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Activated hyaluronan metabolism in the tumor matrix – causes and consequences

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Abstract

Hyaluronan accumulates in the stroma of several solid tumors and promotes their progression. Both enhanced synthesis and fragmentation of hyaluronan are required as a part of this inflammatory process resembling wound healing. Increased expression of the genes of hyaluronan synthases (HAS1-3) are infrequent in human tumors, while posttranslational modifications that activate the HAS enzymes, and glucose shunted to the UDP-sugar substrates HASs, can have crucial contributions to tumor hyaluronan synthesis. The pericellular hyaluronan influences virtually all cell-cell and cell-matrix interactions, controlling migration, proliferation, apoptosis, epithelial to mesenchymal transition, and stem cell functions. The catabolism by hyaluronidases and free radicals appears to be as important as synthesis for the inflammation that promotes tumor growth, since the receptors mediating the signals create specific responses to hyaluronan fragments. Targeting hyaluronan metabolism shows therapeutic efficiency in animal experiments and early clinical trials.

Introduction

Hyaluronan is abundant during the embryonic development of most organ systems, when cells migrate and proliferate before settling into their final position and functional state [1, 2]. The association of hyaluronan with dynamic changes in tissue organization is also evident in adults following inflammation and injury, when hyaluronan is an important component in the provisional matrix needed for wound healing [3, 4]. The role of hyaluronan in the continuous remodeling that takes place in the matrix of malignant tumors can thus be considered similar to these physiological processes in the fetal and postnatal periods.

Animal experiments support the importance of hyaluronan metabolism in the progression of cancer. Experimental overexpression of *HAS*s in cancer cells [5, 6] or their stromal cells [7] enhance tumor progression *in vivo*, while their suppression retards tumor growth [8-11] and similar reports exist on manipulation of hyaluronidases (see below).

Numerous experiments on cell cultures have demonstrated the signaling functions of endogenously synthesized, or exogenously added hyaluronan. These signals facilitate cell proliferation and motility, important for invasion and metastasis, and confer also resistance to apoptosis and cytostatic drugs used in the treatment of cancers [12]. The signals are mostly dependent on hyaluronan receptors, such as CD44 and RHAMM [13]. Especially interesting are the *in vitro* observations that hyaluronan binding to CD44 can increase the activity of many growth

factor receptors [14]. Cell culture models have also demonstrated that endogenously produced hyaluronan by overexpression of HAS2 triggers epithelial to mesenchymal transition (EMT) [15, 16], and provides the cancer with stem cell properties [11, 17], both inherent features of malignant tumors.

The above results from cell cultures and experimental animals are generally in line with the currently available data on human tumors. A high level of hyaluronan in the tumor stroma or cancer cells is a strong indicator of unfavorable outcome in breast [18] (Figs. 1. and 2.), ovarian [19], prostate [20], colon [21], and gastric cancers [22], and lung adenocarcinomas [23]. Accordingly, the first clinical trials suggest that removing tumor hyaluronan by adding recombinant hyaluronidase to the standard chemotherapy regimen improves the treatment response in the hyaluronan-rich pancreatic carcinoma [24, 25].

Cancers arising from stratified epithelium (squamocellular carcinomas) have shown a two-phase change in hyaluronan and CD44 abundance, first a rise, followed by a patchy decline, when tumors progress into more malignant forms (with more unfavorable prognosis). This takes place in cancers of skin epidermis [26], head and neck cancers [27], and also in melanoma [28].

While signals derived from hyaluronan and their functional effects are difficult to study in patients *in vivo*, correlative data from human tissue analyses are mostly in agreement with the *in vitro* and animal data. In the following discussions on recent findings in experimental models and clinical assays we aim at inducing views and ideas on the potential of hyaluronan in diagnostic and therapeutic applications, complementing the excellent reviews on general regulation of hyaluronan synthesis [29] and the hyaluronan-CD44 signaling in cancer [30].

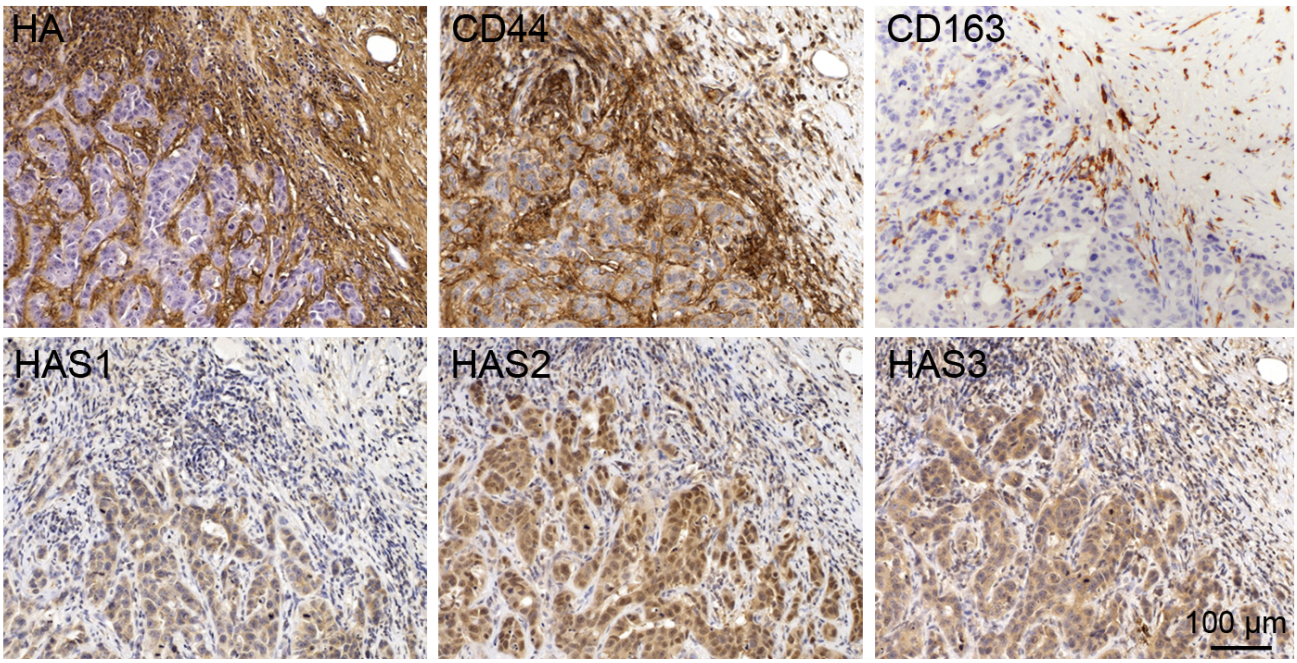

Fig. 1. Hyaluronan (HA), HAS1-3, CD44, and M2 polarized (CD163-positive) macrophages at the edge of a malignant human breast tumor. An unfavorable prognosis of the disease is indicated by strong signals in the tumor stroma for hyaluronan, HAS1, HAS2, and HAS3, CD44, and CD163. Strong hyaluronan and HAS1 signals indicate a bleak outcome also when they are seen in cancer cells.

Pro-cancer signals triggered by hyaluronan

As a resilient gel, hyaluronan allows the cells to move, change their shape, and divide, important functions for the invasive growth. The pericellular hyaluronan matrix also buffers the cells from physical, chemical and biological insults such as cytotoxic lymphocytes and natural killer (NK) cells [31, 32], and therapeutic antibodies targeted to cancer cells [32].

The importance of hyaluronan in the formation of metastases by circulating cancer cells was demonstrated by studies in which the hepatic HARE/Stabilin2 receptor for hyaluronan uptake and degradation was blocked. Preventing hyaluronan uptake dramatically increased its concentration in circulation. The free hyaluronan obviously competed for the hyaluronan on endothelial and/or circulating cancer cells, and thus completely prevented their receptor-mediated adhesion and extravasation [33, 34].

In addition to helping tumor cell entry through endothelium into metastatic sites, hyaluronan, especially when fragmented, supports endothelial budding and capillary formation in 3D matrices [35]. This has been confirmed *in vivo* by overexpression of *HAS2* in stromal fibroblasts, which increases breast tumor angiogenesis and lymphangiogenesis, and also facilitates recruitment of the macrophages that support tumor growth [36].

Epithelial to mesenchymal transition and cancer stem cells - Overexpression of HAS2 induces epithelial to mesenchymal transition (EMT), even in normal mammary epithelial cell lines [11, 15-17, 37]. The molecular mechanisms mediating the EMT-triggering signal from HAS2 are not fully understood, but dependence on type 1 TGFβ receptor, Smad, and p38 MAPK has been shown [16]. Intriguingly, blocking CD44, or treatment with hyaluronidase, did not prevent the induction of EMT, suggesting that HAS2 protein itself is important [16]. The importance of hyaluronan in EMT is also supported by a similar process in the healing of scratch wounds in mesothelial cultures, which involves induction of hyaluronan synthesis, CD44 expression, and secretion of microvesicles carrying these molecules [38].

In established tumors EMT facilitates infiltration into adjacent tissues and favors metastasis, while in the initial phases of oncogenesis it allows cell division beyond the limits of the normal epithelial monolayer. The latter is demonstrated in Madin-Darby Canine Kidney MDCK epithelial cells, which spontaneously form hollow cysts lined by a single layer of epithelial cells when grown in a 3-D matrix. Overexpression of HAS3 in these cells loosens the adhesion between cells, and disturbs the orientation of their mitoses, normally perpendicular to the basal lamina [39]. This leads to uncontrolled proliferation with multiple cell layers and intraluminal invasion of the cells [39], a process that can facilitate the development of a malignancy.

It has turned out that the maintenance of many tumor types, and especially their recovery from treatments targeting the dividing cell population, is due to a subset of tumor cells with stem cell properties. The expression of CD44 and the synthesis of hyaluronan are highest in the primary murine breast cancer cells with stem cell characteristics [17]. This is consistent with the notion that a stem cell niche is often enriched in hyaluronan [40, 41], and that bone marrow mesenchymal stem cells themselves are highly active in hyaluronan synthesis [42]. Thus, hyaluronan is associated with EMT and stem cell properties, both crucial for the progression of malignancies.

CD44-hyaluronan signaling in cancer - CD44 is widely expressed in the tumor cells of breast carcinoma. However, it is in the stroma where differences in CD44 expression correlate with HER2 positivity, and confer an elevated risk of unfavorable outcome [43], a finding perhaps related to the role of stromal macrophages as tumor supporters [44] (Fig. 2). Recent research has discovered specific signals originating from hyaluronan, signals that control the phenotype of the cells, as described above (and reviewed by [45]). The hyaluronan-triggered pro-cancer signals are often mediated by CD44, which associates with, and activates several kinase-associated growth factor

receptors, such as c-met, VEGFR2, PDGF, IGF1R, ErbB family and TGFβ [14, 46-48]. The intracellular signaling pathways thus activated include especially the PI3K-Akt axis, and also Src/Fak, Erk, and Smad (see review by [13]. In addition, CD44 associates with G-protein coupled receptors such as CXCR4, then activating NFkB [35], and with LRP6 in the Wnt signaling system, leading to β-catenin activation [49]. All these signals are known to promote the type of cell behavior required for malignant growth.

CD44 exists as multiple splice variants that differ in the length of the extracellular stem that holds the hyaluronan binding link domain in the N-terminus. The pro-cancer functions of CD44 can be limited to those with specific variant domains, especially v6-v10 [48, 50], while in some cases the standard form of CD44 is important for tumor progression [51].

In molecular terms, however, details of the initiation of the signals from hyaluronan interactions with its receptors are still obscure. Theoretically, a signal can be triggered by clustering or de-clustering of the receptors, changed interactions with cytoplasmic kinases, binding to the cytoskeleton, or reorganization of the cytoskeleton just due to changed adhesion environment in the hyaluronan-enriched matrix.

One of the most puzzling issues that have emerged from the research on CD44 is the huge number of proteins reported to bind and interact with CD44. These include those embedded in plasma membrane, as exemplified above, and numerous cytoskeletal [52] and cytoplasmic proteins [53], as well as extracellular proteins such as proteinases and their activators [54]. Indeed, CD44 appears to be a common signaling platform [55]. A good molecular level explanation for the platform function of CD44 is the recent finding that CD44 bound to cytoskeleton forms “corrals” that limit the free diffusion of membrane proteins [56]. Hyaluronan bound to these CD44 corrals further enhances their barrier function [56]. Limiting the diffusion of proteins susceptible to this catch can dramatically increase or decrease the activities of these proteins.

RHAMM and layilin - While the level of CD44, like that of hyaluronan, is increased in several types of malignant tumors, in some cancers its quantity is not a consistent indicator of unfavorable prognosis [57-60]. In contrast, the reports published so far suggest that elevated expression of the receptor for hyaluronan-mediated motility (RHAMM/HMMR) [45] is associated with bleak prognosis at least in breast [61], lung [62], colon [63] and bladder cancers [64]. As its name implies, it enhances the mobility of cancer cells, but has also other, intracellular functions related to microtubules and cell division [65]. Interestingly, its hyaluronan binding and signaling appears to take place through close interaction with CD44 [66].

Layilin, another cell surface hyaluronan receptor, is usually coupled to cytoskeletal elements as does CD44 [67]. Layilin has been little studied, but its expression is specifically increased in the activated Treg and CD8+ lymphocytes of human lung, colorectal and hepatocellular carcinomas. Layilin suppresses the functions of the CD8+ cells, and brings an unfavorable prognosis [68, 69]. This is interesting considering the recent attempts to uncover the nature of immunosuppression and the high level of hyaluronan in the tumor environment. Silencing of layilin inhibits lymph node metastasis in a lung cancer cell line inoculated subcutaneously [70]. Silencing of layilin also prevents EMT in kidney epithelial cells [71].

In summary, several hyaluronan receptors have turned out to be involved in the progression of cancers, suggesting that many different signals arising from hyaluronan contribute to the growth of malignancies. Importantly, EMT and stemness, the phenotypic cell changes that favor cancer spreading and survival, associate with the level of hyaluronan and its receptors.

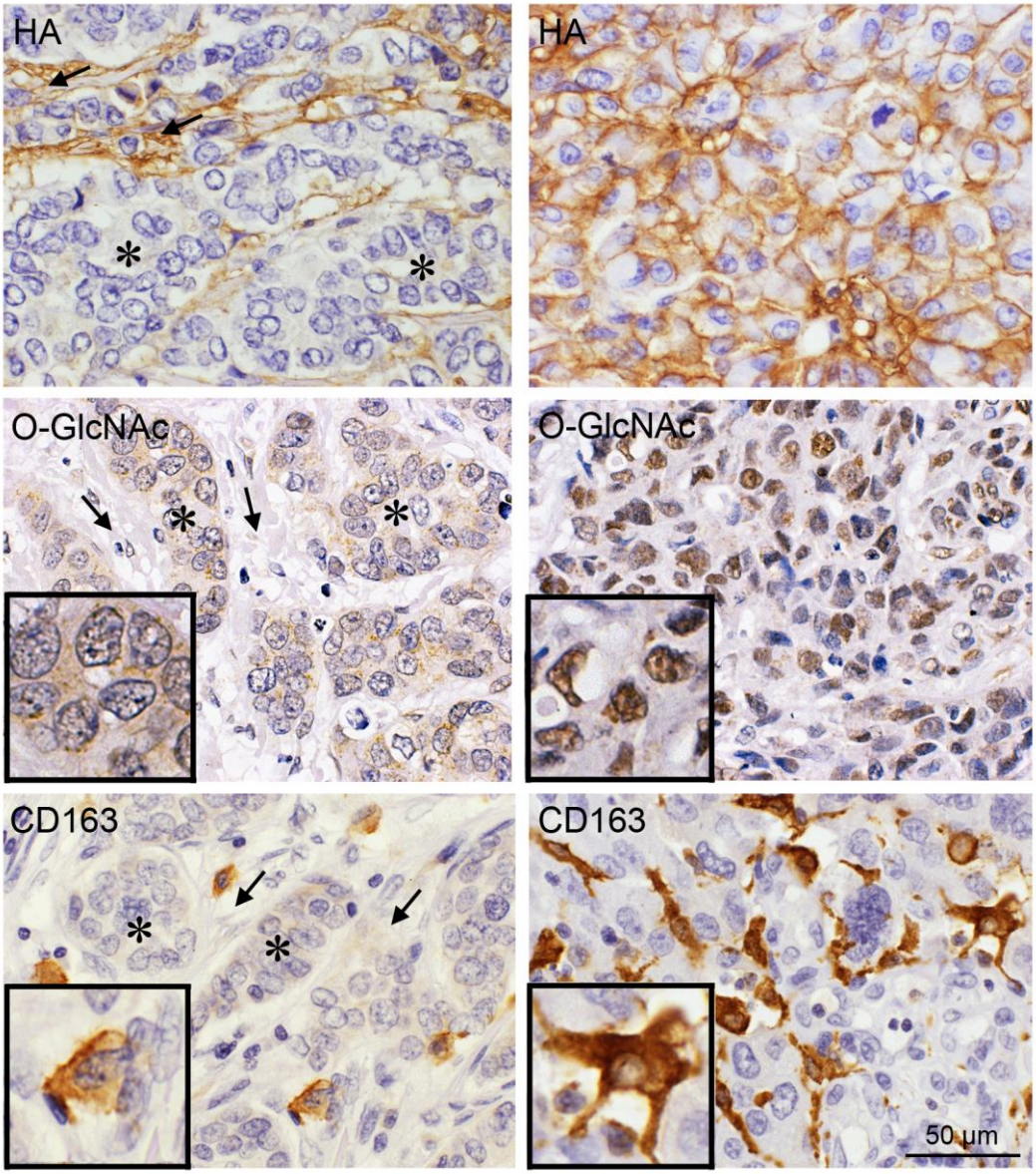

Fig. 2. Localization of hyaluronan, O-GlcNAcylated proteins, and M2-polarized (CD163-positive) macrophages in human breast cancer. Upper panels: On the left, hyaluronan is localized in the strands of stroma, while on the right it is also associated to cancer cells, mostly to their surface. Middle: On the left, the O-GlcNAc signal is limited to the cytoplasm of the cancer cells, on the right it is predominantly in the nucleus. Intense signal in either location indicates a high risk of recurrence and death. Lower panels: Different levels of M2 type macrophages surrounding the tumor cells. The abundance of CD163 positive cells in the right panel indicates poor prognosis. Parts of the O-GlcNAc and CD163 images are shown enlarged in the insets. The breast cancer subtypes in the images are as follows: HA (left), lobular, grade 3, ER+, PR+, HER2+; HA (right), ductal, partly mucinotic, ER+, PR+, HER2+; O-GlcNAc (left), ductal, grade 3, ER+/-, PR+/-, HER2+; O-GlcNAc (right), ductal, grade 3, ER-,PR-, HER2+; CD163 (left) ductal, grade 3, ER-, PR-, HER2+; CD163 (right), squamocellular, grade 3, ER-, PR-.

Activation of hyaluronan synthesis in malignant tumors

While the increase in hyaluronan and its effect as a cancer-promoting factor has been well established in many malignant tumors, it is curious that no consistent upregulation of the *HAS* gene

expression has been found in the numerous genome wide expression analyses on cancers, available in the Oncomine database (Oncomine Research Edition, https://www.oncomine.org). Among 412 reports on *HAS2* mRNA only 25 show a significant increase while at the same time there are 12 reports stating the opposite. The same discrepancy holds for *HAS1* and *HAS3* expression, the majority of reports state decreased mRNA of these genes. For *HAS1*, of 389 reports 3 indicate upregulation, and 15 downregulation. Of the 289 reports for Has3 17 show increased and 34 decreased expression. The logical explanation is that hyaluronan synthesis is stimulated at post-transcriptional level. Of course, this does not exclude *HAS* gene upregulation in individual tumors, known for their genomic heterogeneity.

Indeed, evidence for post-translational upregulation of HAS enzyme activity has been obtained recently by demonstration of increased immunohistochemical signals for HAS enzymes in breast [72], ovarian [73] and endometrial cancers [74], suggesting stabilization of the synthase proteins (Fig. 1). Two specific signals for the stabilization and activation have been described. First, O-GlcNAc moieties coupled to certain serine/threonine residues strongly increase the lifetime of HAS2 and HAS3 in cultured cells [75, 76]. Ubiquitination is another post-transcriptional signaling system that contributes to HAS2 enzyme activity and lifetime [77, 78]. HAS2 enzymes are also phosphorylated, the effect on function depending on the site of the phosphorylation [79-81].

Substrate availability – In addition to the posttranslational modifications of the HAS enzymes, the rate of hyaluronan synthesis is influenced by the supply of substrates, namely UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP- glucuronic acid (UDP-GlcUA) [75, 82, 83, 84]. derived from the glycolysis intermediates fructose-6-phosphate and glucose-6-phosphate, respectively. The high glucose uptake in cancers increases these glycolysis intermediates, their flux into biosynthetic pathways producing the UDP-sugars [85], and eventually hyaluronan (Fig. 3). Indeed, an experimental increase of the glycolysis intermediates is sufficient to promote the synthesis of hyaluronan and growth of cancer in a mouse model [86].

In addition, elevated expression of enzymes in the hexosamine biosynthesis pathway contributes to the enhanced UDP-GlcNAc synthesis in cancers. Expression of GFAT1, a key enzyme regulating the flux through hexosamine biosynthesis, is increased in pancreatic cancer. Furthermore, elevated GFAT1 levels associate with several clinical parameters, including reduced survival [87]. Increased GFAT1 levels predicts poor prognosis also in breast cancer [88], hepatocellular carcinoma [89] and colon cancer [90]. The immunohistochemical signal of GFAT1 protein correlates with that of hyaluronan through different stages of human skin melanomas [75].

We have recently shown elevated expression of GFAT1 and GFAT2 in human breast cancer biopsies, and a 14-fold increase in UDP-GlcNAc as compared with normal breast epithelium. The content of UDP-GlcUA was 4-fold higher, and both UDP-sugars correlated with that of hyaluronan [91]. Since *HAS1-3* mRNA levels in the same biopsies were generally lower than in controls, these findings provide strong evidence for the importance of UDP-sugar supply in tumor hyaluronan synthesis [91].

HAS enzymes are normally active only when transported to plasma membrane [92]. Therefore, intracellular trafficking of the HAS enzymes is an important factor in hyaluronan synthesis regulation [93], and this trafficking is also controlled by UDP-sugar contents [75]. Elevated UDP-GlcNAc content enhances HAS3 plasma membrane residence, and hence increases the activity of the enzyme. In addition, substrate availability stabilizes the enzyme [75].

O-GlcNAc modification - O-GlcNAc modification of certain serine or threonine residues of intracellular proteins controls the activities of enzymes and transcription factors in a plethora of

cellular processes, including those considered as hallmarks of cancer [94, 95]. Changes in global O-GlcNAc modification levels has been reported in many cancers, including those of the breast [44], stomach [96], colon [97], and prostate [98] (Fig. 2.).

Elevated cellular contents of UDP-GlcNAc results in a general increase in the level of O-GlcNAc substitutions. Indeed, both very high UDP-GlcNAc supply and O-GlcNAcylation have been found in human breast cancer using biochemical assays and immunohistochemistry, respectively [44, 91]. The increase in O-GlcNAcylation correlates with HAS enzyme levels, hyaluronan content, and unfavorable prognosis in breast cancer [44]. Indeed, the elevated UDP-GlcNAc content is probably behind the HAS protein increase observed in human breast cancer since *in vitro* studies have shown that the resulting O-GlcNAcylation increases the lifetime of HAS2 and HAS3, their plasma membrane residence, and activity [75, 76].

It has been noted that O-GlcNAc modification can also influence HAS2 gene expression through SP1 and YY1 transcription factors [99] and the natural HAS2-antisense transcript [100].

Ubiquitination - Deregulated ubiquitination is a frequent feature of cancers [101]. The HAS2 enzyme can have both mono- and polyubiquitination. Monoubiquitination of Lys190 is required for the enzymatic activity of HAS2, and replacement of Lys190 with Arg blocks the activity. This mutant also blocks the activity of wild type HAS in a dominant negative manner since the HAS enzymes act as homo- and heteromers [77, 102].

On the other hand, Lys63 or Lys48 polyubiquitination shortens HAS2 lifetime, and hence inhibits the synthesis of hyaluronan [78]. Removal of the HAS2 polyubiquitin chains by the USB17 deubiquitinase stabilizes the enzyme. Supporting the importance of HAS2 ubiquitination in cancer, immunohistochemical signals between USB17, HAS2 and hyaluronan showed positive correlations in lung carcinomas [78].

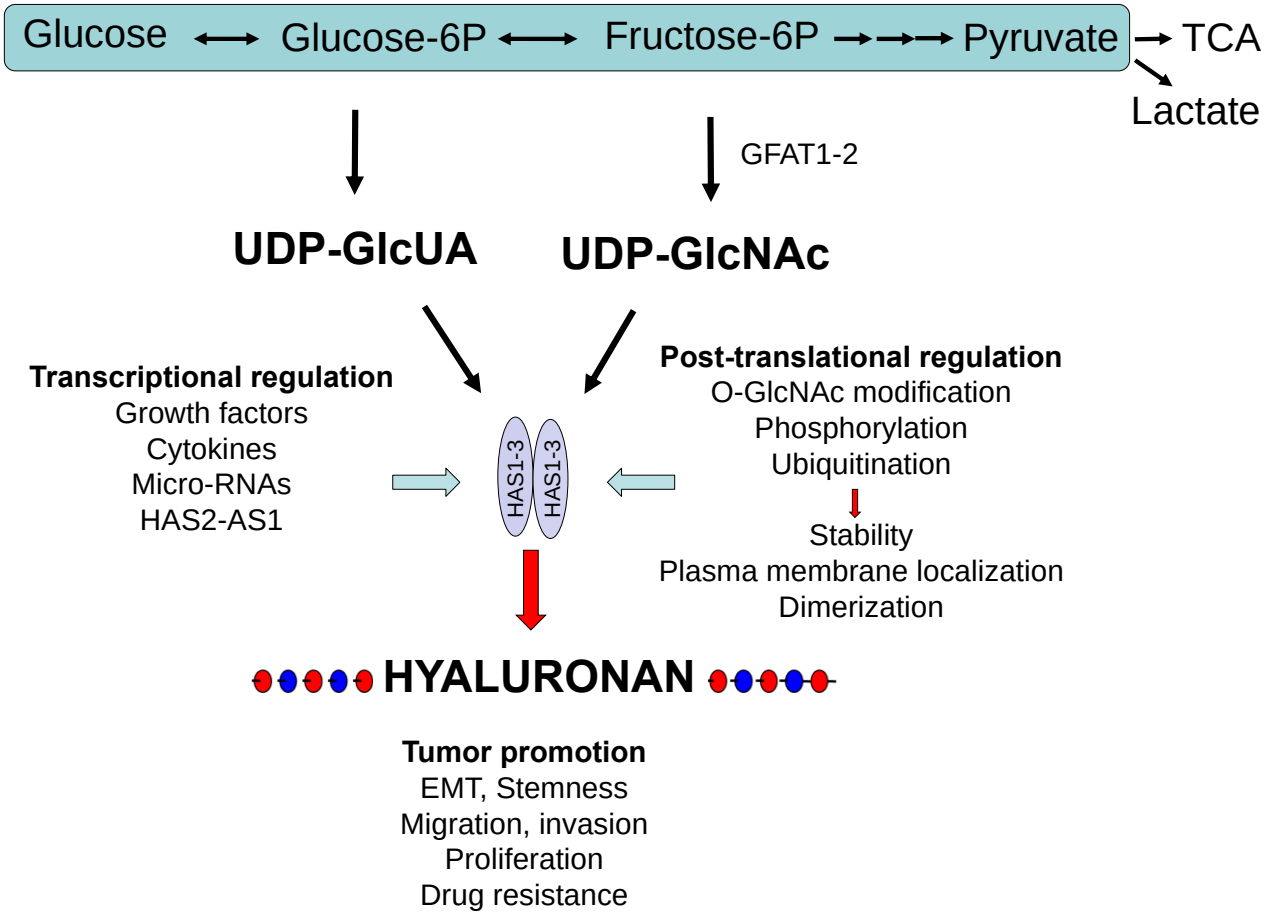

Fig. 3. Glucose metabolism and hyaluronan synthesis in cancer. Malignant tumors show high glucose uptake. Since the flux of glucose to oxidative phosphorylation in tricarboxylic acid cycle (TCA) is partially blocked, glycolysis intermediates shown in the top row accumulate in the cells (Warburg effect). These metabolites constitute a source for several components essential in rapid cell proliferation, including the UDP-sugars. UDP-sugars increase hyaluronan synthesis as substrates, and by stimulating O-GlcNAc modification of HAS2 and HAS3. This modification increases the lifetime of the enzymes, and their trafficking to plasma membrane, where they are activated. The cellular content of UDP-GlcNAc and O-GlcNAc modification also control HAS2 transcription.

Other factors influencing hyaluronan production – In addition to O-GlcNAc modification and ubiquitination, HAS enzymes are activated by protein kinase C (PKC), but the activation might be indirect [81], while the adenosine monophosphate-activated protein kinase (AMPK) seems to directly catalyze the phosphorylation of HAS2, and downregulate the activity [80]. All these post-translational modifications can be associated with other factors controlling HAS activity, such as their dimerization, trafficking, and residence time on plasma membrane [93, 102].

Although there is no consensus in the databases on cancer-associated changes of *HAS* expression, it does not rule out their importance in specific cases. A large number of hormones, growth factors, cytokines and other local factors present in tumors can influence *HAS* mRNA levels. These include platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF2), keratinocyte growth factor (KGF), epidermal growth factor (EGF), transforming growth factor-β (TGFβ), interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), interferon-c, prostaglandins,

corticosteroids and retinoids [103]. In addition, UDP-Glucose, UTP, ATP and their degradation products released from stressed cells can cause a transient *HAS* gene upregulation [104-106]. The effect on hyaluronan production depends on the *HAS* isoform and cell type.

Of the *HAS* isoenzymes, the promoter region of *HAS2* is the most investigated one. It has binding sites for the transcription factors CREB1, retinoic acid receptor (RAR), SP1, STAT1 and YY1 [103]. In addition, at least the FOXOs transcription factor can regulate HAS2 expression [107]. The promoter region of *HAS3* contains binding sites for at least CEBP, SP1 and NFκB [108] and that of *HAS1* is regulated by transcription factors such as Smad3 and SP3 [109]. In squamous cell head and neck cancer, the transcription factor ΔNp63 coordinates the expression of an interesting set of genes: HAS3, HYAL-1, HYAL-3 and CD44 [110]. Besides transcription factors, *HAS2* mRNA levels can be regulated by the presence of a natural antisense transcript (*HAS2-AS1*) [100] and microRNAs [111].

In conclusion, the high level of hyaluronan synthesis in malignant tumors is due to signals for posttranslational activation of the HAS enzymes, metabolic features providing ample precursors for the HAS, and transcriptional programs that maintain the tumor-type metabolism and enzymes supporting HAS activity.

Hyaluronidases and hyaluronan fragments in cancer

Recent years have brought up increasing evidence that the degradation of hyaluronan, has a role in inflammation, including that in cancer. Much hype was created a few years ago on the cancer resistance of naked mole rat, suggested to be the result of exceptionally large hyaluronan, produced by HAS2 [112]. Later research has shown that the size of hyaluronan made by naked mole rat HAS2 is not different from that of other animals. It seems more likely that the naked mole rat somehow avoids the kind of hyaluronan fragmentation that promotes inflammation and malignant growth.

Hyaluronan degradation can occur via enzymatic, or non-enzymatic, ROS-dependent processes. There were traditionally four hyaluronidase genes in humans, of which two (*HYAL1* and *2*) are ubiquitously expressed in most tissues, and their possible contribution to cancer have been studied extensively. Recently, two other proteins, CEMIP and TMEM2, with hyaluronan degrading activity have been discovered, and both are associated with malignancies, as discussed below.

Hyaluronidase 1 and 2 (HYAL1 and 2) – HYAL2, GPI-anchored to plasma membrane, is thought to digest hyaluronan into shorter fragments, which are internalized and catabolized in lysosomes by HYAL1 into oligosaccharides, and eventually into monosaccharides by exoenzymes [113, 114]. The expression *HYAL1* is elevated in bladder and prostate cancers and is a good prognostic marker in both [115, 116]. As compared to benign nevi, *HYAL2* expression is increased in dysplastic nevi, local and invasive cutaneous melanoma, correlating with gradually decreasing hyaluronan contents [28]. *HYAL1* and *HYAL2* decrease in ovarian [73] and endometrial cancers [117] and brings an unfavourable prognosis in the latter. A similar finding was made in pancreatic cancer, in which low HYAL1 expression correlates with poor survival [118].

CEMIP/KIAA1199 and TMEM2 - As indicated above, variable expression levels *HYAL1* and *HYAL2* have been associated in different cancers, yet the turnover of hyaluronan and the formation of HA fragments is probably accelerated in most cancers. The apparent discrepancies may be explained by the recent discovery of two novel proteins involved in hyaluronan degradation. One of them, CEMIP (KIIAA1199) causes cell-associated partial depolymerization of hyaluronan into fragments released back in the culture medium [119]. It has multiple biological functions that favor malignant growth, mostly overlapping with those of hyaluronan, such as angiogenesis, proliferation, motility,

invasion, and EMT [120]. CEMIP is overexpressed in several cancers and associates with poor prognosis in pancreatic [121], lung [122], colon [123] and gastric cancers [124], and likely also oral squamous cell carcinoma [125].

While HYAL1 and HYAL2 degrade hyaluronan only in low pH, the pH optimum of TMEM2, a recently discovered hyaluronidase, is between pH 6 and 7. TMEM2 is located on cell surface and degrades extracellular hyaluronan in a Ca^{2+}-dependent manner into intermediate-sized fragments which can be internalized and completely degraded in lysosomes [126]. Thus, CEMIP and TMEM2 could aid in matrix penetration at the invasive front of tumors. Very little is currently known about the biology of TMEM2, but a report available shows that its expression is an indicator of poor prognosis in breast cancer [127]. Schematic presentation of some of the players in the metabolism of matrix hyaluronan is shown in Fig. 4.

Hyaluronan fragments and inflammation in cancer - Fragmented hyaluronan (<200 kDa) can be created in tissues by the enzymes discussed above, but also by reactive oxygen species (ROS). Interestingly, below 200 kDa hyaluronan adopts a conformation with a rod-like rather than random coil structure [128], which could explain the differences in the signaling. For example, competition by small fragments can prevent cell surface receptor binding of native, high molecular mass hyaluronan and destroy the coat. Fragments can change the signals for example by preventing receptor clustering, dependent on native hyaluronan [129].

In experimental settings, hyaluronan fragments have been reported to trigger an inflammatory response, including activation of the TLR2/4 receptors. Unfortunately, hyaluronan and hyaluronidases used in preparing the fragments are often contaminated by LPS and other bioactive compounds potentially involved in the response. Indeed, highly purified hyaluronidase or hyaluronan fragments neither bind nor activate TLR2 or TLR4 or induce a cytokine response in macrophages or dendritic cells [130, 131]. Therefore, a part of the experiments published on the inflammatory effects of hyaluronan fragments may have to be re-evaluated.

Methodology to assay hyaluronan molecular mass in small tissue samples has just become available [132] but, as far as we know, this has not been applied to tumor tissues. The facile diffusion of small hyaluronan fragments in the extracellular space gives rise to a fast turnover, and hence a low tissue concentration. In plasma, however, elevated levels of hyaluronan fragments have been found in metastasized breast cancer [133].

Macrophages polarized into the alternatively activated, immunosuppressive types (M2) have a key role in maintaining the microenvironment that supports malignant growth. This is done by secretion of growth factors, anti-inflammatory cytokines, and inhibiting the cytotoxic CD8+ T-cells via programmed cell death protein (PD-1).

In breast cancer, the number of M2 type macrophages correlates with high tumor hyaluronan content and poor prognosis [134, 135]. This is consistent with the finding that overexpression of *HAS2* in tumor fibroblasts increases macrophages in mouse breast cancer [36]. Moreover, culture media of breast cancer cell lines with active hyaluronan synthesis convert peripheral blood monocytes towards type M2 macrophages, while inhibition of hyaluronan synthesis by 4-methylumbelliferone, or *HAS2* siRNA, or blocking CD44 with antibodies, prevents the phenotype shift. This M2 conversion is thought to be due to hyaluronan fragments [134, 136]. There are also studies showing that hyaluronan fragments polarize macrophages to M1 phenotype [137]. The conflicting results on M1 vs. M2 polarization are likely accounted by different experimental models and perhaps contaminants in the hyaluronan preparations.

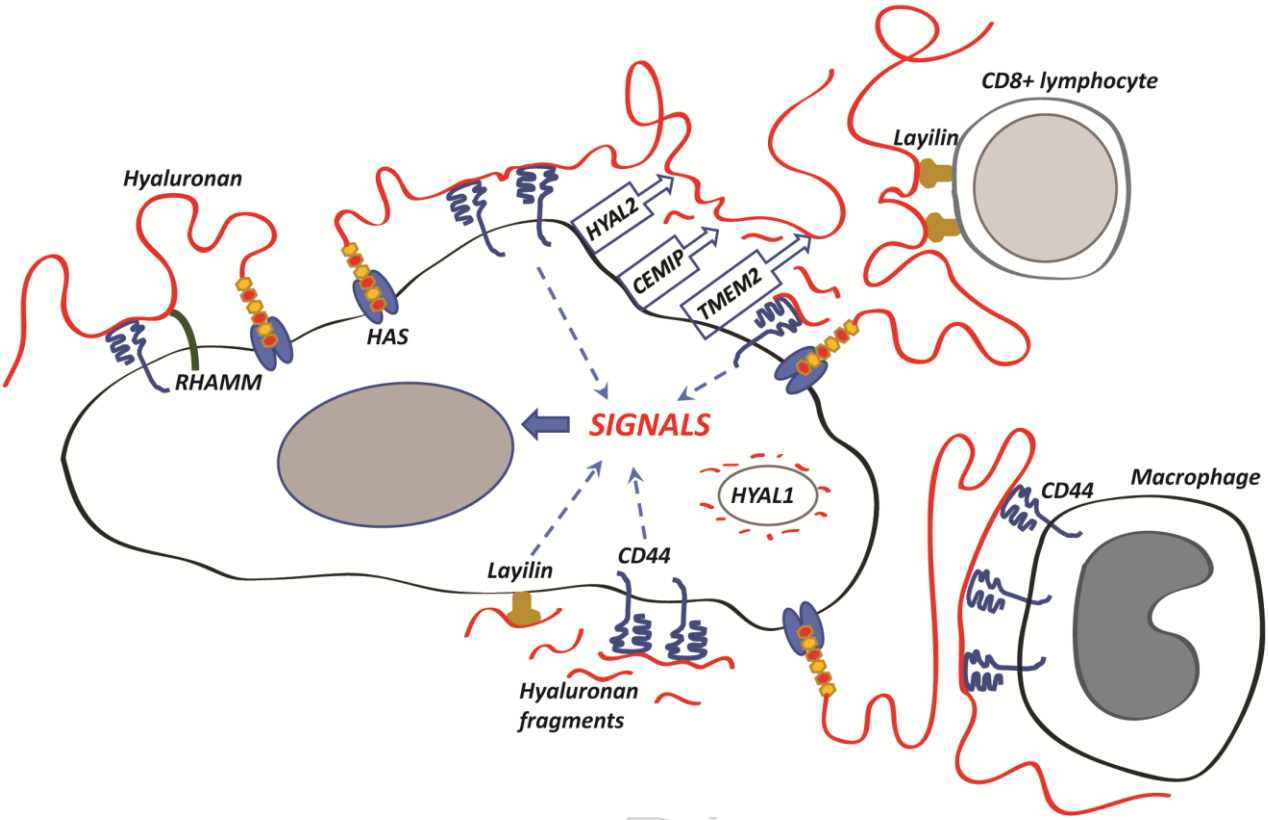

Fig. 4. Major players in the signalling and catabolism of hyaluronan in tumor matrix. The receptors CD44 and RHAMM in the tumor mediate the signals from hyaluronan of different sizes, created by the catabolic activities of HYAL2, CEMIP, and TMEM2. The signals help the tumor in several aspects as listed in Fig. 3. Macrophages are recruited into the hyaluronan-rich matrix and polarize into type M2, creating an anabolic effect and immunotolerance for the tumor. Layilin on CD8+ lymphocytes suppresses their cytotoxic activities, contributing to the immunotolerance.

Hyaluronan fragmentation and angiogenesis - In a hypoxic tumor microenvironment, macrophages release pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor (PDGF), which are essential for neovascularization [138]. In addition, numerous reports have suggested that hyaluronan fragments and oligosaccharides (< 10kDa) induce angiogenesis [139] by a direct impact on endothelial cells through CD44-mediated signals involving ERK, Src, and TGFβ1 [140] or CD44 associated with TLR4, resulting in NFkβ-signaling [138, 139] . Hyaluronan of 4-10 disaccharide units in length also stimulates VEGF secretion by endothelial cells [141]. The tube formation of endothelial cells depends on the expression of HYAL2 and CD44 [35], suggesting they represent the endogenous hyaluronan fragmentation system, and its signaling platform, respectively. This is an important finding considering the doubts of the purity of the exogenously added hyaluronan fragments. Thus, hyaluronan oligosaccharides represent a signal to directly accelerate angiogenesis, or induce the expression of angiogenic growth factors.

In conclusion, it has turned out that both the synthesis machinery and the catabolic systems that degrade hyaluronan are equally important in providing the pro-cancer matrix properties. This implies that interference of not just synthesis, but also some critical factors in catabolism have potential as drugs against malignant tumors.

Shielding against apoptosis

Among the numerous mechanisms malignant cells utilize to survive, synthesis of hyaluronan has been noted in several papers. The same metabolic features that induce hyaluronan synthesis can work against apoptosis. For example, activation of the cyclin D3-CDK6 axis suppresses pyruvate kinase M2 and phosphofructokinase. This leads to flooding of the upstream pool of glycolytic intermediates, resulting in enhanced flow into the pentose phosphate pathway that provides reducing equivalents against the toxic reactive oxygen species (ROS) [142]. At the same time, more glycolytic intermediates flow also into UDP-GlcNAc, which increases hyaluronan synthesis [84, 143], and the O-GlcNAc modification of intracellular proteins. O-GlcNAcylation increases the activity of hundreds of proteins involved in cell survival [144], including the HAS enzymes [75, 100].

Oxidative stress, ROS – Hyaluronan itself, as a coat on cell surface, is important by scavenging toxic ROS such as $^{\cdot}OH$ and $ONOO^{-}$, or inhibiting their formation [145-147]. While scavenging ROS, hyaluronan is fragmented [147], and the fragments form a signal that sustains inflammation. Thus, while protecting cells from the ROS-induced immediate injury to DNA and lipids, hyaluronan fragmentation still tends to maintain the vicious cycle of inflammation [148].

In breast cancer, disappearance of hyaluronan in some of the cancer cells correlates with high levels of nitrotyrosine, a marker of $ONOO^{-}$ formation, suggesting ROS-induced hyaluronan fragmentation into fragments too small for histological detection [149]. Exposure to the UV-radiation-induced ROS increases epidermal hyaluronan synthesis [106], while scavengers of ROS decrease the turnover of hyaluronan in epidermis [150, 151]. Hyaluronan metabolism is thus closely entwined with that of ROS.

CD44 influences the redox balance in cancer cells, reviewed in [152]. For example, in colorectal cancer cells CD44 interacts with, and activates pyruvate kinase M2 and glycolysis, thereby reducing the mitochondrial ROS production in p53 deficient cells [153]. Knockdown of CD44 in p53 deficient cells, or p53 wild type cells exposed to hypoxia, returned their metabolism towards mitochondria, resulting in increased ROS production, decreased level of the antioxidant GSH, and higher sensitivity to cytostatic drugs [153]. In addition, CD44v8-10 stabilizes the cystine transporter xCT on plasma membrane, increases GSH, depletes ROS, and facilitates the growth of gastric cancer [154]. In head and neck cancer cells, inhibition of xCT leads to apoptosis in CD44v8-10_{high} but not in CD44v8-10_{low} cells [50]. In the above studies the role of hyaluronan was not studied, but in an *in vivo* model of glioblastoma CD44-mediated protection against free radicals and cytotoxic drugs was largely dependent on hyaluronan [155].

AKT and prosurvival signals – Many of the cancer-associated cell growth and survival signals involve AKT, which is therefore an attempted target of drug development. Increased expression of hyaluronan leads to the association of CD44 with ErbB2 in a multiprotein complex, including e.g. PI3K, ezrin, HSP90 and cdc37, and resulting in the activation of the AKT cell survival pathway [47, 156]. Hyaluronan synthesis by HAS2 together with CD44 maintains an ErbB2-PI3K/Akt-β-catenin signaling pathway. In colon cancer cells, this activates COX2 and increases cell survival and proliferation [157], while suppression of CD44 downregulates the pro-survival factors Bcl-2 and Bcl-xL, and upregulates BAX and active caspases [158]. MAP-kinases and AKT are also involved in the CD44-dependent survival of lymphatic leukemia [159]. A recent study on breast cancer cells [160] confirmed the previously established positive feedback loop in which hyaluronan binding to CD44 activates PI3K/Akt and maintains hyaluronan synthesis at high level while protecting the cells against apoptosis [156]. Interference of either hyaluronan production or CD44 function sensitizes the cells to cytostatic drugs and apoptosis [156, 160]. Likewise, *Has2* expression and hyaluronan interaction with CD44 maintains viability, while suppression of *Has2* expression by miR26b induces caspase-3 and apoptosis [111]. Hyaluronan interaction with CD44s, but not with CD44E, prevents

anoikis and supports survival of human immortalized breast cells which had undergone EMT [51]. Disruption of the hyaluronan-CD44 complex by reduced expression of either of them, by soluble CD44, by CD44 antibodies, or by short hyaluronan oligosaccharides, have been shown to inhibit the signaling and growth of many other cancer cell types, both *in vitro* and *in vivo* [161].

Multidrug resistance - The finding in cell cultures that induction of hyaluronan synthesis enhances multidrug resistance both through AKT activation [12] and induction of the multidrug transporters MDR1 and MRP2 [156, 162] has considerable clinical potential. Hyaluronan-CD44 interaction has also been reported to augment the expression and activity of other multidrug resistance proteins such as MDR2 in lung cancer cells [163], BRCP/ABCG2 in glioma cells [164], and ABCC1, 2, 3 and ABCB3 in ovarian cancer cells [165]. Upregulation of MDR1 has been suggested to involve PI3K [156], and specific miRNAs [166]. Interestingly, the drug resistance that develops in ovarian carcinoma cells following carboplatin treatment is due to increased expression of CD44, HAS2, HAS3, and secretion of hyaluronan [165]. Moreover, the increased plasma hyaluronan content in patients receiving cytostatic drugs for ovarian cancer suggests that the same mechanism for drug resistance takes place *in vivo* [165]. Synthesis of hyaluronan, and its interaction with CD44 and RHAMM, is important for drug resistance even in leukemia cells, in which the resistance can be cancelled with hyaluronan oligosaccharides [167].

In addition to the transcriptional regulation of the drug transporters, hyaluronan-CD44 interaction may help to retain the drug transporters in the plasma membrane, thereby enhancing their activity [168, 169], perhaps by restricting endocytosis [56].

Potential for therapeutic applications

More than a hundred papers have been published during the last 2 years describing the preparation of hyaluronan-coated drugs vehicles for specific targeting to malignant tumors. The tumor homing of these nanoparticles is presumably based on CD44 receptors in the endothelial, stromal, and actual tumor cells. As far as we know, clinical applications of these drug carriers have not emerged, yet. Interestingly, hyaluronan-coated microvesicles are also released naturally from cells with active hyaluronan synthesis [170], such as mesenchymal stem cells [171] and could have a role in cancer [172].

CD44 is a promising drug target. Especially silencing variant isoforms such as CD44v6 should block tumor growth due to the numerous services they provide to malignant growth. Indeed, that has been shown for example in a mouse colon cancer model [152, 173], see review by Misra et al. [174].

Studies on experimental animals [175] and early clinical studies [24] indicate that intravenous infusion of recombinant human hyaluronidase depletes hyaluronan and reduces the interstitial pressure of hyaluronan-rich tumors like pancreatic carcinoma. Hyaluronan depletion increases the cytostatic drug access to the tumor, resulting in significantly longer survival [24]. Even more improvement of penetration could be expected from hyaluronidase combination with larger molecules, such as antibodies s [32].

Inhibition of hyaluronan synthesis by 4-methylumbelliferone, a coumarin compound that suppresses the expression of *HAS2* and *HAS3*, and depletes the HAS substrate UDP-glucuronic acid (UDP-GlcNAc) [176], strongly retards the progression of many cancer types in experimental animals [10, 177-182]. This drug has been approved in many countries for treating biliary problems [183]. Despite the previous clinical use, and the many successful animal experiments, clinical trials for cancer with this drug have not been reported.

Depletion of UDP-GlcNAc, the other HA precursor and stabilizer of HAS proteins, would also inhibit hyaluronan synthesis. In cell cultures, mannose reduces the cellular UDP-GlcNAc and

suppresses hyaluronan synthesis [139]. *In vivo*, it reduces tissue hyaluronan accumulation and suppresses inflammation and wound healing when injected subcutaneously [184].

Future directions

While it is acknowledged that hyaluronan synthesis drives the progression of certain types of cancers, the possible contribution of hyaluronan to the initiation of carcinogenesis is not known. The western life style, with high sugar consumption, obesity, and type 2 diabetes are known risk factors for malignant tumors like breast cancer, and also associate with unfavourable prognosis. The dependence of hyaluronan synthesis on glucose supply [84, 86, 91] the correlation of hyaluronan content with obesity in breast cancer [43, 72] and the accumulation of tissue hyaluronan during a diabetogenic diet [185] together suggest a role for hyaluronan in the chronic inflammation that disposes to carcinogenesis. For example, the role of hyaluronan metabolites in chronic inflammation, a known risk factor for later development of malignancies, would be worth investigation. Indeed, hyaluronan appears to be involved in metabolic processes that favor mutations and then allow cell proliferation for the selection of those mutations that facilitate uncontrolled growth [186].

In the future it is important to analyze the whole set of genes, proteins and metabolites involved in hyaluronan metabolism of human tumors, since it is obvious that the whole signaling chain with metabolites of hyaluronan are crucial in the maintenance of the pro-cancer conditions. This could also reveal novel points to interfere with the detrimental hyaluronan matrix properties that support cancer.

Clinical applications of drugs suppressing hyaluronan synthesis, clearing the existing hyaluronan, and those involving hyaluronan receptors and hyaluronidases are likely to expand in the future. Since hyaluronan metabolism is deranged in a number of different tumors, it offers a widely useful target of treatment. Considering the extreme diversity of mutations behind individual cancers, developing feasible targets tailored for the mutations is a major effort, as compared to hitting the typical extracellular matrix that feed most tumors. A combination of hyaluronan-targeted therapies for example with those interfering with signaling systems, is also an attractive opportunity. Furthermore, hyaluronan is likely to become an address tag on microvesicles and nanoparticles that are used to deliver drugs to tumors.

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Key words:
Hyaluronan;
Hyaluronidase;
CD44;
RHAMM;
Layilin;
TMEM2;
CEMIP;
UDP-sugar;
Warburg effect

Abbreviations used:
EMT, epithelial to mesenchymal transition; ROS, reactive oxygen species, UDP-GlcNAc, UDP-N-Acetylglucosamine; UDP-GlcUA, UDP-glucuronic acid; O-GlcNAc, O-linked GlcNAc (on Ser/thr residues of intracellular proteins)

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”Activated hyaluronan metabolism in the tumor matrix – causes and consequences”

Highlights:

1. Hyaluronan contributes to most aspects of the malignant phenotype
2. Factors in both hyaluronan synthesis and catabolism contribute to tumor progression
3. Cancer glucose uptake and Warburg metabolism stimulates hyaluronan synthesis
4. Posttranslational regulation rather than expression of HAS enzymes is important
5. Hyaluronan synthesis, degradation and signaling are potential targets of therapy