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Hyaluronan-mediated CD44 activation of RhoGTPase signaling and cytoskeleton function promotes tumor progression

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Abstract

Hyaluronan (HA), a major component of the extracellular matrix (ECM), is enriched in many types of tumors. In cancer patients HA concentrations are usually higher in malignant tumors than in corresponding benign or normal tissues, and in some tumor types the level of HA is predictive of malignancy. HA is often bound to CD44 isoforms which are ubiquitous, abundant, and functionally important cell surface receptors. This article reviews the current evidence for HA/CD44-mediated activation of the ankyrin-based cytoskeleton and RhoGTPase signaling during tumor progression. A special focus is placed on the role of HA-mediated CD44 interaction with unique downstream effectors (e.g., the cytoskeletal protein, ankyrin and/or various GTPases (e.g., RhoA, Rac1 and Cdc42)) in coordinating intracellular signaling pathways (e.g., Ca^{2+} mobilization, Rho signaling, PI3 kinase-AKT activation, NHE1-mediated cellular acidification, transcriptional upregulation and cytoskeletal function) and generating the concomitant onset of tumor cell activities (e.g., tumor cell adhesion, growth, survival, migration and invasion) and tumor progression. I believe this information will provide valuable new insights into poorly understood aspects of solid tumor malignancy. Furthermore, the new knowledge concerning HA/CD44-mediated oncogenic signaling events will have potentially important clinical utility, and could establish CD44 and its associated signaling molecules as important tumor markers for the early detection and evaluation of oncogenic potential. It could also serve as ground work for the future development of new drug targets to inhibit HA/CD44-mediated tumor metastasis and cancer progression.

Keywords

Hyaluronan; CD44; RhoGTPases; Ankyrin; Cytoskeleton; Oncogenic signaling tumor progression

1. Hyaluronan (HA) and CD44 in tumor progression

Tumor invasion and metastasis are the primary causes of morbidity in patients diagnosed with solid tumors such as breast cancer [1], ovarian cancer [2] and squamous cell carcinomas (SCC) [3]. It is now certain that both oncogenic signaling and cytoskeleton functions are directly involved in tumor cell growth, migration, invasion of surrounding tissue, and metastasis [4, 5]. A number of studies have aimed at identifying these molecules which are specifically expressed by epithelial tumor cells which correlate with metastatic behavior. Among such molecules is hyaluronan (HA), a major component in the extracellular matrix (ECM) of most mammalian tissues. HA is a nonsulfated, unbranched glycosaminoglycan consisting of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine [6,7]. HA is synthesized by specific HA synthases [7,8] and digested into various smaller-sized molecules by various hyaluronidases [9]. HA is enriched in many types of tumors [10,11]. In cancer

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patients, HA concentrations are usually higher in malignant tumors than in corresponding benign or normal tissues; and in some tumor types the level of HA is predictive of malignancy [11]. For example, HA levels have been shown to be elevated in the serum of breast cancer patients [12].

HA interacts with a specific cell surface receptor, CD44, which belongs to a family of multifunctional transmembrane glycoproteins expressed in numerous cells and tissues including tumor cells and carcinoma tissues [13–28]. CD44 is often expressed in a variety of isoforms, products of a single gene generated by alternative splicing of variant exons inserted into an extracellular membrane-proximal site [29,30]. In fact, the expression of certain CD44 variant (CD44v) isoforms has been found to be closely associated with tumor progression [13–28]. All CD44 isoforms contain a HA-binding site in their extracellular domain, and thereby serve as a major cell surface receptor for HA [31]. Recently, it has been demonstrated that HA is rich in stem cell niches [32]. CD44 is expressed in both normal and tumor stem cells (displaying unique ability to initiate normal and/or tumor cell-specific properties) and CD44 has been suggested as one of the important surface markers for both normal stem cells and cancer stem cells [33]. The fact that both CD44 and HA are overexpressed at sites of tumor attachment and HA binding to CD44 stimulates a variety of tumor cell-specific functions and tumor progression [13–28] suggests that HA–CD44 interaction is a critical requirement for tumor progression.

2. HA–CD44 interaction with the cytoskeletal protein, ankyrin

CD44 interacts with a number of membrane-associated cytoskeletal proteins, such as ankyrin, which are expressed in a variety of biological systems [14,34]. Ankyrin contains three functional domains: a conserved N-terminal ankyrin repeat domain (ARD) (consisting of 22–24 tandem repeats of 33 amino acids with a consensus sequence such as -G-TPLH-AA-GH—V/A-LL-GA-ND—); a spectrin binding domain; and a variably sized C-terminal regulatory domain [35] (Fig. 1). CD44 binds directly to the ARD of ankyrin through a conserved ankyrin-binding domain in the CD44 cytoplasmic region. This CD44–ankyrin interaction causes cytoskeleton activation and results in several important HA-mediated functions such as cell adhesion, proliferation and migration [14,34,36–38].

In most cell types the plasma membrane contains specialized microdomains, called lipid rafts that have distinct lipid and protein compositions [39]. One type of lipid raft forms invaginated plasma membrane domains, termed caveolae, and is known to contain caveolin, flotillin, cholesterol and sphingolipids [39]. The functional importance of lipid rafts is clearly indicated by the recruitment of key signaling molecules into raft structures during the propagation of signal cascades [39]. Both CD44 and ankyrin have been found to be co-localized with caveolin in lipid rafts [40,41]. Ankyrin also binds to intracellular Ca^{2+} channels such as the inositol 1,4,5 trisphosphate (IP_3) receptor. Most importantly, ankyrin binding to IP_3 receptors is known to modulate IP_3 binding and Ca^{2+} activity [36,42–44]. In addition, ankyrin is involved in regulating the subcellular location of IP_3 receptors [45]. In particular, the C-terminus of ankyrin-B has been implicated in the proper subcellular localization of IP_3 receptors [45,46]. The physical association between IP_3 receptors and ankyrin and the known dysfunction of IP_3 receptors in ankyrin-B knockout mice suggest that the IP_3 receptor and ankyrin are both structurally linked and functionally coupled. Our previous studies have indicated that HA induces CD44–ankyrin interaction and IP_3 receptor-dependent Ca^{2+} mobilization [14,34–38, 40,43,44] leading to several important endothelial cell functions. HA binding to CD44 also promotes recruitment of the cytoskeletal protein, ankyrin, and inositol-1,4,5-trisphosphate (IP_3) receptor into cholesterol-containing lipid rafts. The ARD of ankyrin is responsible for binding IP_3 receptor to CD44 at lipid rafts which subsequently triggers intracellular calcium (Ca^{2+}) mobilization leading to a variety of cellular functions including cell adhesion and

proliferation [40]. These findings indicate that CD44 interaction with ankyrin and IP₃ receptor in cholesterol-containing lipid rafts plays an important role in regulating HA-mediated Ca²⁺ signaling and various important cell functions.

Furthermore, ankyrin is found to be significantly enriched in CD44-containing lipid rafts isolated from three different tumor cell lines (MDA-MB-231 cells (breast tumor cells), SK-OV-3.ipl cells (ovarian tumor cells) and SCC4 cells (HNSCC cells)) [41]. This association of CD44 with ankyrin and signaling molecules in lipid rafts is functionally coupled with HA-induced membrane–cytoskeleton interactions and tumor cell-specific behaviors (e.g., cell survival, growth and migration). Disruption of lipid rafts by the depletion of cholesterol from the raft membrane (using methyl- β -cyclodextrin treatment) not only abolishes the recruitment of ankyrin into CD44/caveolin-containing lipid rafts, but also inhibits HA-mediated tumor cell behaviors [41]. These findings demonstrate that HA–CD44 interaction in caveolin/cholesterol-enriched lipid raft microdomains plays an important role in promoting ankyrin–membrane association and activating intracellular signaling pathways leading to the onset of cytoskeleton function and tumor cell-specific behaviors (e.g., cell survival, growth and migration).

3. HA/CD44-mediated RhoGTPase signaling in regulating tumor progression

RhoGTPases (e.g., RhoA, Rac1 and Cdc42) belong to members of the Rho subclass of the Ras superfamily [47], and are known to cycle between an active GTP-bound state and an inactive GDP-bound state to transmit diverse signals from cell surface receptors to intracellular targets. They function as molecular switches that, in response to external stimuli, regulate key signaling pathways and control a variety of cellular activities (Fig. 2) including gene transcription, cytoskeleton reorganization, cell growth, migration and oncogenesis [47,48]. The high incidence of RhoGTPase overexpression in human cancers suggests these proteins are involved in both cancer onset and progression [48]. In fact, overexpression of certain RhoGTPases in human tumors often correlates with a poor prognosis [49,50]. Coordinated RhoGTPase signaling is considered to be a possible mechanism underlying cell proliferation, migration and invasion, an obvious prerequisite for metastasis [48]. The rationale for our focusing on RhoGTPases is based on previous reports indicating that CD44-associated cytoskeletal proteins [51,52] and tumor cell-specific phenotypes are dependent on RhoGTPase signaling events [19–21]. Here, we summarize recent findings that shed light on the newly recognized RhoGTPase signaling events mediated by HA–CD44 interaction in tumor cells and cancer progression.

3.1. HA/CD44-mediated RhoA signaling

To date, at least 30 different guanine nucleotide exchange factors (GEFs) for RhoGTPases have been identified [54]. Many RhoGEFs participate in oncogenic activation of RhoGTPases and frequently induce malignancy. Presently, at least two RhoA-specific GEFs (e.g., p115RhoGEF and leukemia-associated Rho guanine nucleotide exchange factor (LARG)) have been identified to regulate HA-mediated CD44 signaling during tumor cell activation. Their roles in this process are described as follows.

3.1.1. p115RhoGEF-mediated RhoA/Rho-kinase (ROK) activation and membrane–cytoskeleton function—The p115RhoGEF (also known as the human homolog of the mouse protein Lsc) is involved in RhoA activation [55]. Structurally, p115RhoGEF contains numerous functional domains which include the amino-terminal regulator of G-protein signaling (RGS) domain, a dbl homology (DH) domain, and a pleckstrin homology (PH) domain [55–57]. In particular, the sequence between aa421 and aa635 contains significant sequence homology to the DH domain of many proteins which exhibit GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins [54]. The p115RhoGEF has been clearly shown to act as a GDP/GTP exchange protein for the Rho

subfamily of GTPases, including RhoA [55]. In addition, p115RhoGEF (similar to PDZ-RhoGEF) contains an RGS domain which interacts with Gα13 and regulates the ability of DH to carry out GDP/GTP exchange activity for RhoA [55,56]. One recent study indicates that both CD44 and p115RhoGEF are physically associated as a complex in breast tumor cells. Most importantly, the binding of HA to breast tumor cells stimulates p115RhoGEF-mediated RhoA signaling in metastatic tumor cells [24].

Several enzymes have been identified as possible down-stream targets for RhoGTPases (e.g., RhoA) during the regulation of cytoskeleton-mediated cell motility [23,24,58–61]. One such enzyme is Rho-kinase (ROK, also called Rho-binding kinase) which is a serine–threonine kinase [23,24,58–61]. ROK interacts with RhoA in a GTP-dependent manner and phosphorylates a number of cellular proteins [23,24,58–61]. Structurally, ROK is composed of catalytic (CAT), coiled-coil, Rho-binding (RB) and PH domains. ROK (also called Rho-binding kinase) is known to bind RhoA and participate in cytoskeleton functions and membrane motility [53,61]. ROK phosphorylates myosin phosphatase and myosin light chain [59,60] thereby activating myosin adenosine triphosphatase (ATPase) and generating actomyosin-mediated membrane motility [59,60]. A previous study showed that ROK is responsible for the phosphorylation of CD44-associated cytoskeletal proteins during actin filament and plasma membrane interaction. When ROK is overexpressed or constitutively activated, changes in actin cytoskeleton organization occur which are similar to those observed during normal Rho-activated conditions [60]. ROK is overexpressed in breast tumor cells and is capable of phosphorylating the cytoplasmic domain of CD44 [23]. Moreover, phosphorylation of the cytoplasmic domain of CD44 by ROK enhances its binding interaction with ankyrin [23]. Overexpression of the Rho-binding domain (a dominant-negative form) of ROK by transfecting breast tumor cells with RB cDNA induces reversal of tumor cell-specific phenotypes [23]. These findings support the notion that ROK plays a pivotal role in CD44–ankyrin interaction and RhoA-mediated oncogenic signaling required for membrane–cytoskeleton function and metastatic tumor cell migration.

3.1.2. HA/CD44-mediated RhoA–ROK activation and Gab-1-linked PI3 kinase-AKT signaling in tumor cells—Phosphatidylinositol 3-kinase (PI3 kinase) participates in a CD44-mediated survival pathway in colon carcinoma cells [62]. HA activates PI3 kinase-AKT pathways leading to cell motility and cell survival-signaling pathways [63]. The active mutant of the p110 subunit of PI3 kinase causes the cleavage of CD44 during cancer cell migration [64]. These findings suggest PI3 kinase activation is closely coupled with HA-mediated CD44 signaling. In the pathogenesis of cancer, Gab-1 (Grb-2-associated binder-1) and phosphatidylinositol 3-kinase (PI3 kinase) are key mediators in regulating oncogenesis [65]. Specifically, Gab-1 (a member of the insulin receptor substrate (IRS) family) functions as one of the major adapter molecules downstream of growth factor signaling. Gab-1 also possesses multiple phosphorylation sites that could act as docking sites for PI3 kinase known to consist of a catalytic subunit p110 (α, β and δ) and a regulatory subunit p85 (α, β and p55γ), or a catalytic subunit p110γ and the regulatory subunit p101 [65]. Recently, it has become evident that HA/CD44-activated RhoA also promotes ROK phosphorylation of the linker molecule, Gab-1. Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3 kinase to CD44 and activates certain isoforms of PI3 kinase to convert PtdIns (4,5)P₂ to PtdIns (3,4,5)P₃ leading to AKT activation, cytokine production and tumor cell behaviors (e.g., tumor cell growth, survival and invasion) required for breast tumor progression. These findings suggest that there is a strong connection between RhoA–ROK activation and Gab-1-associated PI3 kinase activation during the stimulation of breast tumor cells by HA–CD44 interaction [24].

3.1.3. HA/CD44-mediated RhoA–ROK activation and NHE1 signaling leading to ECM degradation and tumor invasion—Tumor invasion and metastasis are the major

cause of treatment failure for cancer patients [66]. These processes are often associated with acidification of the tumor microenvironment [67]. In particular, mammary cancer cells acidify the extracellular environment more effectively than normal mammary cells [68]. Moreover, metastatic breast tumor cells (MDA-MB-231 cells) are known to develop acidic microenvironments [69]. Nevertheless, it is clear that the mechanisms responsible for acidification-related tumor progression are quite complex and involve a number of biochemical and cellular events. It is well known that lactate secretion by tumor cells is one of the important causes of acidification of the immediate microenvironment of primary tumors [70]. However, since primary mouse tumors derived from cells lacking lactate dehydrogenase are also capable of acidifying the tumor microenvironment [71], other mechanism(s) must be involved in regulating extracellular acidification during tumor progression.

In this regard, a number of studies have been carried out to identify molecules expressed by tumor cells that correlate with the regulation of pH changes. Among possible candidates is the $\text{Na}^+\text{-H}^+$ exchanger (NHE), a ubiquitous membrane protein known to regulate cellular pH [72]. There are a number of NHE isoforms encoded by different genes [72]. One of the most widely distributed isoform of NHE is called NHE1 which catalyzes the extrusion of intracellular proton (H^+) ions in exchange for extracellular (Na^+) ions, thereby regulating intracellular pH (pH_i) [72]. Previous studies indicate that NHE1 is one of the principal pH_i regulatory molecules in breast tumor cells [73]. NHE1 also appears to be involved in the aberrant regulation of both extracellular pH (pH_e) and pH_i in human breast tumor cells under nutrient-depleted conditions [74]. Certain observations on the localization and regulation of certain NHE isoforms suggest that there are connections between NHE activity and cholesterol-enriched lipid rafts [75].

Interestingly, NHE1 also serves as one of the cellular substrates for RhoA-activated ROK [76]. NHE1 phosphorylation by RhoA-activated ROK induces actin stress fiber assembly [76]. Recent evidence indicates that the binding of HA-CD44 activates RhoA-ROK activity which, in turn, promotes NHE1 phosphorylation and $\text{Na}^+\text{-H}^+$ exchange activity leading to intracellular acidification and creates an acidic ECM environment. These events result in hyaluronidase (Hyal-2)-mediated HA catabolism, HA modification, cysteine proteinase (cathepsin B)-mediated matrix degradation and cancer progression [77].

3.1.4. LARG-mediated RhoA activation and $\text{PLC}\epsilon\text{-Ca}^{2+}$ signaling—Another RhoA-specific GEF, the LARG molecule containing several functional domains (e.g., an amino-terminal PDZ domain, RGS domain, a DH domain and a PH domain) has been identified recently [78]. In particular, the DH domain of LARG by itself exhibits GDP/GTP exchange activity on RhoA, and plays an important role in RhoGTPase signaling [79]. LARG can transduce signals from receptors coupled to the heterotrimeric G-proteins, $\text{G}\alpha_{12}$ and $\text{G}\alpha_{13}$ to Rho [79]. LARG and its close homologs, p115-RhoGEF and PDZ-RhoGEF, all exhibit an RGS-like domain that is involved in a structural linkage with $\text{G}\alpha_{12/13}$ and may function as a GTPase-activating protein for $\text{G}\alpha$ subunits [79]. LARG, unlike the related p115-RhoGEF and PDZ-RhoGEF, also serves as an effector for the $\text{G}\alpha_q$ -coupled receptor and mediates RhoA signaling [79]. Thus, LARG appears to be selectively involved in certain signaling pathways initiated by G-protein coupled receptors (GPCRs) that are linked to different $\text{G}\alpha$ subunits. Furthermore, overexpression of LARG in acute myeloid leukemia (AML) has been shown to exhibit growth-promoting activity [78]. A number of studies show that LARG and its transforming potential can be activated by focal adhesion kinase (FAK) phosphorylation [80] and/or hetero-oligomerization through LARG's inhibitory C-terminal region [81]. Apparently, LARG-RhoGEF activity can be regulated by many different mechanisms in addition to its previously described role in $\text{G}\alpha$ subunit signaling by GPCRs.

A recent study has found that HA binding to CD44 induces LARG-specific RhoA signaling in head and neck tumor cells [82]. In searching for possible new cellular targets for LARG-activated RhoA, phosphoinositide-specific phospholipase C- ϵ (PLC ϵ) was identified. Specifically, it has been observed that HA-CD44 binding stimulates LARG-catalyzed RhoA signaling which, in turn, activates RhoA-association with PLC ϵ in a GTP-dependent manner and promotes PLC ϵ -mediated IP₃ production and Ca²⁺ mobilization [82]. Subsequently, the upregulation of Ca²⁺/calmodulin-dependent kinase-II (CaMKII) occurs leading to phosphorylation of the cytoskeletal protein, filamin. The phosphorylation of filamin reduces its interaction with filamentous actin, promoting tumor cell migration. Further analyses show that the PDZ domain of LARG binds to CD44 directly. Transfection of the head and neck tumor cells (HSC-3 cells) with LARG-PDZcDNA significantly reduces LARG association with CD44. Overexpression of the LARG-PDZ domain functions as a dominant-negative mutant (similar to the PLC/CaMKII inhibitor effects) by blocking HA/CD44-mediated signaling events (e.g., RhoA activation, PLC ϵ -mediated IP₃ production, intracellular Ca²⁺ mobilization, CaMKII activity, filamin phosphorylation and filamin-actin binding) and abrogating tumor cell migration [82]. These findings suggest that CD44 interaction with LARG plays a pivotal role in RhoA activation and PLC ϵ -Ca²⁺ signaling required for CaMKII-mediated cytoskeleton function resulting in head and neck squamous cell carcinoma progression.

3.2. HA/CD44-mediated Rac1 signaling

Signaling by RacGTPase, known to regulate actin assembly associated with membrane ruffling, pseudopod extension, cell motility and cell transformation [83,84], has been found to be abnormal in breast tumor cells as compared to normal breast epithelial cells [83,84]. The fact that Rac1 induces stress fiber formation in a Rho-dependent manner suggests that “cross-talk” occurs between the Rho and Rac1 signaling pathways. At the present time, two Rac-specific GEFs (e.g., T-lymphoma invasion and metastasis (Tiam1) and Vav2) have been identified and shown to be involved in HA-mediated CD44 signaling in tumor cells as follows.

3.2.1. Tiam1-catalyzed Rac1 signaling—Tiam1 has been identified as an oncogene due to its ability to activate Rho-like GTPases during malignant transformation [84]. Specifically, Tiam1 is capable of activating Rac1 *in vitro* as a GEF and induce membrane cytoskeleton-mediated cell shape changes, cell adhesion and cell motility [83,84]. It also acts as a Rac-specific GEF *in vivo* and induces invasive phenotypes in lymphoma cells [85]. These findings have prompted several research groups to investigate the mechanism involved in the regulation of Tiam1. For example, addition of certain serum-derived lipids (e.g., sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA)) to T-lymphoma cells promotes Tiam1-mediated Rac1 signaling and T-lymphoma cell invasion [85]. Tiam1 has also been found to be phosphorylated by protein kinase C (PKC) in Swiss 3T3 fibroblasts stimulated by LPA [86]. Most recently, Exton and his co-workers demonstrate that phosphorylation of Tiam1 by Ca²⁺/calmodulin-dependent protein kinase-II (but not PKC) regulates Tiam1-catalyzed GDP/GTP exchange activity *in vitro* [87]. These findings support the notion that posttranslational modifications of Tiam1 by certain serine/threonine kinase(s) during surface receptor-mediated activation may play an important role in Tiam1-Rac1 signaling. Tiam1 expression has previously been detected in breast cancer cells [25]. Tiam1 contains several functional domains including a DH domain and two PH domains. In particular, the DH domain of these proteins exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins [83,84]. Overexpression of both N- and C-terminally truncated as well as full-length Tiam1 proteins induces the invasive phenotype in otherwise noninvasive lymphoma cell lines [85]. A previous study showed that the N-terminus PH-CC-Ex domain is the primary Tiam1 binding region for CD44 [25]. Most importantly, the binding of HA-CD44v3 of breast tumor cells (SP1 cells) stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration. Transfection of SP1 cells with Tiam1cDNA promotes Tiam1 association with

CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with N-terminus PH-CC-Ex cDNA and Tiam1 cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors [25]. These observations support the notion that a linkage between the CD44v3 isoform and the N-terminus PH-CC-EX domain of Tiam1 is required for HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration during breast cancer progression.

In addition, a previous work demonstrated that the N-terminus PH-CC-Ex domain of Tiam1 also contains an ankyrin-binding site [88]. The structural homology between the ankyrin-binding domain of Tiam1 (the sequence between aa717 and aa727 within the N-terminus PH-CC-Ex domain) and CD44 is quite striking. Most importantly, the Tiam1–ankyrin interaction promotes Rac1 activation and breast tumor cell migration [88]. These observations clearly suggest that Tiam1 contains multiple functional domains (e.g., a CD44-specific membrane localization site and a cytoskeleton binding region for ankyrin) required for the regulation of Tiam1–Rac1 signaling and cytoskeleton function. Taken together, these results suggest that Tiam1 interaction with CD44v3 and ankyrin plays a pivotal role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor progression.

3.2.2. Vav2-catalyzed Rac1 signaling—In the search for other CD44 isoform-linked Rac-specific GEFs which correlate with metastatic behavior, a prime candidate (named Vav2) has been identified. Vav2 is a member of the Vav family of oncoproteins known to act as GEF for Rac1 in a phosphotyrosine-dependent manner [89]. The Vav molecule was first identified as an oncogene based on its ability to induce cell transformation following gene transfer using human tumor DNA [89]. Vav1 is expressed exclusively in hematopoietic cells, whereas the homologous protein, Vav2 is now known to be expressed ubiquitously [90]. Structurally, Vav2 is a 95–100-kDa protein which contains numerous functional domains and structural motifs found in signal transduction proteins and oncoproteins. These motifs include amino-terminal calponin homology (CH)/leucine-rich (LR) and acidic-rich (AD) domains, a DH domain, a PH domain, a cysteine-rich (CR) zinc-binding domain, and two src homology-3 (SH3) domains flanking a single SH2 domain [91]. In particular, the sequence between aa198 and aa469 contains significant sequence homology to the DH domain found in many proteins which exhibit GDP/GTP exchange activity for specific members of the Ras super-family of GTP-binding proteins [91]. *In vitro*, Vav2 has been clearly shown to act as a GDP/GTP exchange protein for the Rho sub-family of GTPases, including Rac1, Cdc42, RhoA, RhoB and RhoG [89,91]. In addition, Vav2 contains one PH domain, which is commonly detected in signaling molecules and cytoskeletal proteins [92]. The PH domain may also mediate the association of Vav2 with the submembrane region of the cell via protein–protein or protein–lipid interactions [92]. The C-terminal region of Vav2 contains an SH2 domain surrounded by two SH3 domains. Although its function is still unclear, this region may facilitate interactions with submembrane signaling complexes. It has been shown that over-expression of Vav2 is sufficient to activate its oncogenic potential [91]. Vav2-induced cytoskeletal changes and cellular transformation are comparable, but not identical, to those induced by other members of the dbl family of oncoproteins [54]. Taken together, these sequence analyses of Vav2 suggest that the association of Vav2 with the transforming phenotype may be mediated via sub-membrane cytoskeletal regulation and/or activation of Rho family GTPases. Immunological data indicate that both CD44v3 and Vav2 are expressed in ovarian tumor cells, and that these two proteins are physically linked as a complex *in vivo*. Using recombinant fragments of Vav2 and *in vitro* binding assays, we have detected a specific binding interaction between the SH3–SH2–SH3 domain of Vav2 and the cytoplasmic domain of CD44 [26]. In addition, we have observed that the binding of HA–CD44v3 activates Vav2-mediated Rac1 signaling leading to ovarian tumor cell migration [26]. Vav2 is also known to interact with adaptor proteins, such as Grb2 [93], that mediate binding to the oncogene product, ErbB2, in transformed cells [94]. CD44–Vav2 interaction with the Grb2–p185^{HER2} complex plays an important role in promoting HA-

dependent Rac1 activation and Ras signaling leading to the concomitant stimulation of human ovarian tumor cell migration and growth [26].

3.3. HA/CD44-mediated Cdc42 signaling

Activation of Cdc42 has been found to produce specific structural and functional changes in the cell membrane–cytoskeleton interaction [47]. Cdc42 signaling is considered to be important in the mechanism underlying cell growth and migration, both of which are obvious prerequisites for metastasis [47,49,50]. The involvement of Cdc42–cytoskeleton interaction during HA–CD44 interaction in tumor cells is described as follows.

3.3.1. Cdc42–PAK1 signaling—In the search for cellular targets of Cdc42 that correlate with metastatic behavior in tumor cells, the PAK family of kinases (e.g., PAK1) was identified and documented to be one of the down-stream effectors of Cdc42 [95]. PAK1 is known to be involved in cortical actin remodeling induced by RhoGTPases such as Rac or Cdc42, and plays a significant role in a variety of biological activities [95]. A recent study indicates that HA/CD44-mediated signaling stimulates both Cdc42–CD44 association and PAK1 phosphorylation [96]. The fact that filamin (a 280-kDa actin-binding protein) is known to bind the p21-binding domain (PBD) of PAK1 (aa52–132) forms a complex with PAK1 during HA/CD44-mediated extracellular signal-regulated kinase (ERK) signaling, suggests that a PAK1–filamin interaction could contribute significantly to cytoskeletal regulation in tumor progression. These findings are consistent with the previous findings that PAK1 activation is closely involved in filamin-mediated motility [96].

3.3.2. Cdc42–IQGAP1 signaling—In the search for additional cellular targets of HA/CD44-activated Cdc42 signaling that correlate with metastatic behavior in tumor cells, a molecule identified as IQGAP1 (a 190-kDa protein—one of the downstream effectors of Cdc42) has been detected [97]. This molecule contains numerous functional domains and motifs found in many signal transduction proteins and oncoproteins. These structural domains include four putative calmodulin-binding (IQ) motifs, a calponin homology (CHD) domain, a poly-proline binding (WW) domain and a Ras-GAP-related (GRD) domain [97]. Some of these motifs are involved in IQGAP1 interaction with specific proteins, such as Cdc42, Rac, actin, calmodulin ERK2, E-cadherin and β -catenin [97–99]. The establishment of a physical linkage between IQGAP1 and its binding partners has been shown to generate several important biological activities. For example, IQGAP1–Cdc42 binding mediates oncogenic signaling events resulting in the activation of actin-cytoskeleton and tumor cell migration/invasion [97–99]. IQGAP1 binding to β -catenin promotes both the rearrangement of the cadherin cell adhesion complex and transcriptional activation. Moreover, the IQGAP1–ERK2 association modulates the Ras/mitogen-activated protein kinase pathways (in particular, ERK signaling) [99]. These findings suggest that IQGAP1 not only serves as a scaffolding protein (mediating multiprotein complex assembly), but also directly participates in cytoskeleton activation and signaling coordination.

ERKs, members of the mitogen-activated protein kinase (MAP kinase) family, are known to be activated by receptor tyrosine kinases, cytokine receptors, and G-protein-coupled receptors [100]. In particular, ERK phosphorylation of certain target proteins is directly involved in transcriptional activation by coordinating extracellular cues and intracellular signals [100]. For example, during EGF stimulation or integrin signaling, ERK activation is coupled with the phosphorylation of specific target molecules, including transcription factors (e.g., the ternary complex factor, Elk-1) [101] and the nuclear hormone receptor (e.g., estrogen receptor) [102], resulting in cyclin D1 expression and cell cycle progression [103]. Furthermore, growth factors have been found to activate ERK and augment estradiol-mediated activation of nuclear hormone receptor, estrogen receptors during malignant transformation [103]. Previous studies

indicate that HA interaction with the hyaluronan receptor, RHAMM, regulates ERK activity [104]. Most importantly, HA-activated ERK signaling participates in the upregulation of certain transcription factors (e.g., NF- κ B and AP-1), cell growth and cell migration [104].

A previous study showed that both IQGAP1 and Cdc42 are physically linked to CD44 in tumor cells (SK-OV-3.ipl cells) following HA stimulation [105]. Furthermore, the HA/CD44-induced Cdc42–IQGAP1 complex regulates cytoskeletal function via a close association with filamentous actin (F-actin) leading to tumor cell migration. In addition, the binding of HA–CD44 promotes the association of the extracellular signal-regulated kinase 2 (ERK2) with the IQGAP1 molecule which stimulates both ERK2 phosphorylation and kinase activity [105]. The activated ERK2 then increases the phosphorylation of both Elk-1 and estrogen receptor α (ER α) resulting in Elk and estrogen responsive element (ERE)-mediated transcriptional upregulation [105]. Downregulation of IQGAP1 (by treating cells with IQGAP1-specific small interfering RNAs (siRNAs)) not only blocks IQGAP1 association with CD44, Cdc42, F-actin and ERK2, but also abrogates HA/CD44-induced cytoskeleton function, ERK2 signaling (e.g., ERK2 phosphorylation/activity, ERK2-mediated Elk-1/ER α phosphorylation and Elk-1/ER α -specific transcriptional activation) and tumor cell migration [105]. Taken together, these findings support the observations that HA–CD44 interaction with IQGAP1 serves as a signal integrator by modulating Cdc42–cytoskeleton function, mediating Elk-1-specific transcriptional activation and coordinating “cross-talk” between a membrane receptor (CD44) and a nuclear hormone receptor (ER α) signaling pathway during tumor progression.

4. Summary

HA/CD44-mediated tumor cell-specific phenotypes are closely linked to cytoskeletal functions which involve membrane-associated cytoskeletal proteins (e.g., ankyrin) and the small GTP-binding proteins such as RhoA, Rac1 and Cdc42. Activation of ankyrin and RhoGTPases (e.g., Rac1, Cdc42 and RhoA) has been shown to produce specific structural changes in actin assembly, cytoskeleton reorganization, transcriptional activation, tumor cell growth, survival, migration and invasion. In summary, a current model for illustrating HA-dependent and CD44-specific signaling pathways is described in Fig. 3. Specifically, the binding of HA to the extracellular domain of CD44 (containing variant exon-coded structures) promotes CD44 interaction with certain cytoskeletal proteins such as ankyrin (A) and RhoGTPases (e.g., RhoA (B), Rac1 (C) and Cdc42 (D)) which then generate specific signaling cascades described as follows.

4.1. Ankyrin-based signaling pathways

- a. HA binding to CD44 stimulates ankyrin-mediated cytoskeleton rearrangement required for cell adhesion.
- b. HA–CD44 interaction also promotes recruitment of IP₃ receptor (located in the intracellular Ca²⁺ storage organelles) into CD44 and caveolin-1/cholesterol-containing lipid rafts. This event is required for the formation of functional IP₃ receptor Ca²⁺ channels (presumably via conformational changes and/or IP₃ receptor tetramerization), and the onset of internal Ca²⁺ release leading to CaMKII (Ca²⁺/calmodulin-dependent kinase-II) activation and filamin phosphorylation as well as cytoskeleton-mediated tumor cell migration.

4.2. RhoA-specific signaling pathways

- (a) HA–CD44 binding is tightly coupled with LARG in a complex which can induce LARG-mediated RhoA activation. HA/CD44 and LARG-activated RhoA then stimulates PLC ϵ -mediated IP₃ production and IP₃ receptor-triggered intracellular Ca²⁺ mobilization resulting

in CaMKII activation. CaMKII then phosphorylates the cytoskeletal protein, filamin, leading to cytoskeleton reorganization and tumor cell migration.

(b-1) The binding of HA–CD44 also induces activation of ROK which, in turn, phosphorylates myosin phosphatase and myosin light chain, thereby activating myosin adenosine triphosphatase (ATPase) and generating actomyosin-mediated membrane motility and cell migration.

(b-2) The binding of HA–CD44 stimulates ROK phosphorylation of NHE1. Most importantly, the phosphorylation of NHE1 by ROK promotes Na^+/H^+ exchange activity, intra-endosomal/lysosomal pH changes and extracellular acidification leading to an activation of low pH-dependent ECM degradation enzymes required for ECM modification and tumor cell invasion.

(b-3) HA–CD44 interaction is tightly coupled with p115RhoGEF in a complex which can up-regulate RhoA signaling and ROK activity. Activated ROK then phosphorylates certain cellular proteins including the linker molecule, Gab-1. Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3 kinase to CD44 and activates certain isoforms of PI3 kinase to convert $\text{PtdIns}(4,5)\text{P}_2$ to $\text{PtdIns}(3,4,5)\text{P}_3$ leading to AKT activation and tumor cell survival required for breast tumor progression.

(b-4) HA binding to CD44 promotes ROK phosphorylation of CD44 which, in turn, recruits ankyrin from the cytosol to the membrane leading to ankyrin–Tiam1 interaction and Rac1 activation as well as cytoskeleton-mediated cancer progression.

4.3. Rac1-specific signaling pathways

The binding of HA–CD44 promotes Tiam1/Vav2-mediated Rac1 activation and downstream effector functions (e.g., PAK (a) and IQGAP1 (b)) leading to actin assembly and cytoskeleton reorganization and cancer progression.

4.3.1. Cdc42-specific signaling pathways—(D-a) The binding of HA–CD44 promotes Cdc42–CD44 association and PAK1 phosphorylation/activation and PAK1–filamin complex formation leading to cytoskeleton activation and tumor cell migration and invasion.

(D-b) HA-activated CD44 is tightly coupled with IQGAP1 in a complex which interacts with both Cdc42 and ERK2. The CD44–IQGAP1–Cdc42 complex is capable of binding to F-actin required for cytoskeletal function and tumor cell migration. At the same time, the association of CD44–IQGAP1 with ERK2 promotes ERK2 phosphorylation and ERK2 activity which, in turn, phosphorylates Elk-1 and ER α . Most importantly, phosphorylation of Elk-1/ER α by IQGAP1-linked ERK2 enhances transcriptional activation. We believe that all these oncogenic signaling events and tumor cell-specific behaviors play a pivotal role in promoting tumor progression.

Our current model suggests that the close interactions between CD44 and its selected binding partners play a pivotal role in promoting “cross-talk” among various intracellular signaling pathways (e.g., ankyrin-based cytoskeleton and RhoGTPases) leading to the concomitant onset of multiple functions. The coordinated HA-mediated CD44 activation of RhoGTPase signaling and the cytoskeleton as described in Fig. 3 may be a possible mechanism underlying various tumor cell-specific behaviors, transcriptional activation, tumor cell adhesion, growth, survival, migration and invasion—all obvious prerequisites for metastasis.

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Abbreviations

HA, hyaluronan
 ECM, extracellular matrix
 ARD, ankyrin repeat domain
 ERM, Ezrin/Radixin/Moesin
 IP₃, inositol 1,4,5 trisphosphate
 GEFs, guanine nucleotide exchange factors
 RGS, regulator of G-protein signaling
 DH, dbl homology
 PH, pleckstrin homology
 ROK, Rho-kinase
 PI3 kinase, phosphatidylinositol 3-kinase
 Gab-1, Grb-2-associated binder-1
 NHE, Na⁺–H⁺ exchanger
 LARG, leukemia-associated Rho guanine nucleotide exchange factor
 Tiam1, T-lymphoma invasion and metastasis
 PLCε, phosphoinositide-specific phospholipase C-ε
 CaMKII, Ca²⁺/calmodulin-dependent kinase-II
 ERKs, extracellular signal-regulated kinases

Fig. 1. Schematic illustration of ankyrin structure. Ankyrin contains ankyrin repeat domain (ARD) (with consensus sequence -G-TPLH-AA-GH—V/A-LL-GA-ND—), spectrin binding domain (SBD) and regulatory domain (RD).

Fig. 2.
Ligand-receptor-mediated RhoGTPase signaling and cytoskeleton activation.

Fig. 3.

A current model for illustrating HA-dependent and CD44-specific signaling pathways during oncogenesis and tumor progression.