatively spliced. (Figureure 2) Exons 1 to 5 form the extracellular domain, exons 6 to 15 (also termed v1 to v10) are the alternatively spliced exons, and 16, 17 form the membrane proximal region of the extracellular domain. The transmembrane region is encoded by exon 18. And the last two exons- 19 and 20- form the

cytoplasmic tail of the CD44 molecule. (reviewed in Naor et al., 1997)

1.3.1 CD44 modifications

Many modifications to the CD44 glycoprotein occur, particularly at the transcript and protein levels. This allows for the formation of many isoforms of the CD44 molecule. The most prevalent form of CD44 is the standard form, (known as CD44s, or CD44H for its prominent expression on hematopoietic cells) (Stamenkovic et al., 1991) which does not contain any alternatively spliced exons. (He et al., 1992) Based on amino acid content, the core CD44H protein is predicted to have a size of 37 to 38 kDa (Screaton et al., 1992), but posttranslational modifications greatly increase the molecular weight. CD44 is a heavily glycosylated molecule, with at least 6 sites for N-linked carbohydrates and 7 for O-linked carbohydrates. (Goldstein et al., 1989) In addition to glycosylation, the CD44 protein can be glycosaminoglycanated by the addition of heparan sulfate (Brown et al., 1991) and chondroitin-4-sulfate (Stamenkovic et al., 1991). The addition of sialic acid to the CD44 glycoprotein can also occur at a number of sites, and causes CD44 to have an acidic isoelectric point of 4.2 (Jalkanen et al., 1988)

Not only are the exons v1 to v10 in the extracellular domain of CD44 subject to alternate splicing, but the cytoplasmic tail (exons 19 and 20) is as well. Screaton et al. (1992) showed that splicing of exons 19 and 20 can form the short (3 amino acid) or long (70 amino acid) "tail" for CD44 molecules; the longer version is found more often than the truncated one. Other cytoplasmic region modifications include phosphorylation on at least 6 sites, some of which are constitutively phosphorylated. (Pure et al., 1995)

As previously mentioned, alternative splicing of the v1 to v10 exons of CD44 can be

instrumental in the creation of CD44 isoforms. These exons can also be modified, furthering the variation in the CD44 glycoprotein molecules. A number of O- and N-linked glycosylation sites are present in the CD44 variable region, most often on exons v2, v8, v9 and v10. (Naor et al., 1997) Several CD44 isoforms have been described, specifically the variants that relate to the metastatic process. Second in occurrence only to CD44 standard form, is the CD44E (epithelial) form. This variant incorporates the v8, v9 and v10 alternatively spliced exons to generate this 130 kDa isoform. (Stamenkovic et al., 1991) Other prominent variants are the pMeta-1 and pMeta-2 "metastatic" variants which contain v4-v7 and v6-7 alternatively spliced exons, respectively, which were discovered to confer metastatic ability to non-metastatic cells (Gunthert et al., 1991) and are of particular interest in cancer invasion and metastasis.

1.3.2 CD44 tissue expression

CD44 molecules are normally expressed on many cell types. For example, CD44E (the v8 to v10 variant) is expressed normally on breast epithelial cells, as is CD44H. (Iida and Bourguignon, 1995) CD44v9 isoforms are widely expressed in organ such as intestine, stomach, regions of the kidney, ovaries, to name a few. (Mackay et al., 1994; Stauder et al., 1995) Another example is a recent paper describing strong v5, v7-8, and v10 variant expression on endothelial cells by immunohistochemistry and FACS analysis, and weaker expression of the v3 and v6 containing variants. (Koopman et al., 1998) CD44 glycoproteins also have been shown to be expressed on non-activated and activated hematopoietic cells. All hematopoietic cells (T, B lymphocytes, macrophages, natural killer cells, granulocytes) preferentially express the standard CD44H form. (Mackay et al., 1994; Arai et al., 1995) But

the CD44 levels on T cells go through a substantial increase when they become activated, and they express v9-containing variants more often. (Mackay et al., 1994) When T cells were stimulated *in vitro* (either by antigen, mitogen or allogen stimulation) v6- and v9-containing variants were transiently upregulated. (Koopman et al., 1993)

1.3.3 CD44 as an adhesion molecule

The primary function on CD44 is to bind hyaluronic acid. Hyaluronic acid (also known as HA, hyaluronate, or hyaluronan) is a glycosaminoglycan formed by repeating disaccharides; (D-glucuronic acid and N-acetyl-D-glucosamine) it is synthesised by fibroblasts (Teder et al., 1995) and functions as a component of the extracellular matrix. Hyaluronan functions as well as a regulator of cell/cell adhesion, cell/cell organisation, and differentiation. (Laurent and Fraser, 1992) Hyaluronate also is involved in such biological processes as wound healing, tissue remodelling and inflammation. (Laurent and Fraser, 1992) There is much evidence that CD44 is the primary hyaluronic acid receptor (reviewed in Naor et al., 1997) including transfection experiments of CD44 cDNA into cells that did not express any form of CD44. This allowed the cells to bind soluble of bound hyaluronan in a CD44 dependant manner. As well, this binding could be inhibited by monoclonal antibodies for CD44. (Lesley and Hyman, 1992)

CD44 is one member in a family of hyaluronan binding proteins called the hyaladherins, which includes RHAMM, (receptor for hyaluronan mediated motility which is important in hyaluronan-induced cell motility) cartilage link protein, and aggrecan. (Knudson and Knudson, 1993) It has been suggested that the basic mechanism of hyaluronan binding by CD44 molecules involves regions of positively charged amino acids (arginines and

lysines) on the extracellular region that bind with the hexuronate groups of hyaluronan which are negatively charged. (Hardingham and Fosang, 1992) These positive amino acids have been observed in two regions of the CD44 molecule, specifically at positions 21-45 and 144-167, and predominately influence hyaluronic acid binding. (Peach et al., 1993)

Hyaluronate binding is regulated by a number of extracellular CD44 protein modifications and motifs, including the cytoplasmic tail region. The CD44 cytoplasmic tail interacts with several cytoskeletal proteins including actin, ankyrin, and the ERM (ezrin, radixin, moeisin) proteins, (Tsukita et al, 1994) and the question as to whether these interactions affected hyaluronic acid binding was raised. When cells lacking CD44 were transfected with CD44 that was missing the cytoplasmic tail (including the ankyrin binding site), the cell's ability to bind hyaluronan (either soluble or bound) was drastically reduced. (Lokeshwar et al., 1994) However, other investigations into cytoplasmic-truncated CD44 have yielded mixed results. What has emerged is that in some cell lines, hyaluronan binding is decreased (Lokeshwar et al., 1994), and a CD44 clustering effect is required for binding of hyaluronan. (Uff et al., 1995) Lastly, phosphorylation of the CD44 cytoplasmic tail may be also required as well. (Pure et al., 1995)

Glycosylation of CD44 has been reported as affecting hyaluronan binding. It seems that too little glycosylation as well as too much glycosylation can interfere with hyaluronan binding. Deglycosylated CD44 (p42) cannot bind hyaluronan, but CD44 molecules with increasing levels of O and N-linked oligosaccharides (p52 to 58) are able to. (Lokeshwar and Bourguignon, 1991) Complete glycosylation of CD44H was shown to interfere with hyaluronan binding when compared to a glycosylation-defective cell line. (Katoh et al., 1995)

CD44 variants also are affected by glycosylation with respect to hyaluronan binding. As the number of exons in a particular variant increases, this provides an increase in sites for carbohydrate addition and decreases hyaluronan binding ability. (Bennett et al., 1995)

CD44 also has affinity for other non-hyaluronan molecules. CD44 is able, with lower affinity, to bind other extracellular matrix molecules such as laminin, collagen, and fibronectin. (Jalkenan and Jalkenan, 1992; Lokeshwar and Bourguignon, 1991) As well, osteopontin, a secreted chemotactic phosphoprotein (secreted by T cells and osteoblasts) (Weber et al., 1996) has been proposed as a CD44 ligand.(Denhardt and Guo, 1993) In fact, a recent paper by Ue et al. (1998) suggests that osteopontin overexpression may be involved in gastric cancers, and that in the case of poorly differentiated stomach cancers osteopontin and CD44v9 coexpression may be involved in metastasis.

1.3.4 Other functions for CD44

Since it's first description as a T lymphocyte antigen (Dalchau et al., 1980), CD44 has been assigned a number of functions. (reviewed in Naor et al., 1997) These include homing receptor, ability to internalise and degrade hyaluronan, transmitter of growth signals and cell motility. A well characterised role for CD44 has been as a lymphocyte homing receptor. This function was one of the earliest proposed for CD44 in 1986 by Jalkanen et al (Jalkanen et al., 1986), and by using an anti-CD44 monoclonal antibody called Hermes-3 they inhibited peripheral blood lymphocytes (PBL's) binding to mucosal high endothelial venules, independently of hyaluronan. This finding was tested *in vivo* to determine if the same results could be achieved. By treating mice with an anti-CD44 antibody prior to antigenic challenge they delayed the early stages of a DTH (delayed-type hypersensitivity)

response by 24 hours, indicating that CD44 is crucial for lymphocyte homing to sites of inflammation. (Camp et al., 1993)

In addition to the binding of hyaluronan as a cell adhesion molecule, CD44 has been observed to internalise and degrade hyaluronan. Although CD44 does not possess any hyaluronidase activity, CD44 can aid in the endocytic breakdown of hyaluronan by macrophages. (Culty et al., 1992) The relationship between CD44 and macrophages was shown initially in mouse lung development. It was observed that CD44 levels increase as hyaluronan levels decrease. And if new-born mice are treated with a CD44 blocking antibody, alveolar macrophages that contain hyaluronan decrease, while surrounding hyaluronan levels increase. (Underhill et al., 1993) Therefore, in mouse lung development at least, hyaluronan degradation by alveolar macrophages is an integral function of CD44.

CD44 has also been observed to aid other molecules in transmitting growth signals from the cell membrane to the nucleus. Many anti-CD44 antibodies have been shown to produce a number of proliferative effects. These include: T cell proliferation and IL-2 production, when anti-CD44 antibodies were used with anti-CD2 or CD3 antibodies, (Sommer et al., 1995) monocyte release of TNFα and IL-β1 (Webb et al., 1990), and triggering of cytotoxic T lymphocyte/natural killer cell cytotoxic cell activity. (Lesley et al., 1993) As well, hyaluronan stimulation of macrophages induces CTL/NK expression of IL-β1, TNFα, and IGF-1;this was mediated through CD44 binding, as anti-CD44 antibodies blocked cytokine production. (Noble et al., 1993)

Cell motility is also linked to CD44. A melanoma cell line expressing high levels of CD44 was shown to migrate more often and in less time than the melanoma cells with low

CD44 levels, when cells were plated on an artificial "wound" made by a cell scraper. (Birch et al., 1991) CD44-dependant lymphoid cell rolling was shown *in vitro* on cultured endothelial cells or on bound hyaluronan. This "rolling" of lymphocytes was shown to be blocked by anti-CD44 antibodies, hyaluronidase enzyme, and soluble hyaluronan. (DeGrendele et al., 1996) Human melanoma cell transfected with CD44H showed enhanced motility on HA-coated plates, but not if a truncated form (lacking exons 19 and 20, or the cytoplasmic tail) of CD44 was transfected, (Thomas et al., 1992) suggesting that for motility, CD44 interaction with the cytoskeleton is required.

1.4 CD44 and Roles in Cancer

CD44 and variant isoforms have long been associated with the malignant process in many types of cancer. There is much experimental evidence to prove CD44 involvement in invasion and metastasis, as well as the fact that changes in CD44 regulation can influence those processes.

1.4.1 Evidence for CD44 involvement in cancer

Perhaps the best example of CD44 as a gene that confers metastatic abilities came in 1991 when Gunthert et al. created a monoclonal antibody against a metastatic rat pancreatic adenocarcinoma cell line, but one that would not react with a similar non-metastatic cell line. (Gunthert et al., 1991) When this antibody was used to screen a cDNA expression library of a metastatic tumour, two clones were isolated, pMeta-1 (CD44v4-v7) and pMeta-2 (CD44v6,7). These variants were found in two metastasising rat cell lines, a pancreatic

carcinoma and a mammary adenocarcinoma. Transfection with these clones into a previously non-metastatic rat pancreatic tumour cell line conferred metastatic potential. Further evidence indicates that antibodies against variant forms of CD44 have been shown to inhibit metastases. Anti- CD44v6 antibodies (against one epitope on pMeta-1 variant) slowed the spread of a metastatic pancreatic carcinoma cell line in rats and prevented growth in peripheral lymph nodes and lungs. (Seiter et al., 1993) By injecting an antibody to the constant region of CD44 into immunodeficient mice, Guo et al. (1994) showed inhibition of metastasis of a human melanoma cell line (especially to the lungs) even if the antibody injection took place seven days after the tumour cells were injected.

Evidence for CD44 involvement in invasion and metastases has led investigators to study the expression of CD44 in many human cancers. Altered expression of CD44 in malignant cells, when compared to the corresponding normal cells, can occur as up-regulation, down-regulation, or altered variant isoform expression. Of the many types of cancers that express CD44, a selection will be discussed here.

1.4.2 CD44 expression in human cancers

In tumours of the brain it has been observed that more aggressive tumours (glioblastomas) have greater CD44 expression than less aggressive tumour types (lower grade astrocytomas), and certainly when compared to normal brain tissue expression of CD44. (Kuppner et al., 1992) Several studies which examined CD44 levels in neuroblastomas have reported that down-regulation of CD44H occurs with the increased grade of tumour cell differentiation (Combaret et al., 1995), indicating the absence of CD44H is associated with more aggressive tumours of that type. But since CD44H is the

principle hyaluronan receptor, it follows that in the more invasive brain tumours, higher CD44H levels are found. (Kuppner et al., 1992)

In lung tumours, it has been observed that there is a down-regulation of CD44v6 expression in the highly metastatic small cell type lung carcinoma, (Ariza et al., 1995) but CD44H the v6 and v3 isoforms are preferentially expressed in lung cancer of the non-small cell type.

Normally, the colon does not express CD44 variants and CD44H levels are practically non-detectable. (Abbasi et al., 1993) But investigators have demonstrated that CD44H and CD44 variants are expressed in primary colorectal tumours as well as nearly half of all colorectal tumours that metastased to liver, lung and lymph nodes. (Orzechowski et al., 1995) When the progression of these tumours was monitored from non-metastatic to a metastatic state, the expression of CD44v6 containing variants was shown to increase in each sample, as well as the overall number of patients which expressed this isoform. (Orzechowski et al., 1995) However, correlations between CD44 expression and stage of disease are conflicting, as some groups reported v6 detection in the early stages of colorectal cancer as well. (Imazeki et al., 1996) Perhaps this difference could be explained by different methods of variant detection.

Investigations into prostate cancers have indicated that there is an inverse correlation between tumour cell differentiation and CD44 expression. (Nagabhushan et al., 1996) Down-regulation of CD44 levels was also noted in lymph node metastases, when compared to primary prostate cancers.

The CD44 variants that are prevalent in gastric carcinomas seem to be CD44v5, v6

and v9. CD44 isoforms are differentially expressed between types of gastric carcinomas; with the v5 isoform expression more prominent in diffuse stomach tumours (Hong et al., 1995) and v5 and v6 containing tumours predominating in gastric adenocarcinomas. (Hong et al., 1995) Metastases to lymph nodes of both types of gastric carcinomas express increased levels of v6-containing isoforms of CD44 (Dammrich et al., 1995), and coexpression of CD44v9 and osteopontin has been correlated with less well differentiated gastric carcinomas. (Ue et al., 1998)

RT PCR analysis has shown that normal pancreas expresses the CD44H and CD44E (v8-10) mRNA transcripts (Chaudry et al., 1994), but that primary and metastatic pancreatic adenocarcinomas express many transcripts that contain the v6 alternatively spliced exons (Gansauge et al., 1995). This indicates once again that cancer cells that overexpress the v6 exon, combined with other exons, are more likely to become metastatic.

1.4.3 CD44 and breast cancer

A number of different results have been observed when investigators have tried to determine the pattern of CD44 expression in breast cancer. Overall CD44 levels in metastatic breast cancers were increased when compared to normal tissue. (Iida and Bourguignon, 1995) In primary tumours, patients with invasive ductal breast cancer expressed CD44 variants containing exons v3, 7/8, 5,6, and 10. (Kaufmann et al., 1995) Similar patterns of CD44 expression were shown in *in situ* (Sinn et al., 1995) as well as invasive lobular carcinomas. (Dall et al., 1995) When studying lymph node metastases of breast cancer, 2 research groups observed that that these cells were positive for exons v5,6,3 and v9/10.

(Kaufmann et al., 1995; Friedrichs et al.,1995) However, they were at odds in terms of conclusions pertaining to the relevance of CD44v6 exon expression and survival rate. Kaufmann et al. (1995) indicated that patients with tumours expressing v6-containing variants had a statistically significant shorter survival time whereas this finding was not reproduced by Friedrichs et al. (Friedrichs et al., 1995) More recently, Tokue et al. (1998) noted that in a panel of breast tumours, patients whose tumours expressed the v2-containing variant of CD44 had a far lower survival time than patients who were CD44v2 negative. They failed to find any such significant correlation with the CD44v6 variant and overall survival, indicating that the v2 variant may be a more relevant prognostic indicator for breast cancer, rather than the v6 variant. A recent paper by Bourguignon et al. (1998) observed that in a highly metastatic breast cancer cell line, the CD44 variant 3,8-10 was overexpressed. Specifically, a segment of this variant protein was determined to bind to ankyrin, as well as being closely associated with the matrix metalloprotease MMP-9 in the plasma membrane. They postulated that the association of the cytoskeleton (via ankyrin) and the matrix degrading enzyme MMP-9 through CD44v3,8-10 may represent a mechanism to enhance tumour cell migration.(Bourguignon et al., 1998) The same authors had previously noted that the CD44v3,8-10 variant expression directly correlated with increased histological grade in breast tumours, and that this isoform may also serve as a predictive factor for overall survival of patients with breast cancer. (Iida and Bourguignon, 1995) It has also been reported that in ER+ (versus ER-) cell lines, there occurred an increase in CD44v7 and v10 containing variants, which was consistent when ER+ versus ER- tumours were also studied. (Hole et al., 1997)

1.5 Gene regulation and breast cancer

As previously discussed, there are several genetic events that can occur in a normal cell which cause the cell to lose control over it's proliferation, possibly leading to cancer. At the DNA level, there can occur gene amplifications, deletions, and mutations. Examples of gene amplification are the c-myc and c-*erb*B-2 oncogene, which have been shown to be amplified in 20-30% of breast cancers. (Berns et al., 1992 and Gusterson et al., 1992) Deletional mutations, also called loss of heterozygosity, have been shown to affect genes such as the tumour suppressor gene p53. P53 falls on chromosome 17, a common region where loss of heterozygosity occurs (Sato et al., 1990) and p53 is lost in 50% of human cancers. (Baker et al., 1989)

Another mechanism of gene regulation is the alternative splicing of mRNA to form variant transcripts. This can lead to multiple splice variants of a single gene occurring in a cell. As discussed in great detail previously, CD44 is an example of one such gene that is alternatively spliced to form variants in many human cancers, such as breast cancer. (Naor et al., 1997) In the same gene family, the receptor for hyaluronan mediated motility, or RHAMM, is also regulated at the mRNA level by alternative splicing. The RHAMM gene is composed of 4 exons ranging in size from 75 to 1099 bp, and form several variants, particularly with the inclusion of exon 4. (Entwistle et al., 1995) This variant has been shown to be overexpressed in transformed cells and with transfection into murine fibroblasts, it will transform them as well. (Hall et al., 1995) Overexpression of RHAMM in breast tumours correlates with lymph node metastasis, indicating the possible value of RHAMM as a prognostic indicator. (Wang et al., 1998)

The oestrogen receptor is also capable of forming variants expressed in breast cancer. Many of these variants are caused by the deletion of one exon such as exons 3, 4, 5, or 7. (reviewed in Dowsett et al., 1997) One of the most studied variants in breast cancer is the deletion 5 (Δ5) variant. This variant was found to have higher mRNA levels than the wild-type ER mRNA in ER- breast tumours, but was not detected due to the deletion of exon 5, which led to a truncated protein without the ligand binding domain. (Fuqua et al., 1991) Other truncated ER variants that have been identified are deleted at exon2/intron or exon 3/intron boundary and are unable to activate transcription when transfected with a reporter gene that was sensitive to estrogen, (Dotzlaw et al., 1992) indicating that even with the DNA binding domain intact, ER truncations lead to non-functional receptors. Deletion 7 ER variants were deemed dominant negative variants, as they were found to be transcriptionally inactive, but could downregulate the transactivational activity of the wild-type ER in an experimental system. (Fuqua et al., 1992)

1.5.1 The spliceosome

In order to form the variant molecules from the CD44 (and other spliced genes such as RHAMM) pre-mRNA, there first has to be assembly of the nuclear proteins that form the spliceosome. The "core" of the spliceosome is composed of the U1,U2, U4/6 and U5 small nuclear ribonucleic proteins. (Roscigno and Garcia-Blanco, 1995) The initial stage of spliceosome assembly is the binding of the U1 snRNP to the pre-MRNA by pairing the 5` end of the U1 protein to a 5` conserved sequence at the splice site. (Kramer, 1996) The U1 binding must occur in order to facilitate the binding of the U2 snRNP, which commits the pre-mRNA to the splicing pathway. The addition of U2 to the pre-mRNA forms the pre-

splicing complex A, (Konarska and Sharp, 1987) which is converted to the early splicing complex B by the binding of snRNP's U4/6 and U5. The complexing of U2 with U4, 5 and 6 forms a loop connecting the 5` splice site with the snRNP's which facilitates the conformational change required to form the late splicing complex C. (Kramer, 1996) These conformational changes include destabilisation of the U4/U6 complex and the binding of the U2 snRNP (bound at the 5` splice site) to the U6 snRNP. (Madhani HD and Guthrie C, 1992) The U5 snRNP serves to link the 5` and 3` splice sites as the "unwanted" sequence is being excised. The final step of RNA splicing involves the removal of the intron sequence by the snRNP's, and the disassembly of the spliceosome. (Kramer, 1996)

1.5.2 SR proteins

Another function of the spliceosome is to generate alternately spliced isoforms of CD44. This is done by a family of proteins called the SR (serine-arginine) proteins which are required to co-ordinate the components involved in pre-mRNA splicing.

In 1990. Ge and Manley discovered and purified a protein called ASF, which, if in excess, could switch between two 5` splice-sites *in vitro* in SV40 pre-mRNA. (Ge and Manley, 1990) At about the same time, Roth et al. purified a monoclonal antibody that stained lateral loops on amphibian chromosomes and recognised a specific family of phosphoproteins in humans. (Roth et al., 1991) These phosphoproteins turned out eventually to belong to the SR protein family. The criteria for defining members of the SR protein family were determined by Zahler et al. (1992) These included:

1. the fact that monoclonal antibody 104 (Roth et al., 1991) could recognise a specific epitope on the proteins in question,

2. the proteins were able to be copurified in a two-step high salt copurification method using 65% ammonium sulfate and 20mM magnesium chloride salts,
3. these proteins all contained a serine/arginine repeat motif and an RNA recognition site.

In addition to the SR proteins there is an increasing family of SR protein related proteins. These include the recently discovered Clk's 1 through 3. (Johnson and Smith, 1991; Hanes et al., 1994) These are cdc2-like protein kinases that are able to autophosphorylate at both tyrosine and serine/threonine amino acids, although the exact effect of phosphorylation on SR proteins and therefore mRNA splicing is not known. Other SR protein related proteins include other essential splicing factors such as the 70 kDa U1 snRNP-associated protein and U2AF65 heterodimer which help to form the spliceosome with SR proteins. (Fu, 1995)

The actual SR proteins include SR's 75, 55, 40, 30a, b, and c, and SR20; with these numbers corresponding to molecular weight of these phosphoproteins. (reviewed in Fu, 1995) The structural domains of these highly conserved proteins includes a RNA binding region that is important in splice site selection and essential for SR activity (Wang and Manley, 1995) as well as the SR-repeat regions which mediate protein/protein interactions. These interactions could be with other SR proteins or with SR protein related proteins that function as associated splice factors. (Kohtz et al., 1994)

Considering that different SR proteins can bind each other, and that each individual SR protein can act to recover splicing in S100 extracts that are splicing deficient, (Zahler et al., 1992) it seems that SR proteins have an overlapping function. On the other hand, SR proteins individually can have unique specificities for splice sites and efficiency for pre-mRNA splicing. In addition to being highly conserved proteins, SR proteins have ubiquitous

tissue expression, but with cell-type specific differences in their relative levels, (Screaton et al., 1995) which may contribute to expression of specific alternatively spliced isoforms in different tissue.

SR proteins have a number of roles in splicing pre-mRNA. (review in Fu, 1995) This begins early on in the ability of SR proteins to commit pre-mRNA to splicing. Fu in 1993 showed that preincubation of pre-mRNA with SR proteins prevented competitor mRNA form blocking spliceosome formation. (Fu, 1993) In this way, SR proteins complex with the pre-mRNA, and allow for further proteins and splicing factors to bind to form the spliceosome. Once the SR proteins are bound to the pre-mRNA at the 5` splice site, the SR related proteins like U1 70 kDA protein and the U2AF heterodimer begin to interact with the SR proteins to form the spliceosome. SR proteins function in this early spliceosome assembly by choosing a 5` splice site with the U1 snRNP, with U2AF SR proteins stabilise the complex at the 3` site, and lastly, SR proteins bridge the 5`/3` sites in order to stabilise the pre-spliceosome complex. (Fu, 1995) The later steps in spliceosome assembly also involve SR proteins. In order to convert the pre-spliceosome to a mature one, three other snRNP (in addition to U1 and U2, already present) called U4, U5 and U6 must be added, and this process has also been recently determined to be under the control of SR proteins. (Roscigno and Garcia-Blanco, 1995)

1.6 Gene expression, cell morphology and the extracellular matrix

The adhesion of epithelial cells to the extracellular matrix is mediated primarily by integrins and their interaction with proteins like laminin, fibronectin, and vitronectin. (Vogel et al., 1990) By linking intracellularily to cytoskeletal proteins such as actin, talin, and

vinculin (Burridge et al., 1988), these adhesions can alter the shape of the cell. In some cases this alters gene expression directly, or as an indirect result of the substrate-induced shape change.

An example of cell-shape dependant gene expression is the alternate splicing of protein 4.1 in human mammary epithelial cells. (Schischmanoff et al., 1997) p4.1 is a membrane skeleton protein found primarily in red blood cells in up to 200 000 copies per cell. By interacting with other plasma membrane proteins such as spectrin, band 3, and glycophorin, as well as with the cytoskeletal protein actin, p4.1 helps to anchor the cytoskeleton to the plasma membrane. Protein 4.1 is a 30-210 kDa (the major form is about 80 kDa) highly phosphorylated, glycosylated protein with several isoforms produced by alternate splicing. (Conboy, 1993) Using normal human mammary epithelial cells, Schischmanoff et al. (1997) plated these cells on plastic or poly(2-hydroxyethyl)methacrylate (pHEMA) coated tissue culture dishes. They observed initially that cells plated on pHEMA exhibited a rounded morphology (as opposed to the fibroblastic morphology of the cells on untreated dishes), and that these rounded cells stopped dividing and aggregated into clusters of 100 or more cells. This alteration in cell morphology also had an effect on p4.1 alternative splicing, as determined by RT PCR (Schischmanoff et al., 1997), of the amino-terminal, spectrin-actin binding, and carboxy-terminal domains, using specific primer pairs. They compared the expression of each of these regions in cells grown on plastic versus pHEMA and made several discoveries. The amino-terminal region of p4.1 showed no changes in splicing patterns, but primers to the actin/spectrin binding site showed a decrease in the expression of the exon 16 deleted amino acid product, which was abundant in the cells

grown on plastic. The most interesting finding was a novel exon in the carboxy-terminal region of p4.1, only on the pHEMA plated cells. The exon, 17b, was inserted in between exons 17 and 18, and has not been previously detected in other studies. This larger, alternatively spliced isoform would "disappear" in cells plated on plastic, then "reappear" in the same cells plated on pHEMA. (This change in gene product was tested with fibronectin and no such changes occurred between plastic and pHEMA plated cells.) This finding directly links changes in gene expression and changes in cell morphology as a result of alterations to the cellular environment.

A further example of cell shape alterations inducing gene expression involves integrin expression and the eventual production of the matrix protease collagenase-1. Kheradmand et al. (1998) have demonstrated that fibroblasts with disrupted actin cytoskeletons and therefore rounded in shape had increased collagenase-1 expression. As well, this increased expression could also be achieved by cells spreading out via the $\alpha_5\beta_1$ integrin on integrin-specific ligands. This shape change leads to a cascade of cell signalling events starting with activation of the GTP binding protein Rac1, which generated reactive oxygen species that in turn allow nuclear factor kappa B to transcribe IL-1α which induced collagenase-1 production in the cells (Kheradmand et al., 1998)

Artificially generated forms of the extracellular matrix have been long able to be manipulated in experimental systems to show how the matrix can influence gene expression in different cell lines. Streuli et al. (1991) showed that single human mammary epithelial cells suspended in Matrigel (reconstituted basement membrane) could produce the milk protein β-casein, and this was dependant on signal s from the basement membrane proteins

themselves, as it was shown that no milk proteins were synthesised by cells grown on plastic. This integrin dependant cell adhesion and signal transduction was also determined to be independent of cell/cell contact or cell polarity. A few years later, Roskelley et al., (1994) observed that there were two components to milk protein production by human breast epithelial cells: a change in cell shape and a biochemical change in the form of β-integrin clustering and an increase in tyrosine phosphorylation. In order to produce β-casein milk proteins, cells needed the extracellular matrix, prolactin hormones, and an induced rounding shape change of the cells. If one of these components was missing, the cells were unable to produce milk proteins. They also observed that by adding the extracellular matrix (by a Matrigel overlay assay), the cells showed an increase in signal transduction by increased β1 integrin clustering, and increase phosphorylation necessary for signal transduction.

Lastly, Streuli et al. showed that the regulation of TGFβ1 cytokine production in human mammary epithelial cells was influenced by the environment. (Streuli et al., 1993) TGFβ1 has been shown to stimulate matrix production and deposition, to increase integrin expression, to increase protease inhibitors, and to decrease matrix degrading proteases. (Kahari et al., 1991) This paper showed that TGFβ1 was downregulated in cells that were grown on Matrigel versus plastic. This was not the case for GAP gene or for TGFβ2 gene transcription levels. It seemed that transcription of TGFβ1 was suppressed when the cells came into contact with basement membrane, not with plastic, and this was independent of hormone addition. As well, this phenomenon did not occur when cells were grown on collagen type I, only when cells were plated on Matrigel. The authors postulated that a feedback loop mechanism of TGF1-mediated stimulation of basement membrane production

occurred until the basement membrane produced, then TGFβ1 transcription was suppressed.

2 Rationale and Hypothesis

As previously discussed, the process of invasion and metastasis is complex requires a number of genes, including genes that code for adhesion molecules, proteases and their inhibitors, and motility factors. Figureure 1 outlines the basic steps of invasion and metastasis, and the previous introduction discussed this process in detail. The initial step in the process in invasion involves altered adhesion of the cancer cell to the basement and extracellular matrix, as well as to the surrounding tumour cells. Integrins, as previously discussed, have been shown to be overexpressed in many cancers, enhancing the tumour cells ability to bind the basement membrane, and decreased expression of cadherins enable the tumour cell to separate from the surrounding cells. CD44 is another cell adhesion molecule that has altered expression in many cancers. Previous work indicates that the CD44 pattern of variant expression has been associated with a number of invasive human cancers, and correlated with some metastatic tumors as well. Gunthert et al. (1991) observed that transfection with CD44 variant cDNA could confer metastatic ability in non-metastatic cell lines, and Seiter et al. (1993) used anti-CD44v6 antibodies to block tumour growth, and metastases in mice, indicating that the CD44 gene could influence invasion and metastasis.

Previous work on CD44 mRNA expression in breast cancer cells has shown a specific pattern of CD44 expression in estrogen receptor positive versus estrogen receptor negative human breast cancer cells. (Hole et al., 1997) In ER+ human breast

cancer cell lines, there were comparable levels of CD44E (the v8 to v10 inclusive variant) to CD44H (standard form) levels. For the ER- human breast cancer cell lines tested, the CD44H levels were much higher than the nearly undetectable CD44E levels. As well, when quantitative reverse transcriptase PCR (and Southern blot) was performed on the cell lines to assess levels of v7 and v10 inclusive variants, it was determined that the ER- cell lines express fewer of these variants than the ER+ cell lines. (Figureure 3) In addition, these CD44 variant levels are not affected by the addition of estrogen to either cell type. These CD44H and CD44 variant levels were also assessed in a panel of both ER+ and ER- breast tumor samples. The CD44v7 and v10 containing variant mRNA's were more abundant in the ER+ tumors, but there was no detectable difference in the CD44H levels. These observations led to the following hypotheses which we examined in this project:

1. Differences in the CD44 patterns of expression influence invasiveness of human breast cancer cell lines. A decrease in CD44E, relative to the CD44 standard form, contributes to invasiveness, therefore increasing CD44E should decrease invasiveness.

2. Since the differences in CD44 patterns of expression in ER+ and ER- breast cancer cells are not influenced by estrogen, we also hypothesize that the observed differences in the patterns of CD44 expression in ER+ and ER- cells may be caused by the microenvironment and it's influence on cell shape, cell density, and cell adhesion.

3 Methods

3.1 Tissue cell culture

MDA-MB-231 (ER- human breast cancer cell line, adenocarcinoma, ATCC), T47D5 (ER+ human breast cancer cell line, ATCC), and NIH 3T3 (mouse fibroblast cell line, ATCC) cell lines were grown and maintained as a monolayer culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum,10 units/ml penicillin, 10 units/ml streptomycin, 35% (weight/volume) glucose and 2 mM final concentration L-glutamine. All of the above reagents were purchased from Gibco Technologies, except for the glucose, which was purchased from Fisher. All cells were grown at 37°C, in 5% carbon dioxide, (unless during the initial stages of the transfection procedure, where the CO_2 level was set for 3%) and 100% humidity.

3.1.1 Splitting Cell Lines

Cell lines were grown to 80% confluence in a T_{150} flask and harvested with 1X trypsin/EDTA (ethylenedinitrolltetracetic acid) by the following method: medium was aspirated off and the cells were rinsed with 4ml of a 1X phosphate buffered saline (PBS) (8g sodium chloride, 0.2 potassium chloride, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, brought to pH 7.4 with HCl) containing 3mM EDTA. Then 4ml of 1X trypsin/EDTA was added, and the flask was then incubated at 37°C, 5% CO_2 for approximately 5 minutes. Cells were harvested using 6ml of supplemented DMEM and shaking. The cell suspension was then passed through an 18 gauge needle to suspend the cells completely. A 1 in 10 volume of this cell suspension was then used to maintain the culture in the new T_{150}

flask.

3.1.2 Freezing Cell Lines

Cells were frozen down for storage in liquid nitrogen by the following method: cells were split as detailed above, and spun down at room temperature for 5 minutes at 1000 rpm. The cell pellet was then resuspended in a 1ml volume of a freezing solution consisting of 10% dimethlysofoxide (DMSO), 10% fetal bovine serum, and 80% supplemented DMEM.

3.2 Stable Transfections

3.2.1 Plasmid Preparation

The *E.coli* bacteria containing the CD44R1 plasmid, containing the hygromycin selectable marker, (Figureure 4) (Dr. G. Dougherty, Terry Fox Laboratories, Vancouver, B.C.) used in the stable transfections were first grown in 5ml of LB (Luria-Bertani) broth. For 600ml LB broth 6g of Bacto-tryptone (Difco), 3g of Bacto-yeast extract (Difco) and 6g sodium chloride (Anachemia Science) were added to 600ml of distilled, deionized water, the pH was adjusted to 7.5, and the media was autoclaved. To the 5ml culture, 0.5ml of a 40 mg/ml carbenicillin (Sigma) solution was added per 1 ml of media. The culture was shaken overnight at 37°C, for approximately 12-16 hours. The next day, the 5ml culture was added to 500ml of sterile LB broth supplemented with 0.5µl of the 40mg/ml carbenicillin solution per ml of media. The flask was then placed at 37°C to be shaken overnight. At the 4 hour time point, 150µl of a 34mg/ml chloramphenicol (Sigma) solution (34mg chloramphenicol in 1ml of absolute ethanol) was added for plasmid

amplification, then the culture was incubated for the rest of the 12-16 hours. The method used for the large scale plasmid preparation comes from the Qiagen Plasmid Mega Kit which is based on the original method by Birnboim and Doly (1979). In this method, the 500ml culture was spun down for 10 minutes at 8000 rpm's in a Beckman J2-21 centrifuge, using a JA-10 rotor, at 4°C. The cell pellet was then placed overnight at -20°C to facilitate the lysis of the cells. The pellets were then resuspended in 25ml of resuspension buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 100mg/ml RNAse A). Next, 25ml of lysis buffer (200mM sodium hydroxide, 1% SDS) was added to the pellets, which were mixed briefly, and incubated at room temperature for 5 minutes. 25ml of chilled neutralization buffer was then added (3.0M potassium acetate pH 5.5) the solutions were mixed, and incubated for 30 minutes on ice. The lysed cell suspension was then centrifuged at 20 000 x g for 30 minutes at 4°C (using a JA-20 rotor) and the supernatant was retained. This centrifugation was repeated for 15 minutes, and the supernatant was again removed. At the same time, the Qiagen anion-exchange column was equilibrated using 35ml of buffer containing 750mM sodium chloride, 50mM MOPS (3-(N-morpholino)propanesulfonic acid) pH 7.0, 15% ethanol and 0.15% triton X-100. The supernatant was then added to the column and washed with four 50ml aliquots of a wash buffer (1.0M sodium chloride, 50mM MOPS pH 7.0 15% ethanol). The DNA was eluted from the column using 35ml of a elution buffer (1.25 M sodium chloride, 50mM Tris-HCl pH 8.5, 15% ethanol.) The DNA was then precipitated with 0.7 volumes of isopropanol at room temperature, and centrifuged at 15000xg for 30 minutes at 4°C, washed with 7ml of 70% ethanol and resuspended after drying for 10 minutes in an

appropriate volume of TE buffer (10mM Tris, 0.1mM EDTA). Quantitation of all DNA was done at 260nm on a Milton Roy Spectronic 601 spectrophotometer.

3.2.2 Stable Transfection

Stable transfectants were created using the calcium phosphate transfection method based on Chen, (1987). Basically, a exponentially growing T_{75} flask of MDA-MB-231 cells was split as before in a volume of 10ml. 0.5ml of the cell suspension was added to 9.5ml of isotone solution, and the cells were counted in a Coulter counter. $5x10^5$ cells were plated on $100mm^2$ dishes, and incubated overnight in 10ml of media, to grow to 10-20% confluence. The calcium phosphate transfection was performed using 25μg of CD44R1 plasmid along with 2μg of RSVNeomycin selection plasmid, which were diluted to 450μl of distilled, deionized water. 50μl of a 2.5M calcium chloride solution and 500μl of a 2X BBS (50mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280mM sodium chloride, 1.5mM Na_2HPO_4 pH 6.95, entire solution to pH 6.95 with 1N sodium hydroxide) solution were added, mixed gently, and incubated at room temperature for 20 minutes. This suspension was then added dropwise to the plates, which were mixed evenly, and incubated overnight in 3% CO_2. 24 hours later, the cells then were rinsed with 1X PBS, fresh media was added, and the cells were grown overnight in 5% CO_2. Selection media was then added the next day, using neomycin (Gibco) or hygromycin (Sigma). For MDA-MB-231, the initial geneticin concentration (used until the control, untransfected cells died) was 800μg/ml, and for hygromycin initially- 400μg/ml. once the control (untransfected) cells died (about 7-10 days later), the final concentrations of selection agents were used. For geneticin, the final

concentration used was 600μg/ml, and for hygromycin- 200μg/ml. The cells were fed every other day, until colonies appeared on the selection plates, about 2-6 weeks later.

3.2.3 Cloning the Transfectants

Whatman paper disks of approximately 0.5mm diameter were soaked in 1X trypsin/EDTA then applied to the colonies on the 100mm plate (after the media was removed) , for about 60 seconds. Using tweezers, the disk and colony were placed into a well on a 96-well dish which contained 250μl of the DMEM (with 800μg/ml geneticin). From there the clones were systematically expanded to get two confluent T_{150} flasks to freeze down cell stocks, as well as to pellet cells for DNA/RNA analysis.

3.3 Genomic DNA Extraction

All the DNA used in PCR or Southern blots was extracted by modifying the method of Hofstetter et al., (1997) Two T_{150} flasks were pelleted down for the genomic extraction. 700μl of the extraction solution (50mM Tris pH 8 or 7.6, 100mM EDTA, 100mM sodium chloride, to 40ml distilled sterile water) as well as 35μl of a 10mg/ml Proteinase K solution (Beohringer Mannheim) were added to the pellets, and incubated in a shaking incubator at 55°C overnight. To each microfuge tube, enough phenol was added to fill the tube to 2-3mm below the rim, the tubes were inverted one hundred times and spun on a at room temperature for 5 minutes at 12500 rpm. The aqueous layer was removed to a new tube, and this process was repeated using a 1:1 phenol/chloroform mixture, and then with chloroform alone. After the last aqueous phase transfer to a new tube, equal volumes isopropanol was added and tubes was inverted several times until a white stringy precipitate appeared. This DNA precipitate was cleaned with 1ml or 70%

ethanol and left at 4°C for 1 to 2 hours, then the 70% ethanol was removed, and 1ml of absolute ethanol was added. The tubes were shaken, and spun for 2 minutes at room temperature at 12500 rpm. After spinning, the pellet was dried and resuspended in 100µl of TE buffer, and quantitation was determined by reading optical densities at 260nm.

3.4 RNA Extraction

RNA was extracted using the Tri-Reagent (Molecular Research Centre Inc., OH) protocol, which is based on the original protocol of Chomczynski and Sacchi (1987) Typically, 1ml of Tri-Reagent was used per 20-100mg of cells. After addition of Tri-Reagent, pellets were homogenized on ice for 15-30s and let stand at room temperature for 5 minutes. Then 200µl of chloroform per 1ml of Tri-Reagent was added to the samples, which were shaken and left at room temperature for 5 minutes. The samples were then centrifuged at 12000 rpm for 15 minutes at 4°C, and the clear, aqueous phase containing the RNA was transferred to a new tube. Precipitation of the RNA occurred by adding 500µλ of isopropanol per 1ml of Tri-Reagent, then incubating the samples at 4°C for a minimum of 30 minutes, then centrifuging at 12000 rpm for 10 minutes at 4°C. The subsequent pellet was washed with 75% ethanol (1ml ethanol per 1ml of Tri-Reagent) and centrifuged at 7500 rpm for 5 minutes at 4°C. After briefly air-drying the pellet (5 to 10 minutes) on ice, the RNA was dissolved in 30µl of DEPC-treated (diethyl pyrocarbonate) sterile water.

For RNA extracted from tissue culture plates, the cells were lysed directly on the dish and 1ml of Tri-Reagent per $10cm^2$ was used.

3.5 RT PCR

100ng of RNA was reverse transcribed using 5X RT buffer, 2.5mM of each nucleotide, 0.1% bovine serum albumin (BSA), 0.1M DTT (dithiothreitol), 10%DMSO, 50μM random hexamer, and 200 units/μl MMLV reverse transcriptase (Gibco BRL) in a final volume of 10ml. This mixture was incubated at 37°C for 1.5 hours, then diluted to 40ml with distilled water.

For PCR using the CD44H, CD44R1, or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers, 2μl of the above diluted cDNA was used in the PCR mixture, which consisted of: 10X PCR buffer, 25mM magnesium chloride, 2.5mM nucleotides, 10% DMSO, 5U/μl Taq DNA 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 Cylcer using the following program: 10 minutes at 94°C, then for 30 to 45 cycles a program of 45 seconds at 93°C, 45 seconds at 56°C , then 1 minute and 30 seconds at 75 °C. Primer extension at 75°C was for 15 minutes, before cooling down to 4°C. For PCR of genomic DNA samples, 1μg of genomic DNA was used in the above PCR mixture. All PCR products were run out on a 1% agarose gel, containing 7μl of ethidium bromide.

For studies using RT PCR for cell density or cell substrate experiments, the cycle number for the CD44 and GAPDH primers was determined for the linear range of PCR amplification. For the CD44H and CD44R1 primers 40 cycles were used in these experiments, and for GAPDH 35 cycles were used.

3.6 Genomic Southern Blot

10 μg of genomic DNA was digested using 4μl of a 60 unit/ml BamH1 restriction enzyme (Pharmacia), 1mM spermidine, 1X enzyme buffer, in a final reaction volume of

25µl. The samples were incubated overnight at 37°C and run out on a 1% agarose gel containing 5µl of ethidium bromide at 22 volts for 18-24 hours. For CD44R1 plasmid digests, 2µg of plasmid was digested with 2µl of 10unit/ml BamH1 in the same mixture as above, but omitting the spermidine. The next day, the gel was photographed under ultraviolet light, measured, and rinsed three times in preparation for blotting. (Southern, 1975) The first rinse was for 15 minutes with 0.25M HCl, then for 30 minutes with a denaturing solution (0.5M sodium hydroxide, 1M sodium chloride), and lastly for another 30 minutes with a neutralizing solution (1.5M Tris pH 7.4, 3M sodium chloride). The DNA was blotted overnight onto a Zeta probe nylon membrane (Bio Rad) via capillary action and using 10X SSC (sodium/sodium citrate solution). The next day, the membrane was baked at 80°C for 30 minutes, then prehybridized in a solution containing 50% formamide, 120 mM Na_2HPO_4 (pH 7.2), 250 mM sodium chloride, 7% SDS and 1mM EDTA. for 30 minutes minimum, before the probe was added. The next day, the blot was rinsed 3 times, first for 30 minutes with a solution containing 2X SSC and 0.1% sodium dodecyl sulfate (SDS), then for 30 minutes using 0.5X SSC and 0.1%SDS, and lastly at 65°C for 10 minutes using 0.1X SSC and 0.1% SDS. The blot was placed in a sealed plastic freezer bag and placed in a cassette with film for an average of one week to expose.

3.6.1 Probe Synthesis

The radioactive probes used in the Northern and Southern blots were synthesized from a genomic DNA template, using PCR techniques and primers for the v8 and v10 CD44 exons specifically, by the RadPrime method (Gibco). Briefly, 25 ng of DNA is

dissolved in 20µl of sterile distilled water or TE buffer, 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 dNTP labeled with ^{32}P were added to the mixture. Lastly, 1µl of Klenow fragment (large fragment of DNA polymerase I) was added, and the solution was incubated for 10 minutes at 37°C. Once the incubation was finished 5µl of the stop buffer (0.5M EDTA, pH 8.0) was added to the tube and the probe was eluted using two 400µl volumes of TE buffer, boiled for 5 minutes, placed on ice, and applied to the blot overnight.

3.7 Northern Analysis

3.7.1 Sample Preparation

20µg of total RNA was diluted to a volume of 10µl using DEPC-treated distilled water. To this dilution 10µl of formamide, 2.73 µl of gel running buffer (5X GRB: 0.2M MOPS pH7.0, 50mM sodium acetate, 5mM EDTA, pH8.0), and 4.73µl of formaldehyde were added (on ice), the samples heated at 65°C for ten minutes, and cooled on ice.

3.7.2 Gel Preparation and Running of Samples

The Northern gel was a 1% (wt/vol.) agarose gel containing 18% formaldehyde, and 20% gel running buffer. The samples were loaded using 3µl of a loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in distilled water to 10ml), and was run overnight at 40 volts. The next day, the gel was rinsed three times: 30 minutes in a 1% (wt/vol.) glycine solution, 20 minutes in a 0.05N sodium hydroxide solution, and 40 minutes in a 20X standard saline citrate (SSC) solution. As for the Southern blot, the Zeta probe (Bio Rad) membrane was blotted, only using 20X SSC; not

10X. The next day, the blot was baked and probed as for the Southern blot.

3.8 Cell Substrate Adhesion Studies

3.8.1 Cell Culture and Plating

MDA-MB-231 and T47D5 cells were grown, harvested and counted as previously described, then plated at increasing cell densities of $3.1x10^3$, $6.25x10^3$, $1.25x10^4$, and $2.5x10^4$ on plastic 24-well dishes (2 cm^2) for two days and harvested for RNA extraction by the same method as mentioned before, except when the cells were plated on the different adhesion substrates. In these cases, MDA-MB-231 and T47D5 cells were harvested and resuspended in Aim-V serum free complete media (Gibco) and plated at a concentration of 25 000 cells/well of a 24-well dish ($2cm^2$) (Costar) in Aim-V media.

3.8.2 Preparation of Substrates

Polyhydroxyethylmethacrylate (p-HEMA) (Sigma) was prepared by suspension in 95% ethanol, incubated at 37^0C overnight and spun at 2500 rpm for 30 minutes (if undissolved material remained). Working stocks were diluted to 12mg/ml and 75µl/cm^2 for a final concentration of 0.9mg/cm^2. (Schischmanoff et al., 1997) The plates were air dried under sterile conditions under ultraviolet light overnight.

Collagen type IV extracts (Sigma) were diluted to 2mg/ml in 0.25% acetic acid and incubated at 4°C overnight to enhance solubility. The collagen solution was then added to sterile plates at a concentration of 0.25 mg/cm^2. (Le Marer and Bruyneel, 1996)

Matrigel (Biocoat/VWR) was prepared by thawing overnight at 4°C and using pre-cooled pipettes, was diluted 1:2 with cold DMEM. 50µl/cm^2 of the dilution were

added to pre-cooled plates and left at 37°C for 30 minutes to solidify, as per manufacturer's instructions.

In the studies using cells grown on collagen type IV and plastic, the RNA was recovered by adding 100µl Tri-reagent directly to the plates, as mentioned before. In order to recover the cells that were growing in the Matrigel, dispase (Collaborative Biomedical) was added. Briefly, the dispase was warmed to 37°C and 200 µl/cm^2 was applied to the plates, and incubated for 2 hours at 37°C. The cells retrieved were pelleted at 1000 rpm for 5 minutes, rinsed with sterile PBS and spun again (this was repeated 3 times to remove any remaining Matrigel) and 1 ml of Tri-reagent was added to the tube. The cells growing on the p-HEMA coated plates were also pelleted in the same manner.

3.9 Excision of DNA Bands from Agarose Gel

DNA used in Northern and Southern probes was excised from a 1% agarose gel using the "GFX PCR DNA and Gel Band Purification Kit" (Pharmacia, Biotech) protocol based on the method of Vogelstein and Gillespie (1979) Briefly, the appropriate band of DNA was cut from the agarose gel, sliced in several thin pieces, and placed in a 1.5 ml centrifuge tube. 10ml of capture buffer (buffered solution containing chaotrope) per 10mg of gel was added to the tube which was vortexed and incubated at 60°C until the gel was dissolved, usually 5 to 15 minutes. The sample was added to the GFX column which contained a glass fiber matrix, and microfuged for 30 seconds at 13000 rpms. 500ml of wash buffer (TE buffer, 80% ethanol) was added, and the column was again spun down for 30 seconds at 13000 rpms. In order to elute the DNA sample from the column, 50ml of a 10mM TE solution (pH 8.0) was applied to the column, which was

incubated for 60 seconds and centrifuged at 13000 rpms and the purified DNA was collected. Quantitation of DNA was performed at 260nm on a Milton Roy Spectronic 601 spectrophotometer.

3.10 SDS-PAGE Western Blot

3.10.1 Preparation of Samples

Proteins were extracted from cell pellets using 200µl (or 1 ml of isolation buffer per 150 mm^2 dish) SDS-isolation buffer containing protease inhibitors (50 mM Tris pH 8.0, 20 mM EDTA, 5% SDS, 5mM b-glycerophosphate, 1mM AEBSF, 5mg/ml aprotenin). The lysates were passed through a 23 gauge needle several times to shear the DNA and centrifuged at 13 000 x g for 20 minutes at room temperature, then 5µl of glycerol and 0.5µl of 100 mM DTT were added to the lysate. Protein concentrations were determined in a protein assay from Pierce. Briefly, protein standards were made using BSA protein, diluted in double distilled water, to final concentrations of 0, 2, 5, 10, 15, 20, 25, 30, 40, and 50µg. 1µl of each experimental protein sample was diluted to 500µl in distilled water. 500µl of a three-part assay reagent solution was added to each sample. This reagent mixture consisted of a 50:48:2 ratio of solution A (sodium carbonate, sodium bicarbonate, tartrate in 0.2N sodium hydroxide), solution B (aqueous solution to BCA detection) and solution C (4% cupric sulfate pentahydrate). After incubation for 1.5 hours at 60°C, samples were read at 562nm on a Milton Roy Spectronic spectrophotometer. Samples were stored at -20°C until needed. Before loading 50µg of protein for the Western assay, the samples were warmed at 65°C to dissolve the SDS and an aliquot was added to an equal volume of 2X SDS-sample buffer

(2.5mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.02% (w/v) bromophenol blue) and boiled for 5 minutes.

3.10.2 Western Gels

Western gel running and blotting techniques were perform as described in the Bio Rad protocol. Briefly, the plates were set up according to manufacturer's instructions. The separating gel contained 4x separating buffer (1.5M Tris, pH 8.8, 0.4% SDS), 30% polyacrylamide (36:5:1, 2.67% cross-linking) 10% APS (ammonia persulfate, Bio Rad) as well as TEMED. (N,N,N',N'-tetramethylethylenendiamine, Sigma) The stacking gel contained 4x stacking buffer (0.5M Tris, pH 6.8; 0.4% SDS), 30% polyacrylamide, 10% APS and TEMED. The percentage gel used for the SR protein Western blot was 12%, for the CD44 v10 Western, 6% was used. 1X SDS-PAGE running buffer (5X concentration: 125 mM Tris, 960 mM glycine, 0.5% SDS, solution pH 8.3) was added to the inner chamber of the Bio Rad running apparatus, and 0.2X running buffer for the outer chamber. The gel was run at 10 mAmps for 1 hour, then 20 mAmps for another hour. For the Western transfer, 0.45μm nitrocellulose (Zeta probe) blotting membrane and Whatman paper were used. The transfer assembly components (membrane, Whatman paper, scrub pads) were presoaked in chilled 1X transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, solution pH 8.3). The transfer took place at 100 volts for 1 hour, with the entire apparatus on ice. Once the transfer was complete, the membrane was blocked in 5% dried skim milk powder in TBST (20 mM Tris, 137 mM NaCl, 0.5% Tween 20, solution pH 7.6) for one hour with shaking at room temperature. The blocking solution was removed, and the primary antibody (for the SR Western the primary

antibody was mAb104) was added to a new aliquot of blocking solution, and left overnight with shaking at 4°C. The next day, the blot was rinsed 6 times with 1X TBST at room temperature (five 5 minute rinses, one 15 minute rinse) and incubated with the secondary antibody for 2 hours at room temperature. The rinses were repeated as before, and the blot was developed using the Pierce "SuperSignal" method. Briefly, equal volumes of the 2 solutions (luminol/enhancer solution and a stable peroxide solution) were pipetted onto the blot (0.125ml/cm^2 of blot surface) and left for 10 minutes at room temperature. The solution was removed and the blot was developed for various time points. For the CD44 VFF-14 primary antibody (CD44v10 specific antibody), the Western blot was set up as for the SR protein blot, but with several changes. The blot was blocked in 10% dried skim milk powder in PBST (phosphate buffered saline with 0.03% Tween 20) for 1 hour at room temperature. The primary antibody (VFF-14, Bender MedSystems) was diluted at 1:1000 in 5% dried skim milk powder in PBST, and incubated with the blot at 4°C overnight. The next day, the blot was again rinsed 4 times for 5 minutes with PBST, and the secondary antibody (HRP-conjugated rabbit/anti-mouse IgG, DAKO) was added for 2 hours at room temperature with shaking. The detection system is described above.

3.11 Growth assays

MDA-MB-231 and the CD44R1 transfected clones, JS11-H2 and H3, were grown as previously described in 35mm^2 dishes at a concentration of 5x10^3 cells per well for 13 days. This was done in triplicate wells, and the growth assay was repeated three times. The cells in each well were trypsinized and counted at days 2,4, 7, and 10 in order to

determine growth curves for each cell line, using a Coulter Counter.

3.12 Invasion Assays

3.12.1 Cell Culture

NIH 3T3 mouse fibroblast cells were grown as above, and media was harvested from these cells after rinsing the 90% confluent T_{150} flask three times with 10 ml of PBS (phosphate buffered saline) and then replacing the last wash with 30 ml of DMEM supplemented with glucose and glutamine (as before) and 10% Nu-Serum (Collaborative BioMed) plus 50μg/ml ascorbic acid (Fisher Biotech). The flask was incubated for 24 hours at 5% CO_2 and 37°C. The next day the media was decanted and filter sterilized.

3.12.2 Invasion Assay

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°C DMEM for 1.5 hours. Cells were counted to a concentration of $1x10^5$ cells per ml, in DMEM supplemented with 0.1% BSA (bovine serum albumin). 750μl of fibroblast cultured media was added to the wells below the invasion chamber, and $5x10^3$ cells of either MDA-MB-231 or T47D5 human breast cancer cell lines, were added to the invasion chamber. The cells were kept at 37°C and 5% CO_2 for 12 to 24 hours. The non-invading cells were carefully removed from the upper surface of the membrane, and the membrane was fixed with methanol, stained with hematoxylin and eosin, then removed from the invasion chamber and placed on a slide for cell counts. Three fields of view of each slide were photographed, and cells were

counted in each. Percent invasion was calculated by dividing the mean of cells invading through the Matrigel coated membrane by the mean of cells migrating through the uncoated control insert membrane and multiplying by 100.

4 EXPERIMENTAL RESULTS

4.1 CD44R1 Transfection Results

The CD44R1 plasmid (Figureure 4) was transfected into the estrogen receptor negative human breast cancer cell line MDA-MB-231 via the $CaPO_4$ method of Chen et al. (1987) which complexes the cDNA with the calcium ion, and facilitates the uptake of the cDNA into the cell for integration.

Several rounds of transfection with either hygromycin or neomycin as the selection agent, as well as several co-transfections with the pMAMneo plasmid (Clonetech) were performed. (Table 1) The neomycin transfections were performed because initially the concentration of hygromycin (400μg/ml initially, then the cells were maintained in 200μg/ml hygromycin) was too high and killed off most of the potential clones. Table 1 indicates that approximately 230 individual clones were expanded from 11 rounds of transfections, including 3 clones from the February 1997 transfection that were selected with hygromycin, not neomycin.

In order to screen a number of clones for gene integrations, a genomic PCR using CD44E primers (which hybridize both to a 22 base pair conserved exon/ intron boundary CD44 site in exon 5, and a specific 21 base pair primer to the v10 exon sequence) was performed. Of the 230 expanded clones, 75 were analyzed using this method and 38 of the clones tested (51%) were positive for the predicted 400 base pair band. As in Figure. 5, the control cell line was the untransfected MDA-MB-231 ER- human breast cancer cell line, which failed to contain the 400 base pair cDNA PCR result, as it did not contain

the transfected plasmid. Figureure 5 shows a representative genomic PCR of several cell clones, as well as MDA-MB-231 untransfected control cells. The PCR generated a 400 base pair band from the CD44E inserted cDNA in the transfectants, but due to the enormous size of the endogenous CD44 gene (which spans about 60 kb; Screaton et al., 1992) the PCR conditions were inefficient to amplify any signal in the untransfected cells.

To confirm the PCR-positive clones for genomic integration, we turned to genomic Southern blot. Table 1 indicates the number of clones tested by Southern blot for integration. As mentioned previously, 10ug of the extracted genomic DNA was digested with the restriction enzyme BamH1, as was the control plasmid, CD44R1. (See Figureure 4 for CD44R1 map) The CD44R1 plasmid insert contains one BamH1 site which cleaves the insert into two fragments of 1.4 and 0.3 kb, as well as cutting the insert out of the multiple cloning site. The probe used was specific for the v8 to v10 exon and was generated by PCR of the CD44R1 plasmid using v8 and v10 specific primers. Figure 6 shows an example of several clones that were analyzed. Table 1 indicates that out of 26 clones tested, 2 were positive by Southern blot for genomic integration. Clones JS-11-H2 and H3 are transfected clones selected using the hygromycin selectable marker, where JS13-C4, C1 and B4 are neomycin co-transfectants. For controls, untransfected MDA-MB-231 ER- human breast cancer cell line and the CD44R1 plasmid were digested with BamH1as with the other samples. The only lanes to express a second band corresponding to the 1.4 kb CD44R1 fragment (the 0.3 kb fragment was run off the gel) were JS11-H2 and H3. The larger fragment band (approximately 2 kb) present in all lanes most likely

corresponds to a digested genomic CD44E fragment.

Next, we needed to determine if the 2/26 positive clones with apparent genomic integration of the plasmid were able to overexpress an mRNA transcript. Figure 7 shows an ethidium bromide gel loaded with 20ug of RNA, and photographed to show loading similarities between lanes. This gel includes the PCR positive clone JS11-B4, which was shown to be negative by Southern blot for CD44E (R1) plasmid integration, as well as the PCR positive, Southern blot positive, clones JS11-H2 and H3, and the untransfected parent cell line, MDA-MB-231. Figure 7 shows three mRNA transcripts for each lane, of approximately 5.4, 2.6, and 2.0 kb. The reason for the multiple mRNA signals is the presence of multiple polyadenylation signals in the CD44 gene. (Harn et al., 1991)

The radiolabelled probe (see Materials and Methods for details) was generated from the CD44R1 plasmid using the same v8/v10 specific primers as for the Southern blot. This exon specific probe recognized three transcripts for CD44E with sizes of 5.4, 2.6 and 2.0 kb. In one of the Southern positive clones, JS11-H2, it was observed that there was overexpression of the 2.5 kb transcript when compared to the MDA-MB-231 untransfected parent cell line. This transcript ran at a slightly larger transcript size, presumably due to a slightly altered transcription start site in the plasmid, or a longer 3'/poly A tail, as compared to the endogenous CD44 gene. JS11-H3 shows the larger middle transcript as well, but with lower expression than JS11-H2 and at a similar level of expression to the control cell line, MDA-MB-231. With the overexpression of CD44E mRNA in the JS11-H2 clone, the next logical step was to confirm CD44E protein overexpression. Using a CD44v10 specific antibody, (VFF-14, Bender MedSystems)

Western blots were performed on both JS11-H2 and H3 clones, as well as two human breast cancer cell lines for controls: untransfected ER- MDA-MB-231 and ER+ T47D5 cell lines.

The results in shown Figure 8 indicate an overexpression of two bands in the JS11-H2 and H3 lanes, of approximately 165 and 155 kDa. These bands are undetectable in the ER- MDA-MB-231 cell line, but are present to a lesser degree in the ER+ T47D5 cell line. This correlates with our previous data that indicates ER- cell lines have very little CD44E mRNA, and that ER+ cell lines possess a higher level of CD44E. The uniformly expressed bands at approximately 160 and 150 kDa are due to non-specific binding by the secondary antibody. The 80kd band may correspond to a small CD44v10-containing isoform, that is not CD44E, which contains v8 to v10. Using the same antibody, Saegusa and Oakyasu (1998) reported expression of major bands of approximately 140 to 250 kDa. This reflects the fact that the CD44 protein is highly posttranslationally modified by glycosylation, glycosaminoglycanation and the addition of sialic acid. (Goldstein et al., 1989; Brown et al., 1994; Jalkanen et al., 1988) as well as the fact that the VFF-14 antibody recognizes an epitope on the v10 exon. This includes, but is not exclusive to, CD44E, which is the most prominent v10-containing isoform. (Saegusa and Okayasu, 1998).

Growth assays were also performed on the JS11-H2, H3 clones as well as the MDA-MB-231 untransfected cell line. Figure 9 shows the resulting effects of CD44E overexpression on cell growth rates, which indicates that the average growth rate of the transfected JS11-H2 and H3 clones appears lower than the untransfected MDA-MB-231

parental cell line, but the difference in growth rates was not statistically significant by t test. (p..05) However, this trend was present in 3 independent growth assay experiments, and therefore the trend of slower growth rates in the transfectants should be taken into consideration in reference to the invasion results.

In order to answer the initial hypothesis as to whether of not an overexpression of the CD44E (v8-v10) variant isoform would in fact decrease invasiveness, invasion assays using reconstituted basement membrane (Matrigel, VWR) were performed. (Figure 10 illustrates the invasion assay.) In duplicate experiments, when the level of invasion was compared between the parental cell line, MDA-MB-231, and the CD44E transfected clone JS11-H2, the results indicated that there was no statistically significant difference in the level of invasiveness between them. The mean values for the invasion assays with the MDA-MB-231 untransfected cell line was 19.5% and for the JS11-H2 CD44R1 transfected clone, 22.8%. (Figure 11)

4.2 Cell Substrate and Cell Density Results

4.2.1 Cell Density Experimental Results

MDA-MB-231 and T47D5 human breast cancer cell lines were plated at increasing cell densities on $2cm^2$ tissue culture plates. The cells were grown for 2 days, when the highest concentration of cells would have just become confluent, and then harvested for RNA extraction. For each primer grouping, the cell lines were plated at increasing cell densities of 50%, 100%, 200% and 400%. Once the RNA was extracted from the cells, RT PCR was performed to assess the levels of expression of CD44H (standard form), CD44E v8

to v10 variant) and GAPDH (housekeeping gene) as a loading control. The primers used for the CD44 standard form are found in the conserved exon 5 (at an intron/exon boundary) and in the conserved exon 17, also at an exon/intron boundary. For the v10 PCR, the upper primer was the same exon 5 primer, but the lower primer hybridised in exon 14, which is the v10 exon. Figure 12 shows the results of a representative replicate of this RT PCR. The gel was photographed under UV light and densitometry was performed for each lane. In order to quantify the PCR results, the value for each CD44H or CD44E band intensity was divided by the value for the corresponding GAPDH band intensity, to control for loading differences on the gel. Figure 12 shows the quantified results of four separate plating experiments, each containing three replicates of the RT PCR experiment on the extracted RNA. (data not shown) The data for all four cell/primer combinations are expressed as a ratio of the standardised levels of CD44E to CD44H. (Figure 13) The 100% plating value ($6.25x10^4$ cells/well) was used as the standard value for which all the rest would be compared to. Figure 12 indicates that there are no significant differences in the ratio of CD44E to CD44H levels (as detected by RT PCR) with increasing cell densities for either the ER+ (T47D5) or ER- (MDA-MB-231) breast cancer cell line.

4.2.2 Cell Substrate Experimental Results

The next study performed was to assess levels of CD44H and CD44E expression by ER+ and ER- cell lines on different substrates. The same cell lines as for cell density studies were plated at a constant cell number on four different substrates: Matrigel reconstituted basement membrane, collagen type IV, poly-2-hydroxyethylmethacrylate, and uncoated plastic plates. Figure 14 shows a representative ethidium bromide gel for the RT PCR

experiments. For statistical analyses, the densitometric values of the CD44 mRNA levels were once again divided by the results of the densitometry for the appropriate GAPDH levels, and expressed as a ratio of CD44E to CD44H. As well as for the cell density results, we set the values for the cells grown on plastic to 1, since plastic was our control substrate. The graphical analyses lead to several results. (Figure 15) Firstly, CD44E to CD44H mRNA ratios of the ER- cell line MDA-MB-231 were not affected by substrate, as there was no statistically significant difference between any of the other three substrates and plastic. In the ER+ cell line (T47D5), there was a decrease in CD44E to CD44H ratios in all three substrates compared to plastic. For Matrigel, the average decrease in splicing was 51.2%, for collagen, 55.1% and for p-HEMA, 47.3%. This decrease was statistically significant for Matrigel ($p<.01$), and p-HEMA ($p<.05$) and nearly significant for collagen ($p=.09$) when compared to plastic as a control. (Figure 15)

4.2.3 Results of SR Protein Studies

Because of the observed significance decrease in CD44E signal in T47D5 cells grown on Matrigel versus plastic, a series of Western blots assessing the effect of cell substrate on SR protein levels was performed. As discussed in detail in the introduction, SR proteins have been shown to affect splicing of several genes, including CD44. (Zahler et al., 1992) Figure 16 shows a Western blot of T47D5 cells grown on the four substrates used in the RT PCR studies. The antibody used (mAb 104) recognises the 5 different SR proteins which are the SR75, 55, 40, 30's and 20 proteins. The results of the SR protein Western blot (as assessed by densitometry, and expressed relative to SR 75) are shown in Figure 17. These

results indicate that there is no differences in the SR protein pattern of expression in cells grown on Matrigel, collagen or p-HEMA, when compared to plastic.

5 DISCUSSION AND CONCLUSIONS

Our previous work (Hole et al., 1997; see Figure 3) has shown that ER- human breast cancer cell lines, which are highly invasive, express nearly undetectable levels of CD44E and 5 to 10 times higher levels of CD44H (Figure 3). In contrast, ER+ cell lines, which are less invasive, had slightly higher levels of CD44E than CD44H. Based on these findings, we hypothesised that overexpression of CD44E in an ER- breast cancer cell line would alter invasiveness of that cell line. By overexpressing the CD44E levels in these cells, we wanted to determine the effect this gene would have on invasive potential. Our second objective was to determine if this pattern of CD44 expression was related to the cell density, cell substrate, cell shape, or different type of breast cancer cell.

To test the first hypothesis, we transfected the ER- cell line (MDA-MB-231) with CD44E rather than the ER+ cell line (T47D5) with CD44H in order to detect a change in invasiveness. The rationale for this was twofold:

1. Since there are nearly undetectable levels of CD44E in the ER- cells, any increase in CD44E mRNA would be more significant than an increase in CD44H in the T47D5 cells, which has more readily detectable levels of both CD44H and CD44E.
2. In order to determine changes in invasiveness, the Boyden chamber assay was used. It was more likely that we might detect a decrease in invasiveness in cells that already exhibited this property than to induce invasiveness in ER+ cells.

The transfectants were initially selected using the hygromycin selectable marker present in the CD44R1 plasmid, pictured in Figure 4. Initially, the hygromycin levels allowed for the selection of only a few individual colonies, so we went to the co-transfection with the

neomycin plasmid, a selection agent more commonly used.

The first step in the process of invasion and metastasis involves a change in adhesive properties. (Figure 1) This includes a decrease in cell/cell contacts, as well as a change in cell/matrix adhesion (Jiang et al., 1994), perhaps attributable to CD44, one of many adhesion molecules whose expression in altered in breast cancer. No significant difference was found in the levels of invasiveness between the untransfected MDA-MB-231's and the CD44E transfected clone, JS11-H2. (Figure 11) Southern, Northern and Western blot data indicate that the CD44R1 cDNA had been integrated, and expressed as both a mRNA transcript and the resulting protein isoform. Noting this, there may be several reasons for the lack of difference in the invasion propensity between the wild type and the CD44E transfected cells.

The invasion assay itself uses reconstituted basement membrane as the substrate that the cells must invade. The proteins included in the Matrigel are primarily type IV collagen, laminin and heparan sulfate proteoglycans, as opposed to the stroma which is mainly composed of fibrillar collagens (type I, II, III, V and XI) as well as the other glycosaminoglycans like hyaluronan, keratin sulfate and heparan sulfate. Although CD44 does bind some other components of the extracellular matrix, like collagen, fibronectin and laminin, it does so with very low affinity. (Naor et al., 1997) Since CD44 functions as the primary adhesion molecule for hyaluronic acid, (Lesley and Hyman, 1992) the effect of the increased levels of CD44E in the MDA-MB-231 cells may not be apparent in the Boyden chamber invasion assay used. Perhaps a decrease in invasive ability would be more apparent in the CD44E clones if we were to examine the ability of the cells to invade through an extracellular matrix. It has been observed that CD44E does not bind hyaluronan as well as

the standard CD44 form. It has been suggested that since there are 3 extra exons in CD44E (compared to CD44H), there are more sites for glycosylation, which has been shown to interfere with hyaluronan binding by the standard CD44 form. (Bennett et al., 1995) It has also been observed that CD44-deficient Namalwa cells (which are unable to bind hyaluronic acid) transfected with CD44E failed to bind hyaluronan as efficiently as the same cells transfected with CD44H. (Bartolazzi et al., 1995) By increasing the protein expression of CD44E (as shown in Figure 8) we could potentially decrease the cells ability to bind to the extracellular matrix proteins and therefore decrease the invasive potential in that manner. Perhaps an alternate assay that could be used would be a hyaluronan binding assay to determine the effect of increased CD44E on the transfected breast cancer cell line.

Another possibility is that the increased expression of the CD44E variant enhances metastasis of cancer cells, more so than invasiveness. Several papers have indicated that the CD44E (v8-10) variant levels correlate with metastatic rather than invasive cancers. Yamaguchi et al. (1998) showed that serum levels of CD44E in patients with colorectal cancer correlated with lymph node and liver metastasis, but not with serosal invasion. A few years earlier, the same group observed that in gastric carcinomas, CD44E variants were also associated with venous invasion and subsequent metastasis. (Yamaguchi et al., 1995) Animal studies using the CD44E transfected clones could evaluate the enhanced metastatic potential of cells overexpressing CD44E. This could be accomplished by measuring the size and frequency of the metastases of the transfected and untransfected cell lines.

A recent paper by Iida and Bourguignon (1997) has suggested that CD44v10 transfected cells display significantly less CD44-specific adhesion to hyaluronan *in vitro*.

Non-tumorgenic human breast epithelial cells (HBL100 cell line) which express CD44H only, were transfected with CD44v10 cDNA. This decreased the CD44-mediated adhesion in the transfected cell line to 0%, from 97% in the parental cell line. They concluded that CD44H was the major hyaluronan receptor. Also the ratio of CD44H to the v10 variant may affect hyaluronan binding by CD44H. In our model, we also altered the ratio of CD44H (which is normally 5 to 10 fold higher in the ER- breast cancer cell line, MDA-MB-231) to another v10 containing variant, CD44E. If the CD44E levels were only marginally increased over untransfected cell line levels, that may not have been enough to affect invasiveness in our transfected cell lines, and may not have had any noticeable effect on the ratio between CD44H and CD44E. This possibility could be tested by comparing the ratios of CD44E to CD44H in the JS11-H2 transfected clone to the less invasive ER+ T47D5 cell line.

Another possibility may be that CD44E has no direct bearing on invasiveness of breast cancer, but may be only one of many genes that has altered expression in highly invasive ER- breast cancer cells. CD44E could enhance the adhesive ability of breast cancer cells, but that may not be enough to cause the observed increase in invasiveness. In addition to CD44, proteases, motility factors and other adhesion molecules have altered expression in breast cancer contributing to a tumour cells ability to invade.

The second hypothesis in this project dealt with how the cellular microenvironment influenced the expression patterns of CD44 in ER- and ER+ cell lines. To assess this, we studied cell density, cell substrate and cell shape as possible factors.

As indicated in the "Results", there were no significant differences in the CD44E to CD44H ratios (as detected by RT PCR) with increasing cell densities for either the ER- or

ER+ cell line. As well, cell substrate had no detectable effect on mRNA expression of the ratio of CD44E to CD44H in the ER- MDA-MB-231 cell line. But in the T47D5 ER+ cell line, the ratio of CD44E to CD44H was decreased nearly by half for all substrates compared to plastic. And lastly, Western blot data indicated no relative change in SR proteins in the ER+ cells grown on Matrigel, when compared to cells grown on plastic.

From these results, several conclusions can be made. The RT PCR results for both cell density and cell substrate should indicate whether there was a change in CD44 splicing levels. Since the CD44E to CD44H ratio was the same for all cell densities and substrates in ER- cells, it is concluded that splicing was not affected by cell density, substrate or shape (indicating an alternative mechanism of splicing regulation in ER- cells). We observed that CD44 splicing in ER+ but not ER- breast cancer cells was influenced by cell substrate. This was noted in Figure 15, which showed a decrease in the CD44E to CD44H ratio on Matrigel, collagen and p-HEMA, for T47D5 ER+ cells. This result suggests that when the ER+ cells are under different adhesive conditions (by using Matrigel or collagen) or under conditions that alter the cell shape, the level of splicing of CD44 is decreased. These four particular substrates were chosen to mimic several conditions a cells would be under *in vivo*. Matrigel most closely represents the basement membrane which the cells would need to cross in order to invade new tissue, and its proteins include collagen, fibronectin and laminin. The coating of plates with collagen type IV represents the most important constituent of the basement membrane. p-HEMA induces the cells to form more rounded colonies and behave as if they were in suspension, allowing us to determine if differences in CD44 patterns of expression could be attributed to changes in cell shape.

The most interesting result was that the ER+ cells grown on the adhesive substrates such as collagen and Matrigel, showed a decrease in CD44 splicing when compared to cells grown on plastic. The use of Matrigel to affect the transcription of several genes has been well studied. Streuli et al. observed that Matrigel , but not plastic, induced human mammary epithelial cells to produce several milk proteins, including β-casein. (Streuli et al., 1991) In 1993, the same group showed that the regulation of TGFβ1 could be mediated by the substrate. (Streuli et al., 1993) Since TGFβ1 stimulates matrix production, the cells experienced a negative feedback loop when grown on Matrigel, and downregulated the TGFβ1 signal. As previously discussed, CD44E has been shown to be associated with more metastatic forms of colon and gastric cancer (Yamaguchi et al., 1998 and 1995), and may be down-regulated during invasion, which is defined by crossing of the basement membrane, in our case, growth on Matrigel. (Jiang et al, 1994)

Cell shape has been implicated in the expression but also the splicing of several genes. Fibroblasts increase collagenase-1 production as a result of integrin binding and the eventual disruption of the actin cytoskeleton. By changing the cells shape via plating on integrin-specific ligands, a signalling cascade beginning with the integrin adhesion molecule caused increased transcription of the collagenase-1 gene. (Kheradmand et al., 1998) Alternate splicing has also been shown to be directed by changes in cell shape. Schischmanoff et al. (1997) showed that p4.1 (a membrane skeleton protein found in red blood cells) could be alternatively spliced differently depending on the shape of the cell. When the cells had a more rounded morphology induced by plating on p-HEMA, a novel exon insertion was observed. A change in cell shape also induced different patterns of CD44

splicing, perhaps because CD44 is also associated with the cytoskeleton through ankyrin and actin. (Tsukita et al., 1994)

The decrease in ER+ cells of CD44 mRNA splicing on Matrigel and collagen did not correlate with any consistent change in any SR protein levels, when compared to cells grown on plastic. (Figures 16 and 17) This finding suggests that the decrease in CD44 splicing due to changes in adhesive properties is not a direct function of the expressed levels of SR proteins themselves, but of some other mechanisms. (see below) The SR proteins are a family of proteins which regulates splice site selection for CD44 alternative splicing, as well as for other genes. (Screaton et al., 1995) SR proteins also have cell type-specific expression, and certain SR proteins have been shown to favour specific-splice sites (Fu, 1995). It has been demonstrated that the splicing pattern of CD44 is altered when the levels of SR proteins are changed, specifically SR40 and 55. (Screaton et al., 1995) Previous work in our laboratory (M. Pind, unpublished data) has indicated that in breast tumours that have increased expression of v7 and v10 inclusive variants of CD44, there is a relative overexpression of SR55. Transient transfection experiments in our laboratory (M. Pind, unpublished data) using SR40 and SR55 in ER+ breast cancer cell lines indicated that there was no direct effects on CD44 splicing. By altering CD44 splicing through adhesion changes, we observed that SR protein levels were unaffected. It's possible that if changes in expression levels of SR proteins do not directly affect the expression of v10-containing variants, including CD44È (v8 to v10), perhaps there is a change in the phosphorylation or activation of the SR proteins (by kinases such as clk's1 through 3 or SRPK1 and 2. (Hanes et al., 1994) that alter the levels of splicing of CD44 variants in breast cancer cells.

In conclusion, we have determined that the differences in CD44E expression in ER- breast cancer cells may not be directly related to invasive propensity of ER- cells. In ER+ cells, the regulation of CD44 splicing can be influenced by changes in cell adhesion and cell shape. Therefore, some of the differences seen in cell lines between ER+ and ER- cells may be consequences of *in vitro* culture conditions.

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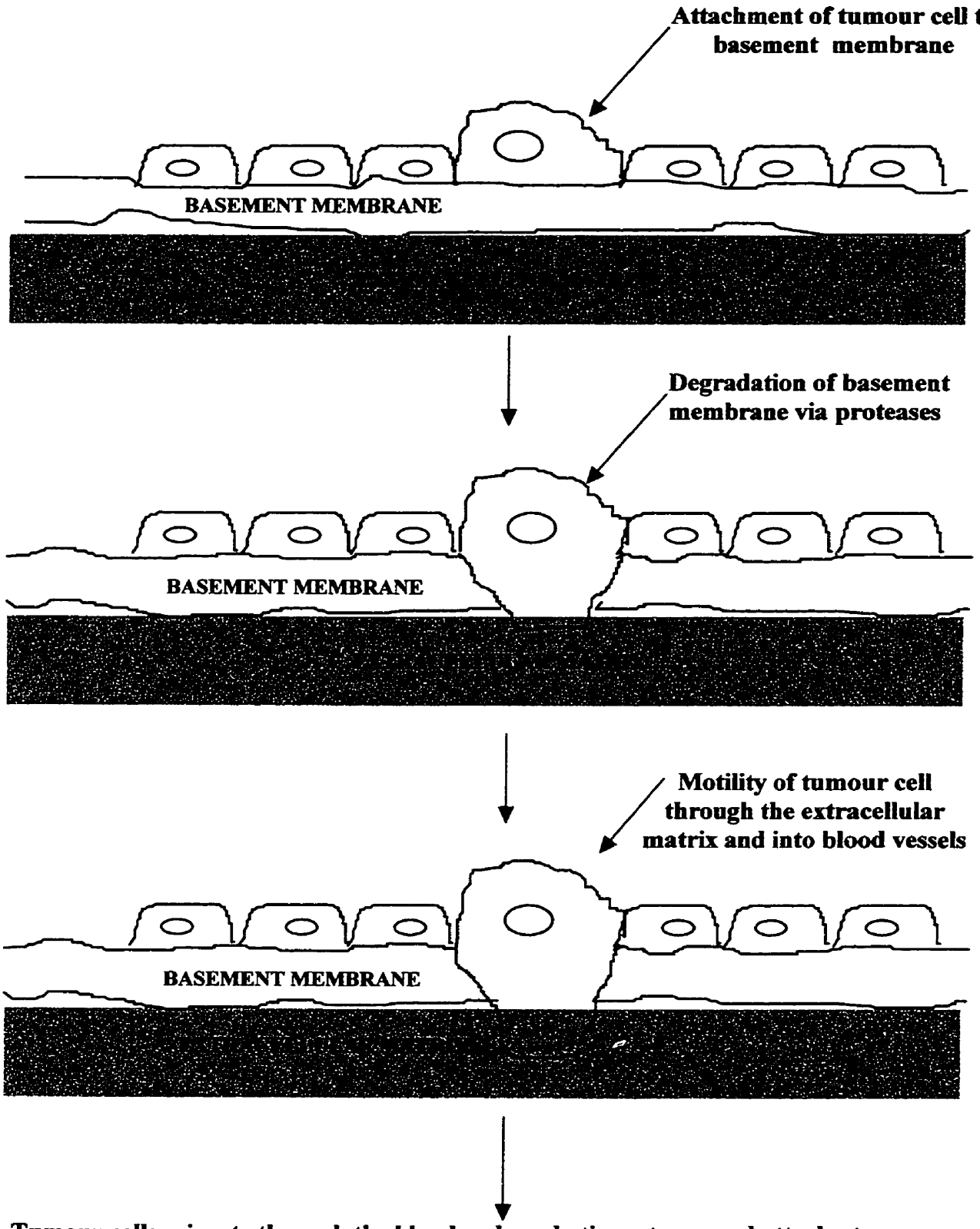


Fig. 1 The process of invasion and metastasis

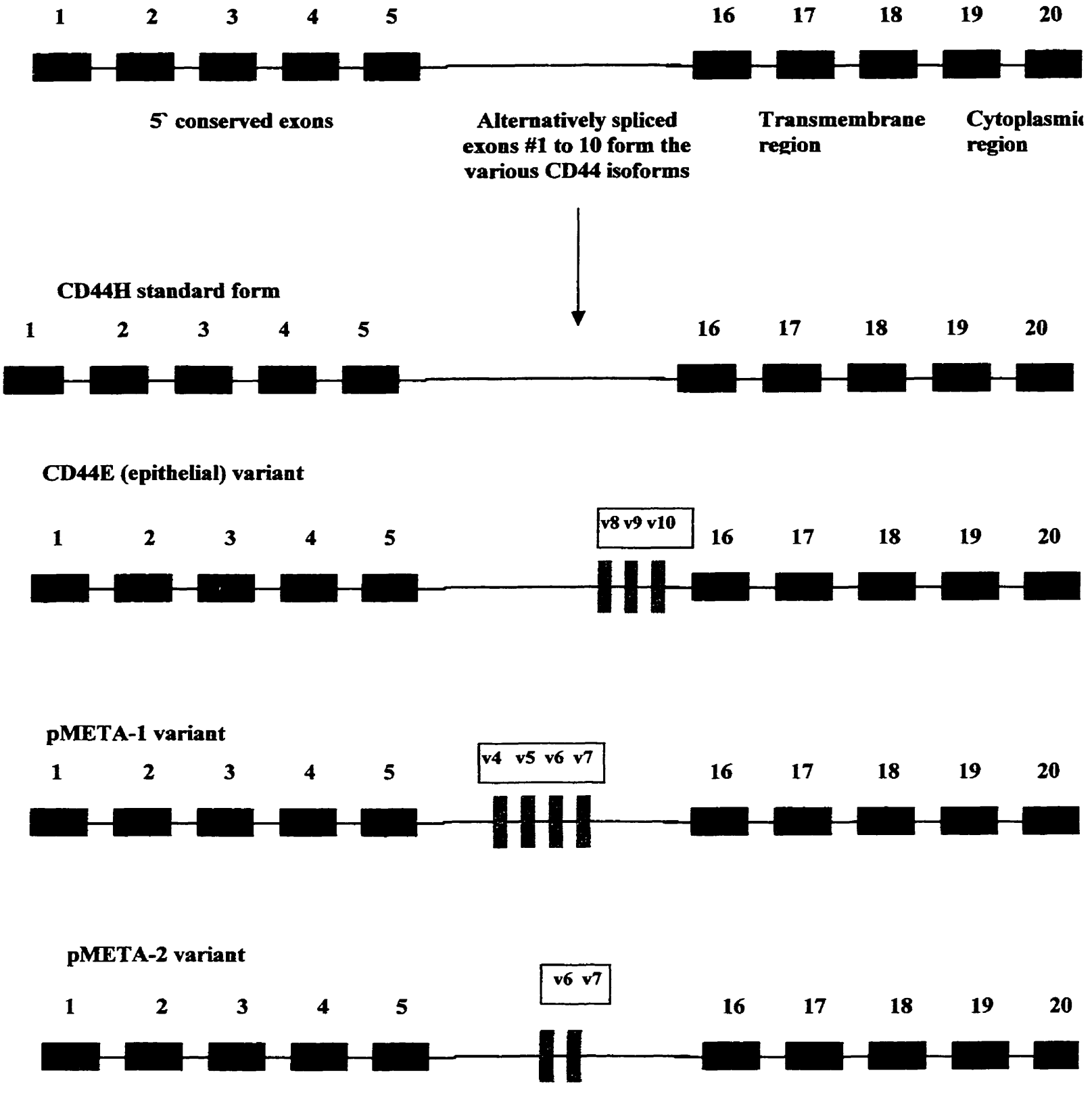


Fig. 2 Exon structure of CD44 and several variants

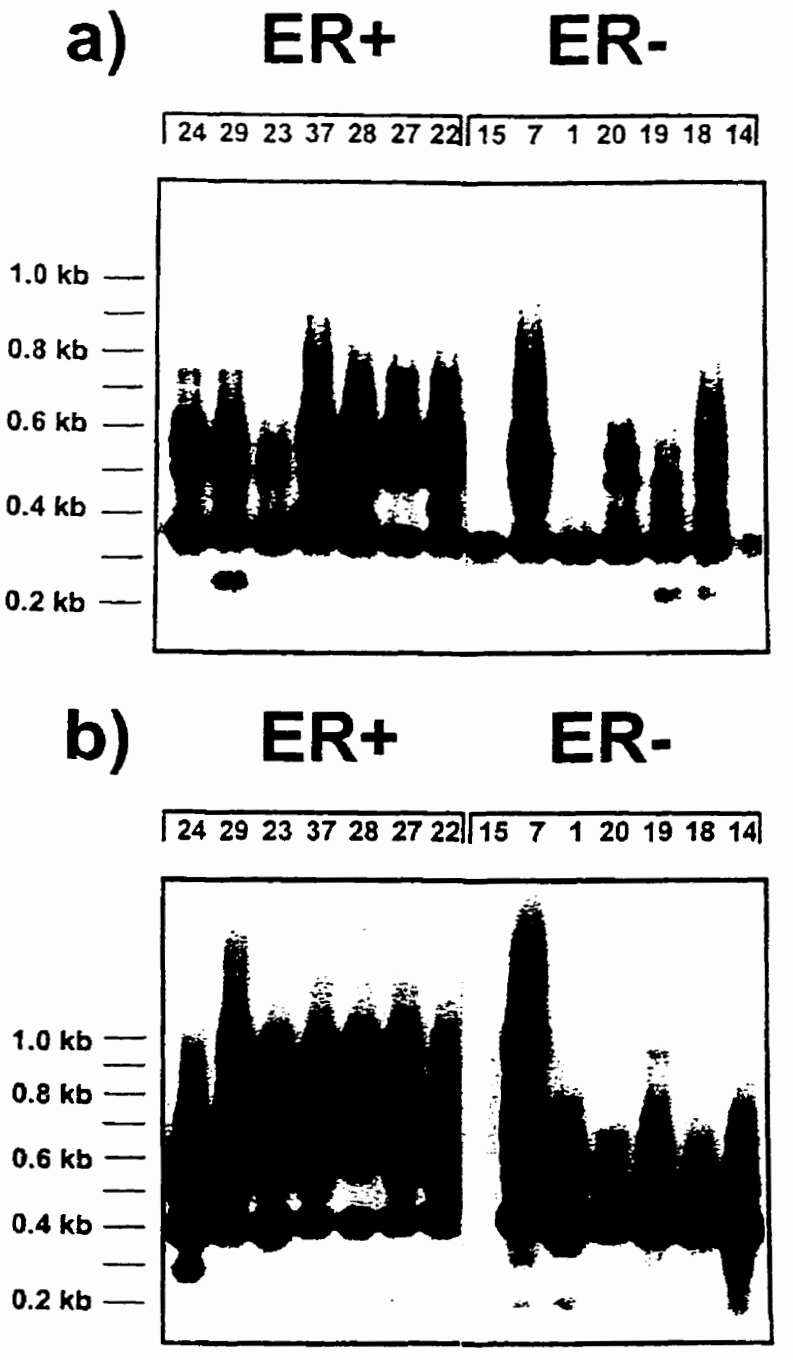


Figure 3. CD44 expression assessed by RT-PCR as described in Figure 2 showing the pattern of expression of CD44 variants incorporating exon v7 (a) or v10 (b) in ER positive and ER negative breast tumors. The lanes correspond to the case numbers in Table 1.

Fig. 3 CD44v7 and CD44v10 pattern of expression in ER+ and ER- tumours. (From Hole et al., 1997)

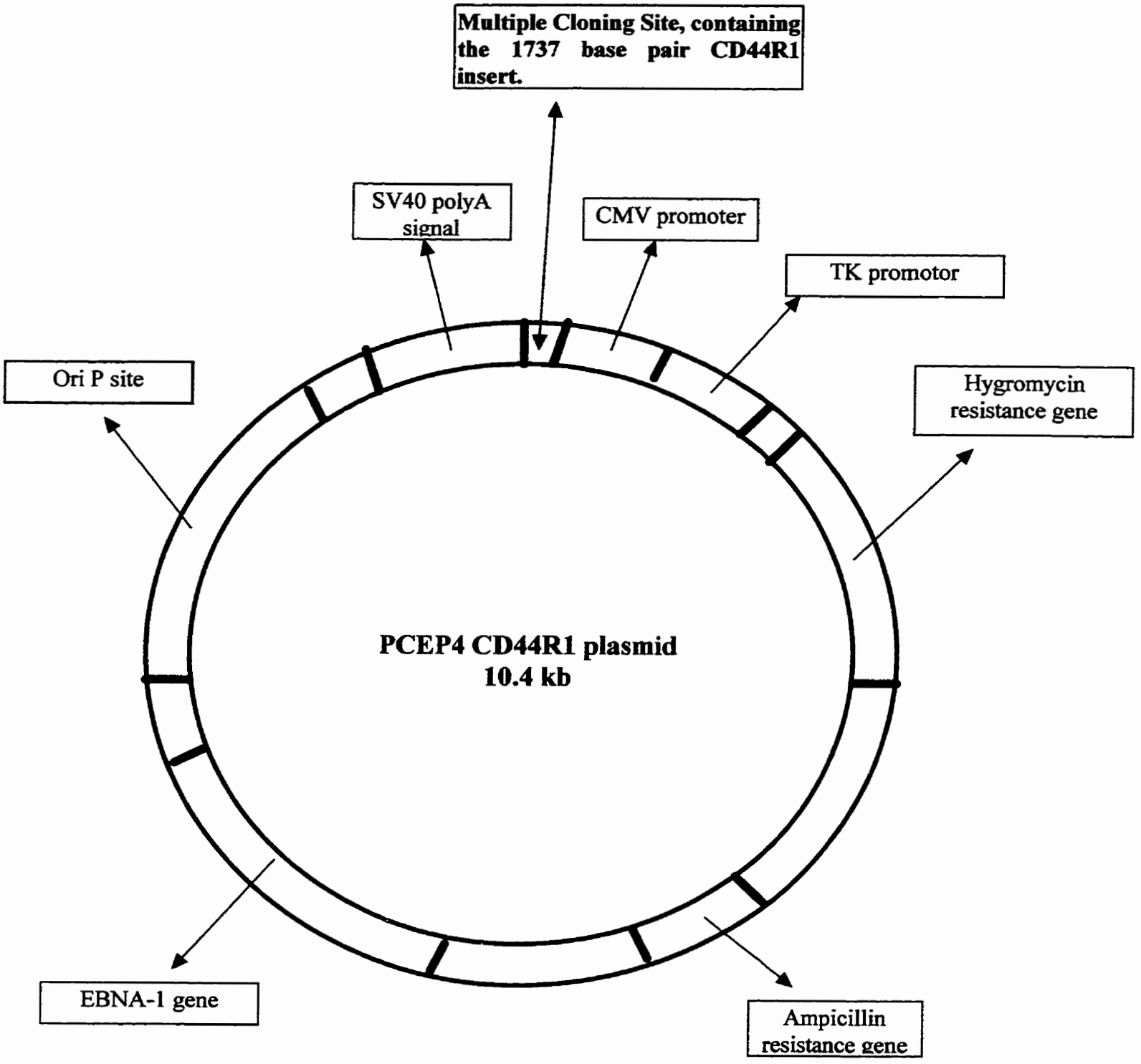


Fig. 4 CD44R1 plasmid map

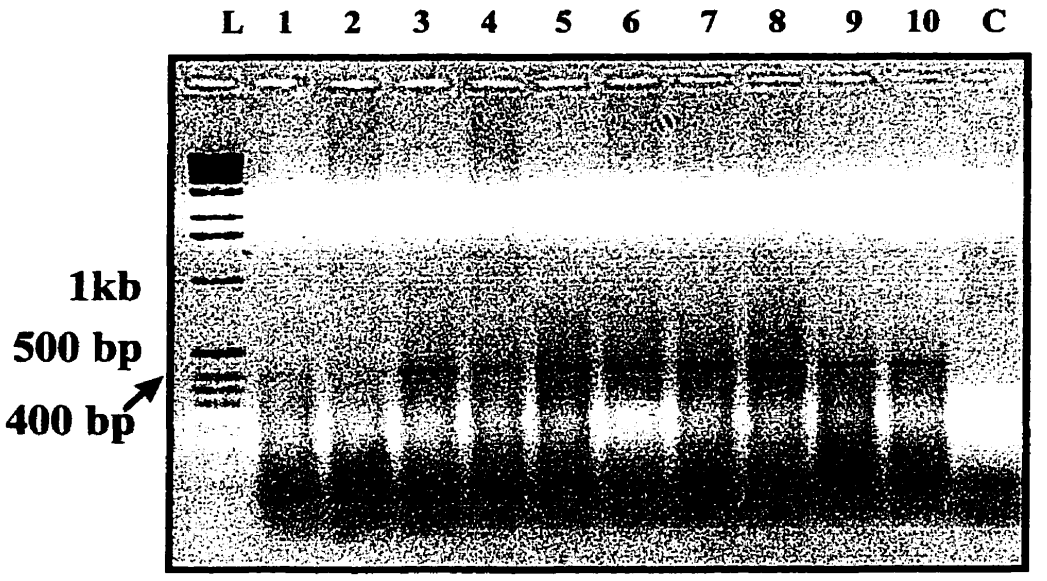


Fig. 5 PCR assay of MDA-MB-231 transfected clones to identify clones containing the CD44R1(E) insert. 1ug of genomic DNA was amplified for 45 cycles at 45 seconds at 93°C, 45 seconds at 56°C , then 1 minute and 30 seconds at 75 °C, for 45 cycles. The primers used anneal to the v10 exon and a commonly expressed exon for all CD44 molecules, generating a 400 base pair signal in the transfected MDA-MB-231 cells (**Lanes 1 to10**) and no signal in the untransfected MDA-MB-231 parent cell line (**Lane C**). **Lane L** is the DNA ladder.

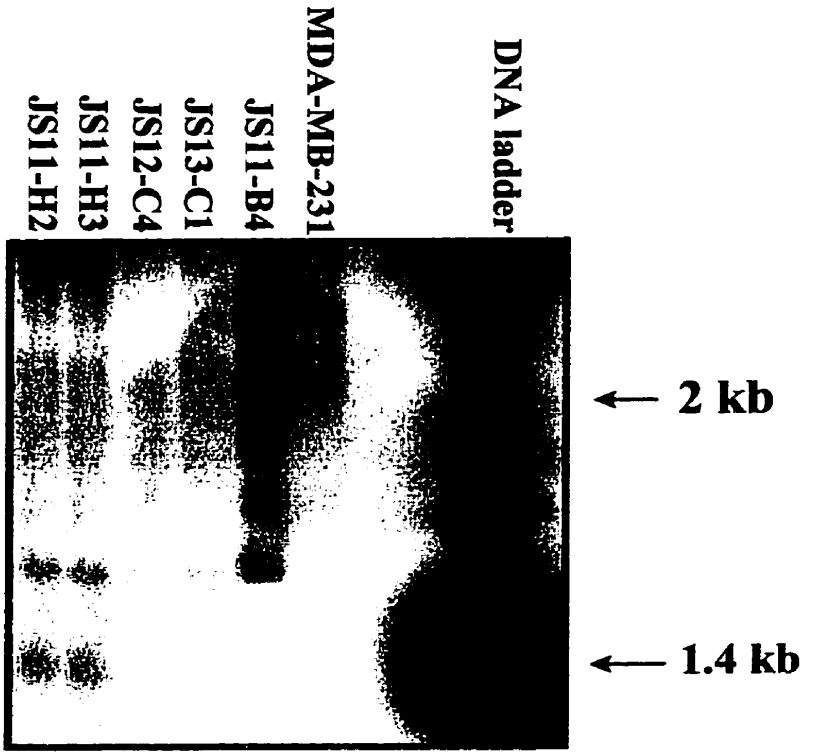


Fig. 6 Genomic Southern blot of MDA-MB-231 transfected clones to assess for CD44E plasmid integration. 10 ug of genomic DNA digested with BamH1enzyme were loaded in each lane, except in lane 7 where 100ng of CD44R1 plasmid was loaded. The radiolabelled probe was specific for the v8-v10 alternately spliced exons of CD44E.**Lanes 1 to 5:**CD44R1 transfected, PCR positive MDA-MB-231 clones.**Lane 6:** MDA-MB-231 wild type cell line. **Lane 7:** CD44R1(E) plasmid. The digested CD44R1 cDNA generates a 1.4kb band which is not present in the control cell line (Lane 6) or in several tested clones. The approximately 1.6kb band corresponds to a digested genomic CD44 fragment.

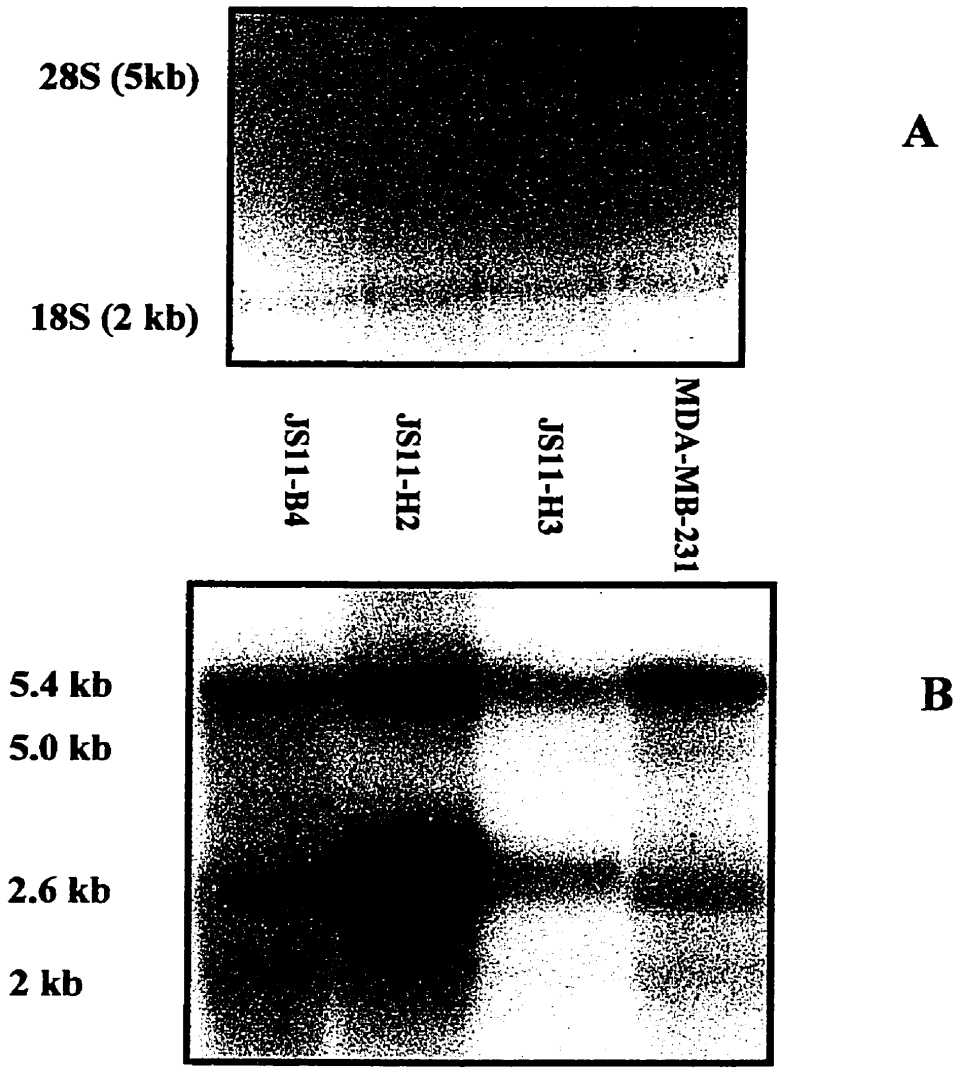


Fig. 7 Northern blot of CD44R1(E) transfectants. 20ug of RNA were loaded into each lane and probed with a radiolabelled probe incorporating the v8, v9 and v10 exons of CD44E. **Lanes 1 to 3:** are CD44R1(E) transfected MDA-MB-231 cells. **Lane 4:** untransfected MDA-MB-231 cell line. **Lane 1:** JS11-B4, a PCR positive, Southern blot negative transfectant, **Lane 2,3:** PCR, Southern blot positive transfectants JS11-H2 and H3. **7A** shows the Northern gel stained with ethidium bromide as the loading control, **7B** depicts the probed Northern blot, with the 3 mRNA signals for CD44E, (5.4, 2.6, and 2.0kb) generated by multiple polyadenylation signals during CD44 splicing. The 5 kb and 2 kb bands correspond to the 28 and 18S band sizes respectively.

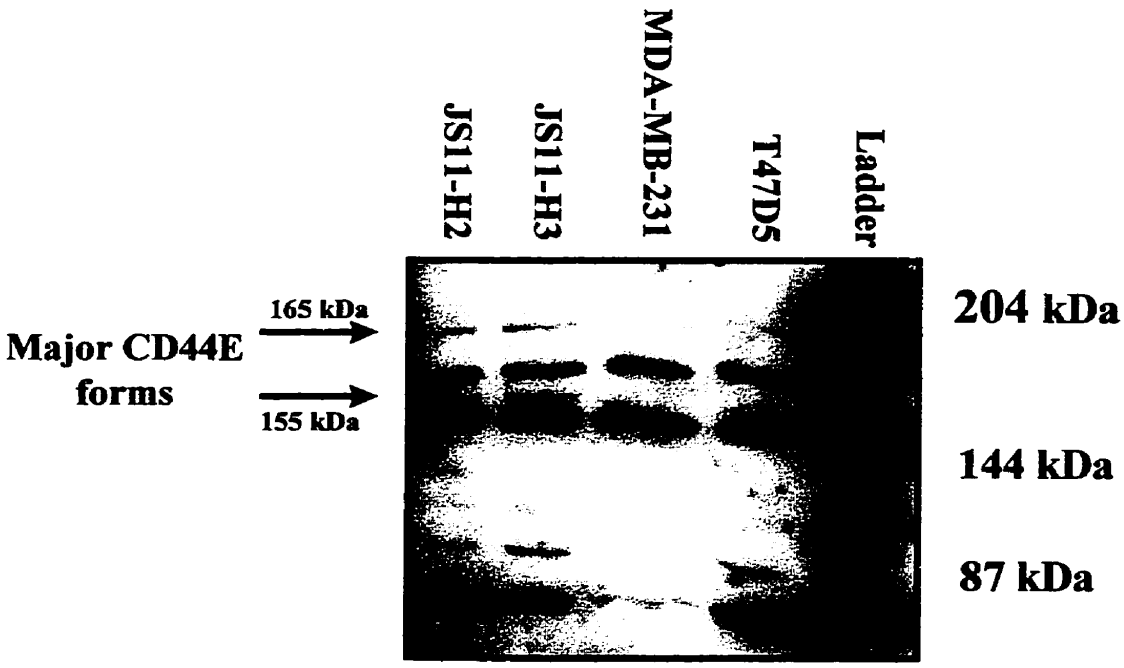


Fig. 8 Western blot of CD44R1(E) transfectants. 50 ug of extracted protein was run on a 6% SDS PAGE gel, and incubated with the VFF-14 antibody specific for an epitope on the v10 exon of CD44E. **Lanes 1 and 2:** are transfected MDA-MB-231 clones JS11-H2 and JS11-H3. These clones arePCR, Southern and Northern blot positive. **Lane 3:** MDA-MB-231 untransfected ER- human breast cancer cell line. **Lane 4:** T47D5 ER+ human breast cancer cell line, untransfected. **Lane L:** protein standard marker. Lanes 1 and 2 show an overexpression of two bands, at approximately 165 and 155kDa that are not detectable in the ER- cell line, and which is expressed in the ER+ T47D5 cell line. These bands correspond to the major forms of the transfected CD44R1 cDNA protein.

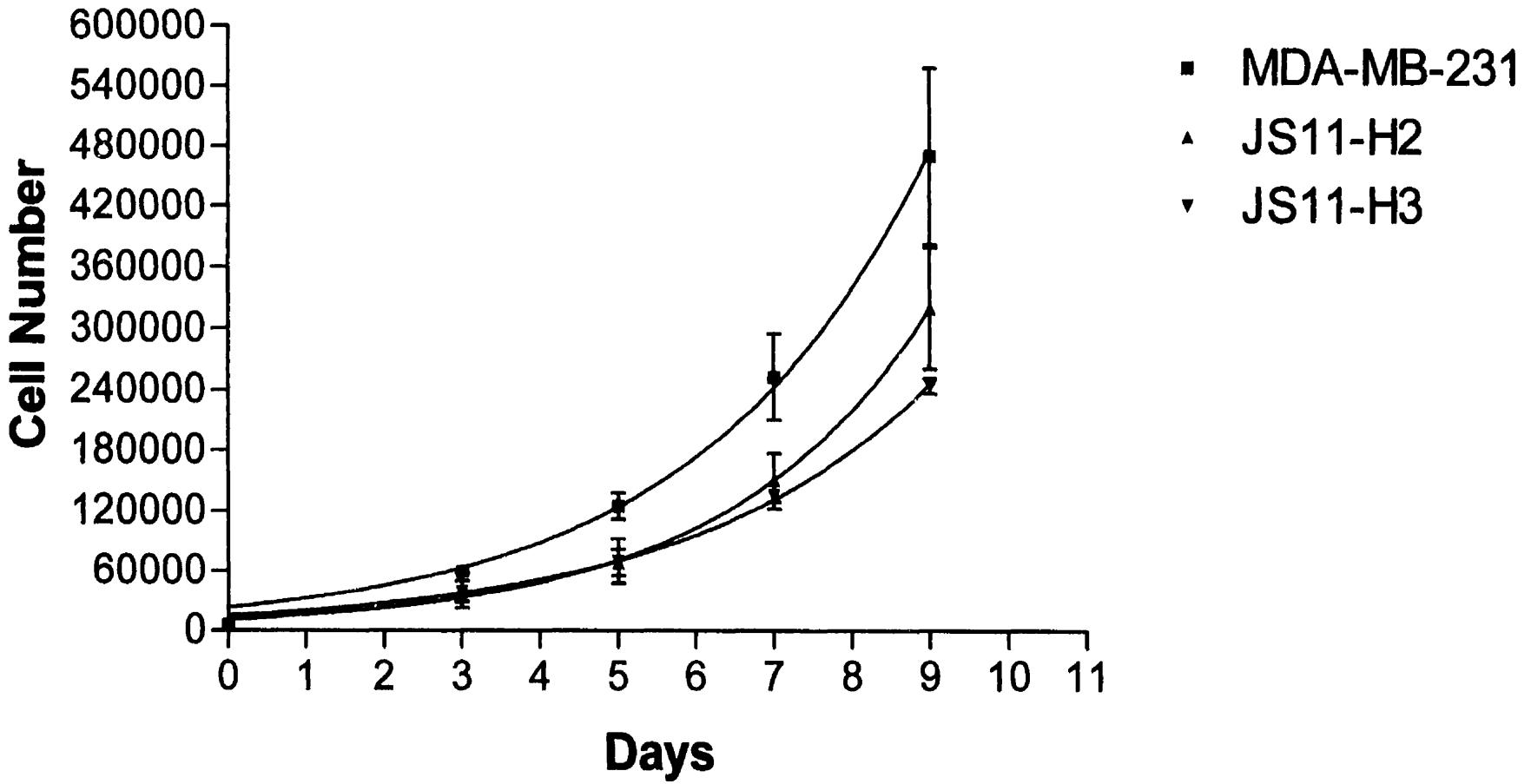
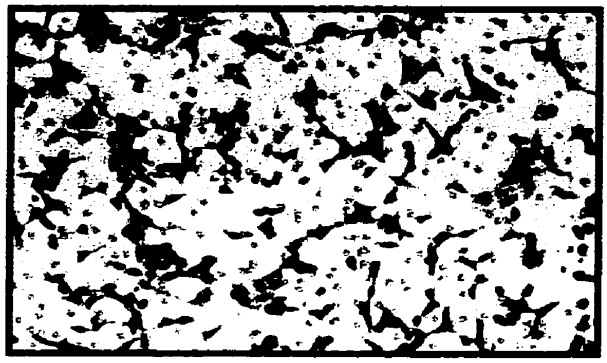
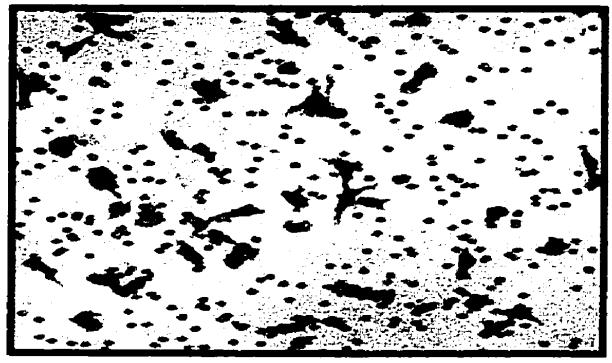


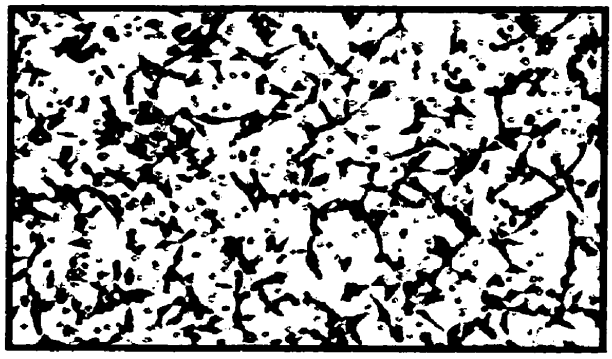
Fig.9 Growth of the MDA-MB-231 parental cell line and the JS11-H2 and JS11-H3 CD44E transfected clones.



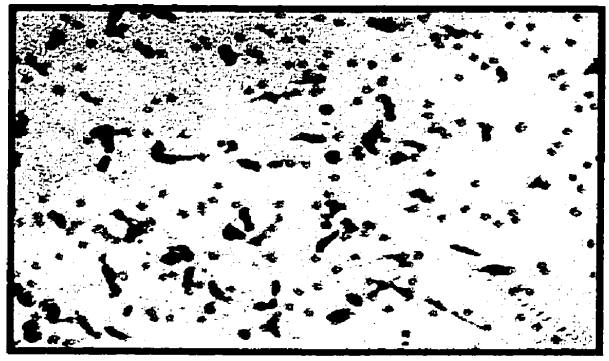
MDA-MB-231 Control Membrane



MDA-MB-231 Matrigel-coated Membrane



JS11-H2 Control Membrane



JS11-H2 Matrigel-coated Membrane

Fig. 10 Invasion assays for MDA-MB-231 parental cell line, and for JS11-H2 transfected cell line. $5x10^3$ cells were plated on either the uncoated or Matrigel coated inserts of 8um pore size, and were grown in 0.1% BSA plus DMEM for 24 hours, using fibroblast conditioned media as the chemoattractant.

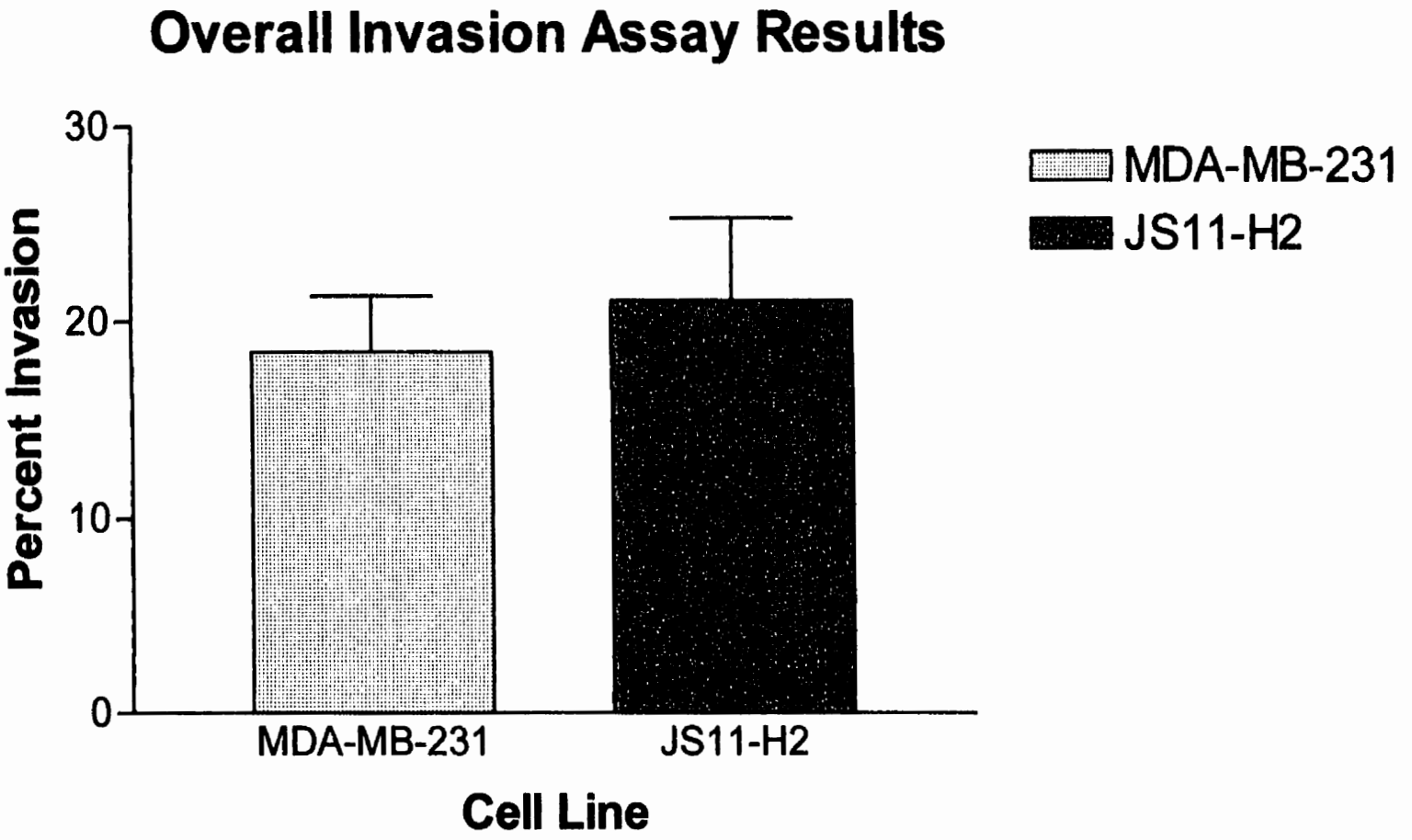


Fig. 11 Invasion assay results for the untransfected MDA-MB-231 and CD44R1 transfected JS11-H2 clone.

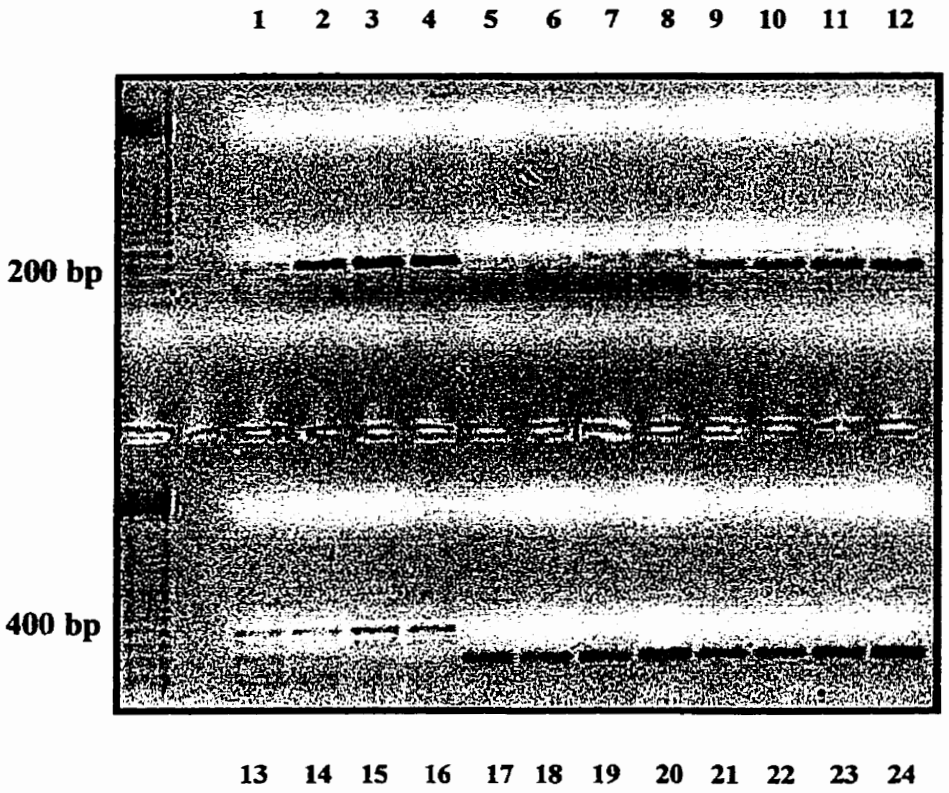
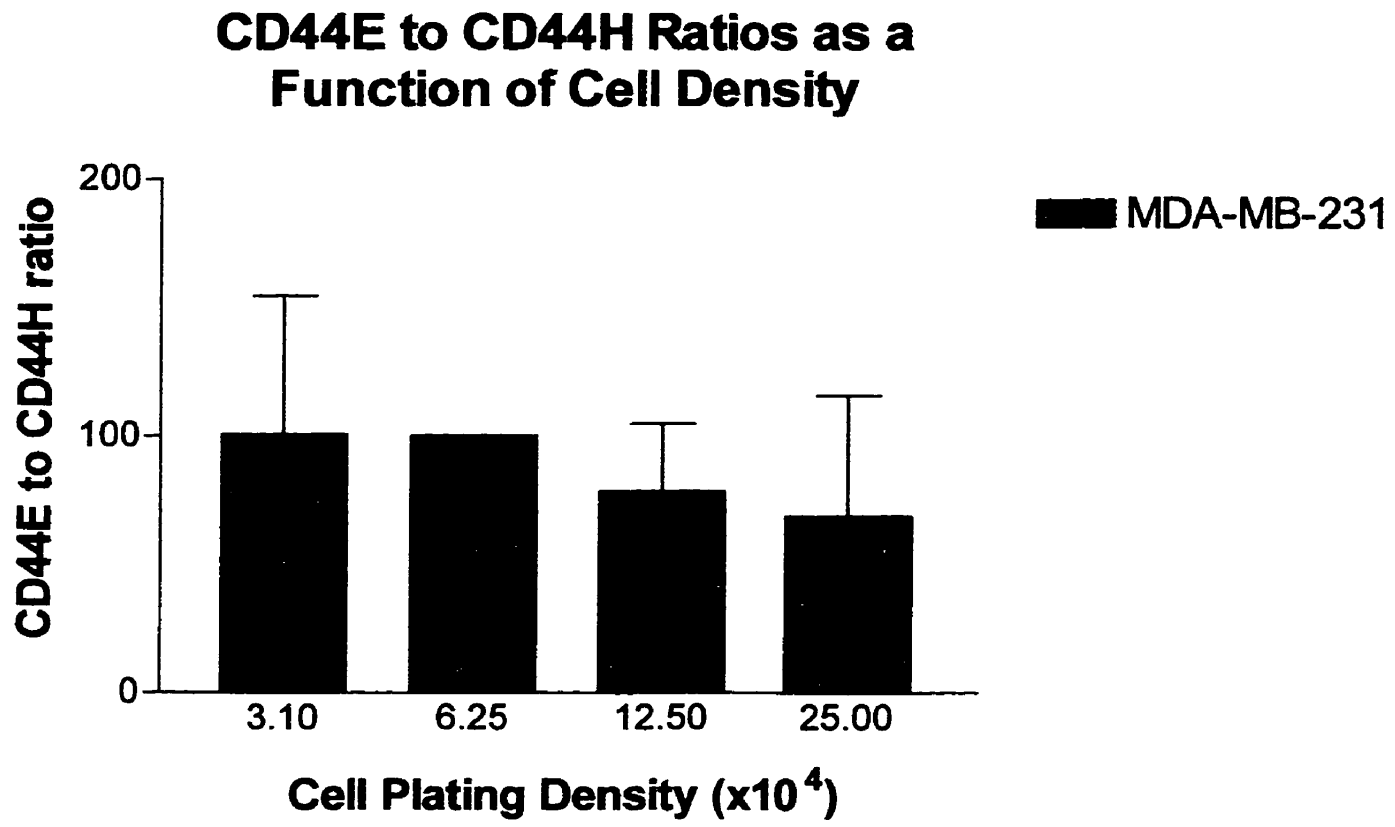


Fig. 12 RT PCR of ER- and ER+ human breast cancer cell lines assayed at cell densities of $3.1x10^3$, $6.25x10^3$, $1.25x10^4$, and $2.5x10^4$ cells per $2cm^2$ dish. Lanes 1-4: MDA-MB-231, ER- cell line, using CD44H (standard form) primers. **Lanes 5-8:** T47D5, ER+ cell line, using CD44H primers. **Lanes 9-12:** MDA-MB-231 cell line, using CD44E (epithelial variant) primers. **Lanes 13-16:** T47D5 cell line, using CD44E primers. **Lanes 17-20:** MDA-MB-231 using GAPDH primers. **Lanes 21-24:** T47D5 cells using GAPDH primers. For each group of 4 lanes, the cells were plated at increasing densities of 50%, 100%, 200%, and 400%. The CD44H band corresponds to approximately 200 bp, while the CD44E band is at 400bp. The GAPDH gene was amplified as a loading control for the CD44H and E PCR reactions. Both CD44 reactions were amplified for 40 cycles at 45 seconds at 93°C, 45 seconds at 56°C , then 1 minute and 30 seconds at 75 °C, for 45 cycles, while the GAPDH was amplified at the same temperatures for 35 cycles.



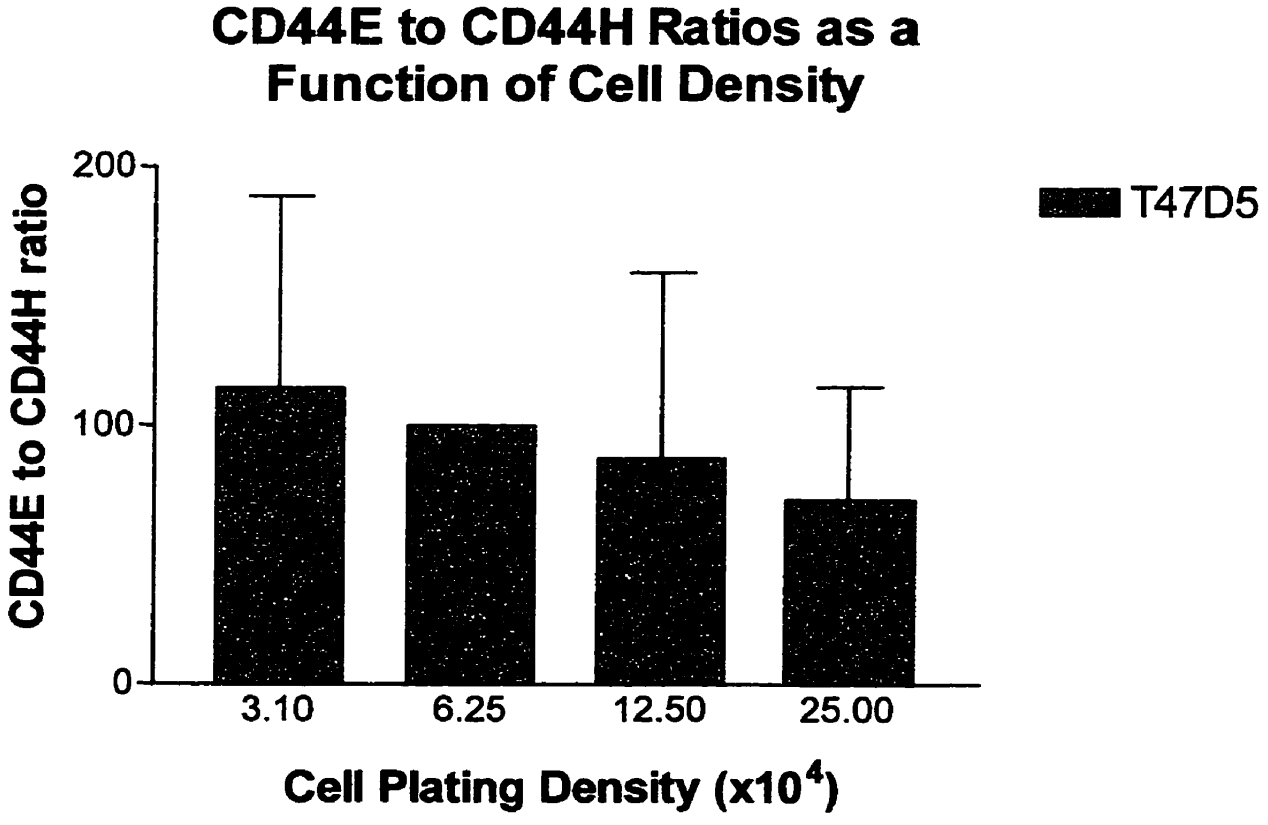


Fig.13 Cell density experimental data

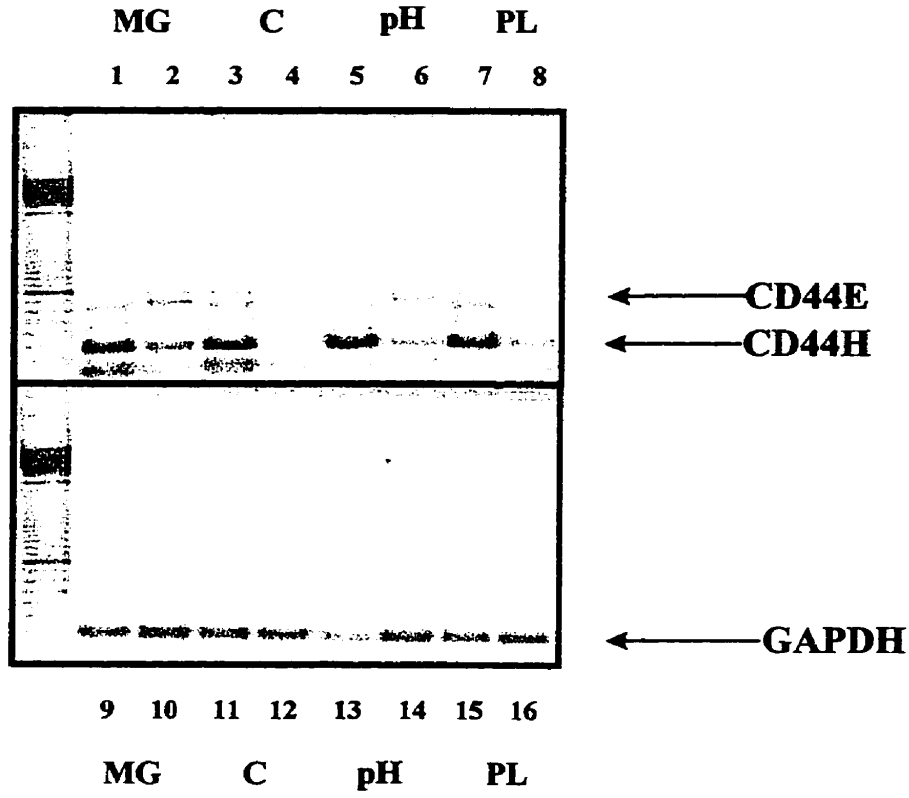
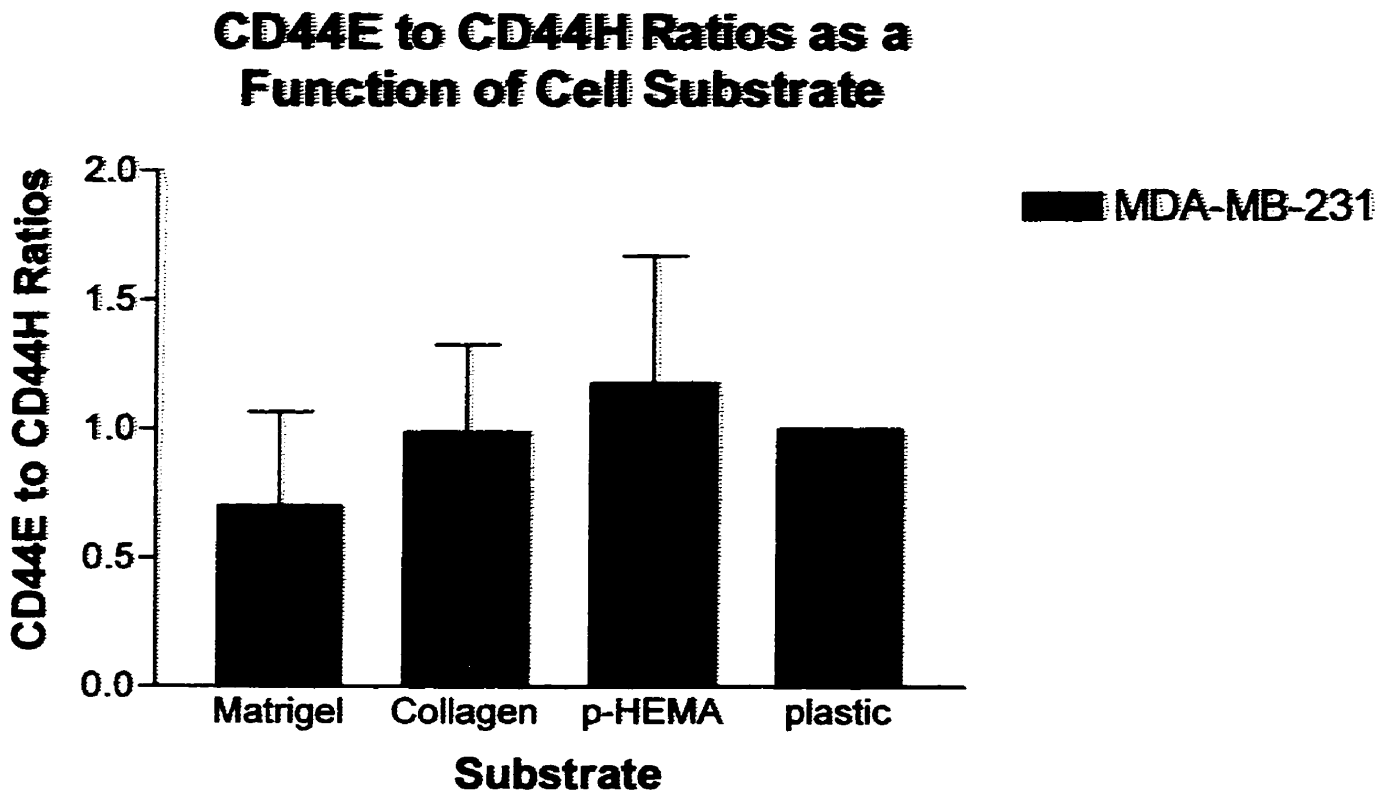


Fig. 14 RT PCR of ER- and ER+ human breast cancer cell lines plated on various substrates. Both the MDA-MB-231 (ER-) and the T47D5 (ER+) plated on four substrates: Matrigel (**MG**), collagen type IV(**C**), p-HEMA (**pH**), and on plastic wells (**PL**). **Even numbered lanes** represent MDA-MB-231 ER- breast cancer cell line and **odd numbered lanes** represent T47D5 ER+ breast cancer cell lines. **Lanes 1 to 8** represent RT PCR using CD44H primers, and CD44E primers. **Lanes 9 to 16** are the same samples using GAPDH primers. Both CD44 reactions were amplified for 40 cycles at 45 seconds at 93°C, 45 seconds at 56°C , then 1 minute and 30 seconds at 75 °C, for 45 cycles, while the GAPDH was amplified at the same temperatures for 35 cycles.



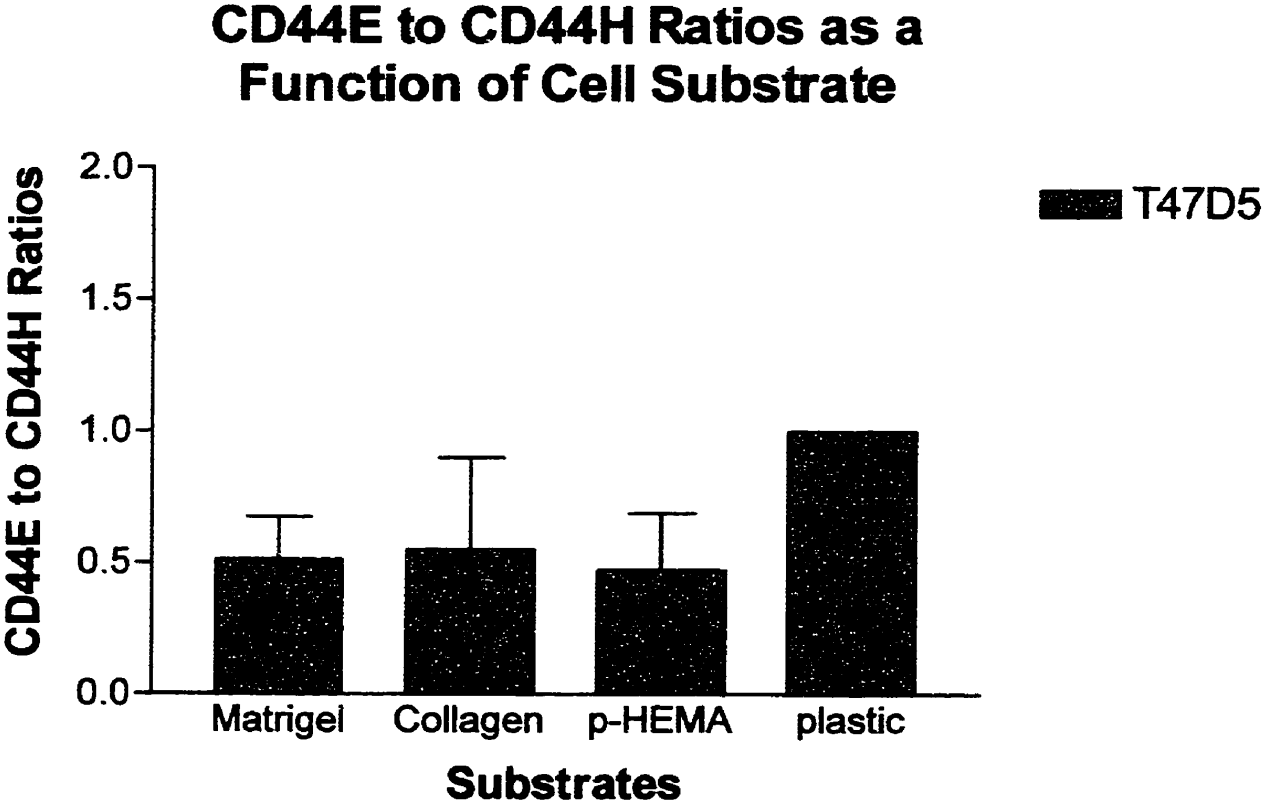


Fig. 15 Cell Substrate experimental data

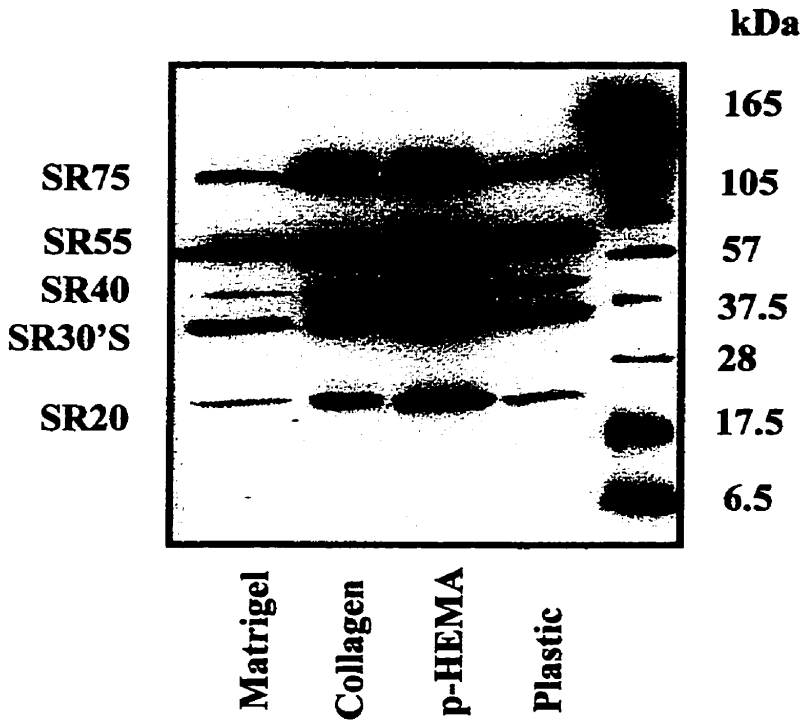
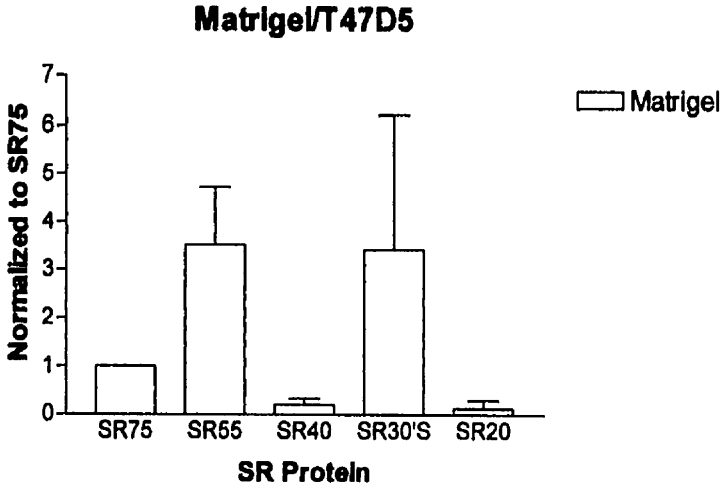
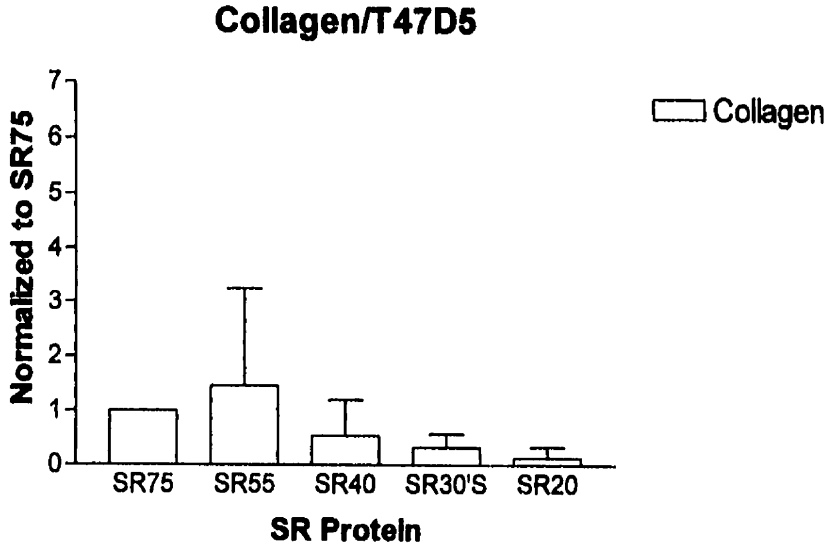
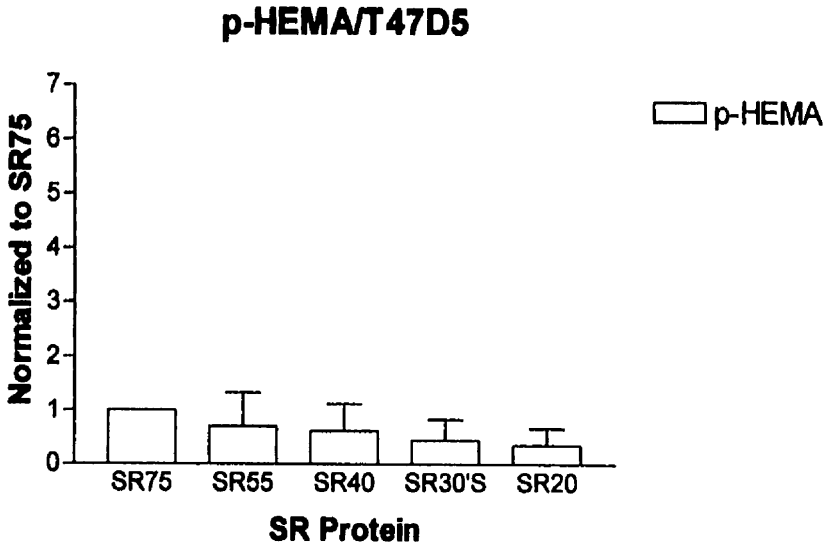


Fig. 16 Western blot of SR protein levels in T47D5 ER+ breast cancer cell line grown on various substrates. 50 ug of the extracted protein samples were run out on a 12% SDS-PAGE separating gel, and incubated with mAb104 which binds to a common epitope on the six SR proteins. **Lane 1:** cells grown on Matrigel, **lane 2:** cell grown on collagen type IV, **lane 3:** cells grown on pHEMA and **lane 4:** cells grown on plastic. **Lane 5:** protein ladder.

Relative SR protein levels in T47D5 cells grown on various substrates.







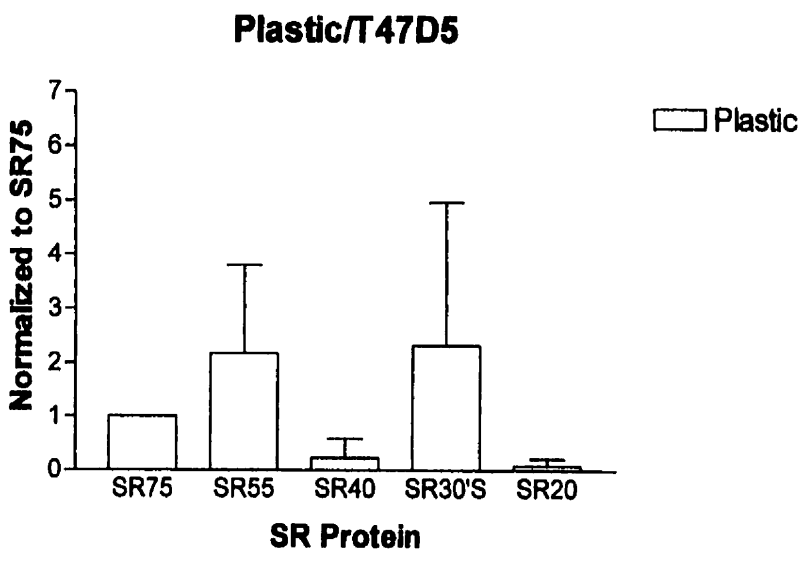


Fig. 17 SR protein Western blot data

Date of transfection	Transfection title	Number of clones	Clones analyzed	Positive clones by PCR (%)	Clones tested by Southern/ Northern blot (% postive)
November 95	JS1-M-#	0[1]	N/A	N/A	(0) (0)
November 95	JS2-M-#	0[1]	N/A	N/A	(0) (0)
December 95	JS3-M-#	0[1]	N/A	N/A	(0) (0)
January 96	JS4-M-#	20	by PCR=20	6 (30)	6 (0) (0)
May 96	JS5-M-#	12	by PCR=12	6 (50)	6 (0) (0)
August 96	JS6-M-#	0[2]	N/A	N/A	(0) (0)
November 96	JS7-M-#	8	by PCR=8	0 (0)	(0) (0)
January 97	JS8-M-#	40	by PCR=5	4 (80)	4 (0) (0)
January 97	JS9-M-#	50	by PCR=23	16 (69.5)	7 (0) (0)
January 97	JS10-M-#	50	by PCR=1	1 (100)	1 (0)
February 97	JS11-M-#	50[3]	by PCR=6	5 (83)	2 (33) (33)

[1] hygromycin selection killed cells
[2] cells died due to infection
[3] denotes that three clones are from a simultaneous transfection using hygromycin as the selection agent.

Table 1: Transfection record of MDA-MB-231 cells transfected with CD44R1(E) plasmid.