10

Hyaluronan Associated Inflammation and Microenvironment Remodelling Influences Breast Cancer Progression

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1. Introduction

1.1 The breast microenvironment

The breast is an organ composed predominantly of glandular, fatty, and fibrous tissues. Glandular tissue is composed of ducts lined by luminal epithelial cells that secrete milk, and is surrounded by a layer of myoepithelial cells that contract to release milk. Myoepithelial cells produce proteases, growth factors and growth factor receptors that contribute to remodelling during breast tissue expansion. Each duct is enclosed by a laminin-rich basement membrane and embedded in extracellular matrix (ECM). Mammary gland ECM and is a mixture of fibrillar proteins such as collagens, laminins, fibronectin, and polysaccharides such as heparin sulphate, chondroitin sulphate and hyaluronan (HA). These collectively provide the mechanical and structural support required for maintaining mammary tissue architecture and for storage of the soluble regulatory molecules needed for tissue homeostasis, plasticity, and remodelling. ECM promotes both the differentiated, homeostatic integrity of mammary tissue and is also a key determinant in branching morphogenesis, response-to-injury and pathological processes such as neoplastic disease. The importance of the ECM in determining homeostatic vs. tumourigenic events was originally demonstrated three decades ago by Beatrice Mintz, who showed that marked embryonic carcinoma cells injected into blastocysts do not give rise to tumours but instead contribute to normal tissue architecture. The same cells injected into adult mice develop into tumours (Mintz and Illmensee, 1975). Components of the microenvironment that support tumour progression have since been identified. For example, chick embryos infected with Rous Sarcoma virus express the oncogene v-src in every cell but tumours develop only at sites of wounding due to the accumulation of TGF-β1 (Weigelt and Bissell, 2008).

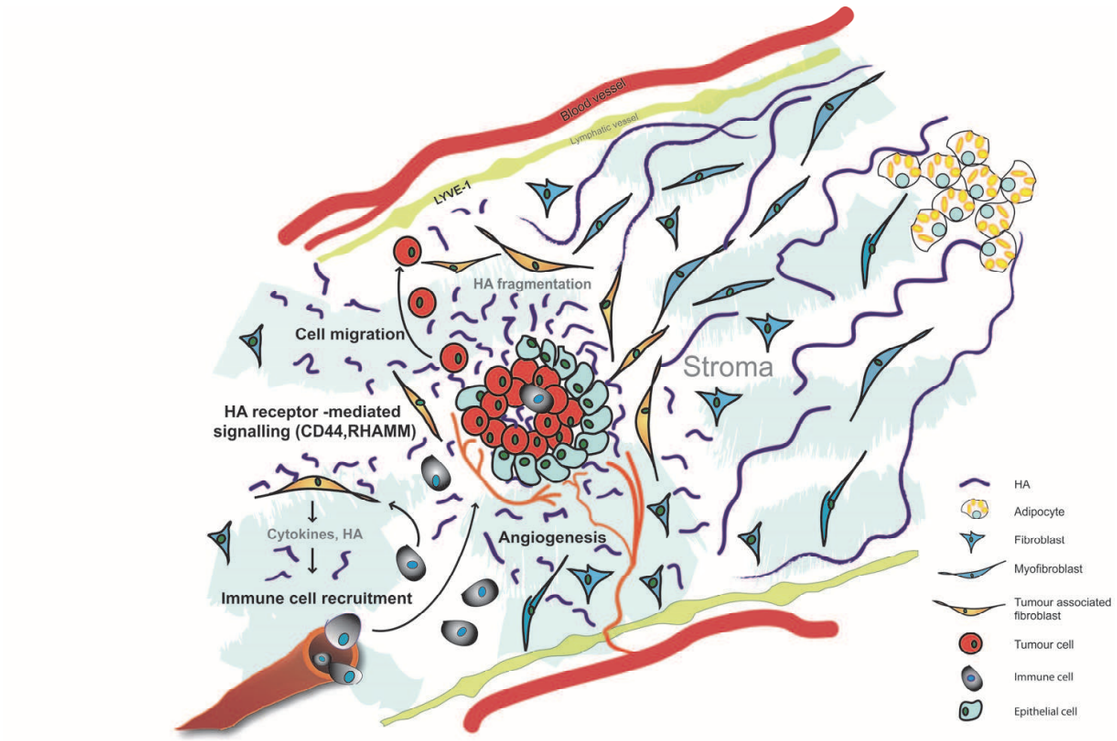

Fig. 1. Breast tumour microenvironment

Conversely, breast tumour cells can be reverted by blocking signalling through ECM receptors, including integrins (Turley *et al*., 2008) and HA receptors such as RHAMM (Hall *et al*., 1995). These and other studies have revealed a key role of ECM in initiating and sustaining breast cancer and introduced the novel concept that transformation can be a plastic rather than irreversible process. Specifically, increased HA accumulation in tumour cells or stroma is associated with poor outcome in Breast Cancer (BCA) (Tammi *et al*., 2008). These studies predict that HA is an important component of ECM that determines a homeostatic vs. tumourigenesis "switch".

2. HA biology

2.1 Biochemical properties

HA belongs to the glycosaminoglycan group of polysaccharides composed of disaccharide units of a hexose linked to a hexosamine. It consists of repeating units of *N*-acetyl glucosamine and β-glucuronic acid (Fig. 2). The native polymer consists of up to 10^6 to 10^7 non-branching disaccharide units. The functions of HA within the ECM and cells depend upon its molecular weight, the type of cell, and the HA receptor(s) that target cells express. High molecular weight HA (e.g. >200 kDa) is a major biomechanical factor in ECM, which contributes to tissue hydration and elasticity by providing a template for the assembly of macromolecular complexes. A well known example is the "bottle brush" complex of aggrecan and link proteins, which provides the visco-elastic nature of synovial fluid. HA fragments provide signalling functions and are usually present during the ECM remodelling that is associated with morphogenesis or disease. Regulated synthesis and degradation are key factors in maintaining a delicate balance between structural (homeostatic) and signalling

(wound and disease) functions of HA (Itano *et al*., 2008, Jiang *et al*., 2007, Veiseh and Turley, 2011). BCA cells are particularly adept at producing and responding to HA fragments. BCA cells produce increased levels of HA by increasing HA synthase expression, rapidly fragmenting HA as a result of increased Reactive Oxygen Species (ROS) production, and increasing hyaluronidase expression and release, and increasing expression and display of HA receptors to elevate the response to these fragments (Simpson and Lokeshwar, 2008, Toole and Slomiany, 2008, Veiseh and Turley, 2011).

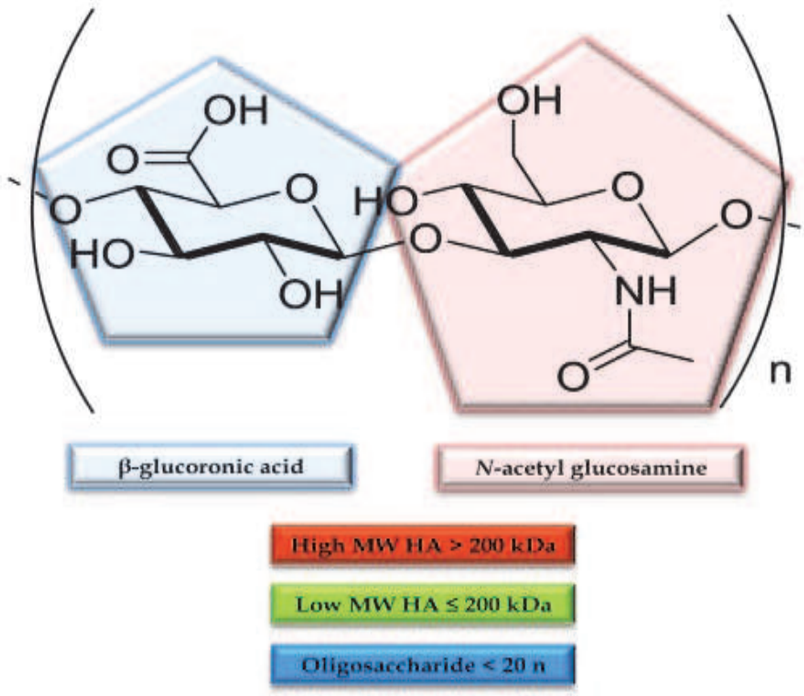

Fig. 2. HA structure and molecular weight ranges.

2.2 HA synthesis and tumourigenesis

HA is synthesized by three HAS isoforms, HAS1-3, which are located on different chromosomes but share from 57 to 80% sequence homology (Weigel *et al*., 1997, Lokeshwar and Selzer, 2008, Stern, 2008). The mature enzymes are multi-pass integral proteins, which are primarily located in the plasma membrane and catalyze polymerization of HA from the uridine diphosphate (UDP) sugars uridine diphosphate glucuronic acid (UDP-Glc-UA) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAC). Synthesis and secretion of HA occur concurrently, allowing for the rapid production and release of large polymers into the ECM (Weigel *et al*., 1997). There is some evidence that HASs are resident in endosomes, ER and the perinuclear membrane although whether or not these produce intracellular HA is not yet clear (Karousou *et al*., 2010, Vigetti *et al*., 2010). HAS1 and 2 are widely expressed throughout the embryo while HAS3 expression is more restricted, for example, to developing tooth-forming neural crest cells and hair follicles. Genetic deletion of HAS2 is embryonic lethal in mice due to severe defects in cardiac tissue development, whereas targeted disruption of the HAS1 or 3 alleles results in fertile viable animals with only minor

aberrations in tooth and follicle development (Weigel and DeAngelis, 2007). It is not fully understood why only HAS2 is absolutely required for organogenesis, but it has been suggested that it produces high molecular weight tissue HA while the other HASs produce the smaller HA sizes (Itano *et al*., 1999). There are differences in the mechanisms by which HAS isoform expression and enzyme activity are regulated that may be relevant to their functions and essential or non-essential roles in organogenesis (Tammi *et al*., 2008).

BCA cells use several mechanisms to rapidly control the synthesis and release of HA, thereby modifying their ECM, including substrate availability, gene expression, posttranslational control of enzyme activity, and differential response to cytokines and ECM signalling. The availability of UDP sugars can profoundly influence the yield of HAS enzymes (Kakizaki *et al*., 2004). This has been demonstrated by the use of 4-Methylumbelliferone (4-MU), which depletes intracellular levels of UDP-Glc-UA (Kakizaki *et al*., 2004) by serving as a glucuronidation substrate. It blocks HA production and reduces BCA tumourigenicty.

The genomic plasticity and instability of cancer cells often leads to chromosomal aberrations that can result in both de-regulation of gene expression and allele duplication. Chromatin breakpoint analysis using a BCA line revealed significant chromosomal rearrangements close to the HAS2 gene. These result in de-regulation of HAS2 expression and significantly higher HAS2 mRNA levels in transformed cells compared to normal breast cells (Unger *et al*., 2009). Detailed *in vitro* and *in vivo* studies of BCA lines and xenografts have provided numerous insights into the effects of genetically modifying HAS expression levels on HA concentration within the tumour and peri-tumoural stroma. Antisense inhibition of HAS2 in MDA-MB-231 BCA cells delays proliferation via a transient arrest of the cell cycle (Udabage *et al*., 2005). Knockdown of HAS expression also results in significant alterations in genes associated with HA metabolism. CD44 and HYAL1 expression are both down-regulated in response to antisense inhibition of HAS2. *In vivo*, MDA-MB-231 cells expressing antisense HAS2 do not form tumours in nude mice after 12 weeks, whereas the parental cell line readily establishes both primary and secondary tumours during this time. This clearly implicates tumour cell HA as a significant driver of BCA formation. Elevated HA accumulation within BCA peri-tumoural stroma is also a prognostic factor and appears to promote a microenvironment suitable for BCA growth. For example, HAS2-/- fibroblasts transplanted with BCA cells into the fat pads of NOD/SCID mice fail to recruit macrophages and promote angiogenesis to the same extent as HAS2+/+ fibroblasts. This defect results in decreased tumour volume (Kobayashi *et al*., 2010).

The expression of all three HASs is controlled by growth factors and cytokines. However, there appear to be subtle differences in the response of each isoform that depend upon the cell type. For example, PDGF and TGFβ induce HAS2 expression in fibroblasts but HAS1 or 3 expression in synoviocytes and keratinocytes, respectively (Karousou *et al*., 2010). H-Ras transformation increases only HAS2 expression in 3Y-1 tumour cells, while transformation with v-src or v-fos increases both HAS1 and HAS2 expression in the same cells (Itano *et al*., 2004). Posttranslational modification of HAS, including phosphorylation by PKC, PKA, and the ERK/ErbB2 MAPK pathways (Goentzel *et al*., 2006, Itano and Kimata, 2008) as well as mono-ubiquitination (Karousou *et al*., 2010) also affects HAS activity. HAS3 serine phosphorylation is enhanced upon treatment with a PKC activator (Goentzel *et al*., 2006). All three HAS isoforms expressed by SKOV3 ovarian cancer cell line are phosphorylated by

ERK1,2 in response to treatment with Heregulin (Bourguignon *et al*., 2007) and mono-ubquitination of K190 on HAS2 rapidly inactivates this enzyme (Karousou *et al*., 2010).

2.3 HA fragmentation and its role in tumourigenesis

In addition to HAS1-3 expression, the amount and polymer size of HA are also affected by reactive oxygen species (ROS) and secreted hyaluronidases (HYALs), which fragment HA to various sizes. Significant levels of ROS can be generated during times of oxidative stress and these are considered critical in cancer initiation, promotion and progression (Karihtala *et al*., 2007). ROS are produced in response to extracellular stimuli such as bacterial infections and environmental toxins, but can also be produced by cellular metabolism (Yu *et al*., 2011). Five HYALs fragment HA: HYAL-1-3, PH-20 and HYAL-5. The HYALs differ in their cellular location and enzymatic properties. HYAL-1 and 2 are the major HYALs produced by somatic tissues whereas HYAL-3 is expressed mostly in bone marrow and testes. Both PH-20 and HYAL-5 expression are normally restricted to testes but PH20 is aberrantly expressed in BCA (Stern, 2008). HYAL-1 and 2 cooperate to degrade HMW HA in a coordinated fashion. HYAL-2, which is GPI anchored to the cell surface, degrades extracellular HA to fragments of 20 kDa, which are then taken up into endocytic vesicles. HYAL-1 present in the lysosome further degrades intracellular HA into tetrasaccharides (Tammi *et al*., 2001, Stern, 2008, Simpson and Lokeshwar, 2008). Coordinated breakdown of HA by HYALs increases the rate of HA metabolism and this appears to be an important factor in tumourigenesis (Veiseh and Turley, 2011). For example, co-expression of HAS3 and HYAL-1 increases the aggressiveness and spread of prostate cancer cells compared to expression of either alone (Bharadwaj *et al*., 2009). In BCA, HYAL-1 and HYAL-2 are often coordinately overexpressed compared to non-malignant breast tissue. Knockdown of HYAL-1, which is overexpressed in MDA-MB-231 and MCF-7 BCA lines, reduces tumour xenograft size (Tan *et al*., 2010).

3. HA receptors detect oligosaccharides and fragments: Control of key signalling pathways by HA fragments

3.1 CD44

CD44 is a class I transmembrane receptor, which binds to HA via a link domain and is expressed by a variety of cells, including fibroblasts, endothelial and epithelial cells, smooth muscle, and haematopoietic cells. A vital role of CD44 is recruiting cells, including immune cells and fibroblasts, to sites of inflammation through HA-mediated signalling. Under homeostatic conditions, CD44 is in a low HA binding state, but during injury and tumourigenesis its binding affinity is increased and it mediates the inflammatory and tissue repair responses (Thorne *et al*., 2004, Naor *et al*., 2008). CD44 is expressed as many different isoforms due to extensive splicing in a region proximal to the transmembrane domain (Thorne *et al*., 2004). The smallest CD44 isoform, CD44s (standard form), skips this variable region. The role of CD44s and variants in BCA progression is still controversial. For example, CD44s expression in $CD44^{low}$ MCF-7 human BCA cells results in xenograft metastasis to the liver (Ouhtit *et al*., 2007) while CD44-/- mice develop more lung metastases than wildtype animals in response to polyomavirus middle T (Lopez *et al*., 2005). Importantly, a recent study by Brown *et al*. (2011) demonstrated that CD44s expression is elevated and required for epithelial-mesenchymal transition of immortalized human mammary epithelial cells and for recurrence of HER2/*neu* induced murine mammary tumours (Lopez *et al*., 2005). HA synthesis is elevated in CD44+ BCAs

compared to CD44- and both CD44+ and HER2+ BCAs are amongst the most aggressive and invasive subtypes of BCA with poor prognosis. Expression of variant exons, in particular exon v6, is associated with increased *in vitro* cell migration and invasion of human BCA cells (Herrera-Gayol and Jothy, 1999). Although CD44v6 expression has been correlated with multiple clinicopathological features (primary tumour size, axillary nodal status, histological grade and pTNM stage) it is not an independent prognostic factor (Ma *et al*., 2005). A study by Rys *et al*. (2003) found a correlation between the expression of CD44 v3 and the presence of BCA metastasis. Additionally, high CD44s expression correlates with increased disease free survival in node negative invasive BCA (Diaz *et al*., 2005). The controversies surrounding CD44 and its role in BCA progression may be caused by a limited number of patient samples in some of these studies, heterogeneity of BCA, and CD44 expression by cancer stem cells. The latter, in particular, has raised much recent interest in CD44 since several groups have identified CD44 as a potential marker for BCA stem cells. This is a highly tumourigenic population of cancer cells that, although only representing a small percentage of cells in the tumour, are thought to be responsible for tumour recurrence, metastasis and treatment failure. Aggressive BCA and BCA tumour progenitor cells have enhanced CD44 expression, associated with an increase in HA synthesis and CD44-HA binding affinity (Heldin *et al*., 2008).

In BCA cells, HA triggers CD44 interactions with a variety of signalling mediators involved in cell proliferation, migration and chemo-resistance. Ankyrin is a membrane-associated component of the cytoskeleton that is involved in regulation of cytoskeleton turnover and IP3 receptor-mediated regulation of intracellular Ca^{2+}. CD44-HA interactions induce CD44-ankyrin coupling and modify receptor-dependent Ca^{2+} mobilization (Bourguignon *et al*., 2008). CD44 also localizes ankyrin and IP3 receptor to lipid rafts, which are cholesterol and caveolin rich signalling microdomains in the plasma membrane (Fig. 3). The Rho GTPases, RhoA, Rac and CDC42, are key regulators of cell migration and HA stimulates RhoA in BCA cells. RhoA activity is regulated by RhoGEF, a guanine nucleotide exchange factor that forms a complex with CD44 in BCA cells. One of the downstream RhoA targets, ROK, phosphorylates the cytoplasmic domain of CD44 thereby increasing CD44-ankyrin interactions. Other targets of ROK are myosin phosphatase and myosin light chain, two important mediators of actin-myosin dependent membrane ruffling required for cell migration. HA also activates the PI3 kinase/AKT pathway: Gab-1 phosphorylation by ROK stimulates PI3 kinase and AKT activation, leading to increased cell proliferation, invasion and cytokine production (Bourguignon *et al*., 2008). Additionally, ROK phosphorylates and activates NHE1, a Na^{+}-H^{+} exchanger, causing intracellular and extracellular acidification leading to HYAL-2 driven HA degradation, ECM breakdown and tumour progression. CD44-HA interactions stimulate signalling through Rac1, another RhoGTPase, via the GEF Tiam1. In MDA-MB-231 cells, CD44-HA interactions also activate c-Src kinase resulting in activation and nuclear translocation of the transcription factor Twist, miR-10b expression and down-regulation of the tumour suppressor gene HOXD10 (Bourguignon *et al*., 2010 Toole, 2004). CD44 undergoes sequential proteolytic cleavages resulting in the release of its ectodomain from the cell surface and formation of a CD44 intracellular domain fragment, which is translocated to the nucleus, acting as a transcription co-regulator (Nagano and Saya, 2004). CD44 ectodomain cleavage is mediated by MT1-MMP and is stimulated by multiple factors, including HA fragments and TGF-β (Kuo *et al*., 2009, Sugahara *et al*., 2006) which, contribute to tumour cell migration and invasion (Fig. 3).

3.2 RHAMM/HMMR

Receptor for HA Mediated Motility (RHAMM/HMMR) belongs to a group of proteins that are found intracellularly as well as extracellularly. RHAMM does not contain a transmembrane domain or classical export signal and is likely exported through an unconventional mechanism that does not involve the Golgi/ER. RHAMM is expressed as multiple isoforms and one of these, an N-terminal truncation that lacks the first 163 aa residues, is transforming in mesenchymal cells (Hall *et al*., 1995). On the cell surface, RHAMM interacts with HA and forms complexes with transmembrane receptors such as CD44, PDGFR, and RON (Maxwell *et al*., 2008). Interestingly, CD44 surface display is reduced in mesenchymal cells isolated from RHAMM-/- mice, demonstrating functional interplay between these two HA receptors (Tolg *et al*., 2006). RHAMM is elevated in most types of cancer in particular breast, ovarian, and prostate cancer, as well as in MM, AML and CML. In BCA, RHAMM is a tumour marker, novel susceptibility factor and prognostic factor for poor outcome (Maxwell *et al*., 2008). Consistent with these clinical correlations, RHAMM has tumourigenic properties in experimental systems that have been linked to its ability to bind HA. In BCA cells, RHAMM/CD44/HA complexes sustain phosphorylation and activation of the Ras/MAPK (ERK1,2) signalling pathway, leading to BCA progression and constitutively high rates of motility and invasion (Hamilton *et al*., 2007). The relationship between RHAMM and ERK1,2 activation has recently been confirmed in BCA samples where concomitant upregulation of phosphorylated ERK1,2 and RHAMM in tumour samples correlates with a high tumour grade (Ward C., in preparation). Intracellularly, RHAMM binds directly to tubulin and is involved in regulation of microtubule stability and turnover as a result of its association with ERK1,2. In mesenchymal cells, the absence of RHAMM increases microtubule stability resulting in reduced cell migration and aberrant mitotic spindle formation (Tolg *et al*., 2010, Groen *et al*., 2004). RHAMM interacts directly with ERK1, inferring that RHAMM may act as a scaffolding protein that directs ERK1 to its substrates including microtubule associated proteins that regulate microtubule stability (Tolg *et al*., 2010). Interestingly, RHAMM expression is downregulated by p53, an important tumour suppressor gene, suggesting that RHAMM may be involved in p53 loss-induced tumour progression (Buganim and Rotter, 2008, Godar and Weinberg, 2008, Sohr and Engeland, 2008). RHAMM also acts on the BRCA1, pathway and may play an important role in BCA tumours arising from loss or inactivation of BRCA1 (Joukov *et al*., 2006)

3.3 TLR2 and TLR4

Toll like receptors (TLR) are part of a cellular defence mechanism that is based on pattern recognition. TLRs recognize and bind bacterial lipopolysaccharides, DNA, and, in the case of TLR2,4, small HA fragments. In general, HA-TLR2,4 interactions control innate immunity through several mechanisms. For example, TLR 2,4 activation results in cytokine and chemokine release and leads to expression of metalloproteinases (MMPs) in immune cells (Voelcker *et al*., 2008). Versican, which is associated with poor prognosis and relapse in BCA, interacts with HA polymers to form cord-like structures that link TLR2 on endothelial cells and fibroblasts. This, in turn, causes the secretion of pro-inflammatory cytokines (Theocharis *et al*., 2010). HA-TLR2,4 interactions also stimulate NFκB signalling and activate TNFα. In BCA cells, TLR 2,4 interact with CD44 and act as co-receptors to stimulate signalling through HA and CD44 regulated pathways which may play a role in breast

tumour cell migration/infiltration. The human BCA cell line MDA-MB-231 expresses mainly TLR4, and siRNA mediated knock-down of TLR4 significantly reduces cell survival and expression of the cytokines Il-6 and Il-8, suggesting that TLR4 is a promising target for BCA therapy (Yang *et al.*, 2010).

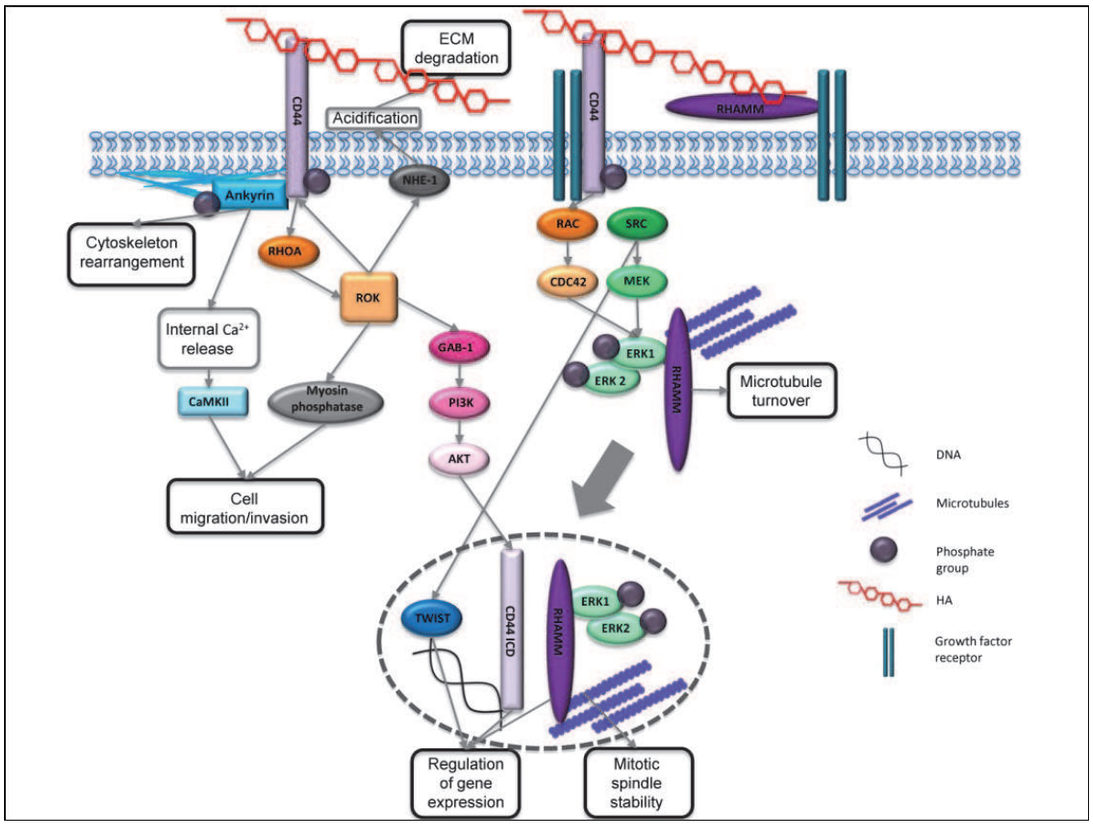

Fig. 3. HA initiates the signalling of RHAMM and CD44 regulated pathways, resulting in a variety of pro-tumourigenic outcomes.

3.4 LYVE-1

HA links the two main functions of the lymphatic system: draining of interstitial fluids and immune surveillance. These functions are achieved through its interaction with the receptor LYVE-1, present in lymphatic endothelia (Jackson, 2009). LYVE-1 is a type I integral membrane polypeptide that exhibits high homology with CD44 (Banerji *et al.*, 1999) and is a homeostatic HA receptor required for liver and lymphatic vessel formation. Its expression does not change as frequently in malignancy as HA receptors involved in response to injury, for example CD44 and RHAMM/HMMR. This does not rule out a role in injury and tumour progression however, as lymphangiogenesis is an important processes in both events, and elevated accumulation of HA in stroma results in lymphangiogenesis *via* signalling through LYVE-1 (Gale *et al.*, 2007).

To further demonstrate the association of LYVE-1 with tumour dissemination through the lymphatic system, (Du *et al.*, 2010) expressed LYVE-1 in COS-7 kidney cells and performed cell adhesion assays with the BCA cell line HS-578T which produces HA. These two cell lines had enhanced adhesion over the control cells, COS-7 not expressing LYVE-1. This

suggests that LYVE-1 plays a role in tumour cell adhesion which is dependent on HA-LYVE-1 interaction. Apart from its effect on tumour cell adhesion, LYVE-1 has also been proven to be a prognostic factor in tongue squamous cell carcinoma and decreased levels of LYVE-1 in the invasive front of tumours predicts cervical lymph node metastasis (Matsumoto *et al*., 2010).

4. HA expression and signalling in different cell types and its relationship to BCA

4.1 HA, inflammation, and the role of inflammatory cells in tumourigenesis

4.1.1 Macrophages

HA has a major role in macrophage biology during inflammation, wound repair, and tumourigenesis and at least part of the detrimental effects of HA accumulation during tumourigenesis is due to the activation of tumour associated macrophages (TAMs). For instance, TAMs preferentially traffic to stromal compartments formed within HA producing tumours (Kobayashi *et al*., 2010). Macrophages are classed into type 1 and 2 according to the adaptive immune polarization with which they associate. Type 1 macrophages are antigen-presenting cells which promote the cytotoxic response, resulting in tumour cell killing. Type 2 macrophages, however, are classically associated with tissue remodelling, angiogenesis, and scavenging/phagocytosis. TAMs are similar to type 2 polarized macrophages which have decreased or inhibited cytotoxic activity (Mytar *et al*., 2003). Kuang *et al*. (2007) found that overexpression of HAS2 was able to polarize macrophages towards a malignant TAM phenotype. Additionally, exposure to solid tumour cell culture supernatant elicits a pro-inflammatory response in monocytes and their subsequent TAM-like polarization, showing that the tumour cells themselves are responsible for the immunosuppressive macrophage phenotype observed in solid tumours (Kuang *et al*., 2007). The importance of TAM recruitment in BCA dissemination was additionally illustrated by CSF-1 null mice crossed with the MMTV transgenic mouse model of BCA. In these mice, a failure to recruit macrophages into the primary tumour results in delayed primary tumour invasion and metastasis to the lungs compared to wildtype MMTV mice. The addition of exogenous CSF-1 rescues macrophage recruitment and restores tumour and metastasis development to baseline levels (Lin *et al*., 2001). After injury, or during tissue inflammation, small fragments of HA associate with TLR4 and control macrophage cytokines and chemokines (Termeer *et al*., 2000). For example, BCA cell associated HA promotes the production of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-12, as well as ROS, by TAMs, an effect which can be alleviated by either blocking CD44 receptors on monocytes, or by the addition of non-BCA cell associated HA (Mytar *et al*., 2001). HA regulation of pro-inflammatory cytokine production also occurs in monocytes pre-exposed to a variety of solid tumour cell types and culture supernatants, including the BCA line MCF-7 (Mytar *et al*., 2003, del Fresno *et al*., 2005), modulating the IRAK family of NFκB regulatory molecules, this further downregulating TNF-α and IL-12 production. HA-mediated CD44 cross-linking induces this activity and is prevented by the addition of exogenous HYAL (Mytar *et al*., 2003). TAMs are recruited and regulated in response to NFκB, whose activation is often HA-mediated through TLR4 (del Fresno *et al*., 2005) and NFκB overexpression results in tumour metastasis (Mantovani *et al*., 2007). Nitric oxide, which is the product of nitric oxide synthase 2 (NOS), is stimulated by hypoxia and CSF-1, among others, and is a signalling molecule integrated within the NFκB inflammatory pathway. NOS2 signals the upregulation of CD44,

c-Myc, MMP, and VEGF, which are all involved in promoting tumourigenesis. In BCA, NOS2 expression within tumour cells themselves is correlated with increased tumour grade and angiogenesis (Ambs and Glynn, 2011).

4.1.2 T Cells

T cells orient their cytoskeleton and migrate towards sites of inflammation, such as those present in a tumour microenvironment (TME), in a PKC-dependent manner as a direct result of CD44 crosslinking by HA (Fanning *et al*., 2005). In BCA, CD8+ T cells are most predominant in advanced cancer stages where their presence in proliferating tumours is a good prognostic indicator. T cells are able to participate in either a Th1 or Th2 polarized immune response and, when polarized to a Th1 response, they express and secrete IFNγ, TGFβ, TNFα, IL-2, resulting in cytotoxic cooperation (T cells and M1). Th2 polarized CD4+ T cells secrete IL-4,5,6,10,13 which leads to an increase in B cell mediated immunity (DeNardo and Coussens, 2007). Because of the anti-tumour effects of T cells, the activation of cytotoxic T cells against HA receptors as immunotherapy in leukemias is currently undergoing clinical trials and will be discussed later in this chapter. On the other hand, the presence of CD4+ T cells correlates with disease progression and metastasis; however, it has been shown by different groups that CD4+ T cells are crucial for mounting an immune response against cancer. For example, tumour growth of EL4 lymphoma cells inoculated into mice is inhibited by the presence of dendritic cells primed against RHAMM protein. This interaction, however, is dependent on CD4+ T cells, as the effect of DC killing of the tumour is significantly reduced with a reduced CD4+ T cell population (Fukui *et al*., 2006). Furthermore, Rakhra *et al.* (2010) showed that in ALL and B-cell leukemia, CD4+ cells were necessary for sustained tumour regression. In mouse models, inhibition of MYC or BCR-ABL rescues tumours from oncogene addiction; however, tumours regress in the presence of TSP-1 induced CD4+ T cells, and knockdown of TSP-1 impairs this ability (Rakhra *et al*., 2010).

Regulatory T cells (Treg; CD4+/CD25+/FOXP3+) play controversial roles in tumour progression and can have both anti- and pro-tumourigenic effects, depending on the chemokines or cytokines produced and the type of solid tumour. Treg cells may be activated in an immunosuppressive manner, preventing cytotoxic immune responses, and allowing the tumours to evade immune attack. For example, in CLL, a large Treg population dampens specific CD8+ T cell responses against tumour associated antigens (Giannopoulos *et al*., 2010). The same may be true for solid tumours. When coordinated, however, with a high T cell density, they may indicate good prognosis and inhibition of metastasis (Camus *et al*., 2009, Carreras *et al*., 2006).

4.1.3 B Cells

Immunoglobulin deposition by B cells in BCA stroma can be detrimental to disease progression and the accumulation of autoantibodies produced by B cells and deposited in the stroma correlates with poor prognosis (Fernandez Madrid *et al*., 2005). An increase in serum IgG correlates with an increase in TAM numbers which, in turn, promotes angiogenesis in mouse mammary carcinoma, a process associated with poor clinical outcome. A proposed mechanism for the involvement of TAMs in B cell processes is the phagocytosis of IgG by macrophages. IgG engages Fcγ receptors, which stimulates VEGF secretion, increases angiogenesis and promotes tumour growth rate (Barbera-Guillem *et al*.,

2002). The majority of stromal B cells localize to perivascular regions within tumours and chronic B cell activation promotes tumours by recruiting macrophages and activating an innate immune response. However, the role of B cells in BCA progression is complicated since, for example, B cells may also recruit antigen presenting cells, such as CD8+ T cells and dendritic cells which help to eradicate neoplasms.

4.1.4 Dendritic cells and mast cells

Dendritic cells (DC) can also exhibit HA dependent characteristics that either promote or inhibit tumourigenesis. HA or chondroitin sulphate, in conjunction with CSF-1, activate DC from an immature to differentiated state via an NFκB regulated process, illustrating the importance of HA in eliciting an immune response (Yang *et al*., 2002). Pedroza-Gonzalez *et al* (2011) recently showed that human BCA produces thymic stromal lymphopoietin (TSLP) which induces expression of OX40L on DCs, polarizing them towards a Th2 inflammatory response. *In vitro* this drives the production of IL-13 and TNF by Th2 polarized T cells (Pedroza-Gonzalez *et al*., 2011). DC also become tumour insensitive and, as a result, do not mature and differentiate into cytotoxic cells. Furthermore, HA fragment build ups are at least partly responsible for preventing DC maturation in tumour bearing animals (Kuang *et al*., 2008).

In BCA, c-kit expression by mast cells, a protein which is usually only present in specific tissue types, such as germ cells, predicts primary tumour recurrence (Khazaie *et al*., 2011). However, an abundance of stromal mast cells in invasive BCA is associated with good prognosis (Rajput *et al*., 2008). The mast cell line HMC-1 expresses high levels of CD44s and, through an interaction with HA, adheres to stromal tissue (Fukui *et al*., 2000). Therefore, in both mast cells and DC, a CD44-HA interaction may result in anti-tumour responses.

4.2 HA regulation of a pro-inflammatory environment by non-immune cells

4.2.1 Breast cancer cells and their contribution to a pro-inflammatory environment

BCA cells secrete a variety of cytokines and chemokines which promote tumour progression. Studies by Tafani *et al*. (2010), showed that MCF-7 cells upregulate pro-inflammatory gene transcription and translation *in vitro*, and a pro-inflammatory gene expression profile can be seen in human BCA tumours even in the absence of an immune infiltrate. This illustrates that BCA cells themselves contribute to the pro-inflammatory/pro-tumourigenic TME. One or both of HER2 and ERα, which are often expressed on BCA cells, promote the expression and secretion of CXCL8 (IL-8) through the PI3K and ERK pathways. CXCL8 is a pro-angiogenic chemokine and secretion of CXCL8 by the MCF7 BCA line (which express both HER2 and ERα) is additive upon stimulation of both of these receptors (Haim *et al*., 2008). The pro-inflammatory chemokines CCL2 and CCL5 are also secreted by BCA cells (Ben-Baruch, 2003) and expression and secretion of all three chemokines requires HA fragment/CD44 interactions on TAMs, tumour associated fibroblasts (TAFs) and BCA tumour cells. Both CCL2 and CCL5 are monocyte-recruiting chemokines and their expression in BCA tumours is correlated with poor prognosis, and in the case of CCL2, pro-angiogenesis factors and vascular invasion (Soria and Ben-Baruch, 2008). TNF-α secretion by TAMs activates a positive feedback loop in BCA tumour cells, stimulating further secretion of growth promoting chemokines (Ben-Baruch *et al*., 2003). Eck *et al* (2009) also showed that conditioned media from BCA cells stimulates the expression of pro-inflammatory genes in normal mammary fibroblasts, polarizing them towards a TAF phenotype. Furthermore, TAF migration is increased, along with the secretion of MMP-1 and CXCR4 (IL-1/SDF-1 receptor), both of which are important factors in BCA progression (Eck *et al*., 2009).

4.2.2 HA/stromal fibroblast/epithelial cell interaction and tumour progression

To begin to define the role played by TAFs in tumour progression, Micke *et al.* (2007) conducted cDNA microarray analyses comparing the transcriptome of TAFs from basal cell carcinoma with normal dermal fibroblasts (Micke *et al*., 2007). This study showed that TAFs overexpress multiple growth factors such as PDGF, EGF, and VEGF, chemokines such as SDF1 and CXCL12 and matrix proteins such as MMP11, LAMA2 and COL5A2. In fact, these TAFs are known to secrete IGF-2, FGF-7, TGF-β, leptin, and NGF, which bind to their cognate receptors on BCA cells to stimulate HA production (Szabo *et al*., 2011). This then promotes expression of cytokines such as TGF-β that attract and stimulate TAFs to proliferate. This paracrine effect is a positive feedback mechanism, because proliferating TAFs secrete additional growth factors, cytokines, chemokines, and MMPs that sustain BCA transformation and promote BCA progression. Additionally, VEGF, produced by TAFs, and HA oligosaccharides induce angiogenesis. HA itself also impairs immune surveillance, and/or activates TAMs and neutrophils that have tumour enhancing potential. Overexpression of HAS in a non-transformed rat fibroblast, 3Y1, increases high MW HA production and the resultant pericellular HA coat provides cells with a proliferation advantage that is accompanied by loss of contact inhibition of growth. This is achieved through HA-mediated activation of PI3 kinase. Lower MW HA also increases proliferation in these cells but has no effect on the HA matrix (Itano *et al*., 2002). TAFs affect not only BCA cells but also normal cells in which the tumour is embedded. For example, TAFs induce stem cell-like behaviour and aberrant differentiation in normal fibroblasts, which can affect BCA progression. TAFs promote the expression of stem-cell markers such as Oct4 and Sox2 in 3T3 cells (Szabo *et al*., 2011) and stimulate trans-differentiation of normal fibroblasts into myofibroblasts when they are confronted with primary BCA cells.

4.2.3 HA, adipocytes and adipose tissue

Adipose tissue in mammary glands is important for its secretory and endocrinal functions as well as metabolism, energy homeostasis and stem cell compartment. Adipocytes contribute to the mammary tissue ECM and this effect is at least partly regulated by HA. There are not many studies that focus on HA and its relationship to adipocytes, however, the importance of this polysaccharide on adipose-stromal interactions in the breast tissue is becoming apparent. For example, HA increases the crosslinking of collagen-HA matrices, supports proliferation and differentiation of pre-adipocytes and induces a higher proportion of cycling cells (Davidenko *et al*., 2010).

Chen *et al.* (2007) also showed that HA extends the lifespan, reduces cellular senescence and enhances differentiation potential of murine adipose-derived stromal cells (mADSCs) *in culture*. Collectively, these results provide preliminary evidence for a key role of HA in controlling the adipose component of the breast tissue and allude to a potential role of this regulation in BCA (Chen *et al*., 2007).

5. HA regulates mammary cell functions that promote BCA progression

5.1 Cell migration

Considerable evidence indicates that HA fragmentation is required for immune cell trafficking, fibroblast migration, stem cell migration from niches to the wound site and endothelial cell migration during angiogenesis. For example, acellular hydrogel matrix composed of fibronectin and HA, which simulates a wound microenvironment, supports

proliferation, migration and spreading of human dermal fibroblasts *in vitro*. HA seems to regulate motility via a variety of mechanisms that include indirect and direct effects on the migrating cell population. An example of an indirect effect was provided by a study of the role of HA on fibroblast migration using a porcine skin wound model. The wound matrix, which contained HA, promoted cell migration and recruitment of fibroblasts. This was shown to be in part due to wounding produced HA, which promotes collagen fibril formation, thus indirectly affecting cell motility (Docherty *et al*., 1989). Direct effects of HA on cell motility can result from its structural properties and from its ability to activate motogenic signalling cascades such as ERK1,2 and PI3 kinase. Both of these effects have been related to an association of HA with cell surface receptors such as CD44 and RHAMM. For example, extracellular HA accumulation induces penetration of stromal cells by increasing turgidity and hydration or disrupting cell-to-cell junctions. These effects may be a result of interactions with CD44 and RHAMM (Itano *et al*., 2008). HA fragments bind to CD44 and/or RHAMM to induce activation of MAPK (ERK1,2) that results in enhanced BCA cell migration and invasion (Hamilton *et al*., 2007). Moreover, upon HA-mediated activation of PI3 kinase, increased HAS2 production induces faster migration in scratch wound assays (Itano *et al*., 2002).

5.2 Angiogenesis

Hypoxic conditions within tumours require neovascularisation of the microenvironment for the tumour to continue to grow and metastasize. Hypoxia, a condition often found within the TME, induces the activation, as seen by nuclear translocation, of either or both of NFκB and HIF-1α. This effect has been shown both *in vitro* in MCF-7 BCA cells, and *in vivo* (Tafani *et al*., 2010). Invasion, migration, and proliferation of endothelial cells, as well as tissue remodelling, are essential processes during angiogenesis, which directly and indirectly help to promote tumour growth and metastasis. Necrotic cells, which have died as a result of hypoxia, also release chemokines that recruit macrophages and a pro-inflammatory response conducive to tissue remodelling. Hypoxia may produce ROS which in turn cause HA fragmentation and Noble *et al.* (1996) showed that NFκB transcription in macrophages is activated by HA fragments (Noble *et al*., 1996). Later, Rockey *et al.* (1998) were the first to show in hepatocytes that HA activation of NFκB induces NOS2 production, which can be synergistically increased in the presence of cytokines such as IFN-γ (Rockey *et al*., 1998). It has since been shown that HA fragments activate the NFκB pathway through TLR4 in both DC and macrophages (Termeer *et al*., 2002). Hypoxia induced activation of HIF-1α and NFκB induces pro-inflammatory gene expression and both mRNA and protein levels of inflammatory mediators such as RAGE, PTX3, NOS2, COX2, and CXCR4 are increased. Increased expression of CXCR4, which is the receptor for SDF-1, is seen on MCF-7 cells subjected to hypoxic conditions (Tafani *et al*., 2010). This increases the migratory and invasive capacity of these cells, which are usually non-invasive. In these same studies it was found that nuclear translocation of NFκB is at least partly dependent on HIF-1α, indicating that it may be under hypoxic regulation, as inhibition of HIF-1α decreases nuclear localisation of NFκB, and in turn RAGE and P2X7R expression, inhibiting cell invasion (Tafani *et al*., 2010).

In general, high MW HA inhibits angiogenesis while fragments promote angiogenesis. Overexpression of HA and HYALs has been linked to an increase in angiogenesis in several types of cancers including breast (Tan *et al*., 2010), bladder (Lokeshwar *et al*., 2000, Golshani

et al., 2008), prostate (Ekici *et al*, 2004, Bharadwaj *et al*., 2007), and endometrial (Paiva *et al*., 2005). Koyama *et al.* (2007) demonstrated that an increase in HAS2 expression by genetic modifications in a mouse model of BCA causes a higher incidence of adenocarcinoma accompanied by an increase in angiogenesis (Koyama *et al*., 2007). An increase in HA by overexpression of HAS2 in transgenic mice induces a more aggressive BCA phenotype and an increase in blood and lymphatic vessels (Kobayashi *et al*., 2010). In these tumours, the stromal cells also secrete a variety of pro-angiogenic factors. Furthermore, HA concentration in stroma and blood vessels is increased, as well as the amount of small HA fragments.

The pro-angiogenic effects of HA fragments result from the display of CD44 and RHAMM (Wang *et* al., 2011, Slevin *et al*., 2007) on the surfaces of endothelial, BCA or leukocyte cells. and Interaction of HA fragments with these cells produces the factors required for stimulating endothelial cells to form new blood vessels. HA fragments stimulate endothelial cell proliferation, migration and tube formation. Increased expression of HYALs in conjunction with MMPs and Cathepsin-D induce a more invasive phenotype in the endothelial cell line ECV-304 as detected by matrigel invasion assay (Wang *et al*., 2009). Additionally, pro-inflammatory cytokines, secreted by leukocytes activated by CD44-HA mediated interactions, stimulate endothelial cells to produce HA. When HUVEC cells are stimulated with IL-1B, TNF-α and β1, they secrete HA. CD44-HA interaction stimulates early morphogenic events, such as tube formation and proliferation in HUVECs (Wang *et al*., 2011). Furthermore, HA works synergistically with macrophage recruitment to promote vascular formation and HA in the stroma promotes lymphangiogenesis at the invasive tumour front in BCA through the activation of endothelial LYVE-1 (Itano *et al*., 2002).

6. HA and multi-drug resistance in BCA

Most tumours initially respond to chemotherapy treatment but later acquire resistance, resulting in treatment failure and tumour recurrence. Some mechanisms by which tumour cells acquire resistance include inhibition of apoptosis, stimulation of cell proliferation and enhanced expression and activity of drug export pumps, particularly ATP driven pumps (ABC transporters), which reduce the intracellular, and therefore active, concentration of several chemotherapeutic agents. HA fragments augment expression and activity of MDR1, a member of the ABC drug transporter family, in primary BCA cells (Toole and Slomiany, 2008). This HA induced upregulation involves the Akt/PI3 kinase signalling pathway and is CD44 dependent. CD44-HA interactions stimulate MDR1 expression via multiple signalling mechanisms including epigenetic gene expression regulation. CD44-HA binding results in activation of PKCε as well as increased phosphorylation and nuclear translocation of Nanog, a stem cell specific transcription factor. Moreover, interaction of Nanog with Stat-3 in the nucleus increases Stat-3 regulated gene expression, resulting in increased expression of MDR1. Activation of Nanog also results in production of the micro RNA miR-21 and down-regulation of PDCD4, a tumour suppressor protein (Bourguignon *et al*., 2008, 2009). CD44-HA interaction increases an association between MDR1 and the cytoskeletal protein ankyrin, resulting in enhanced drug export (Bourguignon *et al*., 2008). Additionally, CD44-HA interactions upregulate the expression of the histone acetyl-transferase, p300, inducing the acetylation of β-catenin and NFκB. This stimulates expression of MDR1 and the anti-apoptotic protein, Bcl-x_L (Bourguignon *et al*., 2009). It is very likely that BCA tumours with high HA metabolisms are also highly resistant to treatment with drugs that can be exported by MDR1.

7. HA and receptor antagonists in clinical trials

Since it is evident that HA and its receptors play an important role in BCA and other tumours, it is unsurprising that reagents blocking HA metabolism are being assessed as therapeutic agents in certain types of cancer. In pre-clinical models, Kultti *et al. (*2009) demonstrated that the HAS inhibitor 4-MU (4-Methylumbelliferone) specifically depletes intracellular levels of UDP-Glc-UA (Kakizaki *et al*., 2004) by serving as a glucoronidation substrate in A2058 melanoma cells, MCF-7, MDA-MB-361 BCA cells, SKOV-3 ovarian, and UT-SCC118 squamous carcinoma cells. Additionally, Lokeswar *et al.* (2010) used 4-MU to block growth of human prostate cancer cell line xenografts in immunocompromised mice. 4-MU induces apoptosis in these tumours and also strongly inhibits cell proliferation, motility and invasion. These effects can be reversed by addition of HA, which demonstrates that, although 4-MU does not specifically block HAS and has other off target effects, its effects on tumour cell growth result from inhibition of HAS (Ekici *et al*., 2004).

HA has also proven to be a good adjunct therapeutic option *in vivo* in human cancers since it promotes targeting of active anti-cancer compounds. For example, when patients with Calmette-Guérin refractory bladder cancer were included in a Phase I clinical trial using Paclitaxel-HA (ONCOFID-P-B™) for treatment of their cancers, 60% of the patients treated exhibited a clinical response with minimal toxicity reported (Bassi *et al*., 2010). HA has been successfully used to carry/target other chemotherapeutics, thus reducing cytotoxic side effects of the active drug. Hyung *et al.* (2008) demonstrated the efficacy of HA-coated drug carriers by delivering doxorubicin to MDA-MB-231 and ZR-75-1 human BCA cell lines (Hyung *et al*., 2008). Similarly, after coating nanoparticles containing paclitaxel with HA, cytotoxicity is reduced while cellular uptake of the drug by S-180 sarcoma cell line is enhanced 9.5 fold *in vitro* and in a mouse model (He *et al*., 2009).

In light of fairly recent evidence for the display of CD44 on BCA tumour initiator cells, interest in developing CD44 targeted therapies has increased. Riechelmann *et al.* (2008) exploited the potential of CD44 in a Phase I clinical trial using an antimicrotubule agent (mertansine) and a monoclonal antibody to CD44v6 (bivatuzumab), (BIWI 1), to treat patients with recurrent or metastatic head and neck squamous cell carcinoma (Riechelmann *et al*., 2008). The response to the treatment was unexpectedly variable and the trials using these agents were stopped after one patient died of toxic epidermal necrolysis (Tijink *et al*., 2006). Targeting the HA binding ability of activated CD44 may result in decreased toxicity.

RHAMM peptide vaccination (e.g. R3, which is HLA-A2-restricted) has recently been assessed in PhaseI/II clinical trials for treatment of MM, AML, and CLL (Giannopoulos *et al*., 2010, Greiner *et al*., 2008, 2010, Schmitt *et al*., 2008). Additionally, vaccination with DC pre-stimulated against the same peptide has also undergone Phase I and II clinical trials for treatment of CLL (Hus *et al*., 2008). Vaccination with RHAMM peptide has the attractive advantage of very low toxicity because it is not expressed in healthy bone marrow tissue. RHAMM vaccination resulted in leukemic blast lysis, blast reduction in the bone marrow and avoided the need for blood transfusions for one patient. Furthermore, an immunological response, marked by an increase in T cell frequency, was observed in 70% of AML, MM, and MDS patients in an initial study (Schmitt *et al*., 2008). Subsequently, RHAMM peptide was shown to be non-toxic at high dosage (1000 μg/vaccination), however, there was no dose-dependent effect, indicating that RHAMM is an effective therapeutic target even at low levels (Greiner *et al*., 2010). A similar response was seen in CLL patients vaccinated with RHAMM peptide, as well as RHAMM peptide-stimulated DC.

Clinical response was correlated with an increase in CD8+ T cell proliferation and in some cases a decrease in Treg population. Interestingly, in B-CLL patients with clinical response to vaccination with stimulated DC cells, the CD8+ cytotoxic T cell and IL-12 anti-tumour response was increased, whereas the Treg cell population was decreased (Hus *et al*., 2008). In a Phase I study of CLL patients vaccinated with RHAMM peptide, there was no correlation between clinical response and Treg population dynamics (Giannopoulos *et al*., 2010). This strategy has not yet been used for BCA, although, as RHAMM is a prognostic marker for BCA. and overexpressed in many cases which currently do not have a specific targeted therapeutic option (e.g. basal subtype) and also given the magnitude of the response, along with such low toxicity, it is an approach which merits further consideration.

8. Conclusion

In summary, HA is a glycosaminoglycan that exerts a critical role in BCA progression by interacting with other ECM components and the tumour cells themselves. HA fragmentation induces inflammation and signalling that results in cancer and immune cell proliferation and migration, which can lead to poor outcome. The links between HA and cancer progression, as well as HA and inflammation have in some aspects been well established. Given the similarities in their signalling cascades and cellular processes, the relationship between HA stimulated innate immunity and the BCA microenvironment should be further considered.

9. Acknowledgments

We would like to thank Kenneth Esguerra and the Translational Breast Cancer Research Unit for their support and assistance.

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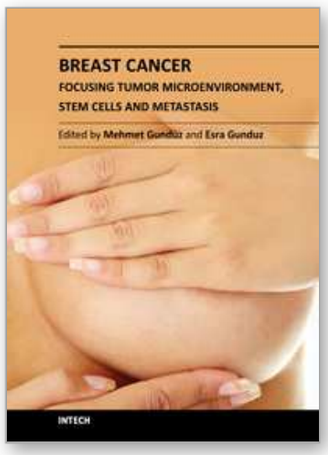

Breast Cancer - Focusing Tumor Microenvironment, Stem cells and Metastasis

Edited by Prof. Mehmet Gunduz

ISBN 978-953-307-766-6
Hard cover, 584 pages
Publisher InTech
Published online 14, December, 2011
Published in print edition December, 2011

Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

How to reference

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Caitlin Ward, Catalina Vasquez, Cornelia Tolg, Patrick G. Telmer and Eva Turley (2011). Hyaluronan Associated Inflammation and Microenvironment Remodelling Influences Breast Cancer Progression, Breast Cancer - Focusing Tumor Microenvironment, Stem cells and Metastasis, Prof. Mehmet Gunduz (Ed.), ISBN: 978-953-307-766-6, InTech, Available from: http://www.intechopen.com/books/breast-cancer-focusing-tumor-microenvironment-stem-cells-and-metastasis/hyaluronan-associated-inflammation-and-microenvironment-remodelling-influences-breast-cancer-progres

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