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REVIEW ARTICLE

Hyaluronan metabolism in remodeling extracellular matrix: probes for imaging and therapy of breast cancer†

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Clinical and experimental evidence increasingly support the concept of cancer as a disease that emulates a component of wound healing, in particular abnormal stromal extracellular matrix remodeling. Here we review the biology and function of one remodeling process, hyaluronan (HA) metabolism, which is essential for wound resolution but closely linked to breast cancer (BCA) progression. Components of the HA metabolic cycle (HAS2, SPAM1 and HA receptors CD44, RHAMM/HMMR and TLR2) are discussed in terms of their known functions in wound healing and in breast cancer progression. Finally, we discuss recent advances in the use of HA-based platforms for developing nanoprobes to image areas of active HA metabolism and for therapeutics in breast cancer.

Introduction

The extracellular matrix (ECM) is an important component of tissue microenvironments, which provide biophysical and biochemical cues that maintain tissue architecture integrity.[1] Modification of these signals appears to be an integral factor in promoting the progression of cancers arising from oncogenic mutations. For instance, forced expression of stromelysin I, an extracellular collagenase, is sufficient to initiate mammary tumors.[2] In some cases, the effects of the microenvironment on tumor progression can even dominate genetic aberrations in controlling aggressiveness of cancer cells.[3] Examples include blocking signaling through β-1 integrin extracellular matrix receptors or HA receptors,[4] such as receptor for hyaluronan mediated motility (RHAMM), which cause reversion of tumor cells to a non-tumorigenic state.[4c] These and other studies (reviewed in ref. 5) have provided compelling experimental evidence that an aberrant tumor ECM is a factor in sustaining tumor initiation and progression. Clinical evidence also supports this concept: epidemiological studies have linked chronic inflammation to cancer initiation while gene signatures derived from either isolated stromal cells (*e.g.* activated fibroblasts and macrophages) or tumor-associated stroma have predicted poor patient outcome and relapse.[5e,f,6] These studies additionally raise the possibility that a tumor microenvironment exhibits properties of wounds that do not heal,[6f,g] one aspect of which is a constitutively remodeling ECM.

Prognostic stromal transcriptomes typically contain high levels of ECM genes including collagens, collagenases, integrins, HA synthases, and hyaluronidases.[5f,6a,7] HA receptors as well as factors that regulate expression of ECM genes, such

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Insight, innovation, integration

Progressing tumors actively remodel their microenvironment like repairing wounds. The mechanisms by which these remodeling processes contribute to tumor progression are still poorly understood. This article reviews the evidence that hyaluronan metabolism, which is one remodeling process that is essential for wound resolution and contributes to breast cancer progression. The role that the breakdown of hyaluronan into bioactive fragments plays in innate immunity and in neoangiogenesis and the impact they have on properties of tumor cells (*e.g.* resistance to apoptosis and enhanced migration) is discussed. The innovative translation of this basic cell biology knowledge to the development of clinically useful hyaluronan-based co-polymers, pro-drugs, nanoprobes and hydrogels is discussed. The application of these novel reagents to imaging and therapeutics in breast cancer is described.

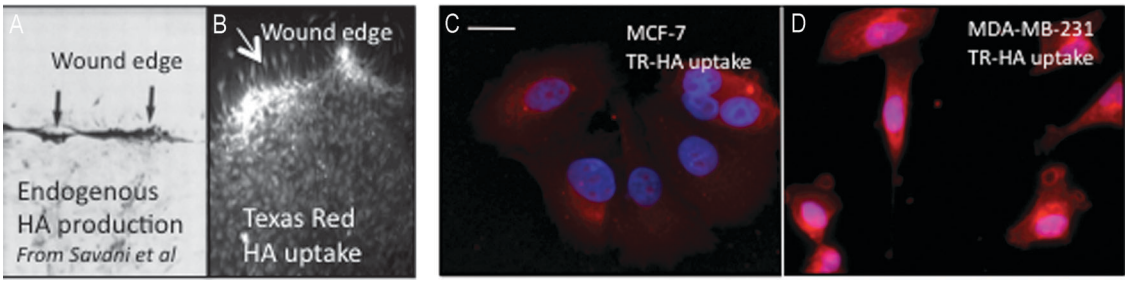

Fig. 1 **HA metabolism** in scratch wounds (left images) and in breast cancer cell lines (right images). A. Endogenous HA accumulates at the wound edge (arrows). B. Texas Red-HA is also largely taken up at the wound edge, suggesting that sites of endogenous accumulation also are sites of active HA uptake/metabolism (arrow in right image of black/white panel). A similarly increased uptake of Texas Red-HA is observed in aggressive human BCA lines (*e.g.* MDA-MB-231 tumor cells), while much lower amounts of HA are taken up by less aggressive human BCA such as MCF-7 tumor cells. Magnification bar = 10 μm. Red color is Texas Red and blue is DAPI.

as transforming growth factor beta (TGFB's) and insulin-like growth factor 1 (IGF-1), are also commonly modified in prognostic stromal gene signatures.[6b,7a] In general, stromal environments that actively promote cancer include those that exhibit aspects of response-to-injury processes including chronic inflammation,[7a,8] repair following chronic radiation,[9] and tissue undergoing involution (*e.g.* involuting mammary gland).[7a,10] Therapeutics targeting specific wound healing processes, such as angiogenesis, have already shown promise in treatment of some cancers.[5e,11] Nevertheless, the key ECM remodeling events responsible for promoting cancer aggression and progression are still not well understood. One process common to wound repair, breast cancer progression, and that of many other cancers is an elevated ability to metabolize HA.[12]

We have developed a method for detecting cells that are actively metabolizing HA and have shown that this property is shared by both normal but injured cells and aggressive BCA cell lines (Fig. 1, and ref. 13). HA metabolism was detected and quantified as the amount of fluorescent, high molecular weight (HMW) HA—referred to as Texas Red-HA (TR-HA)—that can bind to and be taken up by these cultured cells. Quiescent fibroblast monolayers showed little uptake while scratch-wounding these monolayers resulted in high TR-HA uptake at the wound edge (Fig. 1). We verified that the TR-HA probe accurately detected areas of high HA metabolism by demonstrating its rapid and HA-receptor dependent uptake in both liver, which is perhaps the most active of all tissues in metabolizing HA,[14] and injured carotid arteries (*vs.* uninjured contra-lateral carotid artery used as a control), which also exhibited increased HA metabolism.[13,15] Using this TR-HA probe as a marker for high HA metabolism, we showed that aggressive BCA lines such as MDA-MB-231 metabolized HA more actively than less aggressive BCA lines such as MCF-7 (Fig. 1). This finding is consistent with previous evidence that MDA-MB-231 tumor cells produced larger amounts of HA, expressed higher levels of injury-induced HA receptors such as cluster designation 44 (CD44) and RHAMM,[16] and expressed higher levels of hyaluronidases. These results imply that the TR-HA probe is functional because it detects and accurately reports sites of active HA metabolism.

A number of studies indicate that HA metabolism is a transient but essential process for wound resolution.[17]

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However, our results and those of others (see "Clinical uses of HA metabolic activity" in this review) indicate that it is aberrantly and constitutively active in aggressive breast cancer and likely contributes to progression of this disease rather than its resolution.[12a,18] We are currently exploring the flexibility of the HA polymer for developing multi-modality imaging strategies to detect active HA metabolism in remodeling microenvironments of injured and neoplastic tissues *in vivo*. Here we review evidence that the constitutive activation of an HA metabolic cycle is utilized by breast tumors to establish a microenvironment that is permissive for progression. We also discuss potential uses of functional HA-based platforms such as HA nanoprobes for imaging and characterizing breast cancer cells, particularly with respect to properties that are necessary for disease progression.

The HA metabolic cycle in homeostasis and injury

The metabolism of HA is a complicated, multistep and multifunctional process involving coordinated synthesis of HA by one of three HA synthases (HAS1-3) that produce an extracellular HMW polymer. HA uptake is mediated by HA receptors, and the internalized polymer is degraded by lysosomal hyaluronidases.[19] During homeostasis, HMW HA produced in tissues is filtered through lymphatic tissue and then enters the blood vasculature. A small amount of HA produced in tissues is endocytosed and degraded *in situ*, but most accumulates within lymphatic sinuses. Some of this lymphatic HA binds to and is taken up by HA receptors, lymphatic vessel endothelial hyaluronan receptor (LYVE1), Stabilin1 (STAB1) and Stabilin2 (STAB2), which are located on lymph node sinus endothelial cells.[19d,20] The majority of HA escapes from lymphatic sinuses into blood vessels and is rapidly removed by liver endothelial cells as a result of binding to STAB1 and 2 (also known as HA receptor for endocytosis [HARE]) receptors. Kidney tissue participates in HA metabolism but this contribution is minor compared to the amount metabolized by liver tissue.[14,20] The homeostatic HA metabolism scenario is markedly modified when tissues are injured and begin to actively remodel their microenvironment.

During response-to-injury, HMW HA polymers are rapidly metabolized within the injured tissue.[12a,c,15b,18,21] Local HA production and accumulation are increased as a result of elevated HAS expression in injured cells, and HA is then rapidly fragmented by extracellular hyaluronidases, reactive oxygen species (ROS) and mechanical shearing.[19a,22] During wound repair, these LMW HA fragments serve multiple functions including control of innate immune cell function,[15b,21a] promotion of angiogenesis,[17e] and regulation of wound cell proliferation, migration and differentiation.[23] The functional effects of HA fragments and their clearance from the repairing tissue are the consequences of their binding to HA receptors such as CD44, RHAMM and Toll-Like Receptors 2,4 (TLR2,4).[5d,12c,14,17d,18,19c,21b,23b,24] Internalized LMW HA is targeted to the lysosome and further degraded by hyaluronidase 1 and 2 (HYAL1, HYAL2), which are intracellular endoglycosidases, into tetra and hexasaccharides.[22b,25] HYAL1 and HYAL2 are the major hyaluronidases expressed in humans and can equally depolymerize HA, chondroitin, and chondroitin sulfate.[19d] The oligosaccharides produced by HYAL1 and HYAL2 are then further degraded by two lysosomal exoglycosidases, β-glucuronidase (GUSB) and β-*N*-acetyl hexosaminidase (HEXA).[19d,22b,25] A similar process of this injury-specific HA metabolism is constitutively activated in BCA and other tumors.

Functions of HA, HA receptors and HYAL enzymes in homeostasis and tissue repair

The central player of this metabolic cycle, HA, is a relatively simple polysaccharide belonging to the family of glycosaminoglycans that also include chondroitin sulfates, heparins/heparan sulfates, and keratin sulfate.[17c,19b,26] The HA polymer is composed of repeating disaccharide units formed by N-acetylglucosamine and glucuronic acid. These disaccharide units are linked together by β(1–4) bonds (Fig. 2). The HMW HA polymer is synthesized by dual action plasma membrane glycosyl transferases (HAS1-3, named for their order of discovery)[26] that bind both uridine diphospho (UDP)-glucuronic acid and UDP-*N*-acetylglucosamine at the inner membrane surface. The mechanisms for export of growing HA chains to the microenvironment is not understood, but possibilities include extrusion through intra-protein pores formed from synthase oligomers or *via* transporters such as the adenosine-5′-triphosphate binding cassette (ABC) system that are known to export other polysaccharides.[19b,26,27] Mammalian HA synthases are 55–70% identical but only HAS2 is necessary for embryonic development.[19b,c,26] Although the tissue expression patterns of HAS1-3 often overlap, they differ in their rate of synthesis, the size of polymer that they produce, and the type

Table 1 mRNA expression of genes involved in HA metabolism that are upregulated in BCA

Gene name	mRNA expression increased in BCA *vs.* normal ($p < 0.05$)	Expression related to tumor grade and/or poor outcome ($p < 0.05$)
Hyaluronan synthases		
HAS2	16%	+ + +
Hyaluronan receptors		
RHAMM/HMMR	78%	+ + + + +
CD44	11%	+ + +
TLR2	11%	+ +
Hyaluronidase		
SPAM	No change but increased within BCA groups	+

+ to + + + + + is a semiquantitative assessment of the percentage of data sets in which elevated gene expression is related to poor outcome. Poor outcome parameters include relapse, appearance of metastases after treatment, high tumor grade, and death. All data were obtained from Oncomine (www.oncomine.com).

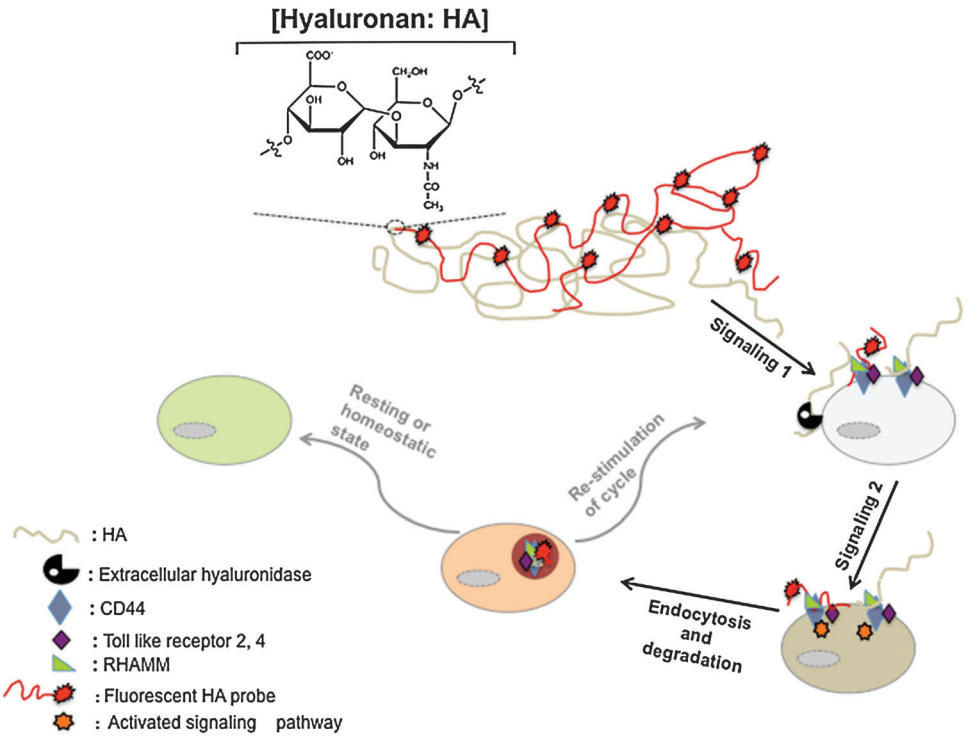

Fig. 2 Schematic representation of injury-induced HA metabolic cycle in wounds. The figure shows the unit structure of HA (*N*-acetyl-glucosamine and β-glucuronic acid) and demonstrates the functions of HA metabolism in BCA and other cancers. HA binding and uptake are tracked by labeling a high molecular weight polymer with a fluorescent dye. HA fragments produced by ROS, shear and extracellular hyaluronidases bind to HA receptors thereby activating their signaling potential. This interaction also results in uptake of HA, its targeting to the lysosome, further degradation by lysosmal HYAL1,2 and ectoglycosidases, GUSB and HEXA. In injured tissues, expression of HA receptors and other genes involved in HA metabolism is down-regulated as tissue regains homeostasis. The cycle is constitutive in transformed cells.

of cancer in which they are aberrantly expressed.[19b,28] For example, HAS2 expression is linked to BCA (Table 1),[3c,29] while HAS2 and HAS3 expression play a role in prostate cancer aggression.[30]

The biological functions of HA are strictly size-dependant (for reviews, see ref. 17*d*,*e*,18,19*b*). HMW HA polymers (*e.g.* >200 kDa) carry out structural and other roles that contribute to tissue architecture and function during homeostasis. One of these is to provide a macromolecular template, which concentrates and organizes the assembly of other proteins in the extracellular and possibly intracellular space.[19b–d] LMW forms, which are generated during tissue repair, activate specific signaling cascades and transcription factors such as extracellular signal-regulated kinases 1 and 2 (ERK1,2), phospho-inositide-3-kinase (PI3K)/ protein kinase B (PKB) and nuclear factor kappa-light-chain enhanced activated B (NFKappaB) cells, and activating protein-1 transcription factor complex (AP1). These pathways control cell processes that are responsible for preserving stem cell compartments, repairing and re-establishing tissue architecture, controlling mesenchymal differentiation (*e.g.* angiogenesis), promoting cell migration/survival/proliferation, and sustaining innate immune function.[12c,17d,18,23b]

During response-to-injury, reduction of HMW HA to sizes that can bind to cellular receptors results from reactive oxygen species, mechanical shear, and extracellular hyaluronidase activity (*e.g.* serum HYAL1, cell surface HYAL2), although the contribution of each is currently not well understood.[22a,25,31] Extracellular hyaluronidases [19a,22a,32] have been reported in injured tissues, although the pH of the microenvironment *per se* is presumably not optimal for their lytic activity. It is likely that cell surface HYAL2 may be active only within cell surface micro-domains that can maintain localized low pH levels as a result of high ion transport activity (Fig. 2).[32a] Regardless of the mechanisms for generating LMW, expression of HA receptors is required for response to these bioactive HA fragments, and this response is required for normal repair.

CD44, RHAMM, and TLR2,4 are the key HA receptors that are activated by LMW HA generated during response-to-injury.[19b,d,33] Our understanding of the wound and, to a lesser extent, tumor functions of these receptors has been greatly enhanced by studying tissue injury in mice or cell lines that lack these receptors. A number of studies indicated that, while genetic deletion of either CD44 or RHAMM kept homeostatic functions intact, it resulted in altered innate immune function and repair, depending on the injury stimulus and responding tissue. For example, CD44 loss was associated with high levels of extracellular bioactive HA fragments, sustained accumulation of macrophages, and unremitting inflammation of bleomycin-injured lung tissue.[17d] On the other hand, genetic loss of RHAMM (but not CD44) resulted in delayed repair of excisional skin wounds and aberrant mesenchymal differentiation within the wound site.[34] In an immune-privileged site such as brain

tissue, deletion of CD44 reduced processes associated with the inflammation stage of tissue repair.[17e] Thus, following cerebral artery occlusion, infarct size was smaller and angiogenic response and neurological damage were less in $CD44^{-/-}$ than in wild type animals. In addition to aberrant response-to-injury, genetic deletion of these HA receptors also affected tumor susceptibility. For instance, loss of CD44 greatly enhanced disease progression in transgenic mice, which are susceptible to BCA due to conditional expression of polyoma middle T-antigen (PyMT) in mammary epithelium. In contrast, loss of RHAMM in a mutant adenomatous polyposis coli gene product (APC)-driven model of fibromatoses reduced tumor invasion.[35] These experimental results predict that injury induced-metabolism of the HA homeostatic tissues is restricted because of the potent biological effects of extracellular HA fragments, which could damage normal tissue architecture, promote inappropriate inflammation, and render tissues susceptible to neoplastic conversion and progression.

HA metabolism in BCA

Clinically, high levels of HA within tumor cells or in the peri-tumor stroma can be observed in many cancers and are strong independent prognostic indicators of poor outcome in breast, ovarian, gastric, and colorectal cancers.[12a,17f,18,19b,36] Normal breast, ovarian, gastric, and colorectal epithelia produce low levels of HA, although HA accumulation can be observed in the corresponding normal stroma.[12a] The percentage of tumors that accumulate higher than normal tissue HA levels is not 100%, but ranges between 50% and 80%, which suggests that HA may be an unfavorable factor in subgroups of cancers. Interestingly, neoplastic conversion of tissues that normally produce high levels of HA, such as the keratinocyte layer of the skin, is associated with loss rather than gain of HA accumulation.[12a]

High accumulation of HA in the tumor and peri-tumor stroma is particularly associated with breast tumor progression (for review, see ref. 12*a*). HA accumulation within BCA malignant stroma and tumor parenchyma is much higher in malignant than in benign lesions, and these levels are correlated with high tumor grade,[37] auxiliary lymph node positivity, and shortened survival.[38] HA accumulation is also correlated with BCA treatment resistance. In women <50 years, tumor HA levels predict occurrence of relapse.[39] In experimental models, HA production by BCA tumor cells contributes to adjuvant therapy (Trastuzumab) resistance likely because it masks ErbB2 antigenic sites that are normally recognized by Trastuzumab.[39b]

Increased HA accumulation and metabolism are constitutive in BCA, and BCA tumors express elevated levels of HA synthases, receptors and hyaluronidases, as identified by both experimental analyses and data mining of data banks such as Oncomine (www.oncomine.com, Table 1).[12a] Table 1 summarizes the mRNA expression of HA metabolic genes that are most commonly increased in BCA. Results were obtained by querying the Oncomine database for genes involved in HA metabolism that were both significantly ($p < 0.05$) increased in breast tumor compared to normal tissue and associated with parameters of poor clinical outcome (*e.g.* recurrence of primary tumors, appearance of post-treatment metastases, and death). Using these criteria, HAS2 is a prominent factor in BCA. This conclusion is supported both by evidence that HAS2 is one of several genes that are commonly re-arranged in BCA cell lines and sporadic BCA[40] and by reports showing that increased HAS2 expression is involved in BCA aggression.[12a,38a] The mRNA levels of RHAMM/HMMR in particular, but also CD44 and TLR2, are increased in BCA compared to normal breast. These mRNA increases link to poor clinical outcome, a finding that is also noted in experimental evidence.[29,41,42] The relationship of CD44 expression with poor clinical outcome (Table 1) may be related to its prominent display on aggressive BCA progenitor subsets.[41a,b,43]

SPAM1 is a glycosylphosphatidylinositol (GPI)-linked cell surface hyaluronidase normally expressed on sperm surfaces and not in breast tissue[44] but is, however, aberrantly expressed in human BCA.[16b,45] Analyses of mRNA expression using Oncomine data banks also show that this hyaluronidase is more commonly increased and linked to poor clinical outcome parameters than other hyaluronidases (Table 1).

HA and HA receptor functions common to wounds and BCA

Since HMW HA is present in biologically active amounts in blood (ng l^{-1}), it is one of the first ECM molecules to rapidly bathe injured tissues. Thus, it is an important participant in initiating remodeling processes necessary for tissue repair.[19b,d] Hypoxic conditions and cytokines released by adherent platelets stimulate further HA production by cells remaining at the wound site or by those adjacent to it.[46] This results in an early but transient production/accumulation of HA within wound sites,[17e,21b,46a] and is a likely constitutive stimulus for HA production in BCA since tumor microenvironments are generally hypoxic. Evidence gleaned from use of modified HA as tissue grafts and wound healing promoters[19c] predict that the functions of HMW HA in wounds are to provide protection against ROS, hydrate tissues, and restrict passage of microbes. The HMW polymer also reduces antigenicity of ECM protein fragments, reduces angiogenesis and inflammation, and protects stem cell compartments.[21b] As repair processes are initiated, HA is steadily fragmented into bioactive LMW HA fragments. These attract and stimulate cells of the innate immune system to produce cytokines that propel wounds into the inflammatory stage of repair. Expression of CD44 is required for leukocyte recruitment to the wound and is also essential for clearing the wound of the immunogenic LMW HA fragments. This ultimately results in the blunting of inflammation so that the fibrogenesis/remodeling stage of the wound site can be initiated.[5e,17b,20] HA fragments stimulate pro-inflammatory cytokine production by macrophages *via* TLR4 and also bind to and activate cell surface RHAMM, which complexes with CD44 and activates ERK1,2 signaling cascades.[16a,34] These last interactions are required for migration and differentiation of wound fibroblasts into myofibroblasts and other mesenchymal cell types within the wound.[34] RHAMM is also present in intracellular compartments, notably the mitotic spindle and nucleus. These intracellular

forms of RHAMM appear to control proliferation and gene expression necessary for cell cycle progression,[33,47] while extracellular forms appear to promote cell migration.[34] Final clearance of HA fragments by injury-induced HA receptors and down-regulation of HA receptor display at the site of injury are necessary for wound resolution.[17d]

Elements of these wound repair processes have been hijacked by breast tumors, and de-regulated expression of HAS, HA receptors and hyaluronidases appear to promote rather than resolve this disease. Experimental tumor models in which these genes have been modified suggest a role for BCA cell HA to directly promote invasion (*e.g.* tumor cell migration) as well as proliferation,[12a,16a,17f] and contribute to stromal changes that promote tumor growth. For example, HA fragments increase host-derived angiogenesis, lymphangiogenesis, and recruitment of macrophages.[19b,48] On the other hand, production of HA by stromal cells also contributes to BCA progression. For example, the development of HA-rich malignant stroma promotes the growth and invasion of BCA cells. It has been shown that combined growth of high HA-producing tumor-associated fibroblasts with MCF-7 BCA in immune-deficient mice strongly promotes tumor growth and this is accompanied by stromal lymphangiogenesis and a stromal reaction.[48] As such, MCF-7 tumors grow slowly in the absence of these genetically modified fibroblasts, stimulate sparse lymphangiogenesis, and evoke a small stromal reaction.[18,48] Similar results were obtained with knockdown of HAS2 in fibroblasts, which prevented stromal angiogenesis/lymphangiogenesis and macrophage recruitment into the stroma of MCF-7 xenografts. HA also participates in the development of BCA drug resistance. HA/CD44 interactions promote drug transporter expression and activity, and affect epitope display of key receptor tyrosine kinases (*e.g.* ErbB2), thereby limiting receptor-oriented therapy.[18,39b]

Collectively, these data indicate that HA performs multiple wound-like functions in BCA progression and that components of the tumor HA metabolic cycle are likely to be useful targets for both addressing drug resistance and designing microenvironment targeted therapeutics.

Clinical uses of the HA metabolic cycle

Because of the unique structural properties of HMW HA, it has long been used as a designer biomaterial for tissue engineering applications.[49] Currently many preparations have been approved as tissue replacement supplements, cosmetic fillers, anti-adhesives to prevent surgical adhesions and drug delivery vehicles (*e.g.* treatment of skin lesions such as actinic keratoses and oral mucositis). Moreover, HA can act as a high-affinity probe for imaging and therapy due to its remarkable ability to target to sites where HA receptors are displayed (particularly CD44 and LYVE1).[50] Traditional approaches for imaging and therapeutic applications have taken advantage of the altered vascular architecture (100–600 nm fenestration) and lymphatic drainage of solid tumors to selectively retain probes at the tumor site, a phenomenon called "enhanced permeability and retention (EPR)".[51] Although some of the accumulation of HA-based therapeutics is probably due to EPR, the enhanced ability of HA-imaging or HA-therapeutic probes to target and to accumulate in tumor tissue is at least partly due to both HA binding to tumor HA receptors and a concentration dependent-promotion of HA half-life in the circulation. For instance, an administered dose of 3 mg kg^{-1} HA has a $T_{1/2}$ = 12 h in human subjects.[52]

Above and beyond these properties, the ability of HA probes to be processed by target cells as part of a metabolic cycle allows for the design of probes that report HA metabolic activity. For example, when fluorescently labeled HA was adsorbed to gold nanoparticles, the fluorescent signal was quenched. When the adsorbed HA was clipped by hyaluronidases, which were elevated in the BCA tumor microenvironment, a fluorescent signal could be detected.[53] In another approach, a HMW tagged HA could accumulate within tumors as a result of being fragmented into LMW HA fragments, which was bound to and was endocytosed by HA receptors displayed on tumor cells (*e.g.* Fig. 2). These biological properties, combined with its non-antigenicity, excellent biocompatibility profile and hydrophilic/anionic nature that can be easily modified with a variety of functional moieties, have facilitated

Table 2 Examples of HA-based platforms for therapy and imaging

Platform	Coupling moiety	Application	Outcome of coupled moiety
HA	Doxorubicin-HPMA[a]	Therapy[56]	Improved disease-targeted delivery
	Sodium Butyrate	Therapy[57]	Improved half-life and disease-targeted delivery
	Taxol	Therapy[58]	Improved disease-targeted delivery
	Hydrogen peroxide	Therapy[59]	Long acting radiosensitization of local tumors
HA nanogel	5-flurouracil,	Therapy[60]	Controlled release
	GFP-siRNA	Therapy[61]	Controlled release and disease-targeted delivery
	Cisplatin	Therapy[62]	Improved disease-targeted delivery and release
	Doxorubicin-PEG-PCL[b]	Therapy[63]	Improved disease-targeted delivery and sustained release
	Doxorubicin-PLGA[c]	Therapy[64]	Improved disease-targeted delivery
HA + nanoliposome	Doxorubicin	Therapy[65]	Long-term circulation and disease-targeted delivery
	Mitomyocin (MMC)	Therapy[66]	Long-term circulation and disease-targeted delivery
HA + nanoparticle	Gold nanoparticles	Imaging[53,67]	Optical detection of ROS[d] and hyaluronidase
	Fe_2O_3 nanoparticles	Imaging[68]	Improved diagnostic MRI[e] imaging
	Gadolinium nanoparticles	Imaging[69]	Detection of hyaluronidase activity
	Quantum dots	Imaging[54b,70,71]	Improved disease-targeted optical imaging

[a] *N*-(2-hydroxypropyl)methacrylamide. [b] Poly(ethyleneglycol)-polycaprolactone. [c] Poly[lactic-*co*-(glycolic acid)]. [d] Reactive oxygen species. [e] Magnetic resonance imaging.

development of HA nanoscale particles and copolymers for imaging and therapeutic use in inflammatory and neoplastic diseases.[54] Conjugation of HA to drugs improves the solubility of hydrophobic drugs, enables controlled release of these drugs, and increases their circulation time within the vasculature.[19c,52,55] Ultimately, these HA-based platforms can also be used to enable the isolation and study of tumor cell compartments/subsets undergoing HA metabolism, which in turn will provide a better understanding of the process and function that HA metabolism carries out in BCA progression. To date, HA in the forms of drug-conjugates/prodrugs, nanogels/hydrogels, nano-liposomes, and nanoparticles have been developed for use in therapy or imaging (Table 2).

Regardless of their physical structures and sizes, all of these site-specific platforms contain HA as a carrier or as a bioactive targeting probe. They are formed either through direct chemical conjugation to functionally active sites of polymer chain or by physical incorporation strategies (Fig. 3). Such HA-based platforms are particularly well suited for treatment/imaging of BCA. HA prodrugs injected directly into breast tumors are well-retained within tumor tissue, which is likely due to the high expression of HA receptors. For example, Kochi Oxydol-Radiation Therapy is a new radiosensitizer containing H_2O_2 that turns radiation-resistant breast tumors into a radiation-sensitive state following its injection into the tumor. Phase I clinical trials of this HA-based therapeutic have revealed no adverse effects.[59] Direct injection of Cisplatin- or doxorubicin-HA nanoconjugates into the mammary fat pad or into nearby subcutaneous tissue has resulted in the accumulation of these conjugates, both in the tumor and in nearby lymphatics. Since aggressive BCA cells initially invade and extravasate *via* local lymphatic tissues, this property of HA nanoconjugates may allow for early treatment of invasive BCA.[62a] The lymphatic accumulation of HA nanoconjugates is likely the result of high LYVE1 expression.[72] These studies collectively point to the specific targeting ability of HA. However, the use of HA for targeting, particularly if it is unmodified, has challenges. These include avoiding rapid clearance from the circulation by the liver as a result of HA endocytic receptors and, to a lesser extent, the reticular endothelial system (RES).[73] Strategies that have been devised for addressing these issues include cross-linking of individual polymer chains, chemical modification of carboxyl groups, and pre-treatment of the liver with chondroitin sulfate before exposure to HA-based nanoprobes. Chondroitin sulfate binds to HA endocytic receptors resulting in their internalization and prolonged blocking of HA uptake by the liver.[54a,73–75]

An alternative approach to using the HA polymer for tumor-targeted imaging or therapy at sites of HA metabolism is to utilize HA receptors that are expressed by BCA cells. Anti-CD44 antibodies have been tested in phase I clinical trials in patients with head and neck cancers and have also been evaluated for imaging these tumors in pre-clinical studies.[75d] However, trials were terminated due to the death of an enrolled patient. Current efforts are focused on designing reagents that specifically block HA/CD44 interactions. As noted above, the relationship between CD44 expression and BCA progression appears to be complex, and use of these types of reagents is therefore limited by our still-rudimentary understanding of the basic biology of CD44/HA interactions. RHAMM is the most commonly over-expressed HA receptor in BCA (Table 1) and has been classified as a tumor marker in this neoplastic disease.[76] However, the biological role of RHAMM in BCA is also not yet well understood,[77] which limits design of therapeutic approaches to target this receptor. Nevertheless, RHAMM is also highly expressed in blood malignancies, and development of RHAMM vaccines currently shows promise for treatment of acute myeloid leukemia and multiple myeloma.[78]

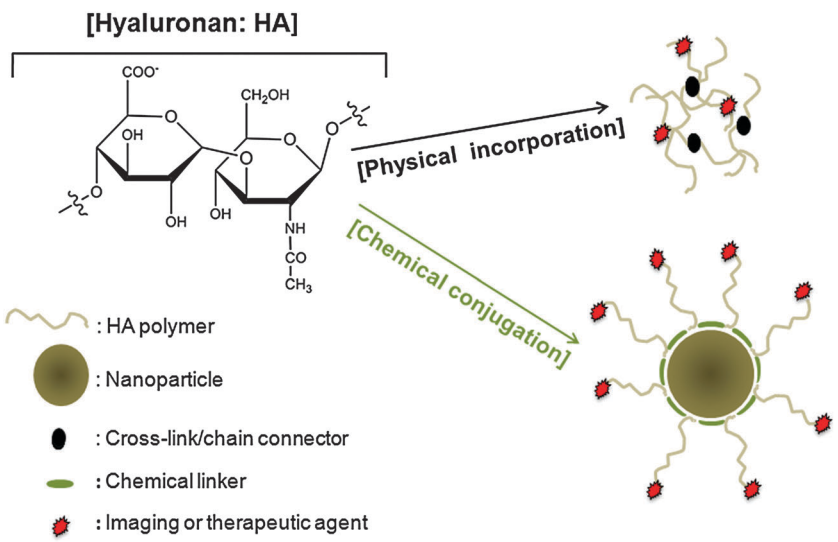

Fig. 3 HA modification for preparation of probes. HA is modified for preparation as a therapeutic or an imaging probe through physical incorporation of moieties within HA polymers (black arrow) or chemical conjugation of HA polymers/HA nanoparticles to moieties (green arrow).

Conclusions

Both clinical and experimental evidence indicate an involvement of HA, HA receptors, and hyaluronidases in BCA malignancy. Data mining confirms that elevated mRNA expression of genes involved in HA metabolism is common in BCA. This and experimental data suggest that HA performs multiple wound-like functions in BCA progression and predict that the components of the tumor HA metabolic cycle are useful therapeutic targets. In addition, HA-based nanoprobes that detect active HA metabolic cycling or directly target HA receptor-bearing cells may be useful tools for diagnostic imaging. However, effective use of these as therapeutics requires much more basic information about the functions of HA metabolism in BCA progression and the types of BCA that display active HA metabolism.

Table of abbreviations

ABC	Adenosine-5′-triphosphate binding cassette
PKB	Protein kinase B
AP1	Activating protein-1 transcription factor complex
APC	Adenomatous polyposis coli gene product
BCA	Breast cancer
CD44	Cluster designation 44
ECM	Extracellular matrix
EPR	Enhanced permeability and retention
ErbB2	Human epidermal growth factor receptor 2
ERK1/2	Extracellular signal-regulated kinases 1 and 2
GPI	Glycosylphosphatidylinositol
GUSB	β-glucuronidase
HA	Hyaluronan, hyaluronic acid, hyaluronate
HARE	Hyaluronan(HA) receptor for endocytosis (liver, Stabilin 1,2)
HAS1-3	HA synthases1,2,3
HEXA	Hexosaminidase
HMW	High molecular weight
HPMA	*N*-(2-hydroxypropyl)methacrylamide
HYAL1-4	Hyaluronidases1-4
IGF-1	Insulin-like growth factor 1
LMW	Low molecular weight
LYVE1	Lymphatic vessel endothelial hyaluronan receptor1
NFKappaB	Nuclear factor kappa-light-chain enhanced activated B cells
PI3K	Phospho-innositide-3-kinase
PyMT	Polyoma middle T antigen
RHAMM/HMMR	Receptor for hyaluronan mediated motility (protein designation)
RES	Reticuloendothelial system
ROS	Reactive oxygen species
SPAM1	Hyaluronidase PH-20
STAB1,2	Stabilin1,2
TGFB	Transforming growth factor beta
TLR2,4	Toll-Like Receptors 2,4
TR-HA	Texas red-HA
UDP	Uridine diphospho [*e.g.* glucuronic acid, N-acetyl-glucosamine]

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