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# Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells

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## Abstract

Hyaluronan is an important structural component of extracellular matrices but also interacts instructively with cells during embryonic development, healing processes, inflammation, and cancer. It binds to several different types of cell surface receptors, including CD44, thus leading to co-regulation of important signaling pathways, notably those induced by activation of receptor tyrosine kinases. Consequently, interactions of both stromal and tumor cell-derived hyaluronan with tumor cells play important cooperative roles in several aspects of malignancy. This review focuses on cell autonomous hyaluronan-tumor cell interactions that lead to activation of receptor tyrosine kinases and enhanced drug resistance. Particular emphasis is placed on the role of hyaluronan-CD44 interactions in drug transporter expression and activity, especially in cancer stem-like cells that are highly malignant and resistant to chemotherapy. Antagonists of hyaluronan-CD44 interaction, especially small hyaluronan oligomers, may be useful in therapeutic strategies aimed at preventing tumor recurrence from these therapy-resistant sub-populations within malignant cancers.

## Introduction: The relationships between drug resistance, malignancy and cancer stem-like cells

Invasion and metastases of cancer cells and the development of resistance to anticancer therapies are the main causes of morbidity and mortality from cancer. Recently, sub-populations of stem-like cells have been characterized within a variety of cancers. These cells are highly malignant in that they can rapidly regenerate a fully grown tumor when implanted in small numbers in an animal host (1-3) and they may be responsible for tumor metastasis (4,5). In addition, these cells usually demonstrate resistance to chemotherapy (multi-drug resistance) (6). Expression of the hyaluronan receptor, CD44, is frequently associated with these stem-like cells (1). Both hyaluronan and CD44 have been shown to play a role in drug resistance (7-9), as well as in malignant cell behavior and cell survival (10). Treatment with hyaluronidase enhances the action of various chemotherapeutic agents (11,12). Moreover, hyaluronan may be extruded from cells by ABC-family drug transporters (13). These and other observations point towards a role for hyaluronan-CD44 interactions in the malignant and drug-resistant properties of cancer cells, and possibly cancer stem-like cells.

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## Hyaluronan in tumor progression

Hyaluronan is a large, linear glycosaminoglycan composed of 2,000-25,000 disaccharides of glucuronic acid and N-acetylglucosamine: $[\beta 1,4\text{-GlcUA-}\beta 1,3\text{-GlcNAc-}]_n$, with molecular weights usually ranging from $10^5$ to $10^7$ Da. Hyaluronan is distributed ubiquitously in vertebrate tissues. In adult tissues such as the vitreous, synovial fluid and dermis, it clearly plays an extracellular, structural role that depends on its unique hydrodynamic properties as well as its interactions with other extracellular matrix components. However, hyaluronan is also concentrated in regions of high cell division and invasion, e.g. during embryonic morphogenesis, inflammation, wound repair, and cancer. Thus, in similar fashion to numerous matrix constituents, hyaluronan has an instructive role in terms of cell signaling via hyaluronan receptors on the cell surface, as well as an important structural role (10,14-17). Although underlying regulatory mechanisms are not well understood, it is clear that hyaluronan-induced signaling is “activated” during dynamic cell processes such as occur in cancer but not under conditions of adult tissue homeostasis.

Considerable experimental evidence implicating hyaluronan in tumor progression has now been obtained in animal models. Several approaches have been used, including manipulation of levels of hyaluronan and perturbation of endogenous hyaluronan-protein interactions. For example, experimental over-expression of the hyaluronan synthase, HAS2, in human fibrosarcoma cells gives rise to elevated hyaluronan production and causes increased tumor cell growth in xenografts in vivo (18). Similar results were obtained in vivo on HAS2 over-expression in a transgenic mouse breast cancer model (19) or on over-expression of HAS3 in human prostate tumor cells (20). On the other hand, transfection of prostate carcinoma cells with antisense *Has2* and *Has3* reduced subcutaneous tumor growth in nude mice xenografts (21). Tumor growth and metastasis are also inhibited in animal xenograft models by perturbing endogenous hyaluronan-cell receptor interactions in various ways. For example, soluble hyaluronan binding proteins such as the ectodomain of CD44 competitively displace hyaluronan from its endogenous cell surface receptors. Thus over-expression of CD44 ectodomain in mouse mammary carcinoma cells or in human malignant melanoma cells has been shown to inhibit growth, local invasion, and metastasis in vivo (22-25). These effects most likely arise due to induction of apoptosis (23) or cell cycle arrest (24) in vivo. No significant effects were obtained in these studies if the CD44 ectodomain was mutated such that hyaluronan binding was reduced. A soluble form of Rhamm, another hyaluronan receptor, also induces cell cycle arrest and inhibits metastasis (26) and a soluble hyaluronan-binding complex derived from cartilage inhibits both tumor growth and metastasis (27). Likewise, administration of antibodies that block hyaluronan binding to CD44 inhibits tumor growth and invasion (28,29). In addition, we have found that treatment with small hyaluronan oligosaccharides (oligomers) retards growth of several tumor types in vivo (30-32). These oligomers most likely compete for endogenous polymeric hyaluronan (see Figure 1), thus replacing high affinity, multivalent and cooperative interactions with low affinity, monovalent receptor interactions (33,34); oligomers containing 6-18 sugar residues are monovalent in their interaction with CD44, whereas larger polymers are multivalent (34).

Although these and many other studies have strongly implicated hyaluronan in tumorigenesis, numerous observations have been made that indicate the role of hyaluronan in cancer is complex, especially with respect to hyaluronan processing. First, lower molecular weight hyaluronan, e.g. 10-100 kDa, stimulates angiogenesis but high molecular weight hyaluronan (>1,000 kDa) is inhibitory (35-37). Second, even though elevated hyaluronan production usually promotes tumor progression, extremely high levels of hyaluronan production can be inhibitory (38). Third, glioma progression is promoted by increased hyaluronan production only when hyaluronidase is expressed concomitantly with hyaluronan (39). Similarly, maximum growth of prostate tumors in xenografts was observed on coexpression of both the

hyaluronan synthase, HAS2, and the hyaluronidase, HYAL1 (40). Fourth, tumor progression often correlates with both hyaluronan and hyaluronidase levels in human cancers (41). These observations have led several investigators to propose that hyaluronan turnover is essential to the promotion of tumor progression by hyaluronan (23,39-43). This idea is compounded by the apparent importance of partial degradation of hyaluronan in signaling pathways implicated in inflammation (17), an important factor in the progression of many tumor types (44,45).

Another complex aspect of the relationship between hyaluronan and tumor progression is the relative contribution of stromal versus cancer cell-produced hyaluronan. In human patients, correlations have been made between increased levels of either stromal or parenchymal hyaluronan and malignant outcomes (reviewed in Tammi et al, Ch. ??). The importance of stromal hyaluronan has been highlighted in a Neu-induced, spontaneous, mouse breast cancer model in which hyaluronan levels were increased by up-regulation of HAS2 (19)(see Kimata and Itano, Ch. ??). Using this model, it was shown that induction of hyaluronan production caused recruitment of stromal cells, deposition of a hyaluronan-enriched stromal matrix and increased angiogenesis, as well as enhanced cell survival signals in the tumor cells themselves (19). However, it was not clear in this study the extent to which stromal cells were the product of epithelial-mesenchymal transition of the carcinoma cells since it is known that hyaluronan can regulate this process (46,47). Many mechanistic studies have addressed the effects of exogenously added hyaluronan, possibly mimicking the influence of stromal hyaluronan (reviewed in Bourguignon, Ch. ??), but the focus of our work has been on manipulating constitutive hyaluronan interactions in cancer cells themselves. Although both approaches have yielded interesting insights, there is an urgent need for further animal studies that distinguish the effects of stromal and cancer cell-produced hyaluronan.

## Cell autonomous regulation of receptor tyrosine kinase activation and anti-apoptotic signaling pathways by endogenously produced hyaluronan

Receptor tyrosine kinases are a class of plasma membrane receptors that bind various regulatory factors, such as EGF, IGF, HGF and PDGF, and activate several intracellular signaling pathways, such as the MAP kinase and phosphoinositide 3-kinase/AKT pathways. Aberrant activities of these receptors, especially members of the ERBB family, have been implicated in the progression of numerous types of human cancers. Increased activity of receptor tyrosine kinases can arise from gene amplification, activating mutations or altered regulation, e.g. by cross-talk between these receptors and integrins or other receptors, or by altered autocrine and paracrine stimulation by various regulatory factors. These changes lead in turn to enhanced tumor cell growth, motility, survival, and resistance to therapies (48-50).

Several reports have documented augmentation of receptor tyrosine kinase and downstream signaling pathway activities after treatment of cancer cells with exogenous hyaluronan (reviewed in Bourguignon, Ch. ??). We have shown that manipulations of constitutive hyaluronan production and interactions in cancer cell themselves also have profound effects on these pathways. We found that constitutively high levels of active, i.e. autophosphorylated, ERBB2 in carcinoma cells are dependent on endogenous hyaluronan-CD44 interaction and that experimentally increased hyaluronan production causes elevated ERBB2 phosphorylation in cells that normally exhibit low levels of ERBB2 activity (51). Furthermore, stimulation of hyaluronan production induces assembly of a constitutive, lipid raft-associated, signaling complex containing phosphorylated ERBB2, CD44, ezrin, phosphoinositide 3-kinase, and the chaperone molecules, HSP90 and CDC37; whereas inhibition of endogenous hyaluronan-CD44 interaction causes disassembly of this complex. Antagonists of hyaluronan interactions used in these studies include hyaluronan oligomers, soluble hyaluronan-binding proteins and siRNA against CD44 (Figure 1), all of which caused disassembly of this complex and inactivation of ERBB2 (51). Recent work in our lab shows that hyaluronan antagonists cause

rapid internalization of ERBB2 and CD44 accompanied by their disassociation from one another (M. Slomiany & B. Toole, unpublished). Based on the previous work of other groups (see Bourguignon, Ch. ??), it is likely that variants of CD44, rather than standard CD44, are involved in these events; this issue is currently under investigation in our lab. Similar influences of constitutive hyaluronan-CD44 interaction occur with other receptor tyrosine kinases, i.e. EGFR, IGF-1R, PDGFR and c-MET (52), and corresponding effects have been shown for downstream anti-apoptotic and proliferation pathways known to be regulated by these receptor kinases. For example, increased hyaluronan production stimulates the phosphoinositide 3-kinase and MAP kinase pathways whereas antagonists of hyaluronan interactions suppress these pathways (31,53).

The phosphoinositide 3-kinase/AKT signaling pathway, up-regulated in most malignant cancer cells, is an anti-apoptotic pathway regulated by several receptor tyrosine kinases, e.g. EGFR, ERBB2 and IGF-1R. These receptor kinases are known to be important in malignant cell properties such as deregulated proliferation, anchorage independent colony formation and invasiveness (48-50). In addition to these pro-malignant and anti-apoptotic activities, this pathway leads to increased expression of broadly distributed ABC family multidrug transporters, e.g. P-glycoprotein (MDR1/ABCB1), multidrug resistance-associated protein-1 (MRP-1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) (8,54,55). Not surprisingly, then, recent publications have demonstrated a close relationship between malignant cell properties and resistance to therapy (6,7,9,56-58).

## Regulation of multidrug resistance by hyaluronan

Drug resistance can arise in numerous ways, e.g. decreased uptake of drugs due to cell and tissue barriers, activation of repair and detoxification mechanisms, increased activities of anti-apoptotic signaling pathways, or enhanced drug efflux via cell membrane transporters (59-62). Drug efflux from cancer cells is commonly mediated by ATP-dependent efflux pumps such as members of the MDR, MRP and other ABC transporter subgroups, and expression of these transporters is frequently elevated in malignant cancer cells (6,59).

The possibility that hyaluronan might influence drug resistance was suggested in the finding that hyaluronidase treatment enhances the action of various chemotherapeutic agents, especially when used locally (12). The relation between hyaluronan and multidrug resistance was also studied in multicellular mammary tumor cell spheroids, known to be enriched in therapy-resistant stem-like cells; dispersion of these spheroids with hyaluronidase reverses drug resistance (63,64). The mechanistic action of hyaluronidase on drug resistance was not understood at the time of these studies but was usually explained in terms of possible effects on cell adhesion barriers (63), drug penetration (12,65), or cytokine diffusion (66) rather than hyaluronan-specific effects on signaling pathways. However, early studies by our laboratory showed that calcium-independent aggregation of transformed cells, such as occurs in multicellular spheroids, can be due to hyaluronan-mediated, multivalent cross-bridging of receptors on adjacent cells (67). This observation and the finding that hyaluronan constitutively regulates cell survival signaling pathways (31) led us to further investigate the possible role of hyaluronan in drug resistance. As such, increased hyaluronan production was found to stimulate drug resistance in drug-sensitive cancer cells, whereas disruption of endogenous hyaluronan-induced signaling suppresses cell resistance to several drugs, including doxorubicin, taxol**,** vincristine, and methotrexate (53). This and other studies show that hyaluronan and CD44 promote drug resistance in a variety of cancer cell types, including breast, lung and head and neck carcinomas, and lymphoma (8,68-71). Although the anti-apoptotic effect of hyaluronan is likely to contribute to these phenomena, hyaluronan-CD44 interactions also regulate expression of drug transporters, including P-glycoprotein (8), MRP2 (8,71) and BCRP (32). Recent work from our lab suggests that the effect of hyaluronan on transporter

expression may be mediated by stabilization of these transporters in the plasma membrane rather than on synthesis. This conclusion is based on experiments in which we show that treatment of cells with hyaluronan oligomers induces rapid internalization of the drug transporters, BCRP and P-glycoprotein (see Figure 2 for details) (M. Slomiany & B. Toole, unpublished).

Activation of the MDR1 upstream promoter, associated with P-glycoprotein overexpression, correlates with metastases to the lymph nodes in breast carcinoma cells (57). In accordance with the role of CD44 in malignant cell behavior and metastases, it was shown by confocal microscopic co-localization and fluorescence resonance energy transfer (FRET) studies in NIH3T3 cells that P-glycoprotein is closely associated with CD44 and other components of plasma membrane lipid microdomains, commonly known as lipid rafts (72). Interestingly, P-glycoprotein was found to be anchored to the cytoskeleton. CD44 is known to bind to the actin cytokeleton through ERM-family proteins (73) or ankyrin (74). Thus, these results suggest that CD44 resides in close molecular vicinity (<10 nm) to P-glycoprotein and may be one of the proteins responsible for the cytoskeletal association of this transporter. Furthermore, raft localization of P-glycoprotein seems to be of functional importance since cholesterol depletion results in strong inhibition of transporter activity (72). In addition, a study comparing multidrug resistant cell lines of breast, oral, and ovarian origin that overexpress P-glycoprotein with their respective P-glycoprotein-negative, drug-sensitive, parental cell lines demonstrated a positive correlation in the expression of CD44 and P-glycoprotein. The two were found to co-immunoprecipitate, and drugs that interfere with the function of P-glycoprotein also interfere with cell motility and invasion, both hallmarks of CD44 receptor activity (7). We also have observed that CD44 co-localizes in the plasma membrane of cancer cells with the transporters, P-glycoprotein and BCRP, and that treatment of the cells with hyaluronan oligomers rapidly induces internalization of the transporters and CD44 into the cell. Interestingly, it has been noted that drugs that interfere with P-glycoprotein can also affect localization of CD44 on the cell membrane and promote CD44 capping, and therefore might act via inhibition of actin polymerization (7). Similarly, we have seen that the CD44 internalization process is inhibited if the cells are co-treated with an inhibitor of actin polymerization, latrunculin, thus suggesting that the transporters and CD44 are anchored to actin filaments (M. Slomiany & B. Toole, unpublished). Our interpretation of these results is shown in Figure 2.

Relatedly, it has been demonstrated that expression of CD44 and P-glycoprotein are co-regulated, whereby modulation of CD44 expression correspondingly affected P-glycoprotein expression (7). P-glycoprotein-targeted siRNA also decreased the rate of cell migration (7), which agrees with the observation that both P-glycoprotein and CD44 need to be active in T lymphocytes for their proper migration to lymph nodes (75). Most likely these effects are due to alterations in CD44 expression, and therefore its migration-inducing activity (76,77), by inhibition of P-glycoprotein expression.

In addition, there may be a direct relationship between hyaluronan and drug transport since manipulation of hyaluronan in a cell-free system inhibits drug transport (S. Misra, S. Ghatak & B. Toole, unpublished). Moreover, based on studies documenting association of polysaccharide export with ABC-transporters in bacteria (78), recent work indicates that hyaluronan might be secreted through multidrug transporters in vertebrate cells (13,79). Studies employing a battery of inhibitors as well as siRNA to sort out possible transporters involved in hyaluronan export led to the conclusion that MRP5 is the most likely hyaluronan transporter in human fibroblasts (13). The MRP5 gene is ubiquitously expressed, with the highest levels of expression found in skeletal muscle and the brain. However, whereas MRP5 knockout mice are viable (80), hyaluronan deficiency is most likely incompatible with life in vertebrates (46). Thus hyaluronan export probably requires alternative or backup transport systems that can compensate for the lack of MRP5. Also, it would not be surprising to find other members

of multidrug resistant transporters as hyaluronan exporters since these transporters are often expressed in a tissue specific pattern. These may include MRP1 (75) or ABCC11 or ABCC12 due to their close phylogenetic relationship (13).

Although this evidence supports a role for drug transporters in hyaluronan secretion, other studies strongly suggest that constitutive export of hyaluronan requires only the hyaluronan synthases themselves (81). Moreover, definitive evidence for hyaluronan export through ABC transporters, rather than regulation by transporter activity, is lacking. Nevertheless it is likely that such export does occur at least under certain circumstances. Indeed, our findings demonstrate that hyaluronan production or export is inhibited by treatment of cells with hyaluronan oligomers (52) and that treatment of cells with hyaluronan oligomers induces rapid internalization of the drug transporters, BCRP and P-glycoprotein (M. Slomiany & B. Toole, unpublished). Thus, the finding that drugs used in the inhibition of drug transporters may also inhibit hyaluronan synthesis may open novel ways for treatment of diseases characterized by hyaluronan overproduction such as edema formation after injuries and inflammation, or in metastasis.

## Hyaluronan-CD44 interactions, cancer stem cells and resistance to chemotherapy

Of particular relevance to the relationship of malignant cell properties to chemoresistance are the properties of a small sub-population of stem-like cells that has now been characterized within many cancers. These cells have been variously named: “cancer stem cells”, “cancer progenitor cells” and “tumor-initiating cells”. These cells are highly malignant in that a very small number can rapidly regenerate a fully grown tumor when implanted in an animal host (1-3) and they may include the metastatic sub-population of tumors (4,5). These cells are also resistant to chemotherapeutic agents (6) and to radiation (82,83).

CD44 is one of the most common markers used for isolation of cancer stem-like cells (84-87), and recent studies of leukemia stem cells indicate that CD44 may be functionally important as well (88,89). Other studies point to a possible role for another hyaluronan-binding protein, Rhamm, in myeloma progenitors (90,91). However, virtually nothing is known about the potential role of hyaluronan, the major ligand for CD44 and Rhamm, although hyaluronan appears to have a role in normal stem cell behavior (92-95) and hyaluronan synthases are altered in myeloma progenitors (96,97).

The exact nature of these tumor sub-populations is controversial, especially with respect to their precise relationship to stem cells (2,98-100) but the presence of highly malignant, therapy-resistant sub-populations within human tumors is reasonably well-established. Consequently, recent work has focused on the nature of their resistance to therapy and in particular, the elevated expression of ABC-family drug transporters by these cells that may be central to tumor recurrence or persistence after chemotherapy (6,58). Recently we have begun to examine the effects of perturbing hyaluronan interactions with hyaluronan oligomers on the malignant and therapy-resistant properties of stem-like cells isolated from cancer cell lines and from patient-derived tumors. As discussed above, these oligomers most likely displace constitutively bound hyaluronan polymer from its receptors, resulting in attenuation of hyaluronan-induced signaling (31,51,52). We find that the oligomers inhibit growth of a very aggressive stem-like sub-population isolated from C6 glioma cells in a novel spinal cord engraftment model that replicates invasive behaviors of human gliomas in the central nervous system (32). The oligomers cause increased apoptosis and decreased proliferation in these tumors. The stem-like cells show elevated activation of EGFR and AKT, expression of the BCRP drug transporter and resistance to treatment with methotrexate, when compared with the parental cells. All of

these parameters were reduced by treatment with the oligomers (32), indicating the potential importance of hyaluronan in the properties of these cells.

Studies in several types of cancer cells have demonstrated that it is possible to define and isolate an enriched tumor-initiating population using the so-called “side population” phenotype (101); this phenotype depends on efflux of the Hoechst 33342 dye by drug transporters, especially BCRP. In a study of lung cancer cells (102), the side population cells were found to be significantly enriched for tumorigenicity and invasiveness, possessed stem cell properties such as multidrug resistance, high telomerase activity, and tumor-repopulating capacity, and consistently expressed ABC transporters, including BCRP, ABCA2, P-glycoprotein, and MRP1 (and related subfamily members MRP2 to MRP9), that consequently increased their resistance to multiple chemotherapeutic drugs. However, expression of CD44, and other commonly used cancer stem cell markers, varied amongst side population preparations and was present in side population and non-side population cells, pointing to remaining heterogeneity amongst these subpopulations and lack of exclusivity of CD44 to a particular subpopulation (102). This is in agreement with recent studies that confirm the difficulty of employing Hoechst dye exclusion alone to isolate homogenous side populations of stem-like cells, as drug transporter type and expression may vary widely, even amongst populations with similar tumorigenicities (98). Thus, the immediate challenge to understanding the relationship between hyaluronan, CD44, and multidrug resistance in cancer stem-like cells will come from the isolation of homogenous populations to better characterize the transporters involved and their relationship to CD44 expression.

Despite these caveats, it is reasonable to expect that antagonists of hyaluronan-CD44 interaction, e.g. small hyaluronan oligomers, may be useful in therapeutic strategies aimed at preventing tumor recurrence from therapy-resistant sub-opulations within malignant cancers.

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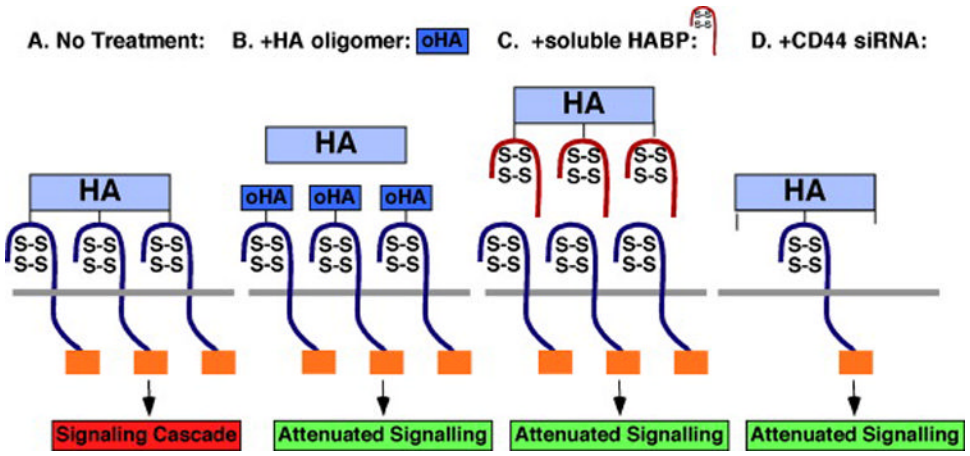


**Fig. 1.**

Antagonists of hyaluronan-CD44 signaling (adapted from refs 10 and 51). HA, hyaluronon; HABP, HA-binding protein.

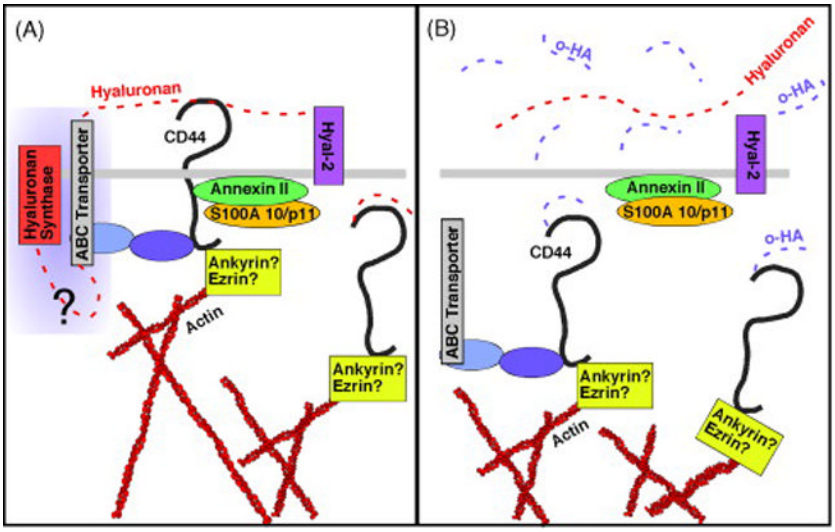


**Fig. 2.**

**A.** Hyaluronan is tethered by CD44 at the plasma membrane whereby it stabilizes actin-linked CD44-transporter complexes in lipid microdomains. Hyaluronan is cleaved by Hyal-2 and internalized via CD44 in an orderly manner. **B.** Oligomers of hyaluronan (o-HA) stimulate CD44 internalization en masse, destabilizing transporter complexes.