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Roles and Targeting of the HAS/Hyaluronan/CD44 Molecular System in Cancer

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Abstract

Synthesis, deposition, and interactions of hyaluronan (HA) with its cellular receptor CD44 are crucial events that regulate the onset and progression of tumors. The intracellular signaling pathways initiated by HA interactions with CD44 leading to tumorigenic responses are complex. Moreover, HA molecules may perform dual functions depending on their concentration and size. Overexpression of variant isoforms of CD44 (CD44v) is most commonly linked to cancer progression, whereas their loss is associated with inhibition of tumor growth. In this review, we highlight that the regulation of HA synthases (HASes) by post-translational modifications, such as O-GlcNAcylation and ubiquitination, environmental factors and the action of microRNAs is important for HA synthesis and secretion in the tumor microenvironment. Moreover, we focus on the roles and interactions of CD44 with various proteins that reside extra- and intracellularly, as well as on cellular membranes with particular reference to the CD44-HA axis in cancer stem cell functions, and the importance of CD44/CD44v6 targeting to inhibit tumorigenesis.

Keywords: Hyaluronan, Hyaluronan synthase, CD44, Tissue-specific-targeting, Cancer, Cancer stem cells

Abbreviations

ADAM a disintegrin and metalloproteinase; ADAMTS a disintegrin and metalloproteinase with thrombospondin motifs; AMPK adenosine monophosphate activated protein kinase; CD44 cluster of differentiation 44; CD44s standard CD44; CD44v CD44 variant isoforms; c-Met hepatocyte growth factor receptor; CSC cancer stem cell; ECM extracellular matrix; EGFR epidermal growth factor receptor; EMT epithelial-to-mesenchymal transition; ERα estrogen receptor alpha; ERβ estrogen receptor beta; ERM ezrin-radixin-moesin; FGF-2 fibroblast growth factor-2; GAG glycosaminoglycan; HA hyaluronan; HAS hyaluronan synthase; HAS2-AS1 natural antisense transcript for HAS2; HB-EGF heparin-binding epidermal growth factor; HGF hepatocyte growth factor; HYAL hyaluronidase; HMWHA high-molecular-weight HA; ICD intracellular domain; IQGAP1 IQ motif containing GTPase activating protein; LMWHA low-molecular-weight HA; LYVE lymphatic vessel endothelial hyaluronan receptor; MIF macrophage-migration inhibitory factor; miRNA microRNA; MMP matrix metalloproteinase; 4-MU 4-methylumbeliferone; O-GlcNAcylation O-*N*-acetylglucosamination; OPN osteopontin; PDGFR platelet-derived growth factor receptor; PEG polyethylene glycol; PEGPH20 pegylated human recombinant HYAL; PEI polyetheleneimine; pFabpl-Cre Fabpl-promoter controlling Cre recombinase; PG proteoglycan; PKC protein kinase C; RHAMM receptor for hyaluronan-mediated motility; RTK receptor tyrosine kinase; shRNA short hairpin RNA; Tf transferrin; TGFβ transforming growth factor beta; TLR-4 toll like receptor 4; UDP-GlcNAc UDP-*N*-acetylglucosamine; UDP-GlcUA UDP-glucuronic acid; uPA urokinase plasminogen activator; VEGF vascular endothelial growth factor; VEGFR-2 vascular endothelial growth factor receptor-2

1. HYALURONAN METABOLISM IN CANCER

Hyaluronan (HA) is an unbranched heteropolysaccharide that is ubiquitously distributed in vertebrate organisms and is highly correlated to rapid tissue turnover and repair conditions. Neoplasia is a pathologic state that has similar characteristics to embryogenesis and is associated with increased levels of HA that enhance tumor cell functions. The contribution of HA to tumor progression is based on two fundamental mechanisms: the first is the HA metabolism, including the regulation of HA metabolic enzymes that define the amount and size of HA; and the second is the binding of HA to cell membrane receptors that activate signaling pathways and modulate cell functions. In this first part of the review, we present main points of the HA structure and metabolism in normal and tumor cells, delineate particular characteristics of HA metabolism in distinct tumors, and define the importance of HA as a biomarker in pleural mesothelioma.

1.1 Hyaluronan Structure and Metabolism

HA is one of the most prominent macromolecules of the extracellular matrices (ECMs) that belongs to the family of glycosaminoglycans (GAGs). The repeating disaccharidic unit of HA is composed of *N*-acetyl-D-glucosamine and D-glucuronic acid, which are linked by beta bonds (-β1,3-*N*-acetyl-D-glucosamine- β1,4-D-glucuronic acid-)$_n$. It can form oligomers or polymers that may reach a size of 6 to 8 MDa with a length of about 1 nm, and these are neither sulfated nor covalently bound onto a core protein, in contrast to proteoglycans (PGs). Even though HA is found predominantly as a secreted molecule, it may also be found in traces intracellularly or on the cell surface, where it is mainly non-covalently linked to glycoproteins. HA is ubiquitously distributed in all connective tissues and ECMs in mammals, it has gel properties and is associated with high hydration [1] and, thus, is more abundant in the rapidly growing fetal tissues, especially in the first weeks of gestation [2], than in mature adult tissues. One role of an HA-rich environment is to promote cell proliferation and migration, which is important during embryogenesis for the quick movement of

stem cells to the location of organ development [3, 4]. Moreover, HA is a necessary regulatory element in bone marrow hematopoiesis [5]. Because of the similarity of development and tumorigenesis in the activation of cell functions, the HA-rich environment was studied by various research groups, demonstrating its importance for several tumor cell functions such as proliferation and metastasis. The size of HA is critical for tumor progression, since high-molecular-weight HA (HMWHA) is functionally linked to cell proliferation and tissue development, whereas the low-molecular-weight HA (LMWHA) modulates angiogenesis and may promote pro-inflammatory events [6, 7]. Recent studies on the naked-mole rat showed that the longevity and resistance to cancer that present these animals are correlated to HMWHA [8]. Specifically, the size of HA in naked-mole rats is at least five times larger than in mouse or human, and the abundant accumulation of HA in the tissues is mainly due to a unique amino acid sequence of the HA synthesizing enzyme HAS2, and to a decrement of the activity of hyaluronidases (HYALs).

Both HMWHA and LMWHA can act as signaling molecules through the interaction with cell surface receptors, such as CD44, receptor for hyaluronan-mediated motility (RHAMM), toll like receptors-2 and -4 (TLR-2 and -4), lymphatic vessel hyaluronan receptor (LYVE) , layilin and ECM molecules, such as collagen VI and proteoglycans [9-12]. The interactions of HA molecules with CD44 are presented in more detail in the following sections. Regarding the other receptors, it has been shown that there is a high association between cancer progression and inflammation with increased levels of RHAMM and TLR-4, respectively [9, 10, 13]. RHAMM exists in several isoforms and interacts with both LMWHA and HMWHA. RHAMM is often highly expressed in later stages of carcinomas and in some cases it is associated to tumor stage and prognosis [14, 15]. Similarly, binding of LMWHA to TLR-2 and -4, which are highly expressed in cancer cells such as ovarian and breast cancers, mediates cell motility, tumor growth and invasion [16-18].

The size of HA depends on the enzymes related to its metabolism. The family of the enzymes that synthesize HA are called hyaluronan synthases (HASes). In mammals, three isoforms exist (HAS1, 2 and 3) that share 55-71% sequence identity [19] and differ in enzymatic ability to form HA matrices and to establish the product size. In particular, HAS1 and HAS2 produce HA with an approximate size of $2x10^6$ Da, whereas HAS3 produces HA of lower sizes that reach $2x10^5$ Da. Moreover, the amount of HA changes, since HAS1 synthesizes lower amounts of HA than the other two HASes [20]. On the other hand, degradation occurs by the endoglucosidases HYALs, which cleave HA to LMWHA fragments that are internalized by endocytosis and are further degraded by other enzymes in endosomes or lysosomes [21]. There are six different HYALs (HYAL-1, -2, -3, -4, -P1, PH-20) that, with the exception of HYAL-P1, share *ca* 40% of amino acid identity, and their activity depends on the pH of the environment and on the binding with other molecules. For instance, it was found that in chondrocytes, HYAL-2 is more active when it is linked to the hyaluronan receptor CD44 at the surface of cells than when it is released within the ECM [22]. Recently, a new HA binding protein called KIAA1199 was found to be involved in HA degradation [21].

The type and expression levels of HA metabolic enzymes are related to the type of tissue and to the specific physio- and patho-logical conditions. Several studies demonstrate that cell de-differentiation, which is firmly related to the initial stages of cancer, is associated to HA aberration in amount and size that in turn are linked to alternate expression and activity of both HASes and HYALs. In the last few years, numerous studies have focused on post-translational modifications and epigenetic control of the HASes enzymes. Thus, it is critical to further highlight on the regulation of HA metabolism as it could be a target from a therapeutic standpoint.

1.2 Hyaluronan Synthases: Regulation of the Enzymatic Activity

Structurally, HAS isoenzymes have molecular masses from 42 to 64 kDa. They are localized within the plasma membrane and consist of both multiple membrane-spanning regions and large cytosolic

loops. The catalytic activity of HASes resides on the inner face of the membrane with active sites for the two precursors, UDP-N-acetylglucosamine and UDP-glucuronic acid, and the product is secreted or translocated to the ECM through the HAS protein complexes [23]. Recent studies demonstrated that a pool of HASes is localized in the ER-Golgi, but with no or limited enzymatic activity which is regulated by N-glycosylation [24, 25]. However, when dividing mesangial cells were grown in hyperglycaemic conditions, it was likely that HA synthesis was initiated within the cell through an activated protein kinase C (PKC)-dependent pathway [26].

HASes have been shown to form homo- and heterodimeric complexes with each other [27, 28], when cells were transiently transfected with plasmids containing the three HASes, whereas complexes of HAS1-HAS2, HAS2-HAS2 and HAS2-HAS3 were observed among endogenously expressed HASes. The interaction occurred mainly via the uncharacterized N-terminal 86-amino acid domain(s). Complexes were detected in both plasma membrane and Golgi apparatus. Interestingly, among all homomeric complexes, HAS1 has the lowest activity, whereas HAS3 has the highest activity. Moreover, HAS1 transfection causes a reduction of the synthesis of HA mediated by HAS2 and HAS3, suggesting a functional cooperation between the isoenzymes [28].

HASes activity is also regulated by the traffic of these enzymes to/from the plasma membrane. Specifically, it was demonstrated that HA synthesis from HAS3 and an enlarged cell surface HA coat were increased when this enzyme was localized in the plasma membrane [29]. The endocytosis of HAS3 is dependent on the Rab10 GTPase, which means that the blocking or the stimulation of this protein controls the trafficking of HAS3 and consequently the HA secretion. Reduction of HA synthesis by enhancement of HAS3 endocytosis may result to a reduced amount of HAS3 in microvesicles which, as demonstrated before, mediate extracellular communication during tumor progression [30].

Post-translational modifications and environmental factors, such as hypoxia or glucose availability, may affect HASes enzymatic activity and HA synthesis [31, 32]. Indeed, HAS2 was found to be active when it is monoubiquitinated at lysine 190 [27]. Under conditions of low cellular energy status, where the ATP/AMP ratio is low, it was shown that HAS2 is phosphorylated by the adenosine monophosphate activated protein kinase (AMPK) at threonine 110, which resides to a cytoplasmic loop, resulting in a decreased HA synthesis [33]. Moreover, in aortic smooth muscle cells, HAS2 can be regulated by O-*N*-acetylglucosamination (O-GlcNAcylation), which increases its activity and stability [34]. This modification has an important impact in altered glucose metabolism, such as in cancer cells where the Warburg effect is observed and hyperglycaemic conditions lead to an increase of UDP-*N*-acetylglucosamine (UDP-GlcNAc) that, in turn, directs to an increase of O-GlcNAcylation [35]. Recently, it was found that O-GlcNAcylation of histone and the opening of chromatin in proximity of HAS2 promoter are enhanced by the natural antisense transcript for HAS2 (HAS2-AS1) [36, 37]. In addition, HA metabolism can be a rheostat for controlling an acceptable normal range of cytosolic UDP-GlcNAc concentrations in order to maintain normal cell functions [38]. The role of the UDP-sugars was intensively studied in COS-1 cells, transiently transfected with different plasmids encoding the three HASes [39]. As shown by this study, HAS2 and HAS3, but not HAS1, are able to synthesize HA and to form an HA cell coat. Increasing the amount of glucosamine in the growth medium, which in turn increases the UDP-GlcNAc, showed a significant increase of HA synthesis by HAS1. Moreover, culture in glucose-free medium resulted in a depletion of UDP-sugars and a decrease of HA synthesis by all HASes, even though this phenomenon was more evident in HAS1 activity, less in HAS2 and almost unaffected in HAS3. Thus, hyper- or hypoglycaemic conditions drastically affect the activity of HASes by both post-translational modification of O-GlcNAcylation and disposition of UDP-sugars. In addition, treatment of cells with the pharmaceutical preparation 4-methylumbeliferone (4-MU) results in a post-

transcriptional suppression of HAS2 and an inhibition of HA synthesis in a UDP-glucuronic acid (UDP-GlcUA)-dependent mechanism [40].

Water homeostasis and oxygen tension also influence HA levels. Renomedullary interstitial cells (RMICs) under hypoxic conditions showed a decrease in HA [41] and, indeed, cancer cells that are localized within a hypoxic area synthesize much less HA than the stromal cells of the tumor-stroma microenvironment.

In the last few years, several studies focused on the regulation of signaling pathways through epigenetic regulation of the synthesis of specific proteins, by the action of microRNAs (miRNAs) [42]. One of these miRNAs, miR-23a-3p, was shown to target HAS2, acting as an inhibitor of HAS2 gene expression, which results in a decrease of HA synthesis [43]. Similarly, miR-7 targets and decreases the expression of the epidermal growth factor receptor (EGFR), negatively affecting the HA-mediated CD44-EGFR pathway and decreasing indirectly the expression of HAS2 [44]. The regulation of HASes and HA synthesis by stimuli deriving from the cross-talk between cancer and stromal cells are summarized in Figure 1, steps 1-4.

1.3 Hyaluronan and Hyaluronan Metabolic Enzymes in Solid Tumors

Several studies regarding the role of HA in tumors have been mainly focused on the enzymes of HA metabolism and on the binding of HA to its cellular receptors. The role of HA in the tumor microenvironment has been also widely studied and it is now clear that it accumulates in the stroma of a variety of tumors, activating signaling pathways that modulate cell motility, proliferation, differentiation and invasiveness [45, 46]. The increased concentration of HA in many cancers is associated with malignant progression and poor survival as in the case of colorectal, breast, prostate, lung, ovarian, and gastric tumors [47]. Moreover, alterations in HA size are closely related to cancer progression, since LMWHA promotes angiogenesis that is required for survival, growth and metastasis of tumors, whereas HMWHA has the opposite effect [48-51]. Notably,

HMWHA through its interaction with its major cellular receptor CD44 is able to induce and maintain epithelial-to-mesenchymal transition (EMT), a critical event in the invasion and the initiation of metastasis [50, 52]. It is well known that EMT is a process that is mainly induced by transforming growth factor beta (TGFβ). It is worth noticing, however, that this process requires the expression of HAS2 [53]. Moreover, TGFβ that can be found within the cancer exosomes can cause differentiation of fibroblasts to myofibroblasts, a phenomenon that is accompanied by an increase of the pericellular HA coat [54]. Thus, cellular processes that are enhanced by secreted factors and favor cancer progression are correlated to an increase of HA in cancer microenvironment. These processes are highlighted in Fig. 1, steps 5-8.

Induction of HASes gene expression is closely related to cancer progression and aggressiveness, even though different types of tumor cells and cancer stages show a preference for HAS stimulation. For instance, high amounts of HA and a preferential HAS2 mRNA overexpression are observed in highly invasive breast cancer cells, whereas low amounts of HA synthesis and a prevalent gene expression of HAS1 and HYAL1 are observed in less invasive breast cancer cells. In fact, silencing of HAS2 in the highly invasive breast cancer cells showed a decreased aggressiveness and migration and a concomitant up-regulation of HAS1, HAS3 and HYAL1, ensuing to a 50% decrease of HA [55]. Clinical studies regarding the HAS2 expression on breast carcinomas of various types showed that HAS2 was expressed in 30.6% of invasive ductal carcinomas and 72.7% of metaplastic carcinomas [56]. Interestingly, this latter carcinoma is a subtype related to EMT that in turn is strictly related to the estrogen receptors alpha (ERα) and beta (ERβ) both of which regulate the synthesis and turnover of tumor ECM [57, 58]. Porsch *et al.* have demonstrated that in NMuMG mammary epithelial cells, the TGFβ-induced EMT depends on the HAS2 expression, but not on extracellular HA [59]. Indeed, HAS2 depletion in TGFβ-stimulated breast epithelial cells resulted in a suppression of the expression of EMT markers fibronectin, Snail-1 and Zeb-1. Moreover, the

induction of the component of intercellular epithelial cell junctions ZO-1 by TGFβ was abolished in HAS2-suppressed cells. Therefore, HAS2 has a dominant role in breast cancer and can be considered a key factor in tumor aggressiveness. Further studies on the importance of HAS2 in cancer cells that undergo EMT will be helpful to understand its role in the metastatic potential.

In breast cancer diagnosis, HA has been used as a biomarker. Specifically, the serum LMWHA level (but not the total HA) of patients was significantly correlated with lymph node metastasis, suggesting that smaller sizes of HA promote the migration and invasion of breast cancer cells [60].

Interestingly, the increment of HA and gene expression of HAS2 and HAS3 in leiomyosarcoma seems to be correlated to the amount of versican, as depletion of versican in *in vivo* experiments showed a decreased production and accumulation of HA, down-regulation of both synthases and lower tumor volume [61]. Furthermore, versican and HA increase ovarian cancer cell migration and invasion in a CD44-dependent manner [61] (represented in Fig. 1, steps 7a and b). Indeed, elevated levels of versican that bind HA promote an inflammatory response, through stimulating cytokine release, which influences the immune response [62-65]. Thus, secreted proteoglycans, such as versican, play an important role in the induction of HASes and, subsequently, of HA synthesis in inflammation and tumors.

HAS3 induction and accumulation of HA in the tumor microenvironment are correlated to an increased tumor growth and aggressiveness in prostate and pancreatic cancer. Specifically, aggressive prostate tumor cells express 20-fold higher levels of HAS3, but the HA produced by HAS3 reduced tumor growth and adhesion [66]. This phenomenon is inversed by the action of HYAL1, showing that the role of HA in tumor growth is associated with its molecular size. HMWHA produced by HAS3 may suppress prostate tumor growth but induce cell migration [67], whereas LMWHA associated with the high expression of HYAL1 favors angiogenesis, which is important for the first stages of tumor (showed in a general scheme in Fig. 1 step 8). Similarly, HAS3 and HYAL1

are significantly increased also in lung cancer [68]. Elevated HAS3 expression is correlated to smoking history, and HYAL1 is strongly related to the lung prognosis [68]. In contrast to prostate cancer, elevated levels of HAS3 promote pancreatic tumor growth by modulating cancer microenvironment, including the increase of HA, collagens and proteoglycans [69]. In particular, *in vivo* experiments showed that overexpression of HAS3 resulted in faster growing xenograft tumors, accompanied with an abundant accumulation of extracellular HA and a decreased adhesion of epithelial cells. The removal of the secreted HA by treatment with pegylated human recombinant HYAL (PEGPH20) showed a significant decrease of the growth rate of tumors by pancreatic cancer cells overexpressing HAS3 compared to parental tumors. Similar treatments of HAS2-overexpressing pancreatic tumors with PEGPH20 showed a weaker effect on growth rate, which still grew more slowly [69]. Notably, there are several studies showing that enzymatic depletion of tumor HA improves the access of chemotherapeutic agents to tumor cells [70-72]. Therefore, the approach of drug designation to PEGPH20 for the treatment of tumors that accumulate HA is promising and now under clinical development.

To sum up, individual solid tumors present different preferences in HASes and HYALs expression but in all tumors described here it is evident that HA is important in tumor cell biology and is associated with cancer prognosis and tumor progression. Detailed studies will improve our knowledge on the mechanisms by which HA is regulated in certain tumor microenvironments and therefore they should be performed in each distinct tumor separately. This will further elucidate the specific characteristics that could be targets for diagnosis and/or treatment of the specific cancer type.

1.4 Hyaluronan as Soluble Biomarker in Malignant Pleural Mesothelioma

Soluble biomarkers are crucial for early detection of various diseases, particularly cancers with deleterious prognosis. Malignant mesothelioma is a mesenchymal tumor originating from the

mesothelial cells covering the body cavities**.** Accumulation of a pleural effusion is one of the first signs of the disease, providing also the first biological material for diagnosis. Biochemical-, ultrastructural-, molecular- and immunocytochemical characterization of the effusion can be used as adjuvant methods to the morphological diagnosis of a malignant mesothelioma [73] allowing differentiation from the main differential diagnostic alternatives comprising metastatic lung adenocarcinoma (AD) or reactive mesothelial hyperplasia. A high HA concentration was recognized early as a specific and reliable biomarker of malignant mesothelioma in malignant effusions [74] and it still constitutes one of the most established soluble biomarkers, also holding promise for future multi-parameter biomarker approaches [75].

The level of HA exceeding 75 μg HA-derived uronic acid (230 μg HA) per mL in effusion is strongly indicating a mesothelioma, whereas benign effusions often contain less than 10 μg HA-derived uronic acid (30 μg HA) per mL. Moderately elevated values are however sometimes seen in effusions caused by other conditions such as bacterial infections, but the HA level measured in these conditions does not reach the above mentioned cutoff levels.

The high HA levels seen in mesothelioma effusions have been regarded as an indication of the mesenchymal origin of this tumor. Both epithelioid- and sarcomatoid mesothelioma cells produce this polysaccharide, with a higher rate in the epithelioid phenotype [76, 77]. HA is the most specific among established mesothelioma biomarkers, but a conclusive diagnosis can be achieved more often if it is combined with mesothelin. Several attempts have been made to combine biomarkers in diagnostic batteries [78, 79]. Such combined biomarker panels have greater diagnostic accuracy than individual biomarkers and they also carry significant prognostic information provided by the addition of HA [80]. The largest of these studies consists of a two-step model combining HA and the serum marker N-terminal fragment expressed in renal carcinoma (N-ERC)/mesothelin. This model predicts the presence of mesothelioma with high specificity.

Biomarkers are also useful for monitoring the results of therapeutic interventions. Serum or plasma HA concentration reflects the tumor burden [81]. The diagnostic value of serum analysis however, seems to be low, depending on the rapid turnover of this polysaccharide in the blood stream and the fact that other conditions such as rheumatoid arthritis and liver disease also will cause elevated serum HA levels [82].

2. IMPORTANCE OF CD44 TARGETING IN TUMORS

CD44 proteins participate in the reception of a variety of microenvironmental signals, thereby regulating cell-cell and cell-matrix adhesion, and controlling cell proliferation, differentiation, migration, and survival. The binding ability of CD44 to ECM components, particularly to HA but also to growth factors, cytokines, and metalloproteinases, as well as the co-receptor functions of CD44 molecules and the multiple binding motifs residing in their intracellular domain (ICD) are features that might explain the nodal contribution of CD44 to tumorigenesis. In this second part of the review, we describe the interactions and roles of CD44 proteins in normal and, especially, in tumor cell biology with particular reference to the functional properties of cancer stem cells. Current CD44/HA-based therapeutic targeting approaches, as a mean for the design and production of specific diagnostic and therapeutic tools, are also discussed.

2.1 CD44: Structure, Diversity and Interactions

CD44 proteins are single-span transmembrane glycoproteins that are encoded by one gene composed of 20 exons that are alternatively spliced. The presence of GAG chains in some CD44 isoforms classifies CD44 molecules as part-time PGs [83]. Structurally, all CD44 proteins consist of three major domains: an extracellular portion or ectodomain, a transmembrane domain, and a cytoplasmic or ICD. CD44 ectodomain is composed of a common N-terminal globular domain and a stem membrane-proximal region, which accounts for the heterogeneity of this protein family,

while the transmembrane and intracellular domains are common in all CD44 proteins. Among the 20 exons of CD44 gene, the ten central exons (exons 6-15) known as “variant” exons (v1-v10) are excised or included in various combinations by alternate splicing in the membrane-proximal stem region [84]. The complexity of the CD44 protein family is further enhanced by post-translational modifications, such as N- and O-glycosylations, and linkage to the GAG chains of chondroitin or heparan sulfates in the extracellular portion [85, 86]. Standard CD44 (CD44s) does not contain any variant exon in its ectodomain and is widely expressed. In contrast, the expression of CD44 variant isoforms (CD44v) takes place only under specific developmental and pathological conditions. Interestingly, specific CD44v are expressed in a variety of different cancers, particularly in advanced stages [87] (Table 1). Specifically, the variant 6 of CD44 (CD44v6, in which exon 6 is expressed) is of particular interest because it is over expressed in various cancers and plays a significant role in disease onset and progression [46, 86, 88, 89]. The complex interplay of CD44 with receptor tyrosine kinases (RTKs) and the role of CD44v are represented in Fig. 2.

CD44 is the main cellular receptor for HA. The binding of HA to CD44 induces conformational changes to the receptor as well as post-translational modifications regulating downstream signaling pathways and, as a result, numerous pathobiological processes including inflammation, wound healing, tumor growth, and metastasis [90, 91]. HA binding is mediated in part by the link domain, consisting of 90 amino acids (residues 32-123), as well as other sequences flanking the link domain that form a lobular extension, creating a larger HA-binding domain [92]. The minimal size of HA fragments binding to CD44 corresponds to three to five disaccharide units. This is important since newly synthesized HMWHA is degraded by HYALs into smaller fragments (LMWHA) that still can bind to CD44 but often exert opposite effects compared to HMWHA molecules giving rise to the known size-dependent functions of HA in physiological and pathological processes [85].

Post-translational modifications of CD44 proteins regulate their ability to bind to a number of ECM components with increased importance in tumor progression, including HA, collagens, fibronectin, laminin [93], fibrin, osteopontin (OPN) [94], and serglycin [95]. In particular, chondroitin and heparan sulfates on amino acid sequences in the CD44 stem region encoded by specific variant exons allow CD44 to interact with collagen, fibronectin, and laminin. The degree of sulfation of CD44 sugar side chains also regulates its ability to bind to fibrin, while sialylation inhibits the interaction of CD44 with HA [96, 97]. OPN, a highly negatively charged matricellular protein, binds cells via integrins and CD44, and these interactions are involved in adhesion, migration, homing, survival, and proliferation of tumor, and stromal cells [94, 98]. Compared to HA, OPN binding to CD44 has different effects on chemotaxis and aggregation. Serglycin, an intracellular proteoglycan secreted by various cell types, interacts with and regulates the activity and availability of numerous inflammatory mediators to their extracellular target molecules, and plays a role in inflammation and tumor progression and metastasis [95]. Importantly, CD44-serglycin interactions have been implicated in the growth of malignant melanomas, in part through the regulation of angiogenesis [99].

At cellular membranes, numerous studies show that CD44 acts as a signaling platform controlling cell surface receptors of diverse structure and function (Fig. 2). Heparan sulfate-modified CD44 isoforms (mainly CD44v3) can bind to growth factors, such as heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) [100] thereby modulating their activities and interactions with their cognate receptors. Subsequently, various CD44 isoforms have been demonstrated to regulate the signaling of receptor tyrosine kinases (RTKs), such as c-Met, vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor (PDGFR), and EGFR in both HS-dependent and independent manners. Moreover, CD44v regulate the activity of matrix

metalloproteinases (MMPs), such as MMP-7, MMP-9, and MT1-MMP, either involved in the maturation of growth factors and subsequent activation of their cognate receptors, or the degradation of ECM for tumor cell invasion and metastasis [101-105]. Furthermore, CD44 isoforms, like CD44v6, act as co-receptors for RTKs such as c-Met exerting a dual function: on one hand, CD44 ectodomain controls c-Met activation in a ligand-independent manner, and on the other hand, the CD44ICD binds to the ezrin-radixin-moesin (ERM) family of proteins and to the cytoskeleton, providing an intracellular platform for c-Met signaling [106]. Moreover, c-Met internalization and its intracellular signaling were shown to depend on CD44v6 [107]. In a similar mode, CD44v6 regulates VEGFR-2 activation [108]. CD44v are also involved in the regulation of PDGFRβ and FGFR activation [109, 110]. Importantly, the specific requirements of a given RTK for particular CD44 isoforms in its signaling (for example, CD44v6 for c-Met, VEGFR-2, EGFR, and CD44v3 for FGFR, ErbB4) as well as the unique ligand-binding properties of specific CD44 isoforms to specifically support ligand-induced RTK activation, allows the design and production of specific diagnostic and therapeutic tools [108, 111, 112].

It is worth noting that CD44 collaborates not only with RTKs, but also with the serine/threonine kinase receptors TGFβ-RI and TGFβ-RII, G protein-coupled receptors (CXCL12-CXCR4 axis), as well as the chondroitin sulfated form of CD74, which acts as a receptor for macrophage-migration inhibitory factor (MIF) (reviewed in [85]). More recently, CD44, which constitutes a Wnt-target gene, was shown to regulate Wnt signaling [113]. The role of CD44 in Wnt signaling was highly dependent on the binding of ERM proteins to CD44ICD, but independent to HA binding.

Although CD44ICD is only 72 amino acid residues long and devoid of any enzymatic activity, it contains motifs implicated in interactions with a plethora of binding partners, including proteins involved in cytoskeletal reorganization, transcription, apoptosis, survival, endocytosis, and intracellular transport [86, 114, 115]. Additionally, the CD44ICD undergoes regulatory

phosphorylation at specific serine residues (Ser291, Ser316, Ser325) that determines the specific interactions between CD44 molecules and effector proteins [115, 116]. Interestingly, CD44 (either full length or cleaved CD44ICD) can translocate to the nucleus, acting, at least in part, as a transcriptional regulator [117, 118].

Several extracellular cues are dependent on the interaction of ankyrin and ERM proteins with the CD44ICD. Numerous studies have suggested that CD44 transduces both contact inhibition cues and HA-mediated signaling through its differential interaction with ERM proteins and merlin (a tumor suppressor ERM-related protein), and these interactions can regulate CD44-HA binding [119-122]. Furthermore, HA/CD44-mediated tumor cell-specific phenotypes and functions are closely linked to the activation of small GTP-binding proteins, such as RhoA, Rac1 and Cdc42 (RhoGTPases) [123]. IQ motif containing GTPase activating protein (IQGAP1), an essential scaffolding protein, was shown to have important roles in HA-induced actin cytoskeleton remodeling, and functional properties through its interaction with CD44ICD by inhibiting the intrinsic GTPase activities of Rac1 and RhoA thereby stabilizing their GTP-bound forms [114, 124, 125].

In the context of carcinogenesis, the contribution of CD44 to the inhibition of apoptosis is very important. Among the underlying mechanisms, the HA-dependent activation of TGFβ1, HB-EGF and OPN by CD44v6 isoforms as well as the inhibition of Fas signaling by CD44 are the most well characterized [86, 126, 127]. Jung *et al.* suggested that CD44v6 isoforms account for the formation of a pre-metastatic niche that promotes survival of tumor cells and induces chemoresistance [128, 129]. Another possible mechanism may involve the inhibitor of apoptosis-stimulating protein of p53 (iASPP), a novel CD44 interacting protein that was revealed by the proteomic approach described in Skandalis *et al.* [114]. iASPP is known to inhibit p53-mediated apoptosis in mammalian cells [130]. Given that CD44 has crucial tumor-promoting functions in tumor cells lacking p53 function [131], and that CD44 confers growth advantage on breast cancer stem cells (CSCs) [132],

this finding may shed light on the mechanisms whereby CD44 is involved in cancer cell survival and evolution.

2.2 The CD44-Hyaluronan Axis in Cancer Stem Cell Function

Given the multitude of functions exerted by CD44 and its ligand at the cell biological level, it is not surprising that they have been linked to stem cell-associated properties. According to the CSC concept, a small proportion of cells within a given tumor has the properties of self-renewal, remarkably high proliferative potential, expresses high levels of multidrug-resistance proteins, shows apoptosis resistance, a highly efficient DNA repair system, and a considerable developmental plasticity [133, 134]. These functional properties distinguish these so-called tumor-initiating cells from the majority of cells within the tumor, and have been linked to increased resistance to chemotherapy, radiotherapy, and even targeted therapies [135-139]. Depending on signals from a specialized cellular and ECM environment, the so-called stem cell niche, CSCs are thought to be responsible for reconstituting after therapy, which has been initially successful in targeting the bulk of tumor cells [140, 141]. Notably, in combination with other markers, CD44 is a CSC marker for a large number of tumor entities, including breast, colon, gall bladder, gastric, liver, ovarian, pancreatic, prostate and head and neck cancer [134].

Given the prominent role of CD44 in signaling pathways that modulate cancer cell behavior, its use as a CSC marker suggests a functional link. Indeed, several studies have shed new light on some of the mechanisms by which CD44 modulates the functional properties of CSCs, thus rendering them attractive targets for antitumoral therapies that may eradicate a tumor cell population particularly relevant for recurrence and therapeutic resistance. In this context, several studies indicate that the interaction between HA and its receptor CD44 is of functional importance even for non-malignant stem cells. For example, human bone marrow-derived mesenchymal stem cells were shown to express high levels of HAS1–3 [142]. Consequently, these cells secrete huge amounts of HA

resulting in the formation of a substantial HA coat functionally interacting with CD44. These findings underscore the importance of autocrine HA secretion for the maintenance of a proper stem cell niche. However, HA is also relevant in a CSC context. For example, porous chitosan-HA scaffolds have been used as a mimic of the microenvironment [143]. Regarding the role of CD44 as marker, in several instances, specific isoforms of CD44 could be linked to the malignant CSC phenotype (Table 1). Lau *et al.* recently demonstrated that CD44v8-10 is the quantitatively most important CD44 variant expressed in gastric cancer cells, verifying its role by limiting dilution and serial transplantation assays [144]. Notably, only exogenous CD44v8-10, but not standard CD44, was able to increase the frequency of tumor initiation in a mouse xenograft model. The tumor-initiating potential of the malignant cells could be markedly reduced by silencing of CD44, and rescued by CD44v8-10 expression, demonstrating the particular relevance of this isoform for gastric CSCs. Todaro *et al.* reported an important mechanistic approach on the role of another CD44 variant and the impact of the microenvironment on its expression [145]. The authors showed that clonogenic colorectal CSCs express CD44v6, and revealed that this CD44 variant played a major role for their ability to migrate and to generate metastatic tumors. Notably, cells in the tumor microenvironment were able to induce CD44v6 expression by secreting HGF, OPN and SDF-1. The resulting activation of the Wnt/beta-catenin pathway promoted migration and metastasis in a xenograft model. Moreover, a clinicopathological study revealed that expression of CD44v6 correlated to a poor patient survival, whereas the finding that phosphatidylinositol 3-kinase inhibition selectively killed the CD44v6 CSC population raises hopes for future therapeutic targeting approaches.

In another landmark study, Bourguignon *et al.* demonstrated the presence of a subpopulation of CSCs characterized by high levels of CD44v3 expression in a human head and neck SCC cell line, HSC-3 [146]. The cell population showed the self-renewal and was capable of generating

heterogeneous cell populations. At a functional level, these properties mark these cells as a CSC population. Notably, these cells not only expressed high levels of the surrogate stem cell marker ALDH1 [134], but also the transcription factors Oct4, Sox2, and Nanog, which are part of the so-called 'Yamanaka-factor'-signature needed to reprogram differentiated cells into an embryonic stem cell-like state [147]. The authors were able to link the interaction of HA with CD44v3 to the formation of an Oct4-Sox2-Nanog complex, and to its nuclear translocation. Further mechanistic investigations revealed a complex regulatory circuit, demonstrating that microRNA miR-302 expression is controlled by Oct4-Sox2-Nanog binding sites in its promoter, and that the CD44-HA interaction resulted in miR-302 upregulation. In turn, the epigenetic regulators AOF1/AOF2 and DNMT1 were suppressed, whereas the survival proteins cIAP-1, cIAP-2 and XIAP were upregulated, leading to increased self-renewal, clonality, and resistance to the chemotherapeutic drug cisplatin. Application of an anti-miR-302 inhibitor could revert this phenotype, suggesting a possible therapeutic approach for the CSC phenotype imposed by the CD44v3-HA interaction. Another link between the interaction of CD44 with HA and a miRNA-dependent mechanism of CSC regulation was recently revealed in a study on colon cancer [148]. Using the cell lines HCT-15 and HCT-116 as an *in vitro* model, the authors demonstrated that CD44 expressing cells formed more colonies in soft agar and displayed increased tumorigenicity *in vivo* compared to cells devoid of CD44 expression. Importantly, the interaction of CD44 and HA stimulated c-Src kinase activity, which resulted in a reduced expression of miR-203 in HCT-15 cells, a miRNA known as an inhibitor of 'stemness' [149]. Interestingly, a part of the mechanism of miR-203 regulation included an interaction of the EMT-related factor snail with its promoter [148]. This is noteworthy, as it was recently shown that massive HA production promotes acquisition of CSC expression signatures in breast cancer through the coordinated regulation of Twist and Snail [150], providing independent proof of concept in a different experimental system.

A link between CD44 and the Nanog, Sox2 and Oct4 transcription factor network was recently also demonstrated for breast cancer CSCs. Cho *et al.* overexpressed the CD44ICD in breast cancer cells, and observed an increase in mammosphere formation as a readout of CSC activity [151]. Downregulation of CD44ICD decreased the expression levels and nuclear localization of the stemness factors, whereas overexpression of CD44ICD reversed these effects. Moreover, an impact of CD44 on transcriptional activation of Oct4 and Sox 2 was demonstrated at the promoter level using luciferase reporter assays. As gamma secretase inhibitor treatment (which blocks the cleavage of CD44ICD) interfered with mammosphere formation, this approach may represent a possible therapeutic strategy targeting the CD44-dependent CSC compartment in breast cancer. Supporting this concept, the relevance of ADAM17-mediated cleavage of CD44 had previously also been demonstrated in HNSCC cancer stem cells [152]. Overall, these studies demonstrate the relevance of CD44 isoform expression, and of functional CD44-HA interactions for the acquisition and maintenance of a CSC phenotype. However, it should be noted that a number of studies have demonstrated that the shift from variant isoforms (CD44v) to the standard isoform (CD44s) was critical for regulating EMT and stemness during cancer progression [153, 154].

2.3 Targeting CD44 in Cancer: Approaches and Perspectives

Cancer cells need constant supply of nutrients through microvasculature networks present in the stroma, where HA serves as one of the critical components [128, 155, 156]. Many pathways in cancer are over activated downstream of CD44 that support tumor progression [88, 110, 155, 157-161]. In animal models, increases in HA production and expression of CD44 splice variants have been observed. In human tumors, overexpression of CD44 variants and the presence of high HA levels have also been found [87, 88, 160, 162].

Why is CD44 a more convenient target in cancer therapy instead of HA? Methods to eliminate HA production in a tissue-specific manner are not available as of today, whereas reagents (antibodies

or HA) are now at hand for targeting CD44 variants. HA is used by several investigators as a vehicle to transport anticancer drugs for at least three reasons. First, HA binds CD44 variants. Second, HA-drug conjugate is concentrated on the cell surface and internalized. Third, toxicity due to conjugated drug is low compared to the free drug. HMWHA binds to a number of CD44 receptors on the cell surface [3]. A simple calculation shows how many CD44 molecules would bind to HA having a molecular mass of 500 kDa. Minimal molecular mass of HA that binds to one CD44 molecule on the cell surface is 2.5 kDa. A molecular mass of 500 kDa could bind up to 200 CD44 receptors. Thus, upon binding HA-CD44 could be internalized and rapidly eliminated from circulation by the liver hepatocytes [163]. Based on these data, it may be suggested that CD44 targeting is considered as one of the best choices.

As mentioned above, serine residues of the CD44 cytoplasmic region are important for signal transduction. A peptide from CD44 cytoplasmic region containing phosphoserine at residue 325 has become of interest to block CD44 signaling and cancer cell functions. Conjugated to cell penetrating protein penetratin, the delivered peptide (pSer325) reduced movement of melanoma cells *in vitro* [164]. In prostate cancer cells, peptides containing either pSer325 or pSer323 and pSer325 interrupted the activation of CD44/MMP-9 complex [156]. An octapeptide (Ac-KPSSPPEE-amide, A6) selected from the non-receptor binding region of human urokinase plasminogen activator (u-PA) that binds specifically to CD44 inhibited migration, invasion and metastasis of cancer cells [165]. One possible explanation is by intervention of the uPA-dependent signaling pathway [166].

It is clear that the peptides that bind to HA have drawn attention because of their similarity to CD44 binding domain. Such peptides are Pep-1 (dodecamer) and BH-P (a 42 amino acid peptide having three BX7B HA-binding motifs present in CD44). Both peptides demonstrated antitumor activities in animal models [167, 168].

It is now established that CD44v6 is a co-receptor for VEGF/VEGFR2 and HGF/c-Met (depicted in Fig. 2) and has been the focus of attention because: (i) transfection of CD44v6 induced metastatic properties on cells which were otherwise non-metastatic , (ii) a five amino acid peptide from v6 exon inhibited the co-receptor function of CD44v6 and as a result stopped the vascularization in tumors, prevented growth, migration and invasion [108, 169], (iii) the availability and use of monoclonal antibodies (e.g., 2F10, VFF4, VFF7, VFF18, U36, BIWA) against specific domains containing 42 amino acids encoded by exon 6 to screen almost 10,000 tumors and metastatic tissues of sections by immunohistochemical analysis showed presence of CD44v6 in >80-90% cases [170], (iv) CD44v6 is also present in non-epithelial tumors [170]. These findings suggested a tumorigenic role of CD44v6 and, thus, became a high value target for antibody-based cancer therapy. Antibodies against the CD44v6 can selectively deliver a radioisotope (chimeric ^{186}Re-U36 antibody, humanized monoclonal antibody ^{186}Re-bivatuzumab (BIWA 4)), toxins and cytotoxic drugs to cancer cells. The phase I clinical trial performed with radiolabeled or toxin conjugated CD44v6 antibodies in HNSCC patients showed promising results. Encouraged by the initial response, a phase I dose escalation trial with anti-CD44v6 antibody-mertansine was conducted on seven patients who had developed untreatable HNSCC or esophagus. One out of seven developed severe side-effects on skin - apoptosis of keratinocytes and epidermal necrolysis, and the patient died. This could be due to expression of CD44v6 in the skin tissue [171]. Therefore, tissue-specific expression of CD44shRNA would be a safe alternative to drug conjugate.

When complexed with polyetheleneimine (PEI), non-viral plasmid vectors mediate unspecific interactions with non-target cells and blood components, which results in a rapid clearance from the circulation. These unfavorable effects can be minimized by “shielding” with polyethylene glycol (PEG). PEGylation of PEI-plasmid polyplexes can protect the plasmid vector from systemic nuclease degradation, increased circulating life-span and reduce the toxicity of polyplexes [172]. To increase

the transfection efficiency in target tumor cells having high levels of transferrin receptors (Tf-R) on the surface, transferrin (Tf), which is an iron-transporting protein, is conjugated to PEI (Tf-PEI) before PEGylation. Therefore, the shielded particles (i.e. plasmid DNA/PEG-Tf-PEI) target more specifically tumor cells than the surrounding normal cells, as Tf-receptor is 100-fold over expressed in tumor cells than normal colon cells. Internalization of plasmid DNA/Tf-PEG-PEI is promoted by receptor-mediated endocytosis [173, 174].

To target CD44v6 one must know: (a) what to deliver – a mixture of conditionally silenced plasmid pSico-CD44v6shRNA and plasmid containing colon-specific Fabpl-promoter controlling Cre recombinase (pFabpl-Cre), (b) how to deliver - both plasmids can be delivered in nanoparticles by coating with Tf-PEG-PEI, and (c) where to deliver – systemically in mice by injecting into peritoneum to target tumor and non-tumor cells.

The conditional expression of the plasmid and the mechanism of selective action on CD44v6 expression are as follows: Fabpl promoter will produce Cre recombinase in the colon and intestine cells since Fabpl promoter is leaky (acts on cells of colon and intestine). Activation of conditional plasmid pSicoCD44v6shRNA takes place by the recombinase, which removes the floxed region, and the U6 promoter works on the CD44v6 sequence. In the case of colon/intestinal colon cancer and since CD44v6 is only present in colon tumor cells, shRNA will form a double stranded mRNA which will be degraded by dicer enzyme. In the normal colon cells, CD44v6shRNA will be also produced. Since no CD44v6 mRNA is formed in these cells the shRNA will do nothing and will decay with time [175]. When these nanoparticles, containing pSicoCD44v6shRNA and pFabpl-Cre, are injected systemically into the peritoneum of the $APC^{Min/+}$ mice, they seek out Tf-receptor-positive cells, undergo endocytosis and then synthesize CD44v6shRNA, knockdown CD44v6 expression, and inhibit CD44v6 signaling. As a result, it prevents tumor cell growth and induces apoptosis [88, 176].

3. CONCLUSIONS

Cancer is characterized by uncontrolled cell growth within a tissue and is further distinguished as non aggressive (*in situ* non metastatic) and aggressive (metastasized to distant sites). Although cancer is extremely complex and tissue specific, it shares common characteristics such as the expression of oncogenes and states similar to inflammation. In the first part of this review, we focused on the alterations of HA metabolism, specifically on the regulation of its synthesis. Alteration of HA homeostasis in tumor microenvironment is highly correlated to tumor aggressiveness and progression and this is dependent on nutrition supplements, post-translational modifications of HASes and the paracrine factors of the cross-talk between tumor and stromal cells (illustrated in Fig. 1). Deeper knowledge of HA regulation in tumor progression may enhance the development of new therapeutic agents that target directly the enzymes that synthesize HA. Acting on the gene expression of HASes in terms of "gene therapy" by using microvesicles that include miRNAs [177], or treating tumor cells with agents that block HA-induced tumor cell functions, such as 4-MU [178], are among the targets of recent studies. As described here, degradation of HMWHA to LMWHA favors tumor progression and, thus, up-regulated HYALs should enhance tumor metastasis. Paradoxically, clinical trials used a combination of chemotherapeutic agents with HYALs for a more efficient treatment of chemoresistant carcinomas that accumulate HA since it was shown that enzymatic depletion of tumor HA facilitated the transport of drugs to tumor cells [70-72].

Activated HA-CD44 signaling regulates a variety of cellular processes, such as cell survival, migration, invasion, adhesion, and differentiation in a cell type- and context-dependent manner. A large number of other cellular receptors, including VEGFR, EGFR, c-Met, and LRP6, determine the functional outcome of HA-CD44 signaling, as illustrated in Fig. 2. As described in the second part of this review, targeting the interaction of HA-CD44 can inhibit the malignant processes at multiple

stages. An interference with this signaling pathway or the use of CD44/HA as targeting strategies for cytotoxic drugs have been the subject of numerous therapeutic targeting approaches. At the level of CSCs, strategies include the use of HA-functional amphipathic and redox-responsive polymer particles for the simultaneous delivery of chemotherapeutics and hedgehog pathway inhibitors to breast CSCs [179], the use of CD44-targeted docetaxel conjugates [180], the functionalization of liposomes with anti-CD44 aptamers [181], intracellular targeting of CD44-positive cells with self-assembling, protein-based nanoparticles [182], the use of T cells targeting the CD44v6 to mediate antitumoral effects against multiple myeloma and acute myeloid leukemia [183], Cdk2 kinase inhibition [184], and even nutritional approaches, such as vitamin D-analog-mediated Notch inhibition as a strategy to diminish the CD44-positive CSC population in breast cancer [185]. The diversity of these approaches and the current intense research in this area generate hope for a successful translation of CD44-HA-mediated CSC targeting approaches into clinical applications in the near future.

Further therapeutic strategies which do not exclusively target the stemness-related functions of CD44 were also explored in this review. Recent advances in nanomedicine have offered new valuable tools for cancer detection, prevention and treatment. HA-CD44 molecular system provides nanoparticles with specific targeting capabilities (reviewed in [186]) and, thus, it constitutes a potential target for translation of research preclinical data to the clinic. Testing ground of the nanoparticle plasmid delivery approach was the colon cancer cell models where transfection of pSV-β-gal/Tf-PEG-PEI-nanoparticles and a mixture of Tf-PEG-PEI-nanoparticles containing pSicoCD44v6shRNA and pFabpl-Cre were first verified [46, 88, 176]. Misra *et al.* successfully demonstrated *in vivo* that the CD44v6shRNA is localized into the colon tumor cells by an end point assay of CD44v6 expression, and by perturbation of HA-CD44v6 interaction, as reflected in the reduction in the number of tumors [88]. Recent unpublished animal studies by Misra *et al.* in the

C57Bl/6 mice demonstrated that systemic delivery of a mixture of two plasmids containing prostate specific Probasin-Cre/Tf-PEG-PEI-nanoparticles and floxed pSTOP-beta galactosidase/Tf-PEG-PEI-nanoparticles, can target prostate epithelial cells in mice. This novel approach opens up novel ways to combat cancer and to understand tumorigenesis *in vivo.* Collectively, the wide role of HA-CD44 interactions in normal cell biologies raises the concern of potential side effects. Therefore, understanding their specific role in various tumors is essential for the selectivity of treatments as demonstrated by the identification of HA interaction with CD44 variants, e.g. CD44v6 being required by the colon cancer cell model and the ability to effectively attack this interaction specifically.

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Table 1. Biological functions of CD44 variants

CD44 variant	Biological Function	Reference(s)
CD44v2-10	Confers a metastatic phenotype when overexpressed in a non metastatic human hepatocellular carcinoma cell line	[187]
CD44v3	Contains HS/CS attachment sites. Co-receptor for FGFR, ErbB4. Associated with HNSCC growth, migration, and identified in lymph node metastasis. Cancer stem cell marker for HNSCC	[100, 146, 188]
CD44v4	Major E-selectin ligand that mediates breast cancer cell transendothelial migration	[189]
CD44v4-7	Introduction into non-metastasizing rat pancreatic cancer cells rendered these cells metastatic	[190]
CD44v4-10	Promotes adenoma initiation in $APC^{Min/+}$ mice in colorectal cancer. Expressed in human intestinal stem cells derived from microadenomas of familial adenomatous polyposis patients	[191]
CD44v6	Contains HS/CS attachment sites. Co-receptor for c-Met, VEGFR-2, EGFR. Detected in cervical, colorectal and pancreatic carcinomas, and correlates with poor prognosis and metastatic potential. Marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Variant isoforms lacking exon v6 are expressed in early adenomas, whereas expression of v6-containing isoforms is specific for advanced stages of tumor development and is linked to metastatic progression. In collaboration with c-Met and VEGFR-2 plays a decisive role in pancreatic cancer. Poor prognostic marker in patients with osteosarcoma. Premetastatic niche formation	[46, 88, 89, 100, 108, 111, 128, 129, 145, 170, 192-195]
CD44v7	Expression on stromal cells supports hematopoietic progenitor cell homing	[196]
CD44v8-10	Associated with progression of myeloid leukemia from the chronic phase to blast crisis chronic myeloid leukemia. Most important variant in gastric cancer stem cells. Correlates with metastatic potential of pancreatic adenocarcinoma. Inhibits oxidative stress in tumor cells by promoting the glutathione synthesis	[144, 197-200]
CD44v9	Recurrence and metastasis of bladder cancer. Important role in colon cancer stem cells	[201, 202]
CD44v10	Mediates preferential binding of multiple myeloma cells to bone marrow endothelium	[203]

Legends to Figures

Figure 1: Overview of HASes regulation and HA levels in paracrine interactions between cancer and stromal cells.

Enzymatic activity of HAS2 enzyme is modified by post-translational modifications and enzyme complex formation, as shown in the following steps (1), (2) and (3). (1) O-GlcNAcylation by elevated amounts of UDP-GlcNAc and ubiquitination induce HAS2 activity and trigger HA synthesis; (2) Dimerization of HAS2 with each of the three HASes stimulates HA synthesis; (3) Contrarily, low levels of energy in terms of ATP/AMP ratio increase AMPK levels resulting to HAS2 phosphorylation which blocks its synthesizing capacity; (4) HAS2 transcription is positively regulated by HAS2-AS1, whereas HAS2 mRNA is targeted directly by miR-23a-3p, which decreases drastically its synthesis and activity; Among various mechanistic roles, CD44 (5a) regulates the secretion and activity of growth and soluble factors, and MMPs, which in turn (5b) act as paracrine factors on stromal cells by inducing HASes expression/activity thus increasing HA synthesis; (6) TGF-beta also induces HA synthesis in a similar manner, whereas it may be implicated in stromal cell differentiation and EMT that enhance tumor progression; (7a) HMW-HA synthesized by activated tumor cell-derived stromal cells is linked to CD44, formulates complexes with RTKs and versican, regulating cancer cell proliferation, and (7b) cell migration and invasion; On the other hand, (8) degradation of HA to LMW-HA by HYALs contributes to inflammatory conditions and high tumor aggressiveness, while binding to CD44 leads to a neoangiogenesis, which is essential for tumor cell survival and metastasis.

Figure 2: CD44v, receptor tyrosine kinases and apoptosis resistance. This figure shows the complex interplay between CD44v6, HA, and various RTKs leading to anti-apoptotic signaling, multidrug resistance and contribution to feed-back information on HA production. CD44v6-HA

binding (Green color), is accompanied by activation of Src→Ras→Erk pathway for anti-apoptotic signaling. Also, the binding activates (via phosphorylation) the cytoplasmic tail, which leads to the attachment of Ezrin, Moesin and Radixin (ERM) as well as the actin cytoskeleton. As a co-receptor, CD44v6 binds either to c-MET or VEGFR or EGFR to form complexes. The co-receptor and receptor kinase function depends on CD44v6 link through its v6 region. Each pair sends a powerful combined signal. For example, CD44v6 binds to hepatocyte growth factor (HGF, a ligand for c-MET) and presents it to c-MET. The ligand activates c-MET and the downstream signaling cascades require sustained activation of CD44-associated phosphorylated ERM and Src signaling via the Ras-MAPK and the PI3K pathways**.** CD44v6-VEGFR activates Erk, sending an anti-apoptotic signal to endothelial cells forming blood vessels**.** β-catenin is one of the key players in cancer progression and is present in normal cells interacting with a destruction complex (consisting of glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1), the tumor suppressor protein APC (adenomatous polyposis coli) and the scaffolding protein, axin) that leads β-catenin to proteasomal degradation. When Wnt proteins bind to frizzled receptor and LRP5/6 co-receptors, the complex releases β-catenin, which activates gene expression of cyclooxygenase 2 (COX2), resulting in the formation of PGE2. HA-CD44v6 interaction also activates COX2, and the subsequent production of PGE2. Particularly in colorectal cancer, the CD44–ERBB2 complex provides a strong apoptotic resistance through stimulation of COX2 transcription via PI3K and β-catenin. In turn, COX2-induced PGE2 stimulates HA synthesis. Plasma membrane contains lipid rafts wherein CD44v6, PI3 kinase, multidrug resistance protein1 (MDR1), and several RTKs (including ERBB2, ErbB3, IGF1R-β, PDGFR-β, VEGFR, EGFR, and c-MET) reside. PI3-kinase promotes phosphorylation and activation of the RTKs. PI3K also activates Akt and downstream anti-apoptotic events that contribute to drug resistance of cancer cells. ERBB2 most likely forms complex with CD44 and PI3K via Gab-1 protein. CD44-HA crosstalk with RTKs, and non-RTKs (Src) regulates the Ras and ERK pathways. However, HA

and PI3K stimulate MDR1 expression, and the stimulatory effects of PI3K would be due mainly to its feedback stimulation of HA production by a positive feedback loop. MDR1 is associated with CD44 in the lipid rafts.

Figure 1

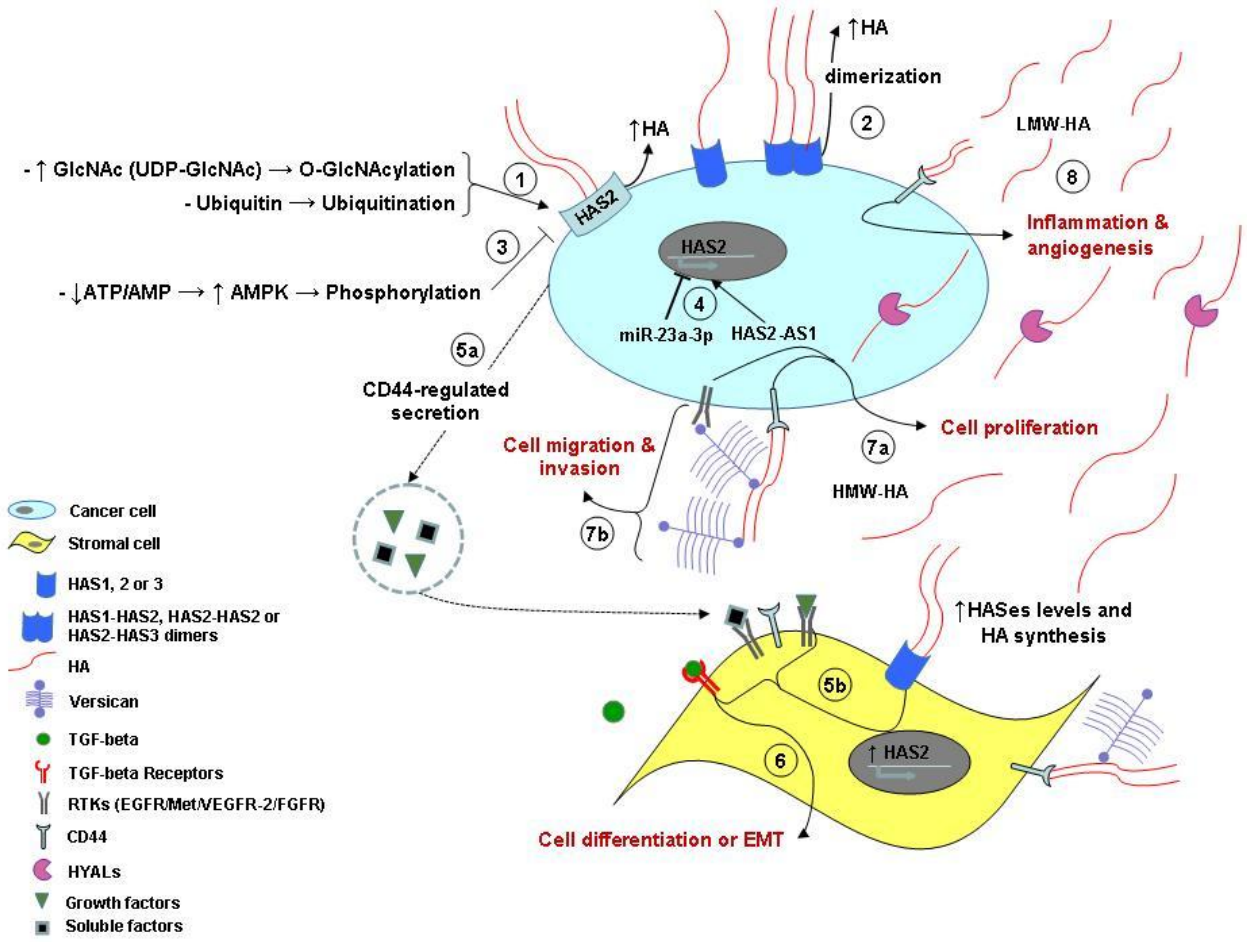
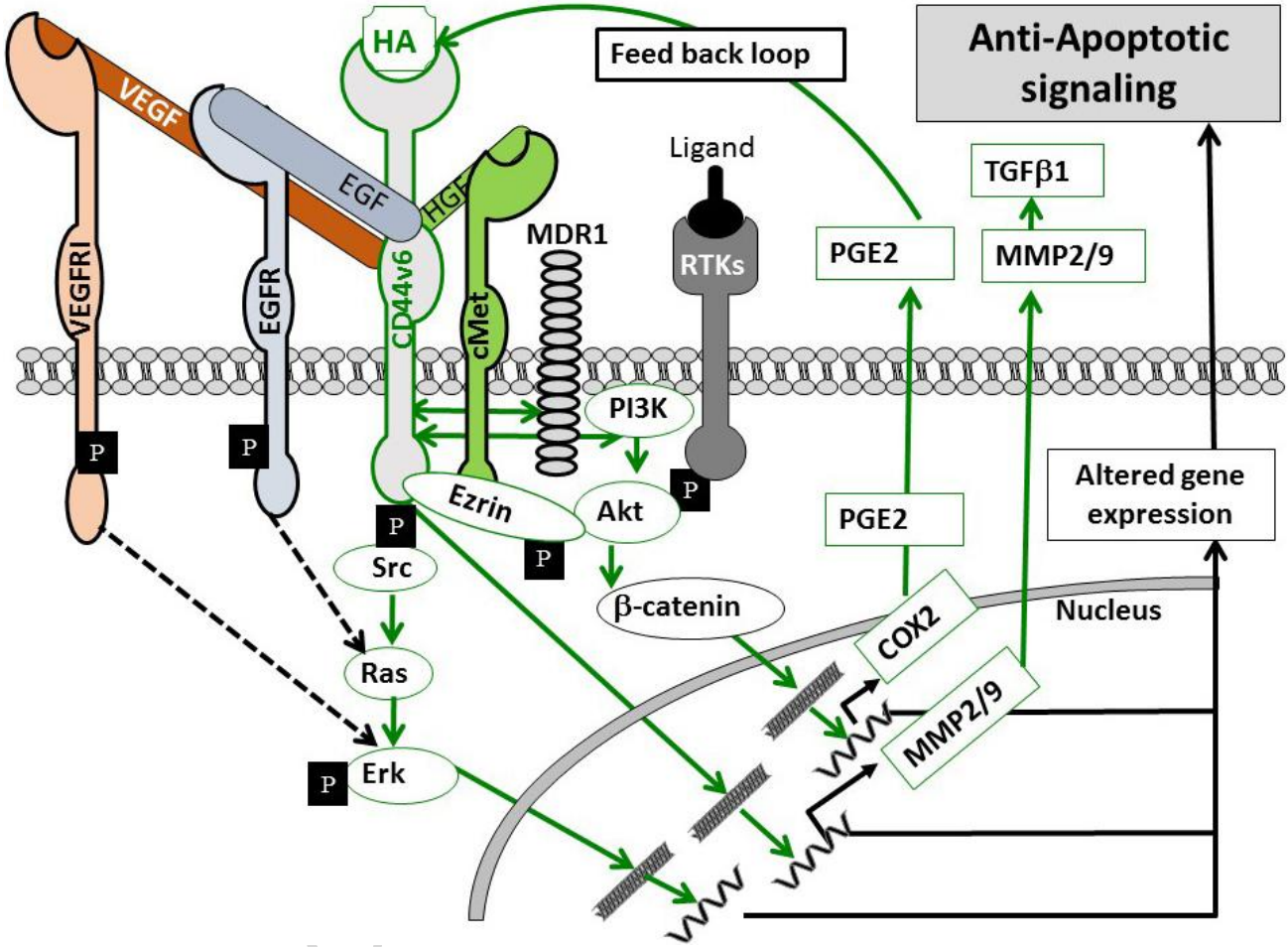


Figure 2



Highlights

- Synthesis, deposition, and interactions of hyaluronan (HA) with its cellular receptor CD44 are crucial events that regulate the onset and progression of tumors.
- HA molecules may perform dual functions depending on their size and concentration.
- The regulation of HA production by post-translational modifications of HASes is highlighted as a strategy to control the properties of tumor microenvironment.
- The relevance of CD44 variants expression and functional CD44-HA interactions for the acquisition and maintenance of a CSC phenotype is presented.
- The understanding of CD44-HA specific role in various tumors is essential for the selectivity of treatments as demonstrated by the identification of HA interaction with CD44v6 being required by the colon cancer cell model and the ability to effectively attack this interaction specifically.