{Frontiers in Bioscience, 3, d637-649, July 1, 1998]

# CD44 isoform-cytoskeleton interaction in oncogenic signaling and tumor progression

**Lilly Y.W. Bourguignon, Dan Zhu and Hongbo Zhu**

*Department of Cell Biology and Anatomy, University of Miami Medical School, Miami, Fl. 33101*

Received 4/27/98 Accepted 5/15/98

**TABLE OF CONTENTS**



## 1. ABSTRACT

CD44, a major hyaluronan receptor, exists as several isoforms and is widely distributed in different cells and tissues. The isoforms of CD44, such as CD44s (the standard form), CD44E (the epithelial form) and CD44v (variant isoforms) (arise from differential splicing of one to ten (or eleven) variable exons that encode portions of the membrane proximal extracellular domain. The molecular diversity of CD44 isoforms is further compounded by differential biosynthetic processes and post-translational modifications [e.g. N–/O-glycosylation or glycosaminoglycan (GAG) addition]. This structural arrangement, which occurs within either the invariant region or the extracellular domain of the variant region, is important for CD44-mediated communication between extracellular matrix materials [ECM-hyaluronic acid (HA), collagen and fibronectin] and intracellular protein components (e.g cytoskeletal proteins and various regulatory enzymes). The 15 amino acid sequence [e.g. NSGNGAVEDRKPSGL (in human) or NGGNGTVEDRKPSEL (in mouse)] residing in the cytoplasmic domain of CD44 isoforms is the ankyrin-binding domain of this family of transmembrane glycoproteins. Biochemical analyses plus in vitro mutagenesis indicate that the ankyrin-binding domain is required for CD44-mediated "outside-in" and "inside-out" cell activation events. Furthermore, CD44s-cytoskeleton interaction is tightly coupled with signal transducing molecules (e.g. p185$^{HER2}$ or Src kinases) during oncogenic signaling. Moreover, the transmembrane linkage between CD44v isoforms (CD44v10 and CD44v3) and the cytoskeleton up-regulates invasive and metastatic-specific tumor phenotypes [e.g. matrix degradation (MMPs) activities, tumor cell invasion and migration]. These findings strongly suggest that the interaction between CD44 isoforms and the cytoskeleton plays a pivotal role in the onset of oncogenesis and tumor progression.

## 2. INTRODUCTION

CD44 denotes a family of glycoproteins that are expressed in a variety of cells and tissues derived from hemopoietic, epithelial, endothelial, and mesodermal origins (1-5). Specifically, CD44s (the standard form) is known to be important in T-cell signaling and a variety of immune cell functions (6-14). In addition, CD44s plays a role in T-and B-cell adhesion, cell aggregation, proliferation and cell migration (15,16). CD44s is also an important cytotoxic triggering molecule on cytotoxic T-lymphocytes (CTL) (7,8), polymorphonuclear cells (PMN) (9) and natural killer (NK) cells (10,11).

Furthermore, CD44s is responsible for inducing human monocytes to release tumor necrosis factor (TNF) and interleukin-1 (IL-1) (13). CD44s expression has also been linked to several autoimmune disorders. For example, overexpression of CD44s has been associated with the autoimmune disease, ulcerative colitis (17). In arthritis, CD44s has been suggested to play a key role in directing the migration of inflammatory leukocytes into the extravascular compartment of the synovium (18). In this regard, treatment with anti-CD44 antibodies has been used to decrease tissue swelling and leukocyte infiltration in an

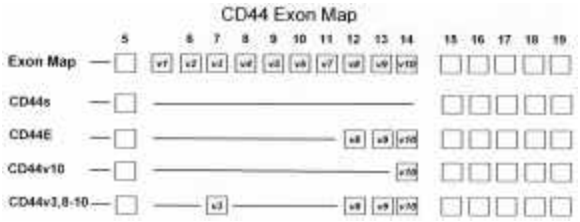


**Figure 1.** CD44 exon map showing all 19 exons (a); the standard form of CD44 (CD44s) (b); the epithelial form of CD44 (CD44E) (c); the v10 isoform of CD44 (CD44v10) (d); and the v3,8-10 isoform of CD44 ($CD44v_{3,8\text{-}10}$).

arthritis murine model system (18). These findings suggest that CD44s plays an essential role in regulating chronic inflammatory responses.

The expression of CD44s also changes profoundly during tumor metastasis, particularly during the progression of lymphoid carcinomas (19-21). In fact, CD44s expression has been used as an indicator of metastasis in non-Hodgkins's lymphoma (NHL). NHL with low level of CD44 expression is less malignant, disseminates less frequently and has a favorable prognosis. However, NHL with high level of CD44s expression is more malignant and metastatic (19,21). Furthermore, cells with a high level of CD44s expression show enhanced hyaluronic acid (HA) binding which increases their migration to various lymphoid tissues (19,21). Therefore, it has been suggested that cells with a high level of CD44s expression play an important role in the dissemination of NHL and, via this mechanism, exert an unfavorable prognostic influence (19). In addition to NHL, many other malignant leukemias [e.g. null acute lymphocytic leukemia (ALL), common ALL, chronic lymphocyte leukemia as well as other types of carcinomas of epithelial and glial origins] also show a high level of CD44s expression phenotype (20,21). Therefore, the presence of a high level of CD44s expression is emerging as an important metastatic tumor marker in a number of carcinomas, and is also implicated in the unfavorable prognosis of these diseases.

A larger protein, CD44E (the epithelial form), results from the alternative splicing of three additional exons [exons 12-14 (v8-10)] into the membrane proximal region of the CD44 molecule and is preferentially expressed on epithelial cells (figure 1c) (22). Other higher molecular weight "variant" isoforms of CD44 (CD44v) are derived from the alternative splicing of up to ten additional exons in various combinations of the extracellular domain of the molecule (figure 1a) (22). Cell surface expression of CD44v isoforms (e.g.$CD44v_{10}$ and/or $CD44v_3$-containing isoforms) appears to change profoundly during tumor metastasis, particularly during the progression of various carcinomas (23-26). Furthermore, CD44v isoforms have been detected on highly metastatic cell lines and transfection of these molecules confers metastatic properties to otherwise non-metastatic cells (27).

HA, the major glycosaminoglycan found in the extracellular matrix of mammalian tissues, is now considered to be both a physiologically-relevant ligand and an adhesion molecule for CD44 isoforms (also known as the hyaluronan receptor) in many cell types (1,5,6,14,28). HA plays an important role in several important physiological functions such as maintaining cartilage integrity, maintaining homeostasis of water and plasma proteins in the intercellular matrix and promoting/inhibiting mitosis (28). The interaction between CD44 isoforms and their physiological ligands (e.g. HA and small HA fragments) activates several chemokine gene family such as interleukine 8 (IL-8), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1ß (MIP-1ß), cytokine responsive gene-2 [crg-2 (interferon-inducible protein-10)], and RANTES (regulated on activation, normal T cell expressed and secreted) (14,29). The binding of CD44 isoforms to HA is known to cause cell adhesion to extracellular matrix (ECM) components (16,30) and is also implicated in the stimulation of cell aggregation (31), cell proliferation (32), cell migration (33) and angiogenesis (34).

Several mechanisms for the regulation of CD44 isoform-mediated function have been suggested. These include modifications by an additional exon-coded structures (via an alternative splicing process) (35), variable N-/O-linked glycosylation in the CD44's extracellular domain (36); and modulations of the CD44's cytoplasmic domain by cytoskeletal proteins such as ankyrin (6). Among the important questions related to the regulation of CD44 isoforms are: (i) what are the similarities and/or differences in post-translational modifications among various CD44 isoforms; (ii) how are CD44 isoforms linked to various cellular components such as cytoskeletal proteins and regulatory enzymes; and (iii) which oncogenic signaling pathways are associated with CD44 isoform-cytoskeleton mediated metastatic tumor behaviors. In this review, we will discuss some recent advances made toward answering these questions.

## 3. BIOSYNTHETIC PATHWAYS AND POSTTRANSLATIONAL MODIFICATIONS OF CD44 ISOFORMS

### 3. 1. CD44s (the standard form)

CD44s [known as $GP90^{Hermes}$ antigen, ECMR III, and homing cellular adhesion molecule (H-CAM)] was first detected during lymphocyte homing to high endothelial venules (37-39). It is comprised of a variable extracellular domain, a single spanning 23 amino acid transmembrane domain and a 70 amino acid cytoplasmic domain (22). The most common CD44s (also called hemopoietic form) contains exons 1-4 (N-terminal 150 a.a.-extracellular domain); exons 5, 15 and 16 (membrane proximal 85 a.a.), exon 17 (transmembrane domain); and a portion of exons 17 and 19 (C-terminal 70 a.a.-cytoplasmic domain) (figure 1) (22). CD44s (M.W. ≈85kDa, so-called GP85) is synthesized as a 42 kDa (p42) unglycosylated polypeptide which is converted to CD44s upon sequential post-translational modifications. Using a standard pulse-chase approach, a 52 kDa polypeptide precursor (p52) containing a high mannose-type N-linked oligosaccharide chains is detected within the first 5min of pusle labeling. The

**Table 1.** Chemical composition analysis of CD44s and CD44v10

| Amino Acid | CD44s (mol/mol Protein) | CD44v10 (mol/mol Protein) |
|---|---|---|
| Asx (AsP + Asn) | 41 | 47 |
| Ala | 23 | 28 |
| Glx (Glu + Gln) | 32 | 38 |
| Thr | 29 | 40 |
| Ser | 39 | 45 |
| Pro | 23 | 27 |
| Met | 8 | 9 |
| His | 11 | 10 |
| Try | 2 | 3 |
| Tyr | 11 | 14 |
| Ile | 22 | 21 |
| Arg | 21 | 24 |
| D-glucosamine | 12 (≈3 N-linked) | 45 (≈8 N-linked) |
| D-Galactosamine | 5 (≈4-5 O-linked) | 11 (≈11 O-linked) |

conversion of p52 to 85kDa CD44s requires futher glycosylation (both complex type N-linked and O-linked) which takes place in the Golgi complex within 10-20 min after p52 is synthesized. CD44s is then incorporated into the plasma membrane where its turnover rate is relatively slow, $t_{1/2}$ of approximately 8h. Following tunicamycin treatment, two other precursor proteins, p42 (an unglycosylated form) and p58 (an O-glycosylated form) were detected. The p42 appears to be an immediate precursor of p52 because p52 is converted to p42 upon deglycosylation. Therefore, the biosynthesis of CD44s appears to occur in the following sequence: p42 ->p52 -> CD44s (40). Further chemical composition analysis of CD44s indicates that this molecule contains approximately 3 N-linked and 4-5 O-linked oligosaccharide chains. O-glycosylation (and to a lesser extent N-glycosylation) of CD44s is involved in cell adhesion binding to ECM such as hyaluronic acid (HA) and collagen (table 1). These findings indicate that CD44s and its biosynthestic precursors play an important role in CD44s-ECM interaction.

### 3.2. CD44v (variant isoforms)

Most often, the alternative splicing of CD44 occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons within the membrane proximal region of the extracellular domain (figure 1). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N-and O-glycosylations (36,45) and glycosaminoglycan (GAG) additions (41,42). A number of studies indicate that certain CD44 variant (CD44v) isoforms are expressed on the surface of tumor cells during metastasis, particularly during the progression of various carcinomas (23,26).

The CD44v10 isoform (M.W, 116kDa-also called GP116 or CD44R2) (44) contains a single insertion of exon 14 (≈62 amino acids) within the CD44s form (figure 1d) and is also the major HA receptor present on a number of cell types including tumor cells (5,24,45). This receptor mediates high affinity HA binding ($K_d \approx 0.8$ nM) and cell adhesion to HA-coated dishes (45). In this regard, CD44v10 appears to be similar to the major HA receptor, CD44s (1,6,30,36). This molecule is the only CD44 variant (CD44v) isoform consistently detected in many human cancer tissues (5,24,25,46) and has been shown to be specifically associated with transformed hemopoietic cells (44), maliganant epithelial tumor cells (5,24,46) and endothelial cells (45).

Biosynthesis studies indicate that CD44v10 is encoded by a CD44 transcript containing exon v10 (ex14). Specifically, CD44v10 is synthesized from a 52 kDa precursor which is larger than p42, the polypeptide precursor of CD44s or GP85 (40). Since v10 encodes for 65 amino acids, a larger precursor for the CD44 molecule containing v10 is expected. Post-translational modification of CD44v10, results in the addition of 8 N-linked oligosaccharides in a tri- or tetra-antenary structure (3000 - 4000 Da per oligosaccharide). N-glycosylation adds ≈ 27-32 kDa of mass to the polypeptide precursor which agrees with the results of our tunicamycin treatment studies (45). O-glycosylation accounts for ≈ 28 kDa of mass for the CD44v10, suggesting this molecule is also extensively O-glycosylated (45). Amino acid composition analysis further confirms this observation since it indicates that CD44v10 contains 11 O-linked oligosaccharide chains (Table 1). It is worth noting that the structure of the O-linked oligosaccharides in CD44v10 appears to differ from those in CD44s (40). For example, CD44v10 (but not CD44s) is resistant to neuraminidase treatment indicating the lack of a terminal sialic acid residue on CD44v10 (unpublished results). However, CD44v10 appears to contain sulfated O-linked oligosaccharides similar to mucin-like molecules (45); but it does not contain any sulfated GAGs as described for the CD44v3 isoform (41,42). These selective post-translational modifications of CD44v10's structure may be required for the function of this molecule in cell adhesion, membrane-cytoskeleton interaction and/or cell proliferation.

HA has been shown to play an important role in several important physiological functions (28,32). In particular, degradation products of HA containing 3-25 disaccharide units have been found to promote gene expression, cell proliferation, migration and angiogenesis (14,29,32,45). Recent results show a correlation between high affinity binding of HA polymer or HA's F1 fragment (≈ 10-15 disaccharide units)[but not smaller HA fragments s such as F2 fragment (≈ 3-6 disaccharide units) or F3 fragment (≈ 2 disaccharide units)] to cells expressing CD44v10 and the occurrence of a mitogenic response (45). These findings suggest that a specific cell surface HA receptor may be responsible for the induction of ligand-induced mitogenic signals. Since anti-CD44 antibody inhibits both HA or the HA's F1 fragment-mediated cell adhesion and the mitogenic response, it has been suggested that CD44v10 is the CD44-related HA receptor involved in HA-mediated functions.

## 4. TRANSMEMBRANE INTERACTION BETWEEN CD44 ISOFORMS AND THE CYTOSKELETON

The intracellular domain of CD44 binds to certain cytoskeletal proteins such as ankyrin (6) and ERM (ezrin, radixin, moesin and merlin) proteins (47). The transmembrane interaction between CD44 isoforms and ankyrin/ERM provides a direct link between the ECM and the cytoskeleton. CD44 isoform-cytoskeleton interaction


**Figure 2.** The ankyrin-binding sequence in the cytoplasmic domain of CD44 from various species (e.g. mouse vs human). The region I of CD44 contains the high affinity binding site (as indicated by the shaded letters with a great deal of sequence homology) for ankyrin. The region II acts as a regulatory domain for CD44-ankyrin interaction. [TM: transmembrane domain].

also influences CD44 function and recent progress in this area will be discussed below.

### 4.1. CD44 isoform and ankyrin

The cytoskeletal protein, ankyrin, originally was determined to cross-link the band 3 membrane protein to the cytoskeleton in erythrocytes (48). Now, ankyrin is known to bind to a number of plasma membrane-associated proteins including two other members of the anion exchange gene family (48,49), $Na^+/K^+$-ATPase (50-52), the amiloride-sensitive $Na^+$ channel (53), the voltage-dependent $Na^+$ channel (54,55), $Ca^{2+}$ channels (56-58) and the adhesion molecule, CD44 (1,6,30,45,59-64). It has been suggested that the binding of ankyrin to certain membrane-associated molecules may be needed for signal transduction leading to a variety of cellular functions.

The cytoplasmic domain of CD44 (approximately 70 a. a. long) is highly conserved (≥ 90%) in most of the CD44 isoforms, and is clearly involved in specific ankyrin binding (1,6,30,45,59-64). The evidence that post-translational modification of CD44 cytoplasmic domain by protein kinase C (61), acylation (62) or GTP binding (63) promotes the binding between CD44 and ankyrin suggests that the interaction between these two molecules is tightly regulated. Deletion mutation analysis indicates that at least two subregions within CD44 cytoplasmic domain contribute to ankyrin binding-region I (i.e. the high affinity ankyrin-binding region) and region II (i.e. the regulatory region) (64,65). In particular, the region I ankyrin-binding domain (e.g. "NGGNGTVEDRKPSEL" between aa 306 and aa320 in the mouse CD44 and "NSGNGAVEDRKPSGL " aa304 and aa318 in human CD44) (figure 2) is required for HA-mediated binding and cell adhesion (64,65). A recent study has also shown that the ankyrin binding domain of CD44s is required for oncogenic signaling and tumor cell transformation (65).

One of the first events to occur following the binding of HA to the CD44 receptor is a marked increase in free intracellular calcium ion ($Ca^{2+}$) concentration (30). Sebsequently, the CD44 molecules are aggregated or "capped" in the plasma membrane and the cells demonstrate the ability to adhere to extracellular HA-coated substrates (30). Using specific inhibitors, it has been shown that the capping and cellular adhesion processes both require $Ca^{2+}$ ion elevation and microfilaments (30). Most importantly, the CD44's ankyrin binding domain shares a large amount of sequence homology with the ankyrin binding domain located in at least two $Ca^{2+}$ channels (e.g. $IP_3$ receptors and ryanodine receptor) (56-58). Ankyrin may play a pivotal role in linking surface CD44 adhesion molecules and intracellular $Ca^{2+}$ storage organelle membrane proteins (e.g. $IP_3$ receptor and ryanodine receptor) to the cytoskeleton. Therefore, the CD44-ankyrin interaction is not only very important for properly presenting CD44 properly for HA binding, but is also required for interactions with other organelles (e.g. $Ca^{2+}$ channels) needed for signal transduction during adhesion, homing, hemopoiesis, migration and tumor metastasis.

### 4.2. CD44 isoform and ERM (ezrin, radixin, moesin and merlin)

Certain CD44 isoforms, such as the CD44E isoform (CD44 epithelial form), have also been found to interact with the ERM (ezrin, radixin, moesin and merlin) family of cytoskeletal proteins (47,66). ERM proteins are now considered part of the erythrocyte band 4.1 superfamily whose members all appear to link the plasma membrane to the actin cytoskeleton (47,66). In vitro binding assays by Tsukita and co-workers show that the CD44 cytoplasmic domain displays very weak binding for the ERM proteins [under physiological ionic strength condition (66)] as compared to CD44 and ankyrin binding (with a high affinity $K_d \approx$1-2nM) (30,64). It appears that ERM proteins must be activated in order to function as cross-linkers between the membrane proteins and actin filaments. The mechanism by which this activation occurs has not been fully determined, but it does involve both tyrosine and serine/threonine phosphorylation plus the presence of $PIP_2$ (phosphatidyl-inositol 4,5-bisphosphate) and small GTP-binding proteins (e.g. rho-like proteins) (66). It is possible that the cytoplasmic domain of CD44 isoforms interacts with several different cytoskeletal proteins with different binding affinities [e.g. a high affinity binding between CD44 and ankyrin (30,45,64) and a low affinity binding between CD44 and ERM (66)]. The differential binding affinities between CD44 isoforms and various cytoskeletal proteins (e.g. ankyrin and/or ERM) may influence selective signaling pathways leading to the onset of different CD44-mediated functions.

## 5. CD44S INTERACTION WITH SIGNALING MOLECULES IN ONCOGENESIS

### 5.1. CD44s and the oncogene product, $p185^{HER2}$

During cell transformation and metastasis, CD44 molecules are often overexpressed. In particular, high levels of CD44 expression occur in carcinomas which are more malignant than those expressing low levels of CD44 (67,68). Several oncogenes, such as src or ras, have been shown to induce overexpression of CD44 in rat intestinal epithelial cells (69). Recent evidence indicates that CD44s expression in $p185^{HER2}$-transfected cells is much higher than that in untransfected cells suggesting that the

oncogene product, $p185^{HER2}$ induces CD44s overexpression (70).

The HER2 oncogene (also called c-erbB-2 or neu) encodes a 185kDa ($p185^{HER2}$) membrane protein that contains a single transmembrane spanning region, two cysteine-rich extracellular domains and a tyrosine kinase-associated cytoplasmic domain (71). This protein belongs to the epidermal growth factor (EGF) receptor subgroup of the receptor-linked tyrosine kinase superfamily (72). Overexpression and amplification of HER2 oncogenes have been found to correlate with poor survival of many known cancers including breast and ovarian cancers (73,74). Hung and his co-worker have shown that HER2 oncogene is also overexpressed in several carcinoma cell lines shown to display high tumorigenic and metastatic potential (75). Furthermore, the $p185^{HER2}$ has been shown to be present in the nucleus of the fibroblasts (76). When the cytoplasmic domain of $p185^{HER2}$ is fused to the DNA-binding domain of GAL4, this fusion protein can function as a transcriptional activator (76). Therefore, $p185^{HER2}$ may be responsible for the induction of CD44s overexpression as a transcription activator.

Cells expressing a high level of CD44 often display enhanced hyaluronic acid binding which is related to tumor migration (77). It has been shown that CD44 overexpression of fibroblasts transfected by $p185^{HER2}$ cDNA displays significantly greater binding affinity for HA-coated plates than untransfected cells (70). These findings suggest that overexpression of CD44-induced by $p185^{HER2}$ oncogene enhances cell adhesion to HA-containing extracellular matrix materials (ECM) which may account for their increased migration capability as shown by other studies (78).

In a previous study we have shown that CD44s-mediated cell adhesion to HA plates by mouse T-lymphoma cells requires the presence of an intact cytoskeletal network containing certain cytoskeletal proteins such as ankyrin (30). The fact that the $p185^{HER2}$-transfected cells display stronger CD44s-ankyrin binding ($K_d \approx 0.19$ nM) than untransfected cells ($K_d \approx 0.3$ nM) suggests that the CD44s' ankyrin binding domain may be modified during the $p185^{HER2}$ oncogene-related activation cascade (70). Double immunofluorescence and confocal microscopy analyses clearly indicate that co-localization of intracellular ankyrin and CD44s at the HA-asssociated adhesion plaques of $p185^{HER2}$-transfected cells (70). These findings strongly suggest that overexpression of CD44s and up-regulation of CD44s-cytoskeleton interaction by $p185^{HER2}$ oncogene may be one of the important steps in regulating cell adhesion to HA-containing extracellular matrix materials during tumor cell migration.

Recently, the interaction between CD44s and the $p185^{HER2}$ was further investigated in the ovarian carcinoma cell line.Specifically, surface biotinylation followed by wheat germ agglutinin column chromatography and anti-CD44-mediated immunoprecipitation indicate that both CD44s and $p185^{HER2}$ are expressed on the cell surface, and most importantly, that these two molecules are physically linked to each other via interchain disulfide bonds (79). Based on the predicted sequence obtained from cDNA cloning of the human CD44s gene, several cysteine residues have been identified including 6-7 cysteine residues at the external domain, one cys(aa 286) in the transmembrane region and one cys(aa 295) in the cytoplasmic domain (22). The functional significance of the cysteine residues at the external domain of CD44s is not clear at the present time. However, point mutation of $^{286}$cys (but not $^{295}$cys) of human CD44s has been reported to cause a reduction (or loss) of hyaluronic acid (HA) binding (80). Certain cysteine residues [e.g. $^{286}$cys (in the transmembrane domain) and/or $^{295}$cys (in the cytoplasmic domain)] in mouse CD44s have been shown to play an important role in signal transduction and cell adhesion. For example, these residues appear to be involved in CD44s' fatty acylation required for CD44s and ankyrin interaction (62). Therefore, it is possible that some of these cysteine residues of CD44s may also be involved in the disulfide linkage with one or more cysteine residues in $p185^{HER2}$. The association of $p185^{HER2}$ with other surface molecules via disulfide linkages has been reported previously (81). Most importantly, HA stimulates CD44s-associated $p185^{HER2}$ tyrosine kinase activity leading to an increase in the ovarian carcinoma cell growth (79).

Following transfection of the ovarian carcinoma cell line with the adenovirus 5 E1A gene, which is known to repress $p185^{HER2}$ expression, both surface CD44s expression and CD44s-mediated cell adhesion to HA are significantly reduced in the transfectant cells compared to the control cells. These data suggest that down-regulation of $p185^{HER2}$ blocks CD44s expression and subsequent adhesion function. These findings indicate that the CD44s-$p185^{HER2}$ interaction is both functionally coupled and biosynthetically regulated (79). We believe that direct "cross-talk" between these two surface molecules (i.e. CD44s and the $p185^{HER2}$) may be one of the most important oncogenic signaling events in human breast and ovarian carcinoma development.

### 5.2. CD44s and Src Kinases

Previously, Taher and co-workers have determined that one of the Src kinase family members, $p56^{lck}$, is closely associated with the CD44s molecule during T-cell activation (82). Therefore, they have suggested that CD44s-mediated cellular signaling may be mediated by this Src kinase family member (82). The mechanisms of CD44s and Src kinase association has also been investigated in epithelial tumor cells, and ankyrin has been found to play an important part. Specifically, it has been shown the binding of ankyrin to the cytoplasmic domain of CD44 is required for Src p60 kinase activity (65).

Anchorage-independent growth in soft agar is generally considered to be a marker for transformation and tumorigenesis (83). Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases, such as those in the Src family (e.g. Src, Fyn, Yes, Lyn, Hck, Fgr, Lck, Blk and Yrk), is also commonly used as a maker for monitoring cell growth and transformation (84-86). In a recent study we

have found that cells containing CD44s (with the ankyrin-binding domain) and Src family (e.g. Src, Yes and Fyn) complex are capable of generating a large number of anchorage-independent colony growth (in soft agar) (65). These data further support the notion that the ankyrin binding domain of CD44s is important for CD44s-Src kinase(s) interaction and onset of tumor cell transformation.

## 6. CD44V ISOFORMS AND TUMOR PROGRESSION

Certain CD44 variant (CD44v) isoforms appear to be expressed at high levels on the surface of tumor cells during tumorigenesis and metastasis (87,88). Moreover, CD44v has been shown to be closely involved in the onset of tumor development and metastasis (5,23-26,35,89-92). For example, one of the CD44v isoforms, CD44v6 has been shown to confer metastatic behavior on rat pancreatic cells in a spontaneous metastasis assay (92); This molecule is also associated with a poor prognosis in human colon carcinomas (89) and non-Hodgkin's lymphoma (90). Nevertheless, the correlation between CD44v6 expression and its prognostic value for the survival in human breast cancer patients is still very controversial (23,25,91). In fact, some studies suggest that CD44v6 is merely an epithelial cell differentiation marker; and the usefulness of this molecule as a specific metastatic tumor cell marker requires further investigation (91).

CD44v5 expression has been proposed as an early tumor marker for colorectal carcinoma since it is detectable on dysplastic colon polyps and carcinoma, yet is not present on normal intestinal epithelium (93). In addition, increased CD44v7,8 expression has been found during the progression of human cervical carcinoma occurring in almost 100% of tissue samples at the stage of carcinoma in-situ (94). A positive association between the presence of CD44v9 and the progression of human gastric carcinoma has also been demonstrated (94).

### 6.1. CD44v10 in tumor cell growth

Recently, CD44v10 has also been shown to be closely associated with breast tumor development and progression (24,27). To further understand the mechanism by which CD44v10 may trigger the onset of the tumorigenesis and/or metastasis, CD44v10 cDNA was recently cloned into a pRc/CMV vector and transfected into non-tumorigenic human breast epithelial cells (HBL100) containing endogenous CD44s. It was found that coexpression of both CD44v10 and CD44s alters several important cellular properties. Specifically, cells coexpressing both CD44v10 and CD44s display a significant reduction in HA-mediated cell adhesion as compared to the parental (untransfected ) cells containing endogeneous CD44s (27). These data indicate that interactions between CD44v10 and CD44s may have altered the HA binding sites on the cell surface of the human mammary epithelial cells.

It has been suggested that several regions in the extracellular domain of CD44 containing clusters of conserved basic residues (95-97) that play an important role in CD44 binding to HA. All CD44v isoforms contain similar HA binding domain(s). However, not all CD44 isoforms constitutively bind HA. Often, certain CD44v isoforms display significantly less HA binding than CD44s (98). Information concerning the regulatory factors or mechanisms responsible for the reduction of HA binding by various CD44v isoforms is currently not available. A previous study showed that CD44v10 contains O-/N-linked glycosylation sites and chondroitin sulfate additions (45). Therefore, it is possible that post-translational modifications of v10-encoded structure may induce surface rearrangements or conformational changes in the HA-binding domains resulting in a loss of HA binding. In addition, it is possible that coexpression of CD44v10 and CD44s could prevent the dimerization (clustering) of CD44s molecules required for high affinity binding to HA as suggested by previous studies (36,99). The reduction of HA-mediated cell adhesion in cells expressing both CD44v10 and CD44s may be one of the earliest events in the onset of tumor migration and invasion processes.

Furthermore, CD44v (v10/ex14)-transfectants (i) display a higher migration/invasion capability, (ii) produce a higher level of certain angiogenic factors, such as basic fibroblast growth factor (bFGF) and interleukin 8 (IL-8); and (iii) exhibit more potent tumor growth potential than either parental (untransfected) or vector-transfected cells (27). Since the cytoplasmic domain of CD44v10 binds to ankyrin and the cytoskeleton, it is speculated that the unique structure of CD44 (v10/ex14) (e.g. O-/N-linked glycosylation or chondroitin sulfate addition) may cause constitutive activation of CD44 isoform-cytoskeleton interactions which induce tumor cell migration and invasion. Most recently, Droll et al. have shown that the chondroitin sulfate moiety attached to the v10/ex14 structure of CD44 is capable of binding to other CD44 molecules (99). This unique adhesive interaction may be critically important for (i) homotypic and/or heterotypic cell-cell adhesion in vitro; (ii) microemboli formation; and/or (iii) the binding between circulating tumor cells and the vascular endothelium in vivo resulting in angiogenic factor production and tumor growth. Therefore, we feel that the introduction of CD44v (v10/ex14) into non-tumorigenic mammary epithelial cells expressing CD44s has helped to elucidate the role of this particular CD44v10 isoform during tumor development.

### 6.2. CD44v3 isoforms in tumor cell invasion and migration

As the histologic grade of each tumor progresses, the percentage of lesions expressing an associated CD44 variant isoform increases. In particular, the CD44v3-containing isoforms are expressed preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (24,100). One study indicates that breast tumor expression of the CD44v3 isoform may be used as an accurate predictor of overall survival (e.g. nodal status, tumor size, and grade) (24,25). It has been speculated that some of these CD44v3

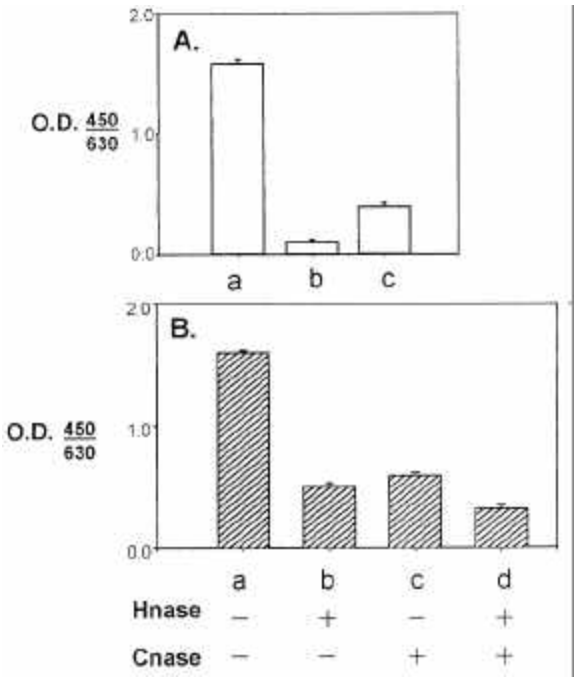


**Figure 3.** Binding of VEGF and b-FGF to $CD44v_{3,8-10}$ by ELISA. A: VEGF binding to purified $CD44v_{3,8-10}$ [without any enzymatic digestion] in the absence (3A-a) and presence of 3mg/ml heparin (3A-c); b-FGF binding to purified $CD44v_{3,8-10}$ (3A-b). B-a: VEGF binding to purified $CD44v_{3,8-10}$ [without any enzymatic digestion]. B-b: VEGF binding to purified $CD44v_{3,8-10}$ [digested with heparitinase]. B-c: VEGF binding to purified $CD44v_{3,8-10}$ [digested with chondroitinase]. B-d: VEGF binding to purified $CD44v_{3,8-10}$ [digested with heparitinase and chondroitinase]. [O.D.450/630 units represent the relative amounts of VEGF or bFGF binding to a constant level of purified $CD44v_{3,8-10}$].

isoforms on epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions (41,42) and subsequent tumor formation. It is also possible that these CD44 variants may interact with extracellular matrix materials (e.g. hyaluronic acid) such that epithelial cells undergo abnormal mitogenesis and migration. This could be one of the critical steps in tumor invasion and metastasis.

Recently, we have found that the CD44v3 isoform (e.g. $CD44v_{3,8-10}$) (figure 1e) is also expressed in breast tumor cells (Met-1 cell line-derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene]) (101). Previously, $CD44v_{3,8-10}$ was shown to contain sulfated oligosaccharides (41,42). Consequently, we have metabolically labeled Met-1 cells with $(^{35}SO_4)^{2-}$ and then looked for the presence of radioactive sulfate label in $CD44v_{3,8-10}$ using anti-CD44v3-mediated immunoprecipitation (102). Our results clearly indicate that $^{35}SO_4^{2-}$ is incorporated into the glycosaminoglycan (GAG) chains of $CD44v_{3,8-10}$ (102). It has also been suggested that the GAG chains of certain CD44v3-containing isoform are involved in the binding of heparin binding growth factors, such as basic fibroblast growth factor (bFGF) (41). Consequently, we have tested the possible interaction between $CD44v_{3,8-10}$ and two heparin binding growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Our results clearly indicate that $CD44v_{3,8-10}$ binds preferentially to VEGF (figure 3A-a; and 3B-a), but not bFGF (figure 3A-b). This binding between $CD44v_{3,8-10}$ and VEGF is reduced in the presence of heparin (figure 3A-c); and appears to be sensitive to the digestion by heparitinase (figure 3B-b) and/or chondroitinase (figure 3B-c and 3B-d), suggesting the GAG chains of $CD44v_{3,8-10}$ are responsible for VEGF binding. It is possible that a different organizational and/or conformational presentation of $CD44v_{3,8-10}$ molecules on the surfaces of Met-1 cells and COS cells may result in the selective binding of certain growth factors to these two types of cells. The fact that VEGF binds $CD44v_{3,8-10}$ on the surface of Met-1 cells suggests that $CD44v_{3,8-10}$ may serve as a modified VEGF receptor on the tumor cell surface. The effects of VEGF on $CD44v_{3,8-10}$-mediated Met-1 cell activation are currently under investigation. VEGF is a specific mitogen for endothelial cells and a potent microvascular permeability factor (103,104). It plays an integral role in angiogenesis and thus in potentiation of solid tumor growth (103,104). Met-1 cells are also capable of inducing a high level of intratumoral microvessel formation (101). Therefore, the attachment of VEGF to the heparin sulfate sites on $CD44v_{3,8-10}$ may be responsible for the onset of breast tumor-associated angiogenesis.

The binding of HA to CD44 is conferred by two regions in the N-terminal extracellular domain (95-97). HA binding can be increased by certain activating antibodies such as rat anti-CD44 antibody (IRAWB 14) (105). While CD44v isoforms all contain similar HA binding motifs (95-97), certain CD44v isoforms have been reported to display significantly less HA binding than CD44s (27,98). Our data indicate that Met-1 cells, expressing the $CD44v_{3,8-10}$ isoform, display a low level of cell adhesion to HA-coated plates (figure 4A). It is possible that post-translational modifications of the $v_{3,8-10}$-encoded structure induce surface rearrangements or conformational changes in the HA-binding domains which result in a reduction of HA-mediated cell adhesion. The fact that rat anti-CD44 (IRAWB-14) is capable of promoting HA-mediated cell adhesion (figure 4B) suggests that the HA binding motifs in the $CD44v_{3,8-10}$ isoform may be readily regulated (either up or down) during the onset of tumor cell transformation leading to increased cell motility (migration) and invasion.

The invasive phenotype of tumor cells characterized by "invadopodia" formation (106,107) and tumor cell motility (108,109) has been linked to cytoskeletal function. Dissection of the transmembrane pathways controlling these cellular processes should aid in understanding the regulatory mechanisms underlying tumor invasion and metastasis. A recent study indicates that the ankyrin-binding sequence (NGGNGTVEDRKPSEL) resides between amino acids 480 and 494 of the $CD44v_{3,8-10}$ molecule (102) and is involved in regulating CD44 isoform-mediated function (5,6). Most importantly,

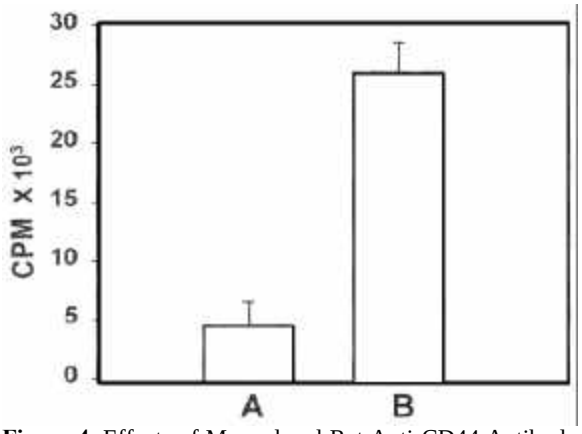


**Figure 4.** Effects of Monoclonal Rat Anti-CD44 Antibody (IRAWB-14) On Cell Adhesion to HA-Coated Plates. Met-1 cells labeled with Tran$^{35}$S-label (9.1 x $10^5$ cpm/$10^5$ cells) were either untreated (as a control) (A) or pretreated with rat anti-CD44 IgG (IRAWB-14)(50 μg/ml) (B). Following treatment, the cells were incubated with HA-coated plates at 4$^o$ C for 30 min. The non-specific binding of cells to HA-coated plates was determined in presence of soluble HA and was subtracted. Each experiment was performed in duplicate and the results represent an average of three separate experiments with a standard deviation <±5%.

treatments of Met-1 cells with certain agents including anti-CD44$v_{3,8-10}$, cytochalasin D (a microfilament inhibitor) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule disrupting agent), effectively inhibit "invadopodia" formation and tumor cell migration (102). Therefore, we believe that CD44$v_{3,8-10}$ plays an important role in linking ankyrin (figure 5), to the membrane-associated actomyosin contractile system required for "invadopodia" formation and tumor cell migration.

It has been suggested that a number of different matrix metalloproteinases (MMPs) [including the 72kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the stromelysins (MMP-3, MMP-11) and the interstitial fibroblast-type collagenase (MMP-1)] are thought to play an important role in degrading extracellular matrix materials (ECM) during tumor invasion and metastases (110-112). The biochemical interaction between MMPs and various cell surface molecules has not been fully investigated. A membrane-type matrix metalloproteinase (MT-MMP, a transmembrane MMP) together with TIMP-2, a tissue inhibitor of metalloproteinase-2 (MMP-2), have been reported to be involved in the activation of MMP-2 on the cell surface (113). Recently, Brooks et al determined that matrix metalloproteinase MMP-2 is localized in a proteolytically active form on the surface of invasive cells based on its ability to bind directly to integrin αvβ3 (114).

Recently, CD44$v_{3,8-10}$ (a surface adhesion molecule) has been found to be closely associated with MMP-9 (gelatinase B) in the plasma membrane of Met-1 cells, based on the evidence provided by anti-MMP-9 and anti-CD44-mediated immunoprecipitation followed by immunoblot and gelatin zymography (102). Furthermore, CD44$V_{3,8-10}$-associated MMP-9 appears to be present in a proteolytically active form and preferentially localized at the "invadopodia" structure of the Met-1 cells. Our results are consistent with previous findings showing that certain protease(s) is(are) localized on "invadopodia" of human malignant melanoma cells (106,107). The close interaction between CD44$v_{3,8-10}$ and the active form of MMP-9 in the "invadopodia" structure of Met-1 tumor cells may be required for the degradation of extracellular matrix (ECM) for tumor cell invasion and metastasis. Therefore, we believe that CD44$v_{3,8-10}$ plays an important role in linking ankyrin to the membrane-associated actomyosin contractile system required for up-regulating metastatic tumor cell behavior (e.g. invadopodia formation and matrix degradation) and promoting angiogenesis during breast cancer progression.

## 7. PERSPECTIVE

A number of studies have been aimed at identifying molecules expressed by cells which correlate with immune dysfunction or cancer development. One such candidate is the CD44 family [e.g. CD44 standard form (CD44s) and variant isoform (CD44v)] of transmembrane glycoproteins. These proteins recognize hyaluronan (HA) at their extracellular domain and contain at least one ankyrin-binding region and a putative ERM binding site in their cytoplasmic domain. Now, it is clear that both external and internal domains influence structural variability and functional heterogeneity associated with CD44 isoforms. Differential biosynthesis and post-translational modifications (N- or O-linked glycosylation and GAG addition) at the extracellular domain of CD44 isoforms provide selective surface recognition for the binding of ECM (e.g. HA). Transmembrane interaction between the cytoskeletal protein, ankyrin and the cytoplasmic domain of CD44 isoforms plays an important role in CD44 isoform-mediated oncogenic signaling. Specifically, deletion mutation analysis indicates that at least two subregions within CD44 cytoplasmic domain contribute to ankyrin binding-region I (i.e. the high affinity ankyrin-binding region) and region II (i.e. the regulatory region) (figure 2). In particular, the region I ankyrin-binding domain (e.g. "NGGNGTVEDRKPSEL" between aa 306 and aa320 in the mouse CD44 and "NSGNGAVEDRKPSGL " aa304 and aa318 in human CD44) is required for the recruitment of Src kinase and the onset of tumor cell transformation. Furthermore, HA binding to CD44s stimulates a concomitant activation of p185$^{HER2}$-linked tyrosine kinase (linked to CD44s via a disulfide linkage) and results in a direct "cross-talk" between two different signaling pathways (e.g. proliferation vs motility/invasion) (see figure 6). Most importantly, certain angiogenic factors (e.g. VEGF or bFGF) and matrix degradating enzymes (MMPs) are also tightly associated with CD44v isoforms and are believed to play a synergistic role in the generation of oncogenic signals leading to tumor-specific behaviors (e.g. invasion and motility/migration) in a cytoskeleton-dependent manner. In conclusion, we believe that CD44 isoform-cytoskeleton interaction plays a pivotal role in transmitting external signals (i.e. ECM-cell or cell-cell) and coordinating intracellular signaling pathways leading to oncogenesis during tumor progression.

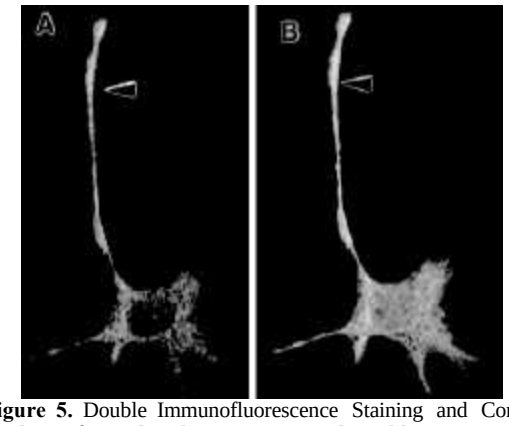


**Figure 5.** Double Immunofluorescence Staining and Confocal Analysis of "invadopodia" Structures (indicated by arrow) in Met-1 Cells. Co-localization of surface CD44$v_{3,8-10}$ (A) and intracellular ankyrin (B) using rat anti-CD44 antibody and mouse anti-ankyrin antibody followed by fluorescence-labeled goat anti-rat IgG and rhodamine-labeled goat anti-mouse IgG, respectively.

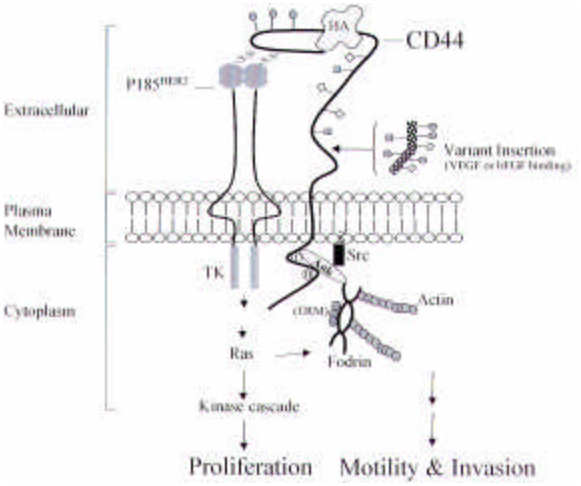


**Figure 6.** A current model for CD44 isoform-cytoskeleton interaction in oncogenic signaling and tumor progression. The binding of extracellular matrix component (ECM) such as hyaluronic acid (HA) to the extracellular domain of CD44 receptor promotes the interaction between CD44 and the cytoskeletal proteins [e.g. ankyrin (Ank) and ERM]. In particular, two subregions within CD44 cytoplasmic domain [e.g. region I (I) and region II (II)] are responsible for the direct linkage of ankyrin to CD44. Region I is critically importnat for the high affinity binding between CD44 and ankyrin (Ank) which, in turn, binds to fodrin and actin-based microfilaments required for cell motility and migration. Region I ankyrin (Ank) binding region is also needed for the proper presentation of CD44 for ECM binding and Src kinase-related oncogenic signaling and tumor cell transformation. In addition, CD44 and the oncogene product, $p185^{HER2,}$ are physically linked via a disulfide linkage. HA binding to CD44 stimulates $p185^{HER2}$ tyrosine kinase activity leading to Ras activation, kinase cascade and tumor cell growth. Finally, certain angiogenic factors (e.g. VEGF or bFGF) bound to variant exon-coded structures may also play a synergistic role in generating a cytoskeleton-dependent tumor-specific behaviors [e.g. "invadopodia formation", association of matrix degrading enzymes (MMPs), invasion and tumor cell motility/migration) as well as angiogenesis. Our current model suggests that the interaction of CD44-cytoskeleton with various oncogenic molecules plays a pivotal role in up-regulating direct "cross-talk" between two different signaling pathways (e.g. proliferative vs motility/invasion) during tumor progression.

## 8. ACKNOWLEDGEMENT

We gratefully acknowledge Dr. Gerard J. Bourguignon's assistance in the preparation of this paper. We would also like to thank Dr. You-Wei Chen for his technical assistance in confocal microscopic analyses. This work was supported by United States Public Health grant (CA66163) and DOD grants [DAMD 17-94-J-4121 (DAMD 17-94-J-4121 and DAMD 17-97-1-7014).

## 9. REFERENCES

1.L. Y.W. Bourguignon, V. B. Lokeshwar, J. He, X. Chen & G. J. Bourguignon: A CD44-like endothelial cell transmembrane glycoprotein (GP116) interacts with extracellular matrix and ankyrin. *Mol. Cell. Biol*. 12,4464-71 (1992*)*

2. T. Brown, T. Bouchard, T. St. John, E. Wayner & W. G. Carter: Human keratinocytes express a new CD44 core protein (CD44E) as a heparin sulfate intrinsic membrane proteoglycan with additional exons. *J. Cell Biol.* 113,207-21 (1991*)*

3. M.B. Omary, I. S. Trowbridge, M. Letarte, M. F. Kagnoff, and C. M. Isacke: Structural heterogeneity of human Pgp-1 and its relationship with p85. *Immunogenetics.* 27,460-4 (1988*)*

4. I.S. Trowbridge, J. Lesley, R. Schulte, R. Hyman & J. Trotter: Biochemical characterization and cellular.distribution of a polymorphic, murine cell glycoprotein expressed on lymphoid tissues. *Immunogenetics* 15,299-312 (1982*)*

5. L.Y.W. Bourguignon, N. Iida, C.F. Welsh, D. Zhu, A. Krongrad, & D. Pasquale: Involvement of CD44 and its variant isoforms in membrane-cytoskeleton interaction, cell adhesion and tumor metastasis. *J. Neuro-Oncol.* 26,201-8 (1995*)*

6. L.Y.W. Bourguignon: Interaction between membrane-cytoskeleton and CD44 during lymphocyte signal transduction and cell adhesion. *Current Topics in Membranes* (Ed: W.J. Nelson) 43,293-312 (1996*)*

7. R. Galandrini, N. Albi, G. Tripodi, D. Zarcone, A. Terenzi, A. Moretta, C.E. Grossi & A. Velardi: Antibodies to CD44 trigger effector functions of human T cell clones. *J. Immunol.* 150,4225-35 (1993*)*

8. A. Seth, L. Gote, M. Nagarkatti & P.S. Nagarkatti: T-cell receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp90MEL-14. *Proc. Natl. Acad. Sci.(U.S.A.)* 88,7877-81 (1991*)*

9. F. Pericle, G. Sconocchia, J.A. Titus & D.M. Segal: CD44 is a cytotoxic triggering molecule on human polymorphonuclear cells. *J. Immunol.* 157,4657-63 (1996*)*

10. P.H.S. Tan, E.B. Santos, H.C. Rossbach & B.M. Sandmaier: Enhancement of natural killer activity by an antibody to CD44. *J. Immunol.* 150,812-20 (1993*)*

11. G. Sconocchia, J.A. Titus & D.M. Segal: CD44 is a cytotoxic triggering molecule in human peripheral blood NK cells. *J. Immunol.* 153,5473-81 (1994*)*

12. R. Galandrini, R. de Maria, M. Piccoli, L. Frati & A. Santoni: CD44 triggering enhances human NK cell cytotoxic functions. *J. Immunol.* 153,4399-4407 (1994*)*

13. D.S.A. Webb, Y. Shimizu, G.A. Seventer, S. Shaw & T.L. Gerrard: LFA, CD44 and CD45: physiologic triggers of human monocyte TNF and IL-1 release. *Science* 249,1295-97 (1990*)*

14.C.M. McKee, M.B. Penno, M. Cowman, M.D. Burdick, R.M. Strieter, C. Bao & P.W. Noble: Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. *J. Clin. Invest.* 98,2403-13 (1996*)*

15. B.F. Haynes, M. J. Telen, L.P. Hale & S. M. Denning: CD44-a molecule involved in leukocyte adherence and T-cell activation. *Immunol. Today* 10,423-28 (1989*)*

16. K. Miyake, K. L. Medina, S. I. Hayashi, S. Ono, T. Hamaoka & P. W. Kincade: Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow culture. *J. Exp. Med.* 171:477-88 (1990*)*

17. K. Mikecz, F.R. Brennan, J.H. Kim & T.T. Glant. Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. *Nat. Med.* 1,558-63 (1995*)*

18. W.M.C. Rosenberg, C. Prince, L. Kaklamanis, S.B. Fox, D.G. Jackson, D.L. Simmons, R.W. Chapman, J.M. Trowell, D.P. Jewell & J.I. Bell. Increased expression of CD44v6 and CD44v3 in ulcerative colitis but not colonic Crohn's disease. *Lancet* 345,1205-09 (1995*)*

19. E. Horst, C.J. Meijer, T. Radaszkiewicz, G.J. Ossekoppele, J.H. Van Krieken, & S.T. Pals: Adhesion molecules in the prognosis of diffuse large-cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA (CD11a/18), and ICAM-1 (CD54*)* *Leukemia* 4,595-9 (1990*)*

20. E. J. Quackenbush, S. Vera, A. Greaves & M. Letarte: Confirmation by peptide sequence and co-expression on various cell types of the identity of CD44 and p85 glycoprotein. *Mol. Immunol.* 27,947-55 (1990*)*

21. S. Jalkanen, H. Joensuu, K.O. Soderstrom & P. Klemi: Lymphocyte homing and clinical behavior of non-Hodgkin's lymphoma. *J. Clin. Invest.* 87,1835-40 (1991*)*

22. G.R. Screaton, M.V. Bell, D.G. Jackson, F.B. Cornelis, U. Gerth, J.I. Bell: Genomic structure of DNA coding the lymphocyte homing receptor CD44 reveals at least 12 alternative spliced exons. *Proc. Natl. Acad. Sci. (USA)* 89,12160-4 (1992*)*

23. P. Dall, K.-H. Heider, H.-P., Sinn, P. Skroch-Angel, G. Adolf, M. Kaufmann, P. Herrlich, & H. Ponta: Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer. *Int. J. Cancer* 60,471-7 (1995*)*

24. N. Iida & L.Y.W. Bourguignon: New CD44 splice variants associated with human breast cancers. *J Cell Physiol*. 162:127-33 (1995*)*

25. M. Kaufmann, K.H. Heider, H.P. Sinn, G. von Minckwitz, H. Ponta & P. Herrlich: CD44 variant exon epitopes in primary breast cancer and length of survival. *Lancet* 345,615-9 (1995*)*

26. C. Rodriguez, G. Monges, P. Rouanet, B. Dutrillaux, D. Lefrancois & C. Theillet: CD44 expression patterns in breast and colon tumors: A PCR-based study of splice variants. *Int. J. Cancer* 64:347-54 (1995*)*

27. N. Iida & L.Y.W. Bourguignon: Coexpression of CD44 variant (v10/ex14) and CD44s in human mammary epithelial cells promotes tumorigenesis, *J Cell Physiol.* 171:152-60 (1997*)*

28. T. C. Laurent & R. E. Fraser: Hyaluronan. *FASEB J.* 6:2397-404 (1992*)*

29. P.W. Noble, C.M. McKee, M. Cowman & H.S. Shin: Hyaluronan fragments activate an NF-κB/I-κBα autoregulatory loop in murine macrophages. *J. Exp. Med.* 183,2373-2378 (1996*)*

30. L.Y.W. Bourguignon, V. B. Lokeshwar, X. Chen & G. L. Kerrick: Hyaluronic acid (HA)-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J. Immunol.* 151:6634-44 (1993*)*

31. S. J. Green, G. Tarone & C.B. Underhill: Aggregation of macrophages and fibroblasts is inhibited by a monoclonal antibody to the hyaluronate receptor. *Exp. Cell Res.* 178,224-32 (1988*)*

32. D.C. West & S. Kumar:The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp. Cell Res.* 183,179-96 (1989*)*

33. E.A. Turley, L. Austen, K. Vandeligt & C. Clary: Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. *J. Cell Biol*. 112,1041-7(1991*)*

34. P. Rooney, S. Kumar, & M. Wang: The role of hyaluronan in tumor neovascularization. *Int. J. Cancer* 60,632-6 (1995*)*

35. P. Herrlich, M. Zoller, S.T. Pals & H. Ponta: CD44 splice variants: metastases meet lymphocytes. *Immunol. Today* 14,395-9 (1993*)*

36. J. Lesley, R. Hyman & P. Kincade. CD44 and its interaction with the extracellular matrix. *Adv. Immunol*. 54,271-35 (1993*)*

37. L.A. Goldstein, D.F.H. Zhou, L.J. Picker, C.N. Minty, R.F. Bargatze, J.F. Ding & E.C. Butcher: A human

lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell* 56,1063-72 (1989)

38. S. Jalkanen, R.F. Bargatze, J. de los Toyos & E.C. Butcher: Lymphocyte recognition of high endothelium antibodies to distinct epitopes of an 85-95kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J. Cell Biol*. 105,983-90(1987)

39. S. Jalkanen, M. Jalkanen, R. Bargatze, M. Tammi & E.C. Butcher: Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. *J. Immunol.* 141,1615-23(1988)

40. V.B. Lokeshwar & L. Y. W. Bourguignon: Post-translational protein modification and expression of ankyrin binding site(s) in GP85(Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biosynthesis. *J. Biol. Chem.* 266,17983-89 (1991)

41. K. Bennett, D. Jackson, J. Simon, E. Tanczos, R. Peach, B. Modrell, I. Stamenkivic:CD44 isoforms containing exon v3 are responsible for the presentation of heparin-binding growth factor, *J. Cell Biol.* 128,687-98 (1995)

42. D. G. Jackson, J.I. Bell, R. Dickinson, J. Timans, J. Shields & N. Whittle: Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J Cell Biol* 128,673-85 (1995)

43. S. P. Jalkanen & M. Jalkanen: Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol.* 116,817-25 (1992)

44 . G.J. Dougherty, P.M. Lansdorp, D.L. Cooper & R.K. Humphries: Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. *J. Exp. Med.* 174,1-5 (1991)

45. V.B. Lokeshwar, N. Iida & L.Y.W. Bourguignon:The cell adhesion molecule, GP116 is a new CD44 variant (ex14/v10) involved in hyaluronic acid binding and endothelial cell proliferation. *J. Biol. Chem.* 271,23853-64 (1996)

46. C.F. Welsh, D. Zhu & L.Y.W. Bourguignon: Interaction of CD44 variant isoforms with hyaluronic acid and the cytoskeleton in human prostate cancer cells. *J. Cell Physiol.* 164,605-12 (1995)

47. T. Tsukita, K. Oishi, N. Sato, J. Sagara, A. Kawai & S. Tsukita: ERM family members as molecular linkers between CD44 and actin-based cytoskeletons. *J. Cell Biol.* 126,391-421(1994)

48. V. Bennet: Ankyrins. *J. Biol. Chem.* 267,8703-6 (1992)

49. J. Davis, S.E. Lux & V. Bennet: Mapping the ankyrin binding site of human erythrocyte anion exchanger. *J. Biol. Chem.* 264,9665-72 (1989)

50. W. J. Nelson & P.J. Veshnock: Ankyrin binding to $Na^+/K^+$-ATPase and implications for the organizations of membrane domains in polarized cells. *Nature (Lond)* 328,533-6 (1987)

51. J. S. Morrow, C.D. Cianci, A.S. Ardito & M. Kashgarian: Ankyrin links fodrin to the alpha subunit of $Na^+/K^+$-ATPase in Madin-Darby Canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108,455-65 (1989)

52. M.M. Shull, D.G. Pugh & J.B. Lingrel: Characterization of the human $Na^+/K^+$-ATPase $\alpha 2$ gene and identification of the intragenic restriction fragment length polymorphism. *J. Biol. Chem.* 264,17532-43 (1989)

53. P.R. Smith, G. Saccomani, E.H. Joe, K.J. Angelides & D.J. Benos: Amiloride sensitive sodium channel is linked to the cytoskeleton in renal epithelial cells. *Proc. Natl. Acad. Sci. (USA)* 88,6971-75 (1991)

54. E. Kordeli & V. Bennet: Distinct ankyrin isoforms at neuron cell bodies and nodes of Ranvier resolved using erythrocyte ankyrin-deficient mice. *J. Cell Biol.* 114,1243-59 (1991)

55. E. Kordeli, S. Lambert & V. Bennet: AnkyrinG, A new ankyrin gene with neural-specific isoforms localized at the axonal initial segment and node of Ranvier. *J. Biol. Chem.* 270,2352-59 (1995)

56. L.Y.W. Bourguignon, H. Jin, N. Iida, N. Brandt & S.H. Zhang: The involvement of ankyrin in the regulation of inositol 1,4,5-triphosphate receptor-mediated internal $Ca^{2+}$ release from $Ca^{2+}$ storage vesicles in mouse T-lymphpma cells. *J. Biol. Chem.* 268,7290-7 (1993)

57. L.Y.W. Bourguignon & H. Jin: Identification of the ankyrin-binding domain of the mouse T-lymphoma cell inositol 1,4,5-triphosphate ($IP_3$) receptor and its role in the regulation of $IP_3$-mediated internal $Ca^{2+}$ release. *J. Biol. Chem.* 270,7257-60 (1995)

58. L.Y.W. Bourguignon, A. Chu, H. Jin & N.R. Brandt: Ryanodine receptor-ankyrin interaction regulates internal $Ca^{2+}$ release in mouse T-lymphoma cells. *J. Biol. Chem.* 270,17917-22 (1995)

59. L.Y.W. Bourguignon, G. Walker, S. Suchard & K. Balazovich: A lymphoma plasma membrane-associated protein with ankyrin-like properties. *J. Cell Biol.* 102,2115-24 (1986)

60. E. L. Kalomiris & L.Y.W. Bourguignon: Mouse T-lymphoma cells contain a transmembrane glycoprotein (GP85) which binds ankyrin. *J. Cell Biol*. 106,319-27 (1988)

61. E.L. Kalomiris & L.Y.W. Bourguignon: Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85 and may function in GP85-ankyrin binding. *J. Biol. Chem.* 264,8113-19 (1989)

62. L.Y.W. Bourguignon, E. Kalomiris & V.B. Lokeshwar: Acylation of the lymphoma transmembrane glycoprotein,

GP85, may be required for GP85-ankyrin interaction. *J. Biol. Chem.* 266,11761-5 (1991*)*

63. V.B. Lokeshwar & L.Y.W. Bourguignon: The lymphoma transmembrane glycoprotein GP85(CD44) is a novel guanine nucleotide-binding protein which regulates GP85(CD44)-ankyrin interaction. *J. Biol. Chem*. 267,22073-8 (1992*)*

64. V.B. Lokeshwar, N. Fregien & L.Y.W. Bourguignon: Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *J. Cell Biol*. 126,1099-1109 (1994*)*

65. D. Zhu & L.Y.W. Bourguignon: The ankyrin-binding domain of CD44s is involved in regulating hyaluronic acid-mediated functions and prostate tumor cell transformation. *Cell Motility and the Cytoskeleton* 39:209-22 (1998*)*

66. S. Tsukita, S. Yonemura & S. Tsukita: ERM (ezrin/radixin/moesin) family : from cytoskeleton to signal transduction. *Curr. Opin. Cell Biol.* 9,70-75 (1997*)*

67. M. C. Kuppner, E.V. Meir, T. Gauthier, M.F. Hamou & N.D. Tribolet: Differential expression of the CD44 molecule in human brain tumours. *Int. J. Cancer*, 50, 572-7 (1992*)*

68. M.S. Sy, Y.J. Guo & I. Stamenkovic: Distinct effects of two CD44 isoforms on tumor growth in vivo. J. *Exp. Med.* 174, 859-66 (1991*)*

69. H. H. Jamal, D.F. Cano-Gauci, R.N. Buick & J. Filmus: Activated ras and src induce CD44 overexpression in rat intestinal epithelial cells. *Oncogene*, 9, 417-23(1994*)*

70. D. Zhu & L.Y.W. Bourguignon: Overexpression of CD44 in $p185^{neu}$-transfected NIH3T3 cells promotes an up-regulation of hyaluronic acid-mediated membrane-cytoskeleton interaction and cell adhesion. *Oncogene* 12,2309-14 (1996*)*

71. C. Bargmann, M-C. Hung & R.A. Weinberg: The neu oncogene encodes epidermal growth factor receptor-related protein. *Nature (Lond)* 319,226-30 (1986*)*

72. E. Peles & Y. Yarden: Neu and its ligands: from an oncogene to neural factors. *BioEssays* 15,815-24 (1993*)*

73. A. Berchuck, A. Kamel, R. Whitaker, B. Kerns, G. Olt, R. Kinney, J.T. Soper, R. Dodge, D.L. Clarke-Pearson, P. Marks, P. McKenzie, S. Yin & J.R.C. Bast: Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res.* 50,4087-91 (1990*)*

74. M.C. Hung, X. Zhang, D.H. Yen, H.Z. Zhang, G.P. He, T. Q. Zhang & D.R. Shi: Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Lett.* 61,95-103 (1992*)*

75. D. Yu, J.K. Wolf, M. Scanlon, J.E. Price & M.C. Hung: Enhanced c-erbB-2/neu expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res.* 53,891-98 (1993*)*

76. Y.M. Xie & M.C. Hung: Nuclear localization of p185neu tyrosine kinase and its association with transcriptional transactivation. *Biochem. Biophys. Res. Comm*., 203, 1589-98 (1994*)*

77. A. Bartolazzi, R. Peach R, A. Aruffo & I. Stamenkovic: Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. *J. Exp. Med*., 180, 53-66 (1994*)*

78. L. Zhang, C.B. Underhill & L.P. Chen: Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer Res*., 55, 428-33 (1995*)*

79. L. Y. W. Bourguignon, H. Zhu, A. Chu, N. Iida, L. Zhang & H.C. Hung: Interaction between the adhesion receptor,CD44 and the oncogene product, $p185^{HER2}$, promotes human ovarian tumor cell activation. *J. Biol. Chem.* 272, 27913-18 (1997*)*

80. D. Liu & M.S. Sy: A cysteine residue located in the transmembrane domain of CD44 is important in binding of CD44 to hylauronic acid. *J. Exp. Med.* 183,1987-94 (1996*)*

81. M. A. Adelsman, B.K. Huntley & N.J. Maihle: Ligand-independent dimerization of oncogenic v-erbB products involves covalent interactions. *J. Virol*. 70,2533-44 (1996*)*

82. T.E.I. Taher, L. Smit, A.W. Griffioen, E.J.M. Schilder-Tol, J. Borst, & S.T. Pals: Signaling through CD44 is mediated by tyrosine kinases. *J. Biol. Chem*. 271,2863-7 (1996*)*

83. V. H. Freeman & S. Shin: Cellular tumorigeneicity in nude mice: Correlation with cell growth in semi-solid medium. *Cell* 3,355-9 (1974*)*

84. R. Klausner & L.E. Samelson: T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell* 64,875-8 (1991*)*

85. B. J. Maye & D. Baltimore: Signalling through SH2 and SH3 domains. *Trends Cell Biol*. 3:8-13 (1993*)*

86. T. Hunter & J.A. Cooper: Protein-tyrosine kinase. *Annu. Rev. Biochem.* 54:897-930 (1985*)*

87. Y. Matsumura & D. Tarin: Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 340,1053-8 (1992*)*

88. K. K. Tanabe, L.M. Ellis & H. Saya: Expression of CD44R1 adhesion molecule in colon carcinoma and metastases. *Lancet* 341,725-6 (1993*)*

89. J.-W. R. Mulder, P. Kruyt, M. Sewnath, J. Oosting, C.A. Seldenrijk, W.F. Weidema, J. Offerhaus & S.T. Pals: Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 344,1470-2 (1994*)*

90. R. Stauder, W. Eisterer, J. Thaler & U. Gunthert: CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. *Blood* 85,2885-99 (1995*)*

91. K. Friedrichs, F. Franke, B.-W. Lisboa, G. Kugler, I. Gille, H.-J. Terpe, F. Holzel, H. Maass & U. Gunthert: CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. *Cancer Res.* 55,5424-33 (1995)

92. U. Gunthert, M. Hoffman, W. Rudy, S. Reber, M. Zoller, I. Haubmann, S. Matzku, A. Wenzel, H. Ponta & P. Herrlich: A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 65,13-24 (1993)

93. P. Herrlich, S. Pals & H. Ponta: CD44 in colon cancer. *Eur J. Cancer* 31A,1110-2 (1995)

94. P. Dall, A. Hekelf, H. Ikenberg, A. Goppinger, T. Bauknecht, A. Pfleiderer, & J. Moll: Increasing incidence of CD44v7/8 epitope expression during uterine cervical carcinogenesis, *Int. J. Cancer* 69,79-85 (1996)

95. B. Yang, B. L. Yang, R.C. Savani & E.A. Turley: Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44, and link protein. *EMBO J.* 13, 286-96 (1994)

96. R. J. Peach, D. Hollenbaugh, I. Stamenkovic & A. Aruffo: Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J. Cell Biol*. 122,257-64 (1993)

97. H.X. Liao, D.M. Lee & B.F. Haynes: N-terminal and central regions of the human CD44 extracellular domain participated in cell surface hyaluronan binding. *J. Immunol.* 155,3938-45 (1995)

98. I. Stamenkovic, M. Amiot, J.M. Pesando & B. Seed: The hemopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronan-bearing cells. *EMBO J.* 10,343-347 (1991)

99. A. Droll, S.T. Doughery, R.D. Chiu, J.F. Dirks, W.H. Mcbride, D.L. Cooper & G.J. Dougherty: Adhesive interactions between alternatively spliced CD44 isoforms. *J. Biol. Chem.* 270, 11567-73 (1995)

100. H.P. Sinn, K.H. Heider, P. Skroch-Angel, G. von Minckwitz, M. Kaufmann, P. Herrlich & H.Ponta: Human mammary carcinomas express homologues of rat metastasis-associated variants of CD44 Breast *Cancer Res. and Treatment* 36,307-13 (1995)

101. A.T.W. Cheung, L.J.T. Young, P.C.Y. Chen, C.Y. Chao, A. Ndoye, P.A. Barry, W.J. Muller & R.D. Cardiff: Microcirculation and metastasis in a new mammary tumor model system. *Int. J. Oncology* 11,69-77 (1997)

102. L.Y.W. Bourguignon, Z. Gunja-smith, N. Iida, H.B. Zhu, L.J.T. Young, W.J. Mulle& R.D. Cardiff: CD44v3,8-10 is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J. Cell. Physiol.* 176,206-15 (1998)

103. H.F. Dvorak, L.F. Brown, M. Detmar & A. M. Dvorak: Review: vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* 146,1029-1039 (1995)

104. J. Folkman: Tumor angiogenesis. *Adv. Cancer Res.* 43,175-203 (1985)

105. J. Lesley, Q. He, K. Miyake, A. Hamann, R. Hyman & P. Kincade: Requirement for hyaluronic acid binding by CD44: A role for the cytoplasmic domain and activation by antibody. *J. Exp. Med.* 175,257-66 (1992)

106. W. L. Monsky, C.Y. Lin & W.T. Chen: A potential marker protease of invasiveness, is localized on invadopodia of human malignant melanoma cells. *Cancer. Res*. 54,5702-10 (1994)

107. S.C. Mueller & W.T. Chen: Cellular invasion into matrix beads:localization of beta 1 integrins and fibronectin to the invadopodia. *J. Cell Sci*. 99,213-25 (1991)

108. W.G. Jiang, M.C. Puntis & M.B. Hallett: Molecular and cellular basis of cancer invasion and metastasis: implications for treatment. *Bri. J. Surg*. 81,1576-90 (1994)

109. D.A. Lauffenburger & A.F. Horwitz: Cell migration: A physically integrated molecular process. *Cell* 84,359-69 (1996)

110. D. Boyd: Invasion and metastasis. *Cancer and Metastasis Reviews* 15,77-89 (1996)

111. W.T. Chen: Proteases associated with invadopodia, and their role in degradation of extracellular matrix. *Enzyme & Protein* 49,59-71 (1996)

112. P. Mignatti & D.B. Rifkin: Biology and biochemistry of proteinases in tumor invasion. *Physiol. Revs.* 73:161-95 (1993)

113. M. Polette, B. Nawrocki, C. Gilles, H. Sato, M. Seiki, J.M. Tournier & P. Birembaut: MT-MMP expression and localisation in human lung and breast cancers. *Virchows Archiv*. 428,29-35 (1996)

114. P. C. Brooks, S. Stromblad, L.C. Sanders, T.L. von Schalscha, R. T. Aimes, W.G. Stetler-Stevenson, J. P. Quigley & D.A. Cheresh: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvß3. *Cell* 85,683-93 (1996)

**Key Words:** CD44s, Ankyrin Binding, Hyaluronic Acid (HA), Cell Adhesion, $p185^{HER2}$, Src Kinases, Metalloproteinases (MMPs), Tumor Cell Growth, Tumor Metastasis

---

Send correspondence to: Dr. Lilly Y. W. Bourguignon, Department of Cell Biology and Anatomy, University of Miami Medical School, 1600 N. W. 10th Avenue, Miami, FL. 33101, Tel: (305) 243-6985, Fax: (305) 243-7166, E-mail**:** Lbourgui@mednet.med.miamai.edu