Hyaluronan as a therapeutic target in human diseases

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Abstract

Accumulation and turnover of extracellular matrix is a hallmark of tissue injury, repair and remodeling in human diseases. Hyaluronan is a major component of the extracellular matrix and plays an important role in regulating tissue injury and repair, and controlling disease outcomes. The function of hyaluronan depends on its size, location, and interactions with binding partners. While fragmented hyaluronan stimulates the expression of an array of genes by a variety of cell types regulating inflammatory responses and tissue repair, cell surface hyaluronan provides protection against tissue damage from the environment and promotes regeneration and repair. The interactions of hyaluronan and its binding proteins participate in the pathogenesis of many human diseases. Thus, targeting hyaluronan and its interactions with cells and proteins may provide new approaches to developing therapeutics for inflammatory and fibrosing diseases. This review focuses on the role of hyaluronan in biological and pathological processes, and as a potential therapeutic target in human diseases.

Graphical abstract
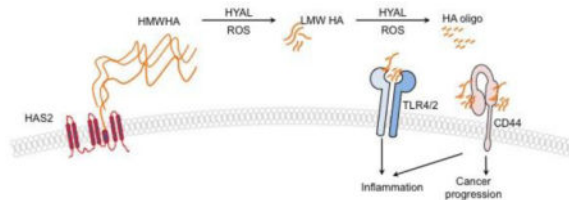
1. Introduction

Hyaluronan (hyaluronic acid, HA) is a major component of extracellular matrix and is a non-sulfated glycosaminoglycan composed of repeating polymeric disaccharides D-

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Chemical compounds studied in this article

Sodium hyaluronate (PubChem CID: 3084049)
Hyaluronic Acid (PubChem CID: 453618)
4-methylumbelliferone (PubChem CID: 5280567)
Glycyrrhizin (PubChem CID: 3495)

glucuronic acid and *N*-acetyl-D-glucosamine linked by a glucuronidic β (1→3) bond. In humans, HA exist in all tissues and is abundant in the vitreous of the eye, the umbilical cord, synovial fluid, heart valves, skin, and skeletal tissues. HA can be produced by many cell types [1, 2], although mesenchymal cells are believed to be the predominant source of HA [3]. HA has multiple functions in normal biological states, such as space filling, hydration, lubrication of joints, and provision of a matrix through which cells can migrate [3]. HA is actively produced during tissue injury [4], regulating tissue repair and disease processes, such as activation of inflammatory cells to mount an innate response to injury [5] and regulation of behavior of epithelial cells [6–10] and fibroblasts [11, 12]. HA has been investigated in a wide range of biological and medical fields, and the research articles referring to HA have grown exponentially in recent years (Figure 1).

There are many excellent reviews on the roles of HA in different fields, such as in angiogenesis [4], reactive oxygen species [13], HA digestion [14], cancer [3, 15, 16], cancer therapeutics [17], cancer metastasis [18], chondrocytes [19], lung injury [20–22], wound healing [23], diabetes [24], leukocyte trafficking [25], and in immune regulation [5, 26, 27]. The current review will summarize the role of HA in biological and pathological conditions, and will emphasize the role of HA in human diseases.

1.1. HA synthases

HA is synthesized by membrane-bound synthases, and there are three mammalian hyaluronan synthases (HAS1-3) [28]. All three proteins are bona fide HA synthases. In vitro transfection experiments showed that HAS1 and HAS3 generated HA with broad size distributions (molecular masses of 2×10^5 to approximately 2×10^6 Da), whereas HAS2 generated HA with a broad but extremely large size (average molecular mass of $> 2 \times 10^6$ Da) [29]. Subsequent studies suggested that all three HAS enzymes drive the biosynthesis and release of high molecular weight (HMW) HA (1×10^6 Da) [30]. Deletion of murine Has1 [31] or Has3 [12] or Has1-Has3 double knockout [32] did not reveal a significant phenotype under homeostatic conditions, whereas Has2 deletion generated an embryonic lethal phenotype due to impaired cardiac development [33]. With tissue injury, both Has1 [32] and Has3 deletions [12, 32] showed dysregulated tissue repair. Wound closure was significantly faster in Has1 and Has3 double null mice [32]. HAS2 protects skin fibroblasts against apoptosis induced by environmental stress such as UV exposure and serum starvation [34]. Furthermore, Has3 deficiency causes reduction in brain extracellular space leading to altered neuronal activity and seizures [35]. These studies generate important insights into the roles of hyaluronan in disease states.

1.2. HA degradating enzymes

Hyaluronidases (also called hyaluronoglucosaminidases) hydrolyze the hexosaminidic β(1–4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA and release disaccharide D-glucuronic acid-N-acetyl-D-glucosamine or HA fragments. In humans, there are six members of a gene family containing hyaluronidases identified: hyaluronidases 1 – 4, PH-20, and HYALP1 [14, 36]. Although Hyal1 deficient mice are viable, fertile and show no gross abnormalities, these mice do develop osteoarthritis [37].

HA fragments control dendritic cell migration from the skin, and HYAL1 expression activated migration and promoted loss of dendritic cells from the skin [38].

Hyal2 deficient mice displayed a significant increase in plasma HA and increased HA in the interstitial extracellular matrix of atrial cardiomyocytes [39, 40], suggesting that HYAL2 is essential for the breakdown of extracellular HA. Hyal2 deficient mice showed severe cardiopulmonary dysfunction [40]. Platelet-derived hyaluronidase 2 cleaves HA into fragments that trigger monocyte-mediated production of pro-inflammatory cytokines [41].

Hyal3 deficient mice showed a subtle change in the alveolar structure and extracellular matrix thickness in lung-tissues at 12–14 months-of-age, although there was no evidence of HA accumulation, suggesting that HYAL3 may not play a major role in constitutive HA degradation [42].

PH20 is elevated in demyelinating lesions and increased PH20 expression is sufficient to inhibit oligodendrocyte progenitor cell maturation and remyelination [43].

1.3. HA binding proteins

HA regulates an array of the biological and pathological processes, depending on its interacting proteins (Figure 2). Several cell surface receptors and secreted proteins have been suggested to be HA binding proteins (HABPs). CD44 is the major cell-surface HA binding protein [44]. It is widely expressed in almost every cell type in human and in mouse [45]. HA-CD44 interactions play an important role in development, inflammation, T cell recruitment and activation, and tumor growth and metastasis [46]. Although the genetic disruption of Cd44 in the mouse did not show much abnormality [47], the mice showed defective hematopoietic progenitor egress from bone marrow [47]. Under stress conditions, deficiency in CD44 results in some severe phenotypes. In a non-infectious lung injury mouse model, we showed that deficiency in CD44 resulted in exaggerated lung injury with increased HA accumulation, suggesting that CD44 is required to clearance of HA for the resolution of pulmonary inflammation [48]. Moreover, CD44 splicing variants have been shown to have a role in cancer metastasis [49, 50], and the strategies are developed to inhibit cancer metastasis with neutralizing antibodies against CD44 variants [51].

HA binds to HMMR (Hyaluronan-mediated motility receptor, or RHAMM, for receptor for hyaluronan-mediated motility expressed protein, also called CD168) [52]. HMMR is a functional receptor in many cell types, including endothelial cells [53]. HMMR-HA interactions play a role in Ras-dependent oncogenesis [54] and in tissue injury and repair [55]. Mice with deletion of HMMR gene were viable with normal appearance, but the deletion of the gene showed reduced formation of aggressive fibromatosis when crossed with mice that harbor a targeted mutation in the tumor suppressor *APC* gene [56]. RMMR deficient fibroblasts fail to repair wounds in an *in vitro* wound healing assay [57].

Lecticans (or hyalectans) are a family of HA-binding proteoglycans, including aggrecan, brevican, neurocan, and versican, mostly expressed in the brain. Lecticans interact with HA and tenascin-R to form a ternary complex [58]. HA binds to brevican, a brain-specific proteoglycan [59]. Brevican binds not only to HA [60] but also to chondroitin sulfate [61].

Neurocan is a nervous tissue-specific proteoglycan of the aggrecan family which interacts with neural cell adhesion molecules. Neurocan binds to HA as well as chondroitin sulfate in the brain [62]. Versican also binds to HA [63] and chondroitin sulfate [64]. Studies have shown that hyalectants have a role in neural development and brain injury [58].

HABP1 (hyaluronan-binding protein 1, also called C1QBP, for complement component 1, q subcomponent binding protein) binds to HA [65]. C1QBP is a multifunctional and multicompartmental protein involved in inflammatory regulation and mitochondria protein synthesis [66]. The extract sequence in HABP1 protein for HA binding is less clear.

HARE (hyaluronan receptor for endocytosis, also called stabilin-2 and FEEL-2) was identified in the abundant expression of 175- and approximately 300-kDa HARE species from sinusoidal endothelial cells of the liver, lymph node, and spleen [67]. HARE protein binds to HA and other sugar species [68] and acts as a systemic clearance receptor for heparin [69]. Deletion of stabilin-2 in mice resulted in a dramatic elevation in circulating HA levels without any overt phenotype [70].

LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1, also called CRSBP-1 for cell surface retention sequence binding protein-1) was cloned as a lymph-specific HA receptor on the lymph vessel wall in human [71] and in mice [72]. Conditional ablation of LYVE-1+ cells showed defensive roles of lymphatic vessels in the small intestine and lymph nodes, but lymphatic vessels in the other organs were relatively intact [73].

SHAP (serum-derived hyaluronan-associated protein) was originally identified as a serum-derived HA-associated protein since it covalently binds to HA [74]. It was later found that SHAP is identical to inter-α (globulin) inhibitor (IαI) heavy chain 2 (ITIH2), which belongs to the inter-α-trypsin inhibitor (ITI) family of structurally related plasma serine protease inhibitors involved in extracellular matrix stabilization.

TNFIP6 (tumor necrosis factor α-induced protein 6, also called TSG-6 for TNFα-stimulated gene-6), contains a Link module binding to HA [75]. TNFIP6 has been shown to play a key role in ITI heavy chain transfer [76]. TNFIP6 amplifies HA synthesis by airway smooth muscle cells [77]. Knockout of Tnfip6 in mice with a BALB/c background did not change the onset of proteoglycan-induced arthritis, but progression and severity were significantly greater in Tnfip6-deficient mice when compared to wild type BALB/c mice [78]. An early and more extensive infiltration of the synovium with neutrophils was the most prominent histopathologic feature of proteoglycan-induced arthritis in Tnfip6-deficient mice [78].

CEMIP (cell-migration induced protein hyaluronan binding, aka, KIAA1199) was recently identified to encode a new HA binding protein. The protein lacks a typical HA binging motif, but contains four PbH1 (parallel beta-helix) repeats, suggesting the protein have hyalunidase activity catabolizing HMW HA to an intermediate-sized product in an endo-β-*N*-acetylglucosaminidase–dependent manner via the clathrin-coated pit pathway [79]. The CEMIP protein is also presented in endoplasmic reticulum having a role in cancer cell migration [80] and invasion [81]. Furthermore, KIAA1199 was overexpressed by synovial fibroblasts and synovial tissues from patients with osteoarthritis or rheumatoid arthritis [79], suggesting a role of HA catabolism in the dermis of the skin and arthritic synovium.

1.4. Biology of HA polymer size

HA plays different roles depending on its molecular weight [82–87] (Figure 3). In its native state, such as in normal synovial fluid, HA generally exists as a HMW polymer, usually in excess of 1000 kDa. The fragmentation of HA occurs during many pathological conditions, a possible consequence of dysregulated expression of HA synthases and HA degradation enzymes during tissue injury and inflammation. Mammalian hyaluronidases are hyaluronate-4-glycanohydrolases degrading HA into medium and small size fragments, but exhaustedly degrade HA to tetrasaccharides as the end product, while bacterial hyaluronidases can produce unsaturated disaccharides [88]. HA fragmentation can also be a result of the release of reactive oxygen species [89] and reactive nitrogen species such as peroxynitrite [90] during tissue injury. We have found that lower-molecular-weight (LMW) forms of HA (<500 kDa, many preparations in 100 – 250 kDa), but not the native form (>1000 kDa), induce inflammatory responses during tissue injury [5, 22]. The role of disaccharides generated during bacterial infection is unknown. Thus, HA fragments can be generated through multiple processes and are biologically active.

Chemically modified LMW HA fragments retain their ability to regulate inflammatory responses. Selectively N-butyrylated LMW HA shows promise as an example of a novel semisynthetic anti-inflammatory molecule. Chemically modified N-acylated HA fragments modulate proinflammatory cytokine production by stimulated human macrophages [91]. Sulfated HA as a hyaluronidase inhibitor was showed as a potent inhibitor of prostate cancer [92]. Therefore, the modified HA preparations may have a broad use as therapeutics and as drug carriers.

1.5. HA as a signaling molecule

HA fragments are able to stimulate an array of cell types to regulate gene expression. The signaling pathway(s) of HA has been extensively investigated. In tumor cells, HA fragments may signal through CD44-dependent tyrosine kinase pathways to promote tumor cell growth [4, 93]. However, HA is able to stimulate chemokine production by macrophages in the absence of CD44, suggesting that the presence of CD44 is not required to mediate HA signaling [6]. The cumulative interpretation of these data is that the role of CD44 in regulating HA interactions depends on the cell type.

We found that HA fragment-induced chemokine and cytokine expression was completely abolished in TLR2$^{-/-}$TLR4$^{-/-}$ peritoneal macrophages [6]. HA oligosaccharides induce the maturation of dendritic cells via TLR4 [94]. Priming of alloimmunity by HA-activated dendritic cells is dependent on signaling via TIRAP, a TLR adaptor downstream of TLR2 and TLR4 [95]. These studies demonstrated HA fragments signal through TLRs in inflammatory cells.

Studies also suggest CD44 may modulate other receptor signaling. We found that CD44 negatively regulated lipopolysaccharide-TLR signaling in mouse macrophages [96]. Engagement of CD44 by HA suppresses TLR4 signaling and the septic response to LPS [97]. CD44 acts as a co-receptor for several cell surface receptors including RTKs, G-

protein coupled receptors, and LRP6 [98]. These studies point to a complex biology in HA-TLR4-CD44 signaling.

2. HA in biological processes

HA signaling involves a wide range of pathogenic process of diseases as well as providing a framework for ingrowth of blood vessels and fibroblasts, activating inflammatory cells to mount an immunological response [5] and regulating behavior of epithelial cells [7, 9] and fibroblasts [12](Li 2011). HA-stem cell interactions have been particularly explored in hematopoietic stem cells [99–101], mesenchymal stem cells [102–104], and adult multipotent progenitor cells, indicating that HA and its binding proteins may regulate tissue injury and repair processes through their interactions with a variety of stem cells. Elucidation of the role and mechanisms of HA in the stem cell niche is crucial in aiding the development of novel therapy for many diseases.

2.1. HA in development

HA, HA synthases, hyaluronidases, and HA binding proteins have been identified in many developmental processes, as well as in different organ development. HA and hyaluronidase activity were found in the chick embryonic heart [105] and kidney [106], suggesting a role for HA in the development of these organs. The direct demonstration of the role of HA in development came from a deletion study in mice. Targeted deletion of Has2 showed major abnormalities in heart and blood vessel development, resulting in an embryonic lethal phenotype [33, 107]. Has2-deficient embryos at embryonic day 9.5 completely lack endocardial cushions [33, 107], consistent with early observations that a high content of HA is present in normal human heart valves. These defects resemble the features in occurring versican knockouts [108]. These studies identified a role of HA in mediating epithelium to mesenchyme transition [33]. Moreover, HAS2 may be regulated through a Tbx 2-Bmp/TGFβ-Smad pathway in the development [109]. These reports suggested that HA and HA synthases play a critical role in the organ development.

HAS2 has a role in limb development. Overexpression of Has2 in the mesoderm of the chick limb bud in vivo results in the formation of shortened and severely malformed limbs that lack one or more skeletal elements [110]. Conditional inactivation of Has2 showed a role for HA in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb [111]. Furthermore, sonic hedgehog signaling directly targets HAS2 in regulating phalangeal joint patterning [112].

HA has also been suggested in myogenesis and bone marrow hematopoiesis. Intrinsic HA synthesis is necessary for myoblasts to differentiate and form syncytial muscle cells, but the HA-dependent pericellular matrix is not sufficient to support differentiation alone [113]. By triple deletion of all three HA synthases, a report showed that HA expressed by the hematopoietic microenvironment is required for bone marrow hematopoiesis [114].

In mice, treated with exogenous HA at age 3 to 8 weeks increased intestinal and colonic epithelial proliferation, resulting in hyperplasia. Pep-1 (a peptide that blocks the binding of HA to its receptors) treatment markedly decreased intestinal and colonic length, crypt depth

and villus height in the intestine, crypt depth in the colon, and epithelial proliferation in the intestine and colon, suggesting a role for HA in normal intestinal and colonic growth [115].

2.2. HA in growth and differentiation

HA promotes tumor cell growth through multiple signaling pathways. CD44 acts as a co-receptor for several cell surface receptors including Erb2/EGF receptor, G-protein coupled receptors, and Wnt signaling receptor LRP6 [98, 116]. Furthermore, HA strongly promotes anchorage-independent growth and the resistance of cancer cells to growth arrest and apoptosis under anchorage-independent conditions [3]. Therefore, strategies targeting HA and its signaling have being developed for patients with various cancers [3, 15–17].

HA and its binding proteins have been shown to have a role in fibroblast proliferation. HA facilitates TGF-β1-mediated fibroblast proliferation [117]. LMW HA or HA oligosaccharides stimulate fibroblast proliferation in various conditions [118, 119], suggesting a role for HA in tissue fibrosis.

HA fragments induce myofibroblastic differentiation [120, 121], endothelial cell differentiation [122], chondrogenic differentiation [123]. But, TNFIP6 inhibits osteoblastic differentiation of human mesenchymal stem cells (MSCs) induced by osteogenic differentiation [124], and HA inhibited the myocardiocyte differentiation [125]. These data suggest that HA and HA binding proteins regulate cell differentiation in a complex manner, which may be related to cell type and microenvironment.

2.3. HA in immune responses

HA and HA-binding proteins regulate inflammation, tissue injury, and repair through regulating inflammatory cell recruitment, release of inflammatory cytokines, and cell migration. Therefore, HA acts as an immune regulator in human diseases [5]. HA-CD44 interaction regulates T cell activation [126], Th1 generation [127], B-cell activation [128], and regulatory T cell functions [129]. A recent report suggested that the role of CD44 in adaptive regulatory T cells was through its interaction with Galectin-9 [130]. HA and CD44 modulate neutrophil adhesion and recruitment [131]. Endothelial CD44 rather than neutrophil CD44 mediates neutrophil migration [132]. Furthermore, HA is able to trigger inflammasome in response to injury [133].

While lymphocytes from CD44$^{-/-}$ mice preferentially homed to lymph nodes, their entry into the inflamed synovial joints was delayed when compared to wild type cells [134]. We showed that CD44 plays a role in HA fragment clearance during tissue injury [48]. CD44-deficient mice succumb to unremitting inflammation following noninfectious lung injury, characterized by impaired clearance of apoptotic neutrophils [48].

Moreover, HA fragments can influence dendritic cell maturation. For example, LMW HA fragments induce dendritic cell maturation and initiate alloimmunity [95]. Also, HA oligosaccharides are potent activators of dendritic cells [87]. The generation of small HA fragments, rather than the loss of large HA molecules, promotes DC migration and subsequent modification of allergic responses [38].

2.4. HA in senescence

HA and HA binding proteins participate in cellular senescence. Diminished versican deposition increases free HA fragments that interact with CD44 and increase phosphorylation of ERK1/2, leading to premature cellular senescence in embryonic fibroblasts [135].

The inhibition of HA synthesis by 4-methylumbelliferone induces senescence [136]. Targeting HAS2 by an miRNA caused cellular senescence, suggesting a role of HA in skin aging [137]. On the other side, HA concentrations in extracellular and pericellular matrices was greatly reduced in senescent mesenchymal stem cells, mainly due to the decreased expression of HA synthases [138]. An HA-based compound inhibited fibroblast senescence induced by oxidative stress in vitro [139]. Therefore, HA may participate in senescence in normal and pathological processes. Further investigation into the role of HA in senescence in disease states may shed light on the pathogenesis and help develop therapeutics for these diseases.

2.5. HA in apoptosis

We have reported that HA protected lung epithelial cells from apoptosis in bleomycin induced lung injury in mice [6]. HMW HA decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells [140]. Has1/3 null skin fibroblasts, with higher levels of Has2 gene expression, are resistant to stress-induced apoptosis, suggesting that HAS2 protects skin fibroblasts against apoptosis induced by environmental stress [34]. For inflammatory cells, HA seems to induce apoptosis. HA induces apoptosis of activated T cells through CD44 [141]. LMW HA-TLR4 interaction induced inflammatory neutrophil apoptosis [142]. In vivo, 1600 kDa HA administration yielded significantly less smoke-induced neutrophil infiltration, lung edema, airway apoptosis and mucous plugging in rats, suggesting a potential beneficial therapy of HMW HA for smoke induced lung injury [143]. Moreover, the cross-linking of specific epitopes of the CD44 molecule rapidly induced neutrophil apoptosis in vitro and inhibited neutrophil-dependent renal injury in rats in vivo [144].

2.6. HA in cell migration and invasion

HA mediates inflammatory cell migration to regulate inflammatory responses and tissue injury. CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice [145]. TSG6 is a potent inhibitor of neutrophil migration in an in vivo model of acute inflammation [146]. Exogenous LMW HA or LMW HA generated by HYAL1 over-expressing promoted dendritic cell migration from the skin and subsequent modification of allergic responses at TLR4 dependent manner [38].

HA and HA binding proteins have a role in fibroblast migration as well as smooth muscle cell migration. Abnormal accumulation of HA matrix promotes fibroblast cell migration [11]. Specific sizes of HA oligosaccharides stimulate fibroblast migration and excisional wound repair [147]. Both CD44 [148] and HMMR [57] have been shown to have a role in fibroblast migration during tissue injury. Furthermore, myofibroblast migration can be regulated by HA [149]. These studies suggested a role for HA in tissue injury and fibrosis.

Formation of HA- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells [150]. HMMR is also necessary for the migration of smooth muscle cells during wound repair [151].

CD44 and HA cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow [101]. HA promotes a CD44-dependent migration of the MSCs [152], suggesting a role for HA and CD44 in moving stem cells to a neighborhood in need of regeneration.

CD44 also regulates directional motility. The interaction between CD44 and the cytoskeletal linker protein ezrin is critical for CD44-dependent directional cell motility [153]. Fibroblast migration is mediated by CD44-dependent TGF-β activation [154], and the migration of CD44-deficient fibroblasts was increased in velocity, but was directionless [154].

HA regulates fibroblast invasion [148] ad tumor invasion [155, 156]. HA-mediated invasive cell motility is regulated by TGF-β [156, 157] and Src activation [157]. Furthermore, cellular heterogeneity profiling by HA probes reveals an invasive but slow-growing breast tumor subset [158]. We showed that fibroblasts isolated from transgenic mice overexpressing HAS2 had a greater capacity to invade matrix. Conditional deletion of HAS2 in mesenchymal cells abrogated the invasive fibroblast phenotype, impeded myofibroblast accumulation, and inhibited the development of lung fibrosis [159].

2.7. HA in stem cells

HA-stem cell interactions have been explored in hematopoietic stem cells [99–101], mesenchymal stem cells [103, 104], and adult multipotent progenitor cells, indicating that HA and its binding proteins may regulate tissue injury and repair processes through their interaction with a variety of stem cells.

During stem cell differentiation, HA synthesis was enhanced by 13- and 24-fold, most likely due to increased expression of HAS2 [160]. HA is required for generation of hematopoietic cells during differentiation of human embryonic stem cells [99, 161]. HAS2 knockdown results in suppression of human embryonic stem cell differentiation [162]. HA oligosaccharides increased epidermal cell stemness by modulation of integrin expression [163]. HA promotes a CD44-dependent migration of the mesenchymal stem cells [152].

CD44 has long been used as a marker for stem cells [102], including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), as well as cancer stem cells (CSCs) [164, 165]. In addition, CD44 may have a role in regulating stem cell functions. A recent review suggests that although CD44 is not a master stem cell gene, CD44 contributes to the generation of a stem cell niche and homing of stem cells in their niche [166]. HA is required for the regulation of the hematopoiesis-supportive function of bone marrow accessory cells and participates in hematopoietic niche assembly [167].

2.8. HA in angiogenesis

Partial degradation products of HA, between 4 and 25 disaccharides in length, induced an angiogenic response on the chick chorioallantoic membrane [82]. HA oligosaccharides

enhance angiogenesis to promote excisional wound healing [119], while HMW HA is anti-angiogenic [168]. Both endothelial CD44 [169] and HMMR mediated angiogenesis [53]. A deficiency in stromal HA in Has2−/− mice attenuated tumor angiogenesis and lymphangiogenesis [31]. Given its role in tumor angiogenesis, targeting HA is proposed for tumor chemotherapy [17].

3. Hyaluronan in human diseases

3.1. Lung fibrosis

Idiopathic pulmonary fibrosis (IPF) is the most common form of interstitial lung diseases. The central hallmark of IPF is excessive fibroproliferation, a chronic, progressive disorder that results in impaired gas exchange and respiratory failure. IPF portends a poor prognosis, with a mean survival of 2–5 years from the time of diagnosis [170]. HA levels were higher in BAL fluid in patients with IPF than in healthy controls [171], and were also correlated with the severity of the disease [171]. Fibroblast clones derived from primary fibroblast cultures from the lung tissue of patients with pulmonary fibrosis secreted much greater amounts of HA and proteoglycan decorin than the clones from normal individuals [172]. Fibroblasts isolated from lungs of patients with IPF had increased HAS2 [159]. IPF fibroblasts also showed increased invasive capacity, an important fibroblast phenotype that is required for development of severe pulmonary fibrosis [159].

In vitro studies suggested a role of HA in fibrosis. HA facilitates transforming growth factor-beta1-mediated fibroblast proliferation [117], as TGF-β signaling plays a central role in fibrogenesis. HA orchestrates TGF-β 1-dependent maintenance of myofibroblast phenotype [173]. Age-related changes in pericellular HA organization leads to impaired dermal fibroblast to myofibroblast differentiation [174].

Elevated HA levels have been reported in animal models of pulmonary fibrosis. There was a transient histological accumulation of HA in the alveolar interstitium, corresponding to increases in HA levels in BAL fluid and lung tissue extracts in bleomycin-induced alveolitis in rats [175]. HA was found not only accumulate in mouse lungs after bleomycin injury, but also fragmented in the injured lung [159, 176]. We showed that over expression Has2 under control of β-SMA promoter resulted in worsened lung fibrosis after bleomycin injury indicated a direct association between HA accumulation and fibrosis [159]. We further showed that conditional knockout Has2 in Col1α2 expressing cells or FSP-1 expressing cells reduced bleomycin induced lung fibrosis in mice [159], demonstrating a causative role for mesenchymal Has2 in the pathogenesis of pulmonary fibrosis.

An anti-fibrotic effect of hyaluronidases was reported in the recent studies. Hyaluronidase-loaded PLGA microparticles reduced neutrophil recruitment and collagen deposition after bleomycin treatment [177]. Intranasal therapy with immobilized and testicular hyaluronidases prevented the deposition of collagen in the parenchyma of bleomycin-treated lungs [178]. Hyaluronidase attenuation of bleomycin-induced lung fibrosis might be due to promoting a population of mesenchymal stem cell-like cells in the bleomycin injured lung [178]. Further study is needed to dissect the role of hyaluronidase in pulmonary fibrosis and the potential molecular mechanisms.

3.2. Asthma

The characteristics of human asthma are chronic inflammation and airway remodeling. HA is an important mediator in asthma. A genomewide association study identified HAS2, the major HA synthesis gene as the strongest statistical significance associated with asthma for adult asthma in Japanese population [179]. The level of HA in lavage fluid from patients with allergic asthma was increased [180]. HA levels were also closely correlated with the severity of asthma, and with the pulmonary function of the patients [181].

The molecular mechanism of HA accumulation and its biology function in the disease is complicated. LMM HA functioned through a CD44-mediated mechanism to elicit chemotaxis of B lymphocytes. HA stimulates B lymphocyte chemotaxis and cytokine production ex vivo in a murine model of fungal allergic asthma [182]. We found that fibroblasts isolated from human asthmatic lung had elevated HAS2 expression, without significant changes in Hyal1 and hyal2 expression levels [183]. Asthmatic fibroblasts produced increased amount and LMW HA compare to that of the fibroblasts from health lungs [183]. One of the reasons for increased HA levels in asthma is an increase in TGF-β [184] and IL-13 levels [185], which promote airway smooth muscle cells to secret glycosaminoglycans such as HA.

Asthmatic airway epithelial cells differentially regulate fibroblast expression of extracellular matrix components. Lung fibroblasts co-cultured with alveolar epithelial cells from asthmatic patients showed differential expression of the ECM constituents COL1A1 and COL3A1 and HAS2 [186], supporting a role for altered ECM production in asthmatic airway remodeling, regulated by unbalanced alveolar epithelial cell signaling.

3.3. Chronic Obstructive Pulmonary Disease and Emphysema

Chronic obstructive pulmonary disease (COPD) is characterized by infiltration of inflammatory cells, alveolar wall destruction, and airway remodeling. HA levels were significantly higher in patients with COPD than in those from controls [187–189]. Some reports showed that HA levels were decreased in COPD [190] and in COPD with alpha-1 antiprotease deficiency [191]. The decrease of HA was associated with a significant reduction in the expression of HAS1 and HAS2 and a significant increase of hyaluronidase-1 [190]. Lower HA levels possible happen in some COPD/emphysema where inflammation is not predominant.

HA plays multiple roles in the progression of COPD. LMW HA promotes lung inflammation. Cigarette smoke exposure leads to enhanced deposition of mostly LMW HA in alveolar and bronchial walls by altering the expression of HA modulating enzymes [192]. Both collagen and HA was increased in mouse models of COPD induced by exposure to cigarette smoke, chronic intratacheal lipopolysaccharide instillation, or over expression TNF-α in SPC-expressing cells in mice [193]. LMW HA contributes to persistent inflammation in the lung [183]. Systemic administration of Hyaruonidase to rats caused alveolar distension and larger functional residual capacity [194].

HA protects elastic fibers by its hydrating effect, and erosolized HA may be an effective means of preventing pulmonary emphysema and perhaps other lung diseases that involve

elastic fiber injury [195]. Aerosolized HA limited airspace enlargement in a mouse model of cigarette-induced emphysema [196] and smoke-induced elastic fiber injury [197]. The effect of HA on inflammation appears to be related to its molecular size, with larger polysaccharide chains having anti-inflammatory activity and smaller ones having proinflammatory properties. The breakdown of inhaled HA into smaller fragments could possibly induce an inflammatory reaction in the lung that counteracts any beneficial effect [198]. Consequently, the proposed therapeutic use of HA will require the development of treatment strategies aimed at minimizing its pro-inflammatory activity [198]. Increased studies of biological functions of HA during the past years provided better understanding of the roles in human diseases, and to develop HA as a therapeutic reagent is in need.

3.4. HA in allograft rejection and BOS

Lung transplantation is a therapeutic option for patients with end stage lung diseases, but long-term survival remains very poor due to chronic allograft rejection. Bronchiolitis obliterans syndrome (BOS), a fibrotic process resulting in progressive narrowing of bronchiolar lumens and airflow obstruction, is a manifestation of chronic allograft rejection. HA as an immune regulator plays an important role in lung allograft. Lung transplant patients with acute rejection had significantly higher levels of HA in BAL compared to the patients with no rejection [199]. HA localized within areas of intraluminal small airways fibrosis in BOS lung tissue [200]. The transcripts of HA synthases were significantly elevated in BOS versus normal lung tissues [200]. HA concentrations were increased in lavage fluid and plasma of recipients with BOS [95, 200].

Elevated HA level was also observed in murine skin transplant rejection model. [95]. In experiment cardiac allograft rejection rat model, HA concentration in serum significantly higher in the rejecting group than that in the non-rejecting group [201].

Treatment with various HA preparations showed contradictory results. LMW HA abrogated tolerance in murine orthotopic lung recipients [200], and prolonged renal and cardiac allograft survival in experimental animals [202, 203], while HMW HA attenuated basal allograft inflammation [200]. Hyaluronidase treatment reduced HA contents of the graft and ameliorated rejection-induced edema in a heterotopic heart transplantation model in rats [204]. More studies are in need to better understand the role of HA in allograft and develop therapeutic strategies.

3.5. Pneumonia

Serum HA in patients with chronic interstitial pneumonia was significantly greater when compared with healthy controls [205]. In mouse models, CD44 deficiency mice showed increased neutrophil accumulation in the lung and edema formation in *Escherichia coli*-induced pneumonia [145], but no difference was observed between these genotypes in *Streptococcus pneumoniae*-induced pneumonia [145]. CD44 deficiency is associated with increased bacterial clearance but enhanced lung inflammation in *Klebsiella pneumonia*-induced pneumonia [206].

As HA accumulates, HA fragmentation occurs during infections. HA is extremely sensitive to ROS-mediated degradation [89]. In vitro, oxidative fragmentation of HA resulted in neutrophil chemotaxis and extracellular superoxide dismutase prevented this effect [207]. CD44 plays a key role to clear HA in the lung to resolve lung inflammation [48]. HA fragment promotes inflammation by down-regulating the anti-inflammatory adenosine A2a receptor through CD44 [207]. Mice with CD44 deficiency showed increased HA accumulation in the lungs and increased inflammatory chemokine and cytokine levels with both bleomycin and LPS induced lung injury [48, 96].

3.6. Hepatitis and liver fibrosis

Elevated serum HA levels have been found in patients with chronic viral hepatitis and cirrhosis, and often correlate with the disease progress [208]. A recent study performed a systematic review showing that serum HA can be used as a noninvasive biomarker to assess the presence of liver fibrosis, and to monitor disease progression [209]. Serum HA in combination with other measurements form a score system [210] or an algorithm model [211] may be used as a clinic tool to monitor liver fibrosis. However, serum HA alone may not be regarded as reliable marker for clinical decisions [212], because of the heterogeneous nature of liver fibrosis.

In mouse models, LMW HA induces NF-κB-dependent resistance against TNF-α mediated liver injury [213]. CD44-deficient mice exhibited suppressed liver inflammation during the early phase after CCl_4 injection due to reduced inflammatory cell infiltration and cytokine production, but showed severe liver inflammation with increased numbers of apoptotic hepatocytes at the late phase [214]. Therefore, HA and CD44 may have multiple function depending the progress of the disease.

3.7. Heart diseases

HA is expressed in all aortic layers. In atherosclerosis, HA associates with leukocytes and vascular smooth muscle cells, and is involved in vascular remodeling [215]. HAS1 and HAS2 are upregulated in response to prostaglandins in human vascular smooth muscle cells [216]. Overproduction of HA in the genetic background of the ApoE-deficient mouse strain promoted atherosclerosis development in the aorta, suggesting that accumulation of HA accelerates the progression of atherosclerosis [217]. However, systemic inhibition of HA synthesis by 4-MU interferes with the protective function of the endothelial glycocalyx, thereby facilitating leukocyte adhesion, subsequent inflammation, and progression of atherosclerosis [218]. Therefore, an HA synthase inhibitor that avoids adverse effects such as impacting the endothelial glycocalyx may be needed for blockage of HA synthase pathways in patients with atherosclerosis.

CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation [219]. Furthermore, CD44 expressed on both bone marrow-derived and non-bone marrow-derived cells promotes atherosclerosis in ApoE-deficient mice through regulating macrophage and T cell recruitment; leukocyte-endothelial cell adhesion and transendothelial migration; and HA-dependent migration of vascular smooth muscle cells [220].

3.8. Diabetes

Increased HA levels were seen in diabetic patients [221] and in the glomeruli from diabetic rats [222]. Serum HA levels correlate with poor blood glucose control and diabetic angiopathy and could be used as a marker of diabetic angiopathy [221]. However, others found a decrease of HA in skin in diabetic patients [223] or rats with chronic diabetes mellitus [224]. This discrepancy may be due to the measurement of HA during different stages of the disease, inflammatory status and treatments.

HA plays an active role in the development of diabetes. HA production in response to a raised glucose environment in diabetes can contribute to mesangial hypercellularity [225]. HA increases vascular smooth muscle cell expression of PAI-1, altering the balance between proteolysis and its inhibition in vessels of patients with type 2 diabetes, thereby contributing to the acceleration of macroangiopathy [226].

In addition, HA binding proteins were also increased in patients with diabetes and in experimental diabetic animal models. Expression-based genome-wide association study (GWAS) links the receptor CD44 in adipose tissue with type 2 diabetes [227]. Injection of anti-CD44 monoclonal antibody or administration of hyaluronidase induced appreciable resistance to insulin-dependent diabetes mellitus in mice [228], suggesting that the CD44-HA interaction is involved in the development of the disease.

3.9. Kidney diseases

Spatially restricted HA production by Has2 drives epithelial tubulogenesis suggesting a role for HA in kidney development [229]. In disease state, marked accumulation of HA was demonstrated in developing and sclerosing crescents, in association with local infiltration of T cells and monocyte/macrophages in the rats [230].

Serum HA was increased in lupus nephritis patients [231] as well as dermatomyositis patients [232]. Serum HA levels were significantly increased in patients with renal insufficiency and with end-stage renal failure, and were correlated well with the degree of impaired renal function [233]. Patients with deteriorated clinical conditions often had greater HA levels, suggesting that HA may be a biochemical marker of patients in these conditions [234], and also a risk predictor of poor survival in dialysis [235]. Fibroblasts derived from active lesions of nephrogenic fibrosing dermopathy synthesize elevated levels of HA when compared with normal controls [236].

CD44 is dramatically up-regulated in a rat model of crescentic anti-glomerular basement membrane disease, suggesting a role for the CD44-HA interaction in leukocyte recruitment and renal fibrosis [237]. Administration of anti-CD44 to mice reduced the influx of neutrophils into the postischemic tissue with associated renal function preservation [132]. Furthermore, sulfated HA as a selectin-blocking agent inhibits intraglomerular infiltration of macrophages and prevents the progression of experimental crescentic glomerulonephritis [238].

3.10. Intestinal inflammation

During homeostasis, endogenous HA regulates normal intestinal and colonic growth [115]. In mice, treated with exogenous HA at age 3 to 8 weeks increased intestinal and colonic epithelial proliferation, resulting in hyperplasia [115]. Pep-1 treatment markedly decreased intestinal and colonic length, crypt depth and villus height in the intestine, crypt depth in the colon, and epithelial proliferation in the intestine and colon [115]. It is not known if the effect was dependent on the receptor CD44 or TLR4.

It is believed that inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis results from exaggerated activation of the mucosal immune system by the flora found within the intestinal lumen [239]. Cytokines, especially Th1 cytokines, have been suggested to have a key role in intestinal inflammation [239]. HA accumulates in the colon in the nonvascular space of patients with chronic intestinal inflammation, such as Crohn's disease and ulcerative colitis [240]. HA deposition within blood vessels of the colon was observed well before any significant inflammatory infiltration and likely promoted leukocyte infiltration in the dextran sulfate sodium (DSS)-induced experimental mouse model of colitis [241]. Indeed, mononuclear leukocytes bind to HA on human intestinal mucosal smooth muscle cells via CD44 after virus infection or treatment with poly(I:C) [242], indicating a role of HA in leukocyte retention and recruitment during intestinal inflammation. STAT1 plays a role in HA-mediated leukocyte adhesion, since both tissue damage and HA deposition were attenuated in STAT1-null mice compared with that in wild-type control mice in the DSS-induced colitis mouse model [243]. In line with the deteriorative role of HA in intestinal injury, *Has*3 null mice showed significant reduction in HA deposition in the colon tissue and thus protected from DSS-induced colitis [244]. The role of HA in intestinal inflammation may function through its receptor CD44. In a TNF-driven mouse model of chronic ileitis, soluble HA levels and expression of *Has*1 were increased coincided with increased expression of CD44 on CD4+ T cells, and deficiency in CD44 resulted in attenuated the severity of ileitis of the mice [245].

Interestingly, reports revealed that platelet-derived HYAL2 is able to cleave HA into fragments that stimulate mononuclear leukocytes in the immediate microenvironment to produce proinflammatory cytokines [41]. However, a recent study showed that patients with IBD had lower platelet HYAL2 levels and activity than healthy controls [246]. Platelet activation caused the translocation of HYAL2 from α-granules to platelet surfaces where it exerts its catalytic activity [246].

In addition, HA interacts with TLR4 to protect intestinal epithelium. Endogenous HA expression was markedly increased in DSS-induced colitis and preserved the epithelium through TLR activation [247]. Furthermore, intraperitoneal administration of HA was able to reduce DSS-induced colitis [247] or radiation-induced apoptosis and increased crypt survival [248]. The roles that HA plays in regulating intestine inflammatory response and in protection of intestinal epithelium through different HA binding proteins and different signaling pathways. Moreover, HA may function differently at different cell compartments. Further studies are needed to dissect the mechanism that HA promote inflammation and

protect intestine epithelium. Targeting HA may present a potential strategy in treatment of inflammatory intestinal diseases.

3.11. Neoplasia and metastasis

A body of work has demonstrated a role of HA, HA synthases, hyaluronidases, and HA binding proteins in cancer initiation and progression, metastasis, cancer therapeutics and chemoresistance. There are many excellent reviews on the topic [3, 15–18, 249]. An example of the role of HA in cancer can be seen in a recent report on naked mole rats. Naked mole-rat fibroblasts make extremely HMW HA (6–12 MDa) that accumulates abundantly in naked mole-rat tissues, conferring cancer resistance [250]. Absence of the CD44 gene prevents sarcoma metastasis [251]. The de-repression of CD44 resulting from inactivation of p53 can potentially aid the survival of immortalized, premalignant cells [252]. Therefore, therapeutics targeting HA and its binding proteins are in the development. For example, a humanized monoclonal antibody specific for CD44 is to target chronic lymphocytic leukemia [253].

4. HA as therapeutic

4.1. HA species as drugs

Native HA products such as Healon (Abbott) and Hylan GF-20 (Genzyme) were approved for eye surgery and osteoarthritis, respectively. In other areas, HA treatment showed a dramatic decrease of oral antibiotic need in a cystic fibrosis patient with asthma-like symptoms [254]. A multicenter, randomized, controlled clinical trial in Italy showed the addition of HA to hypertonic saline reduces the prevalence and severity of cough, throat irritation, and saltiness in the chronic treatment of cystic fibrosis patients [255]. Intravesical HA therapy shows long-term (4.9 years) efficacy, with a high rate of acute symptom remission, in bladder pain syndrome/interstitial cystitis [256].

Preclinically, HMW HA inhibited smoke inhalation-induced lung injury and improved survival [143] and sepsis-induced lung injury with mechanical ventilation in rats [257]. HMW (1,600 kDa) HA, not LMW (35 kDa) HA blocked both monocyte and neutrophil infiltration and decreased the lung injury [257]. Aerosolized HA limited airspace enlargement in a mouse model of cigarette-induced emphysema [196]. However, the proposed therapeutic use of HA will require the development of treatment strategies aimed at minimizing its proinflammatory activity [198].

LMW HA has been shown to prolong renal and cardiac allograft survival in experimental animals [202, 203]. Furthermore, hyaluronidase treatment reduced HA contents of the graft and ameliorated rejection-induced edema in a heterotopic heart transplantation model in rats [204]. These studies suggest that LMW HA may present a nontoxic and non-immunogenic immunotherapy.

Given the role of HA and HA binding proteins in cancer, either HA oligomers or soluble hyaluronan-binding proteins may antagonize HA-HABP interactions for cancer therapy [3]. HA oligosaccharides (6–18 disaccharides), but not large polymers, can kill many types of cancer cells by triggering apoptosis while leaving normal cells unaffected [258]. Moreover,

when co-treated with HA oligosaccharides, chemoresistant cancer cells become drug-sensitive [258].

Chemically modified HA preparations have been investigated in their role in inflammatory responses. Selectively N-butyrylated LMW HA showed an anti-inflammatory effect in human macrophages [91]. Sulfated HA as a selectin-blocking agent inhibited intraglomerular infiltration of macrophages and prevented the progression of mesangial proliferative glomerulonephritis [259], as well as crescentic glomerulonephritis in rats [238]. In an animal model, sulfated HA as a hyaluronidase inhibitor was showed to block the proliferation, motility, and invasion of prostate cancer cells, acting as a potent inhibitor of prostate cancer [92]. Modifications of HA with sulfate groups influence the interaction with recombinant human BMP-4 [260]. The high-sulfated HA preparations exhibited the tightest interaction with BMP-4 [260] and with TGF-β1 [261]. The modified HA preparations may have a broad use as therapeutics and as drug carriers (Figure 4).

4.2. HA as therapeutic carrier

HA has been investigated as a drug delivery agent for ophthalmic, nasal, pulmonary, parenteral, and dermal routes, as shown in a review [262]. Conjugation of chemodrugs to HA has been shown to have a beneficial effect. HA conjugation significantly increased drug uptake and extended the residence time of drugs in tumor cells in mice [263]. For example, conjugation of irinotecan to HA significantly improved the profile of in vivo tolerability for ovarian cancer [264]. Conjugation of paclitaxel improved therapeutic efficacy for peritoneal tumor carcinomatosis [265] and ovarian cancer [266]. An intralymphatic drug delivery method using an HA-cisplatin conjugate was proposed in breast cancer to preferentially treat at-risk regional lymph nodes and avoid systemic toxicities [267]. Furthermore, HA-based nanocarriers were investigated as drug vehicles for systemic targeting of CD44-overexpressed cancers [268, 269]. HA-coated superparamagnetic iron oxide nanoparticles conjugated anticancer drug doxorubicin was delivered at much higher levels and distributed wider in the tumor tissue than intravenously injected free doxorubicin, leading to significant reduction of tumor growth [270]. HA-Irinotecan was tested in a phase II trial to determine progression-free survival in 5-fluorouracil refractory patients with metastatic colorectal cancer [271].

In addition to cancer therapy, HA conjugation has been investigated in other fields. HA-TRAIL (tumor necrosis factor-related apoptosis-inducing ligang) conjugate was reported for targeted treatment of liver fibrosis in rats [272]. An HA-insulin complex was developed to test whether it could be used to treat diabetic patients by oral administration. Glucose-lowering activity was demonstrated after oral administration of the HA-insulin complex to diabetic rats [273]. The HA-insulin complex was active after oral administration and the complexed insulin significantly decreased blood glucose concentrations within 1 hour after oral administration in rats [274].

4.3. Targeting HA and HABPs

4.3.1. 4-methylumbelliferone—4-methylumbelliferone (4-MU, aka hymecromone) was showed to reduce the HA production of fibroblasts markedly [275], by depletion of cellular

UDP-glucuronic acid and down-regulation of HAS2 and HAS3 [276]. 4-MU treatment has viewed as a potential therapeutic strategy in inflammation, autoimmunity, and cancer [277]. Unrelated its role in HA inhibition, 4-MU is used in clinic for bile duct motor disorders [278].

Several studies with inhibition of HA synthesis by treatment with 4-MU showed a beneficial effect in various tissue injury and inflammation. 4-MU suppresses staphylococcal enterotoxin B- [279] and LPS-induced lung inflammation [280]. 4-MU treatment in vivo protected against central nervous system autoimmunity and increased CXCL12 expression in the inflamed nervous system [281]. 4-MU was protective against renal ischaemia-reperfusion injury, and significantly attenuated inflammation [282]. Treatment with 4-MU dramatically decreased the severity of ollagen-induced arthritis in mice [283]. Therefore, development of therapeutics of 4-MU and its analogs can be used for these inflammatory conditions.

In addition, 4-MU inhibited hepatocellular carcinoma growth by decreasing IL-6 production and angiogenesis [284]. Tumors systemically treated with 4-MU showed the extensive areas of necrosis, inflammatory infiltrate and 2–3-fold reduced number of tumor satellites [285], suggesting inhibition of HA synthesis can be a means to treat certain cancers.

4-MU belongs to the family coumarin derivatives and is structurally simple, so that the inhibition of HA synthases may not be very specific. Therefore, modification of 4-MU or designing new class of inhibitors may provide better ways to treat conditions such as cancer and tissue remodelling.

4.3.2. Hyaluronidase and inhibitors—Hyaluronidase is commonly used as an adjuvant to local anesthesia in ophthalmic surgery [286]. Early studies have suggested hyaluronidase may reduce acute myocardial infarction in animals and in humans [287, 288]. However, a large trial did not find a beneficial effect of hyaluronidase on mortality or infarct size in patients with acute myocardial infarction [289]. Recently, immobilized hyaluronidases have been investigated. Pegylated hyaluronidase reduced bleomycin-induced lung fibrosis in mice [178]. Hyaluronidase-loaded poly(D,L-lactide-co-glycolide) (PLGA) microparticles reduced neutrophil recruitment and collagen deposition after bleomycin treatment in mice [177].

Hyaluronidase inhibitors have also been investigated and suggested having roles in treatment of human diseases [290]. Glycyrrhizin from liquorice root acts as a potent inhibitor of hyaluronidase in vitro and has anti-inflammatory effect [291]. Hyaluromycin isolated from sea squirt *Streptomyces sp.* was even more potent to inhibit hyaluronidase activity than glycyrrhizin [292]. Glycyrrhizin has been used to treat patients with chronic hepatitis C [293] as well as upper respiratory tract infections [294]. Inhibition of hyaluronidase activity with 6-O-Palmitoyl-L-ascorbic acid (VCPAL) promoted remyelination which may benefit patients with multiple sclerosis [43].

Many studies suggested an oncogenic role of hyaluronidases in many cancers. Therefore, various strategies with inhibition of hyaluronidases have been investigated. Sulfated HA as a hyaluronidase inhibitor was shown to block proliferation, motility, and invasion of prostate

cancer cells [92]. On the other hand, digestion of HA by hyaluronidases may remodel the tumor environment and favor chemotherapeutics to access tumor cells. Combination of PEGylated human recombinant PH20 hyaluronidase (PEGPH20) with gemcitabine showed that PEGPH20 rapidly and sustainably depleted HA, and improved drug delivery in a mouse model of pancreatic cancer [295, 296].

4.3.3. Targeting HABPs and HA-HABP interactions—Using phage display, a 12-mer (GAHWQFNALTVR) peptide inhibitor, Pep-1, was identified to inhibit HA binding in vitro [297]. Systemic, local, or topical administration of Pep-1 was able to block skin-directed homing of inflammatory leukocytes and inhibited the expression of contact hypersensitivity responses in mice [297, 298]. Pep-1 efficiently inhibited dendritic cell-dependent cluster formation, IL-2 and IFN-γ production, and T cell proliferation [299]. Moreover, Pep-1 ameliorated naphthalene-induced lymphocytic bronchitis in a mouse model [300].

Single injection of Pep-1 significantly reduced the incidence of lung metastasis of melanoma cells and prolonged the survival of the tumor-bearing animals [301]. Furthermore, Pep-1 treatment maintained the effectiveness of IL-2 and prevented the metastasis of melanoma [302].

A 15-mer peptide with homology to HA binding sequences of HMMR blocks HA signaling and reduces inflammation and fibrogenesis in excisional skin wounds [303]. The peptide was also able to prevent nuclear accumulation of HA [158] and to inhibit prostate tumor cell migration toward to fragmented HA [304].

Some new peptide inhibitors such as A-6 peptide [305] and P6 peptide [306] have recently been reported. A6 is a capped eight amino acid peptide (Acetyl-KPSSPPEE-NH2) derived from human urokinase plasminogen activator (uPA). A6 binds to CD44 resulting in the inhibition of migration, invasion, and metastasis of tumor cells, and the modulation of CD44-mediated cell signaling [307]. P6 peptide containing FDAIAEIGNQLYLFKDGKYW sequence from human MMP-9 hemopexin domain specifically interacts with CD44, possibly interfering MMP9-CD44 interaction in chronic lymphocytic leukemia cells [306]. CD44 has been shown to provide a cell surface docking receptor for proteolytically active MMP-9, and localization of MMP-9 to cell surface is required for its ability to promote angiogenesis and tumor invasion [156].

Blocking CD44 with neutralizing antibodies has been investigated in many fields. Anti-CD44 monoclonal antibodies induced differentiation and apoptosis of human myeloid leukemia cells [308, 309]. Anti-CD44 monoclonal antibodies inhibited human melanoma growth and metastasis in mice in vivo [310], and disrupted intracerebral progression of rat glioblastoma in rats in vivo [311]. Blocking CD44v6 with humanized anti-CD44v6 monoclonal antibody U36 has been tested in patients with head and neck cancer [51].

It has been suggested that HA-binding proteins play a role in arthritis. CD44 is up-regulated on many synovial cell types in patients with rheumatoid arthritis, and the level of CD44 present in synovial tissue is correlated with the degree of synovial inflammation [312]. An anti-CD44 antibody abrogates tissue swelling and leukocyte infiltration in mice [313].

Consistent with the role of CD44 in mediating fibroblast invasion and subsequent tissue fibrosis, immunohistochemical analysis of lung tissue from patients who died from acute alveolar fibrosis after lung injury reveals CD44-expressing mesenchymal cells throughout newly formed fibrotic tissue [148]. Anti-CD44 antibody blocked fibroblast migration [148], and induced fibroblast apoptosis [314]. Study results from our lab showed that systemic administration of neutralizing CD44 antibody to mice reduced invasiveness of fibroblasts and attenuated lung fibrosis after bleomycin lung injury [159].

Given the role of HA and HABPs in allograft rejections, blocking CD44 with neutralizing antibodies has been investigated. Anti-CD44 monoclonal antibody down-modulated host reactivity in skin transplantation in rats [315], and inhibited heart transplant rejection in nude mice [316].

In order to move into clinic, fully human anti-CD44 antibodies are developed. A fully human IgG2 anti-CD44 neutralizing antibody (PF-03475952 developed by Pfizer) was reported for therapeutic applications in inflammatory diseases such as rheumatoid arthritis [317]. A humanized anti-CD44 neutralizing antibody (RG7356 developed by Roche) directly induced chronic lymphocytic leukemia cells to undergo caspase-dependent apoptosis [253]. Administration of RG7356 at 1 mg/kg to immune-deficient mice engrafted with human chronic lymphocytic leukemia cells resulted in complete clearance of engrafted leukemia cells [253]. These studies suggested that these humanized antibodies have therapeutic activities in patients with inflammatory diseases and cancer.

5. Conclusion and outlook

HA, HA synthases and degradating enzymes, and HABPs play an important role in many biological and pathological processes. The function of hyaluronan depends on its size, location, and interactions with its binding patterns. Dysregulated HA activities involve in multiple human disease processes. Significant progress has been made in dissecting the role of HA in human diseases and in designing and investigating strategies of targeting HA system for human diseases. High molecular weight HA and chemically modified HA preparations, HA inhibitor, HA blocking peptides, and hyaluronidases have been tested as therapeutics on inflammation, fibrosis, and cancers. Especially the humanized antibodies have showed therapeutic activities in patients with inflammatory diseases and cancer. Therefore, understanding the roles of HA and underlying mechanisms in the normal and disease settings may provide a new way to develop therapeutics for these diseases.

Acknowledgments

Abbreviations

HA   hyaluronan or hyaluronic acid

HAS   hyaluronan synthase

HYAL	hyaluronidase
HMW	high molecule weight
LMW	low molecule weight
HMMR	hyaluronan-mediated motility receptor
HARE	hyaluronan receptor for endocytosis
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
TNFIP6	tumor necrosis factor α-induced protein 6
CEMIP	cell-migration induced protein, hyaluronan binding
IPF	idiopathic pulmonary fibrosis
COPD	chronic obstructive pulmonary disease
BOS	bronchiolitis obliterans syndrome
4-MU	4-methylumbelliferone
IBD	inflammatory bowl disease
DSS	dextran sulfate sodium

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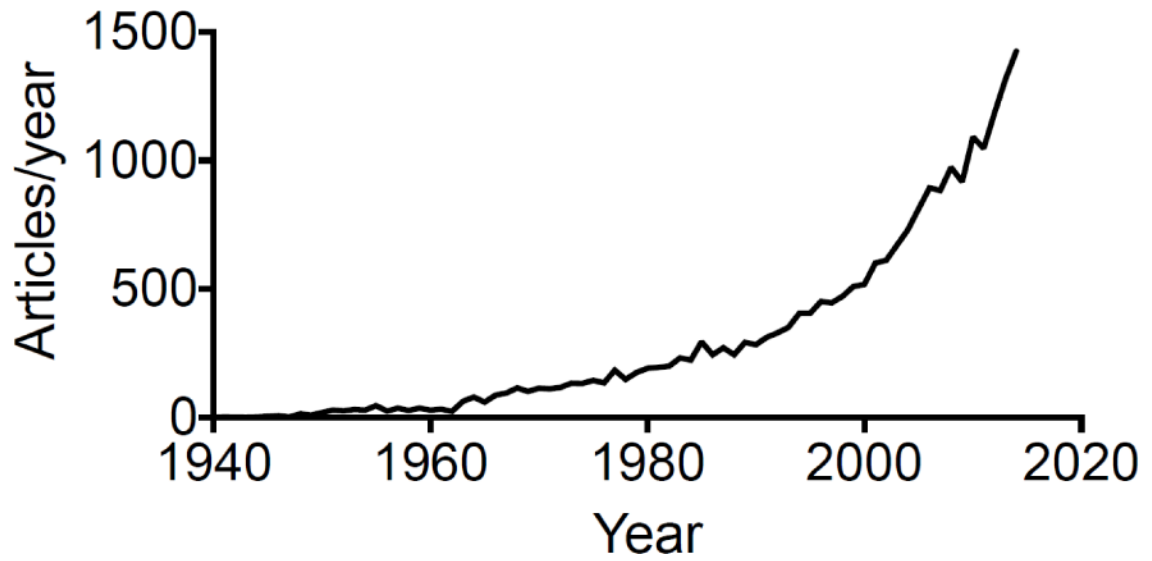
Figure 1. Scientific articles published referring to "hyaluronan" from 1940 to 2014
The number of articles published in each of the past years from 1940 to 2014 was identified by searching the PubMed database (http://www.ncbi.nlm.nih.gov/sites/entrez) using the search terms (hyaluronan OR hyaluronic acid) queried on August 8, 2015.
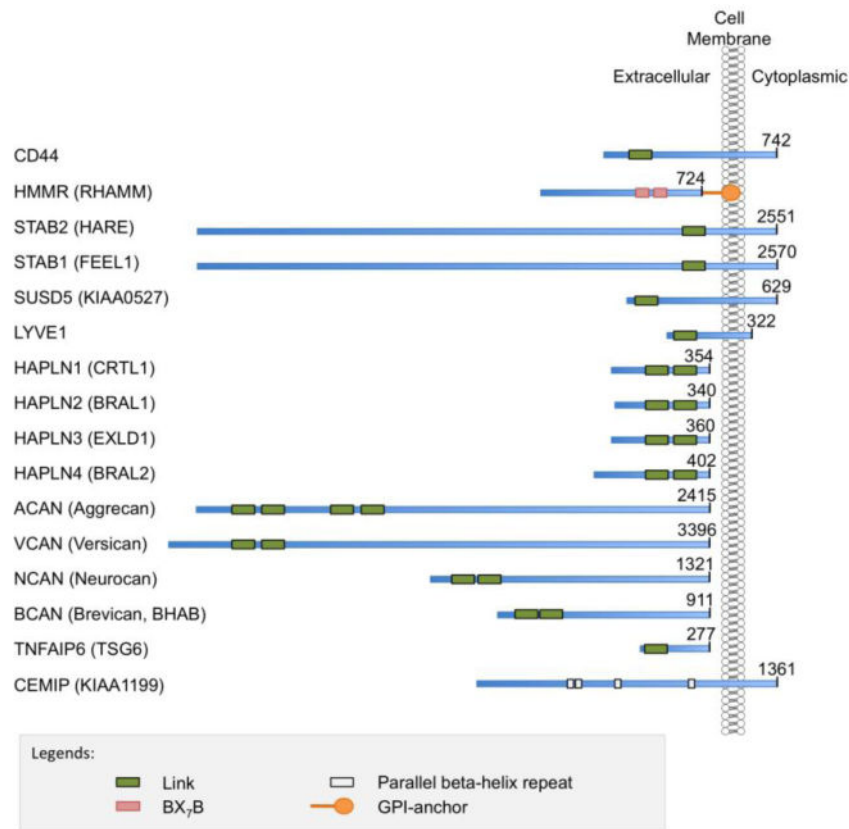
Figure 2. HA binding domains in HA binding proteins
The locations of HA-binding domain Link modules were shown on the majority of HABPs. HA binding sequence, BX_7B, was shown on HMMR. Protein CEMIP contains neither Link domain nor BX_7B sequence, but four parallel beta-helix repeats, which are usually presented in the enzymes with polysaccharide substrates. CD44, STAB1, STAB2, SUSD5, LYVE1 and CEMIP are the single-pass transmembrane proteins, while others are secreted as extracellular matrix proteins. HMMR is a GPI-anchored protein. The proteins are drawn roughly to scale, except VCAN. Protein size was indicated with the number of total amino acids on last residue.
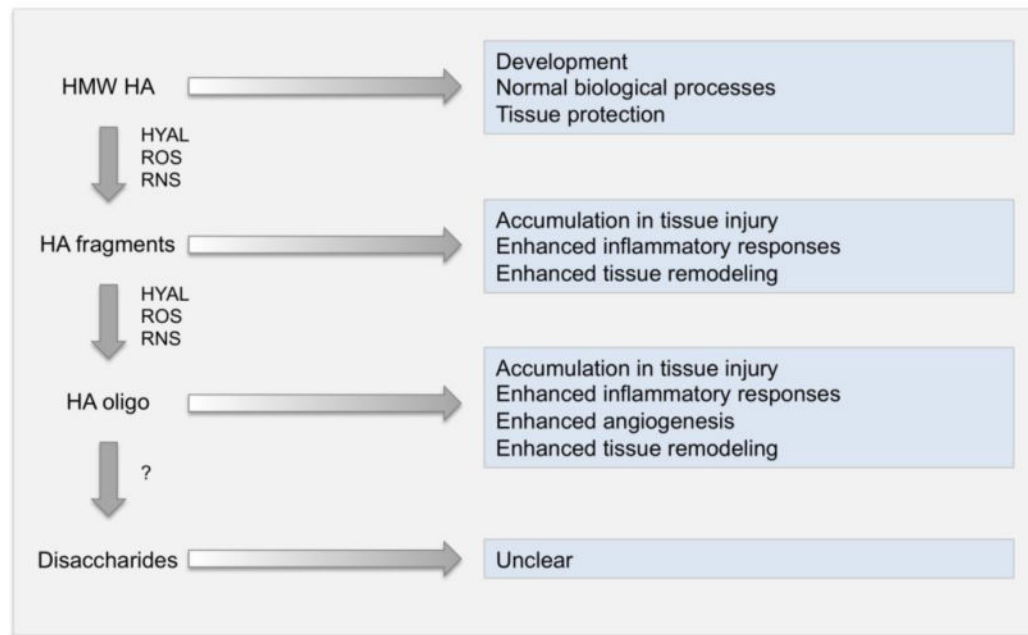
Figure 3. HA size
Native HA exists usually in HMW form, having a role in normal development, biological processes and tissue protection. HMW HA can be degraded by hyaluronidases, ROS, and RNS to LMW HA and HA oligomers. The bacterial, not mammalian, hyaluronidases, produce disaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine. The role of disaccharides in vivo is not clear.
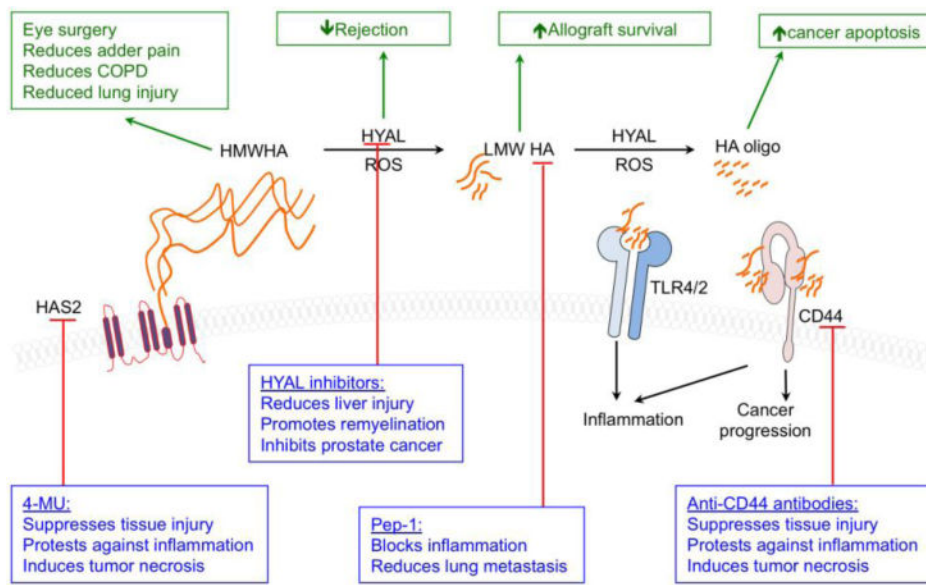
Figure 4. Targeting HA biology for human diseases
Unmodified or modified HA species and hyaluronidase have a role in inflammation and cancer (in green), while blocking HA system can be used to develop therapeutics for human diseases (in blue).

Table 1

Genetically modified mice of HA metabolic enzymes

Gene	Genotype	Phenotype	References
Has1	*Has1⁻/⁻*	Viable, no obvious abnormity	[31]
Has2	*Has2⁻/⁻*	Heart defect leading to embyonic lethality	[33]
	Col1a2-Cre;Has2flox/flox	Embryonic lethal	[159]
	FSP-1–Cre+;Has2flox/flox	Inhibited lung fibrosis in bleomycin model	[159]
	ASMA-HAS2⁺	Promoted atherosclerosis	[217]
	ASMA-HAS2⁺	Accumulated HA, increased lung fibrosis and mortality after bleomycin	[159]
Has3	*Has3⁻/⁻*	Viable, no obvious abnormity, normal lifespan	[12]
		Reduction in brain extracellular space leading to seizures	[35]
Has1, Has3	*Has1⁻/⁻;Has3⁻/⁻*	Viable, no obvious abnormity	[32]
		Enhanced wound repair	[32]
Has1, Has2, Has3	*Prx1-Cre;Has2flox/flox; Has1⁻/⁻;Has3⁻/⁻*	Decreased hematopoietic activity in long term bone marrow cultures	[114]
Hyal1	*Hyal1⁻/⁻*	Viable, fertile and show no gross abnormalities	[37]
		Accumulation of cytoplasmic HA, and osteoarthritis	[37]
	Krt14-CreERT;Hyal1tg	Decreased dendritic cells and suppressed contact hypersensitivity	[38]
Hyal2	*Hyal2⁻/⁻*	An increase in plasma hyaluronidase activity and plasma HA	[39, 40]
		Severe cardiopulmonary dysfunction	[40]
Hyal3	*Hyal3⁻/⁻*	A subtle change in the alveolar structure and extracellular matrix thickness in lung-tissues at 12–14 months-of-age	[42]
		No evidence of HA accumulation	[42]

Table 2

Genetically modified mice of major HA-binding proteins

Gene	Genotype	Phenotype	References
CD44	*Cd44*$^{-/-}$	No obvious developmental defects; altered tissue distribution of myeloid progenitors; highly tumorigenic of CD44-deficient fibroblasts	[47]
		Exaggerated lung injury; failed to clear HA	[48]
		Enhanced hepatitis	[318]
		Reduced atherosclerosis	[219]
		Increased resistance to collagen-induced arthritis	[319]
		Reduction in ischemic infarct	[320]
		Enhanced lung injury in *E. coli* pneumonia	[145]
		Exaggerated acute pulmonary inflammation	[96]
		Ameliorated insulin resistance and adipose tissue inflammation	[227]
HMMR (RHAMM)	*Hmmr*$^{-/-}$	Viable, normal lifespan; fewer litters	[56]
		Attenuated the formation of aggressive fibromatosis	[56]
		Defective skin wound repair	[57]
		Aberrant mitotic spindles and increased multinucleated cells	[321]
		Decreased macrophage chemotaxis	[322]
		Prevented constrictive artery wall remodeling	[323]
		Reduced LMW HA-mediated fibrosarcoma cell adhesion	[324]
Versican	*hdf*	Defect in right cardiac chamber and endocardial cushion formation leading to lethality	[325]
		Defect in limb chondrogenesis	[326]
Versican V2	*VcanV2*$^{-/-}$	Viable and fertile, have a normal life span; defect in extracellular matrix assembly in the central nervous system	[327]
	Prx1-Cre;Vcan$^{flox/flox}$	Viable and fertile; distorted digits	[328]
	VcanV2$^{-/-}$	Viable and fertile, have a normal life span; defect in extracellular matrix assembly in the central nervous system	[327]
Aggrecan	*Cloe*	Develop normally with no skeletal deformities	[329]
	Jaffa	Protected against cartilage erosion and may potentiate cartilage repair.	[330]
HARE (Stab2)	*Stab2*$^{-/-}$	No obvious physical or behavioral abnormalities	[331]
	Stab2$^{-/-}$	The metastasis of B16F10 melanoma cells to the lungs was markedly suppressed	[70]
	Stab1$^{-/-}$*Stab2*$^{-/-}$	Developed severe glomerular fibrosis and mild perisinusoidal hepatic fibrosis	[331]
LYVE1	*Lyve1*$^{-/-}$	Normal; increased interstitial-lymphatic flow and lack of typical irregularly-shaped lumens	[332]
	Lyve1-Cre;iDTR	Lymphatic vessel defect in the intestine and lymph nodes.	[73]
Tnfip6 (TSG-6)	*Tnfip6*$^{-/-}$	Impaired cumulus mucification and female sterility	[333]
		Enhanced neutrophil extravasation and rapid progression of proteoglycan-induced arthritis	[78]
		Severe arthritis	[334]
		Attenuated inflammation in asthma	[335]

Notes:

hdf mouse line is a transgenic mouse with official name Vcan$^{Tg(Hoxa1)1}$. The transgene consisted of 3.5kb of the Hoxa1 regulatory region gene with a lacZ reporter sequence. The transgene was inserted into intron 7 of Vcan gene on chromosome 13 and disrupted the two chondroitin sulfate binding domains of VCAN. The insertion of the transgene caused a recessive lethal mutation, displaying cardiac defect in homozygous mice.

Cloe is an aggrecan knock-in mouse line, in which MMP cleavage site of ACAN was mutated, leading to resistant to proteolysis by MMPs.

Jaffa is an aggrecan knockin mouse line, in which aggrecanase cleavage site of ACAN was mutated, leading to resistant to proteolysis by aggrecanases.