HYALURONAN: FROM EXTRACELLULAR GLUE TO PERICELLULAR CUE

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Hyaluronan is an extracellular and cell-surface-associated polysaccharide that is traditionally regarded as a biological 'goo' that participates in lubricating joints or holding together gel-like connective tissues. Although these are common physiological roles of hyaluronan in adult organisms, hyaluronan also functions as a microenvironmental cue that co-regulates cell behaviour during embryonic development, healing processes, inflammation and tumour development. Recent work highlights a key role for interactions between hyaluronan and tumour cells in several aspects of malignancy and indicates the possibility of new therapeutic strategies.

PROTEOGLYCANS
Specialized glycoproteins with polysaccharide side chains known as glycosaminoglycans. Glycosaminoglycans — such as chondroitin sulphate, heparin and hyaluronan — are composed of repeating disaccharides, which are highly negatively charged as they contain carboxyl and/or sulphate groups. Proteoglycans are characteristic components of extracellular matrices and the cell surface.

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Hyaluronan (which is also known as hyaluronic acid or hyaluronate) is an unusual polysaccharide that has a simple chemical structure but extraordinary properties. It is synthesized as a large, negatively charged, unbranched polymer that is composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine: $[\text{-}\beta(1,4)\text{-GlcUA-}\beta(1,3)\text{-GlcNAc-}]_n$. In normal physiological conditions, hyaluronan consists of 2,000–25,000 disaccharides, which corresponds to polysaccharides with relative molecular masses of 10^6–10^7 and polymer lengths of 2–25 μm. Although hyaluronan belongs to the family of glycosaminoglycans, which includes heparan sulphate and chondroitin sulphate, it differs from these in many ways. Other glycosaminoglycans are made as PROTEOGLYCANS that are synthesized and assembled in the rough endoplasmic reticulum and Golgi apparatus, and are secreted in a similar way to other glycoproteins. Hyaluronan, however, is synthesized as an unmodified polysaccharide by one of three different, but related, hyaluronan synthases — HAS1, HAS2 and HAS3. These are multipass transmembrane enzymes, the active sites of which protrude from the inner face of the plasma membrane. Hyaluronan is extruded through the plasma membrane onto the cell surface or into the extracellular matrix (ECM) while it is being synthesized[1,2] (BOX 1). Hyaluronan is cleaved by enzymes known as hyaluronidases; in humans, there are six hyaluronidase genes, which encode hyaluronidases that have different properties and cellular locations[3].

Hyaluronan has remarkable hydrodynamic characteristics, especially in terms of its viscosity and its ability to retain water. It therefore has an important role in tissue homeostasis and biomechanical integrity, and these properties form the basis of its widespread use in tissue engineering[4]. Hyaluronan also forms a multivalent template for interactions with proteoglycans and other extracellular macromolecules that is important in the assembly of extracellular and pericellular matrices[5] (FIG. 1). These properties of hyaluronan help to regulate the porosity and malleability of these matrices, which are important factors in determining whether cells invade tissues during development, tissue remodelling and cancer progression (BOX 1). This function of hyaluronan, and of pericellular matrices in general, no doubt contributes to the 'permissive' or 'landscaping' role of the microenvironment in which cancer cells proliferate and metastasize[6,7]. However, hyaluronan also has a direct, instructive role in determining the malignant state through its interactions with the surfaces of tumour cells.

Hyaluronan interacts with cell surfaces in at least two ways (FIG. 1). First, it can bind to specific cell-surface receptors, such as CD44 and RHAMM (receptor for

hyaluronic-acid-mediated motility), to induce the transduction of a range of intracellular signals, either directly or by activating other receptors[8]. Surprisingly, however, hyaluronan can also be retained at the cell surface by sustained transmembrane interactions with its synthases. Either means of retention can generate a voluminous pericellular matrix, or 'coat', that incorporates several other hyaluronan-binding molecules[5]. Although it is now well established that hyaluronan-induced signalling occurs through receptor interactions, the role in signalling of the hyaluronan synthases themselves, or of secondary interactions of hyaluronan with other proteins within pericellular coats, has not been established.

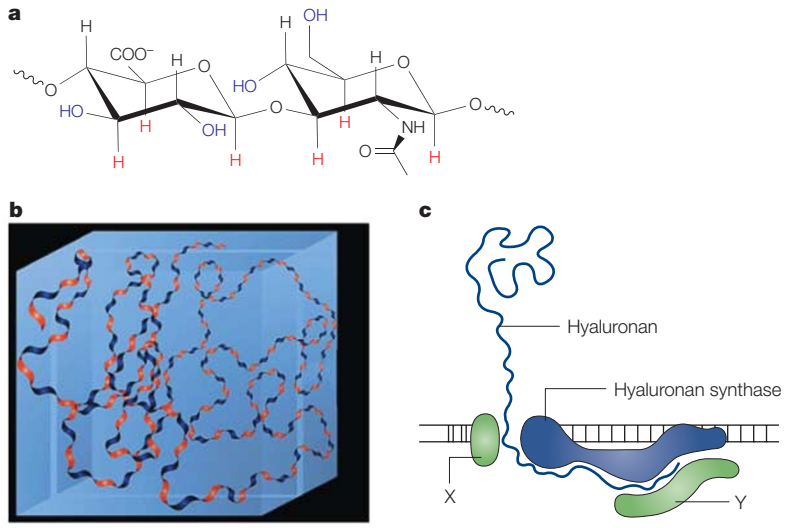
Hyaluronan is overproduced by many types of tumour and, in some cases, hyaluronan levels are prognostic for malignant progression. Moreover, manipulations of hyaluronan concentration or interactions can markedly alter the activities of signalling pathways that are commonly associated with oncogenesis and, accordingly, can also alter the course of progression of several tumour types in experimental animal models. In this regard, hyaluronan functions in a similar way to other non-genetic, microenvironmental cues that have fundamental and profound influences on the initiation, persistence and progression of cancer[9,10]. The body of data that supports a key role for hyaluronan in malignancy is convincing and indicates that this molecule could be a viable therapeutic target.

Box 1 | **Structure and synthesis of hyaluronan**

Part **a** in the figure shows the structure of hyaluronan, which is composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine . The polymer has charged and hydrophobic faces, due to the carboxyl groups of glucuronic acid and a cluster of hydrogen atoms on one face of the disaccharide, respectively. Axial hydrogen atoms that contribute to the hydrophobic face are shown in red.

As shown in part **b** in the figure, the domain that is occupied by each hyaluronan molecule in dilute solutions expands because of mutual repulsion between carboxyl groups, and therefore occupies a large volume, with water trapped inside the structure. In more concentrated solutions, the hyaluronan molecules become entangled, forming a continuous but porous meshwork. This meshwork exerts a so-called 'swelling pressure' because of increased mutual repulsion between and within molecules. When external pressure is applied to a hyaluronan meshwork it will contract, but when this external pressure is withdrawn the hyaluronan meshwork will spring back into its original shape, due to the internal swelling pressure, or will acquire a new shape if new restrictions or boundaries are applied that counterbalance this pressure. This property provides resilience and malleability to many tissues. In addition, hyaluronan-rich areas within developing tissues exert internal pressures that can cause the separation of physical structures and create 'highways' for cell migration[5]. A dramatic example of this is the migration of mesenchymal cells into the cornea following increased hyaluronan deposition, hydration and concomitant swelling of the migratory pathway[171]. This phenomenon has been demonstrated experimentally using glioma cells migrating through a fibrin gel[129]. On a smaller scale, these properties can facilitate cell-shape changes that are required for cell division and movement by providing a highly hydrated zone around the cell that separates it from adjacent cells[128].

As shown in part **c** in the figure, hyaluronan synthesis takes place at the inner surface of the plasma membrane and nascent hyaluronan is extruded onto the plasma membrane while it is still attached to the synthase that produces it[1]. This ensures that hyaluronan has an intimate relationship with the cell surface and can readily participate in the creation of a pericellular hydrated zone (FIG. 1). X and Y are putative regulatory proteins. Part **b** is reproduced with permission from REF. 185 © (1997) Glycoforum. Part **c** is modified with permission from REF. 186 © (2000) Marcel Dekker.



Hyaluronan levels in tumours

The association of hyaluronan with tumorigenesis has been known for some time[11]. Several studies have reported a relation between hyaluronan content and invasiveness, and a greater enrichment of hyaluronan in the STROMA that surrounds tumours than in PARENCHYMAL regions[12,13]. Other studies have shown that hyaluronan production by stromal cells is stimulated by interactions with tumour cells[14,15], but that synthesis is also increased in malignant tumour cells themselves[16–18].

In patients with cancer, hyaluronan concentrations are usually higher in malignant tumours than in corresponding benign or normal tissues, and in some tumour types the level of hyaluronan is predictive of malignancy[19]. However, consistent with the studies that are described above, hyaluronan levels can be increased around tumour cells themselves or within the tumour stroma. For example, in patients with breast and ovarian carcinomas, high levels of hyaluronan in the stroma are associated with low survival rates[20,21]. It is particularly interesting that the shape of invasive human breast carcinomas, as visualized by sonography, corresponds closely to the shape of the hyaluronan-enriched zone that is associated with these tumours. Because of this, it was proposed that sonography might be a more reliable technique for detecting invasive tumours than mammography, which sometimes fails to detect these tumours[22].

High levels of stromal hyaluronan are also associated with malignancy in patients who have non-small-cell lung adenocarcinomas[23] and prostate cancer[24,25]. However, in patients who have breast and prostate carcinomas, both hyaluronan that is associated with tumour cells and stromal hyaluronan are linked with cancer progression[21,26]. Levels of parenchymal hyaluronan also correlate with malignancy in patients with gastric and colorectal cancers[27,28]. Hyaluronan and its receptors are associated with cancers of circulating cells[29–31], as well as with solid tumours. In addition, it has been shown that

Summary

- Hyaluronan is a large, negatively charged polysaccharide that participates in defining the properties of pericellular matrices and in transducing signals in proliferating and migrating cells.
- Hyaluronan and hyaluronidase are overproduced in many types of human tumour.
- Experimentally increased hyaluronan production stimulates tumour growth and metastasis in xenograft models, whereas antagonists of hyaluronan synthesis or of the interactions between hyaluranon and its receptors suppress these phenomena.
- Interactions between hyaluronan and tumour cell-surface receptors influence many intracellular signalling pathways, notably ERBB2 activity and anti-apoptotic pathways.
- Increased production of hyaluronan induces drug resistance, whereas hyaluronan antagonists suppress multidrug resistance.
- Hyaluronan promotes cell invasiveness and epithelial–mesenchymal transition.
- Breakdown products of hyaluronan stimulate angiogenesis.

hyaluronan levels are increased in the urine of patients with bladder carcinomas[32], in the serum of patients with breast cancer[33] and in the saliva of patients with head and neck cancer[34]. However, hyaluronan levels do not correlate with progression in melanomas[35] or in some epidermal carcinomas[36].

Because of the close association of high hyaluronan levels with malignancy in many tumour types, several groups have used experimental manipulations in animal models to test whether hyaluronan is important in tumour progression. Increased hyaluronan production, which was induced by transfection with cDNAs encoding *HAS1*, *HAS2* or *HAS3*, caused increased growth or metastasis of tumours in xenograft models of fibrosarcoma and prostate, colon and breast cancer[37–40]. Correspondingly, a reduction of hyaluronan production in prostate carcinoma cells using *HAS* antisense mRNAs caused decreased tumour growth[41]. A recent study confirmed that reducing HAS levels using antisense techniques suppresses tumour growth, but showed that extremely high hyaluronan levels also inhibit tumour growth[42].

Observations that the experimental overproduction of hyaluronidases suppresses the growth of colon and breast carcinomas in xenografts[40,43], and that one of the hyaluronidases is likely to be a tumour suppressor[44], support a role for hyaluronan in tumour progression. However, some reports indicate that overexpression of hyaluronidases can promote, rather than suppress, tumour progression[45–47]. In addition, levels of hyaluronidases (usually HYAL1), as well as hyaluronan, are often increased in malignant tumours, for example in bladder[48], prostate[24], head and neck[34], colorectal[49] and brain[50] cancers. Consequently, it has been shown that a combined assay for hyaluronan and HYAL1 provides a reliable indication of malignancy in some types of tumour[24,32,34].

The studies described above overwhelmingly support the hypothesis that high levels of hyaluronan correlate with and actively promote tumour progression. In addition, however, products that are generated by the degradation of hyaluronan can stimulate other tumour behaviours, such as angiogenesis (see below).

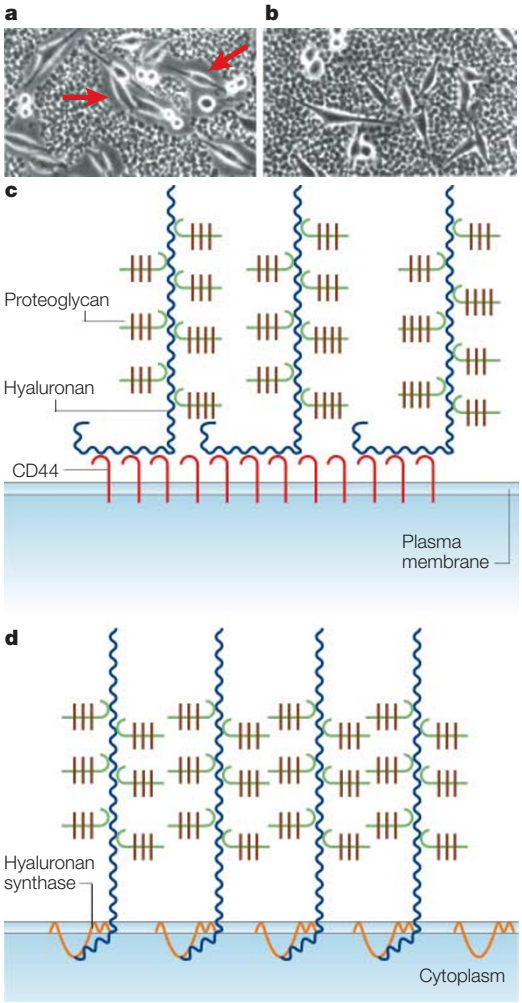


Figure 1 | **Hyaluronan interactions with the cell surface.** **a** | A hyaluronan-enriched pericellular matrix surrounds fibrosarcoma cells (long, 'coated' cells; indicated by arrows). This matrix, or 'coat', can be visualized by the exclusion of particles, in this case fixed red blood cells (small round cells). **b** | Treatment of these cells with hyaluronan-specific hyaluronidase removes the coats, showing that their structure is dependent on hyaluronan. **c** | Representation of the coat, showing hyaluronan tethered to the cell surface through interactions with receptors such as CD44 (REF. 176). Presumably, hyaluronan could also be tethered by other receptors, such as RHAMM (receptor for hyaluronic-acid-mediated motility), but this has not been shown. Hyaluronan binds many proteoglycan molecules, which are highly negatively charged and repel each another, causing hyaluronan to extend out from the cell surface in a 'brush' configuration and to exclude particles[177]. Other hyaluronan-binding proteins — such as TSG6, link proteins and inter-α-inhibitor — can also be retained within this coat[51]. The composition of the pericellular matrix/coat varies with cell type, but its assembly is always dependent on the tethering of hyaluronan to the cell surface. Hyaluronan also forms a template for interactions of proteoglycans and other factors in extracellular matrices, but in this case hyaluronan is not tethered to the cell surface. **d** | The coat is shown with hyaluronan tethered to the cell surface through retention by hyaluronan synthase[178,179]. In this case, the hyaluronan is tethered to the enzyme on the cytoplasmic side of the plasma membrane, but projects through pores in the membrane to the cell surface[1,2]. Parts **a** and **b** are reproduced with permission from REF. 5 © (2001) Academic Press. Parts **c** and **d** are modified with permission from REF. 187 © (1998) Glycoforum.

STROMA
Most organs are composed of two associated compartments — the parenchyma and stroma. In adult organisms, the stroma is composed of connective tissue and contains fibroblasts, cells derived from the circulation, blood vessels, nerves and associated extracellular matrices. Carcinomas usually contain an extensive stromal compartment.

PARENCHYMA
The parenchyma is regarded as the 'business' part of an organ. It is composed of epithelial or epithelial-like cells that produce the characteristic structures of the differentiated organ.

Hyaluronan-induced signal transduction

Hyaluronan receptors. Hyaluronan interacts with many proteins (termed hyaladherins), several of which are known or potential cell-surface receptors[8,51]. Of these, CD44 and RHAMM are established signal-transducing receptors that influence cell proliferation, survival and motility, and are known to be relevant to cancer. Other cell-surface hyaladherins, such as lymphatic-vessel endothelial hyaluronan receptor 1 (LYVE1) and TOLL4, might also have roles in cancer pathogenesis.

CD44 is a cell-surface glycoprotein that contains an ectodomain, a transmembrane domain and a cytoplasmic domain[52–54]. The ectodomain includes an amino-terminal hyaluronan-binding domain that is related to the 'link modules' of hyaluronan-binding proteoglycans and link proteins[51]. The region of the *CD44* gene that encodes the ectodomain contains a site into which many exon products are spliced in numerous combinations. Although hyaluronan is the main ligand for CD44, several other molecules interact with this protein, many of which bind to carbohydrate side groups that are attached to the 'spliced-in' regions. Among these other ligands, fibroblast growth factors, osteopontin and matrix metalloproteinases (MMPs) are particularly important in terms of relevance to cancer[54]. In response to hyaluronan binding, and depending on the cellular context, the cytoplasmic tail of CD44 interacts with many regulatory and adaptor molecules, such as SRC kinases, RHO GTPases, VAV2, GAB1, ankyrin and ezrin[54–56]. CD44 also mediates the cellular uptake and degradation of hyaluronan, which in turn affects growth regulation and tissue integrity[57,58].

RHAMM is alternatively spliced and the different forms of the resulting protein are found both on cell surfaces and inside cells. Although *RHAMM* mRNA does not contain a recognizable leader sequence, the protein is transported to the cell surface, where it binds hyaluronan and — like CD44 — transduces signals that influence growth and motility[8]. There is no link-module domain in RHAMM, but it does include a hyaluronan-binding motif that is present in several hyaladherins and contains the sequence B[X_7]B (where 'B' represents arginine or lysine, and 'X' represents any non-acidic amino acid)[59]. Although RHAMM can bind to other extracellular macromolecules, the significance of this binding is not clear. Intracellular RHAMM interacts with several signalling proteins and cytoskeletal components, including SRC, extracellular-signal-regulated kinase 1 (ERK1), actin and microtubules[8,60,61]. It will be of great interest to determine whether RHAMM is transported to different cellular compartments in response to its interaction with hyaluronan and whether it interacts with both cell-surface and intracellular pools of hyaluronan[8].

Interactions of hyaluronan with CD44 and RHAMM lead to numerous cellular responses, including those that involve tyrosine kinases, protein kinase C, focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase, nuclear factor-κB and RAS, as well as cytoskeletal components[8,54–56]. Although it is clear that CD44 and RHAMM can participate independently in proliferative and migratory phenomena, their relative contributions to any given event have not been fully resolved in most cases, and it is likely that they have redundant or overlapping functions in some situations. In general, the interactions of hyaluronan with CD44 and RHAMM are of obvious physiological importance, and their normal activities seem to be disrupted in cancer cells. The other ligands of CD44 and RHAMM compound this complexity[54]. The interactions of hyaluronan with these receptors, particularly with CD44, regulate two specific cellular functions that are especially important for tumorigenesis — cell survival and ERBB-family signalling.

Cell-survival signalling. One of the best-studied hallmarks of the behaviour of malignant cells is the ability to survive under conditions that would lead to growth arrest or apoptosis in normal cells[7]. This is reflected by the observation that cancer cells are usually able to grow as colonies in soft agar or in suspension — anchorage-independent conditions in which normal cells, especially epithelial cells, would undergo apoptosis (a phenomenon that is sometimes known as anoikis)[62]. Mechanistically, the phenomenon of anchorage-dependent growth in normal epithelia is now known to be due to the requirement for cell-survival signals from ECM components, as well as from growth factors. These signals are usually transduced through interactions of matrix molecules — such as fibronectins, laminins and collagens — with integrins. Cooperative interactions between growth-factor receptors and integrins initiate complex signalling pathways that regulate cell survival and proliferation. Escape from the requirement for properly regulated anchorage to the ECM presumably allows malignant cells to grow outside normal matrix microenvironments and to travel though the circulation to sites of metastasis.

Recent studies have shown that hyaluronan strongly promotes anchorage-independent growth[37,42,63,64] and that the resistance of cancer cells to growth arrest and apoptosis under anchorage-independent conditions is dependent on constitutive interactions between hyaluronan and CD44 (REFS 65,66). Consistent with these results, several groups have shown that hyaluronan activates the PI3K–AKT signalling pathway[64,67–69], which promotes cell survival. Hyaluronan also stimulates the phosphorylation of FAK and BAD, which also promotes cell survival[69–71]. These effects are reversed when constitutive hyaluronan–CD44 interactions are inhibited[66,69]. Interestingly, FAK activation can be induced by the interaction of hyaluronan with either CD44 or RHAMM[70,71]. Recent studies have shown that hyaluronan promotes the interaction of the cytoplasmic domain of CD44 with the p110 subunit of PI3K through the adaptor protein GAB1 and activates this pathway, providing a direct mechanism by which hyaluronan–CD44 interactions regulate cell survival[72]. RHAMM interacts with and activates ERK1 (REF. 61), which — similarly to AKT — can phosphorylate BAD[73], and consequently maintain cell survival. So, the interaction of hyaluronan with CD44 or RHAMM can promote cell survival.

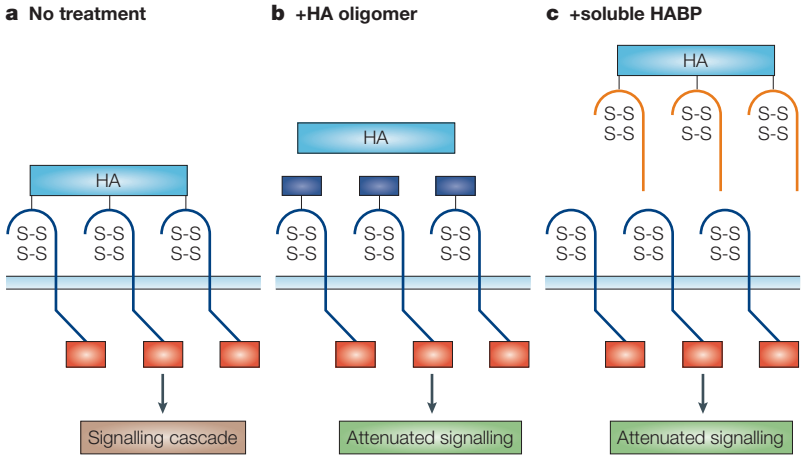


Figure 2 | **Perturbation of hyaluronan function.** Hyaluronan (HA) function can be antagonized by hyaluronan oligomers or by soluble hyaluronan-binding proteins (HABPs). **a** | Polymeric hyaluronan binds multivalently to cellular receptors, such as CD44 or RHAMM (receptor for hyaluronic-acid-mediated motility), to stimulate intracellular signalling. **b** | Addition of hyaluronan oligomers (dark blue boxes) can displace multivalent hyaluronan polymers from cells. These oligomers bind monovalently and attenuate hyaluronan signalling. **c** | HABPs function as decoy binding proteins for endogenous hyaluronan, displacing it from its cell-surface receptors and blocking hyaluronan signalling. Other manipulations that have been used to perturb hyaluronan function are small interfering RNAs and blocking antibodies that are directed against specific receptors.

Many of these studies address the effects of experimentally increased hyaluronan synthesis or treatment with exogenously added hyaluronan. However, these experiments do not necessarily reveal the constitutive effects of hyaluronan in cancer cells. To do this, several methods of disrupting the endogenous interactions between tumour cells and hyaluronan have been used, as shown in FIG. 2. One method is treatment with small hyaluronan oligomers that competitively displace endogenous polymeric hyaluronan from its binding partners, replacing an endogenous, multivalent, high-affinity ligand with a monovalent, low-affinity ligand[66,74]. Another method is treatment with or induced expression of various soluble hyaluronan-binding proteins that act as competitive decoys for the binding of endogenous hyaluronan[65,75–80]. These two methods can disrupt most known hyaluronan-binding events. In addition, treatment with small interfering RNAs (siRNAs) or antibodies against specific receptors has also been used. All of these approaches have been shown to inhibit constitutive PI3K–AKT pathway activities in tumour cells (REFS 66,80; S. Ghatak, S. Misra and B.P.T., unpublished observations).

The concept that perturbation of hyaluronan-induced signalling suppresses cell-survival pathways is strongly supported by studies that have been carried out in animal models. For example, experimental overexpression of soluble CD44 induces apoptosis in TA3/St mammary carcinoma cells as they enter the lung interstitium after injection into the circulation[76] and causes growth arrest of these cells when they are grown in ASCITES[65]. These effects were not obtained with a mutant form of CD44 that does not bind hyaluronan. Consistent with these findings, soluble RHAMM also induces growth arrest[79].

It was recently shown that a synthetic peptide that contains three copies of the hyaluronan-binding motif B[X_7]B inhibits tumour growth. This is thought to occur through hyaluronan-mediated uptake into cytoplasmic compartments, especially into the mitochondria, followed by interaction with BCL2 and induction of apoptosis[81]. An area of increasing interest in the field of hyaluronan research is the potential functions of intracellular hyaluronan and hyaluronan-binding proteins. Intracellular as well as extracellular hyaluronan levels increase during cell division[82] and motility[83]. Several intracellular, B[X_7]B-containing, hyaluronan-binding proteins have been characterized. RHAMM, which was the first protein in which the B[X_7]B motif was characterized[59], can be present both inside cells and on cell surfaces[8]. In addition to interacting with ERK and SRC kinases, intracellular RHAMM binds to both actin filaments and microtubules in the cytoskeleton[8,84]. RHAMM is also targeted to centrosomes, where it participates in the maintenance of spindle integrity[85]. CDC37, which is another intracellular hyaluronan-binding protein[86], is required for the chaperone functions of HSP90 (REF. 87), and therefore for the activity of several cell-cycle-related kinases and oncoproteins[88]. Other intracellular hyaluronan-binding proteins might also be required for essential cellular functions that are related to the cell cycle and motility[89,90]. However, the relation between hyaluronan binding and the mechanisms of action of these intracellular proteins has not yet been elucidated.

ERBB signalling. The ERBB family of transmembrane proteins comprises four members — ERBB1 (the epidermal growth factor receptor), ERBB2 (also known as HER2/NEU), ERBB3 and ERBB4 (REF. 91). ERBB1 and ERBB2 are frequently overexpressed or mutated in breast, ovarian and colorectal cancers and are thought to be important contributors to malignancy. Consequently, several current cancer therapies are targeted to the ERBB family[92]. Analyses of *Has2*-null mice have shown severe defects in morphogenesis, especially in cardiac development, that lead to embryonic lethality[93]. Among these defects is the failure of the cardiac endothelium to undergo an EPITHELIAL–MESENCHYMAL TRANSITION (EMT) and to initiate tissue invasion, which occurs because of inactivation of ERBB2/ERBB3 and RAS signalling[93,94]. Similarly, biochemical studies of breast, ovarian and cervical carcinoma cells, and of glioma cells, have shown that CD44 interacts with ERBB1 and ERBB2, and that hyaluronan–CD44 interactions regulate the activities of ERBB proteins[95–97] (FIG. 3). The cytoplasmic adaptor molecules GRB2 and VAV2 mediate the ERBB2–CD44 interaction and the resulting activation of RAS signalling[95].

We have recently shown that perturbing constitutive hyaluronan–CD44 interactions with hyaluronan oligomers, soluble hyaluronan-binding proteins or siRNAs directed against CD44 dissociates a signalling complex that contains CD44, activated ERBB2, ezrin and PI3K, and therefore inhibits downstream ERBB2 signalling (S. Ghatak, S. Misra and B.P.T., unpublished

ASCITES
When tumour cells accumulate in the peritoneal cavity, a voluminous fluid exudate forms — known as ascites —in which the cancer cells are suspended. This phenomenon is common in ovarian carcinomas and mesotheliomas.

EPITHELIAL–MESENCHYMAL TRANSITION
Conversion from an epithelial to a mesenchymal phenotype, which is a normal process of embryonic development. In carcinomas, this transformation results in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness.

observations)(FIG. 3). It therefore seems that hyaluronan–CD44 interactions regulate constitutive ERBB2 signalling in cancer cells. A similar relation might occur between hyaluronan and other receptor kinases that regulate cell behaviour. For example, the interaction of hyaluronan with CD44 elicits high-affinity binding between the cytoplasmic domains of CD44 and transforming growth factor-β receptor I (TGF-βRI), leading to increased SMAD2/SMAD3 signalling and other downstream events[98]. In addition, CD44 is required for c-MET signalling in response to the interaction of c-MET with its ligand, hepatocyte growth factor (HGF)[99]. When it is associated with the plasma membrane, the oncoprotein Tpr-MET stimulates PI3K activity, which in turn induces hyaluronan and CD44 production and leads to cell transformation[100].

Hyaluronan and regulation of multidrug resistance

Drug resistance can arise in numerous ways, such as through decreased access to or uptake of drugs, activation of repair and detoxification mechanisms, and increased drug efflux. 'Classic' multidrug resistance is due to increased drug export through the action of ATP-dependent efflux pumps, such as MDR (for multidrug resistance), MRP (for multidrug-resistance protein) and other members of the ATP-binding cassette (ABC) transporter families[101]. However, it has become apparent in recent years that alterations in cell survival and apoptotic signalling pathways are connected with drug resistance in cancer cells, and that drug resistance in patients can sometimes be overcome by therapeutic interventions that induce downstream events in the apoptotic cascade[102,103]. It is therefore possible that the effects of hyaluronan on cell-survival signalling might alter drug resistance (FIG. 4).

Treatment of tumour cells with hyaluronidase increases the activities of various chemotherapeutic agents, especially when it is used locally[104]. Of particular interest was the observation that dispersion of multicellular spheroids of EMT-6 mammary tumour cells with hyaluronidase reverses MDR1-based multidrug resistance[105,106]. The mechanistic effect of hyaluronidase was not understood at the time, but has usually been explained in terms of decreasing cell-adhesion barriers[105], increasing drug penetration[104,107] and restricting cytokine diffusion[108], rather than in terms of hyaluronan-specific effects on cell-survival signalling. However, other studies have shown that calcium-independent aggregation of transformed cells, such as that which occurs in multicellular spheroids, is due to hyaluronan-mediated, multivalent cross-bridging of receptors on adjacent cells[109]. This observation, and the finding that hyaluronan stimulates cell-survival signalling, led to the further investigation of the possible role of hyaluronan in drug resistance[69]. Increased hyaluronan production was found to stimulate drug resistance in drug-sensitive cancer cells. In addition, disruption of endogenous hyaluronan-induced signalling suppresses resistance to several drugs, including doxorubicin, paclitaxel, 1,3-bis(2-chloroethyl)-1-nitrosurea, vincristine and methotrexate[69]. Although the anti-apoptotic effect of hyaluronan probably contributes to these phenomena, it also known that lipid products of PI3K, such as phosphatidylinositol-3,4-diphosphate and phosphatidylinositol-3,4,5-triphosphate, directly mediate the function of ABC transporters that are involved in bile transport[110]. Because hyaluronan stimulates PI3K activity, it might also influence drug resistance by stimulating drug transport (FIG. 4). Of particular interest is recent work indicating that inhibitors of multidrug resistance block hyaluronan synthesis and secretion, and that hyaluronan might be secreted through multidrug transporters[111]. This is consistent with the finding that manipulation of hyaluronan in a cell-free system inhibits drug transport (S. Misra, S. Ghatak and B.P.T., unpublished observations).

Extracellular-matrix metalloproteinase inducer (EMMPRIN; also known as CD147 or basigin) — which is a cell-surface glycoprotein and a member of the immunoglobulin-like superfamily[112] — stimulates the production of hyaluronan[113] (BOX 2). It is expressed at high levels in many types of malignant tumour, including melanomas, gliomas, lymphomas, and breast, lung and kidney carcinomas[114]. In addition,

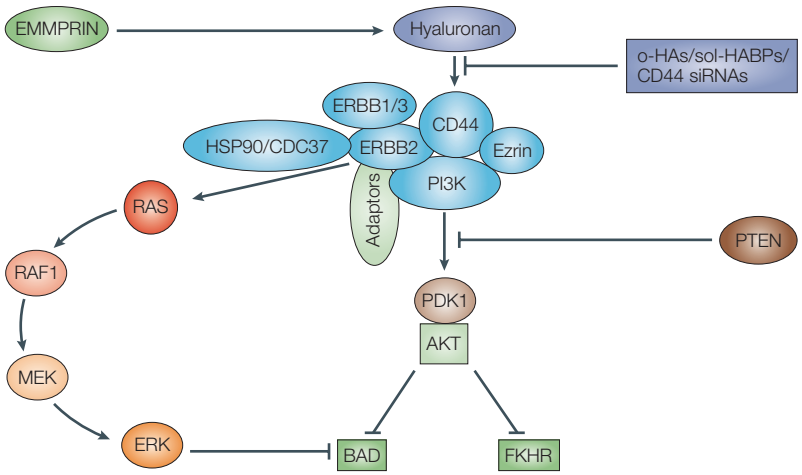
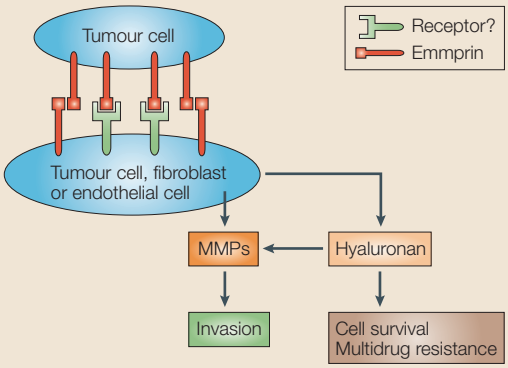


Figure 3 | **Cooperative signalling by hyaluronan receptors and ERBB2.** Hyaluronan induces the formation of a complex that contains CD44, ERBB2, ezrin, phosphatidylinositol 3-kinase (PI3K) and heat-shock protein 90 (HSP90)/CDC37 (a chaperone that is required for ERBB2 activity[180]). Adaptor molecules, such as GRB2 and GAB1, mediate the interactions between some of the components within this complex[72,95]. It is likely that this complex is assembled within a lipid raft and might include other CD44-associated signalling molecules, such as the inositol-1,4,5-trisphosphate receptor[181] and the Na^+–H^+ exchanger NHE1[144], which are also known to reside in rafts. Formation of the complex activates ERBB2, which then promotes cell survival through the PI3K/AKT and mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathways. Both of these pathways phosphorylate the pro-apoptotic factor BAD to inactivate it[73]. AKT also inactivates FKHR (a member of the forkhead family of proteins), which is a pro-apoptotic transcription factor[182]. PTEN is a tumour suppressor that functions by dephosphorylating the lipid products of PI3K activity[183]. Interaction of these components with ezrin probably links the complex to the cytoskeleton and promotes cell migration[146]. Another cytoskeletal linker, ankyrin (not shown), has a similar role[145], but it is not known whether ankyrin also resides within this complex. Extracellular-matrix metalloproteinase inducer (EMMPRIN) stimulates hyaluronan synthesis[113] and thereby induces these signal-transduction pathways. Inhibition of the hyaluronan–CD44 interaction by hyaluronan oligomers (o-HAs), soluble hyaluronan-binding proteins (sol-HABPs) or small interfering RNAs (siRNAs) that are directed against CD44 causes dissociation of this complex in cancer cells, and therefore inhibits ERBB2 activation (S. Ghatak, S. Misra and B.P.T., unpublished observations) and downstream signalling responses[66]. Similar types of interaction might occur with other receptor kinases, such as c-MET[99,100] and transforming growth factor-β receptor I (REF. 98). PDK1, phosphoinositide-dependent kinase 1.

Box 2 | **EMMPRIN**



Extracellular-matrix metalloproteinase inducer (EMMPRIN; also known as CD147 or basigin), which is an immunoglobulin-like superfamily glycoprotein, was originally identified as a factor that is present on the surface of tumour cells and that induces matrix metalloproteinase (MMP) production in fibroblasts and endothelial cells through heterotypic cell interactions[112,172,173]. EMMPRIN stimulates MMP production in tumour cells through homotypic cell interactions[174]. These homotypic and heterotypic interactions are shown in the figure. A putative EMMPRIN receptor has not been identified on fibroblasts and endothelial cells, but signalling might involve homophilic EMMPRIN interactions, as tumour cells induce increased EMMPRIN synthesis in fibroblasts[175]. As a consequence of its effect on MMP synthesis, EMMPRIN stimulates invasion *in vitro* and *in vivo*[116]. It was recently shown that EMMPRIN also stimulates the production of hyaluronan in mammary carcinoma cells[113]. Consequently, EMMPRIN promotes cell-survival signalling and induces multidrug resistance in a hyaluronan-dependent manner[69]. In addition, other studies have shown that EMMPRIN levels on tumour-cell surfaces correlate with multidrug resistance[115]. Hyaluronan might also stimulate MMP production, MMP presentation on cell surfaces and invasiveness, independently of EMMPRIN[136–141]. EMMPRIN is therefore a potent inducer of malignant cell properties that functions at least in part through the stimulation of hyaluronan synthesis.

EMMPRIN levels on the surfaces of tumour cells correlate with multidrug resistance[115]. Experimental overexpression of EMMPRIN in MDA-MB-436 human mammary carcinoma cells, which are relatively less aggressive than most malignant lines and express lower levels of EMMPRIN, results in the ability of these cells to form large, malignant tumours in nude mice[116]. Interestingly, a transcriptome analysis of single cells that were isolated from micrometastases showed that EMMPRIN is one of the most highly expressed proteins in these disseminated cells, indicating that it has an important function in metastasis[117]. EMMPRIN has also been shown to induce drug resistance in a hyaluronan-dependent manner[69].

Hyaluronan in invasion and metastasis

Metastasis. Manipulations of hyaluronan in animal models have shown its importance in metastasis. For example, mouse mammary carcinoma cell lines that were selected for low levels of hyaluronan production were shown to give rise to fewer lung nodules after intravascular injection than lines that produce high levels of hyaluronan. Stimulation of hyaluronan production by transgenic expression of *Has1* in the cell lines that showed low levels of hyaluronan production caused them to form an increased number of nodules[38]. Consistent with these results, transfection of highly metastatic cells with cDNAs that encode soluble CD44, which is an antagonist of constitutive hyaluronan–receptor interactions (FIG. 2), inhibits lung-nodule formation[76], as does antisense inhibition of CD44 expression[118]. This is supported by the observation that the absence of CD44 suppresses metastasis in mice that have mutated *Apc* or *Trp53* genes[119], and by the fact that tumour cells that are treated with soluble RHAMM are unable to form lung metastases[79]. However, similar manipulations were not effective in all studies, indicating that some tumour types might be less dependent on hyaluronan than others[120,121].

Several cellular activities are required for successful metastasis, including intravasation into the blood or lymphatic system, survival in the circulation, extravasation, and initiation and maintenance of growth at the secondary site[122]. The ability of hyaluronan to activate cell-survival signalling pathways might contribute to some of these steps, especially during transit in the circulation and initial re-growth. Extravasation is thought to involve the attachment of tumour cells to endothelial cells at metastatic sites. Metastatic prostate carcinoma cells preferentially and rapidly adhere to bone-marrow endothelial cells. Recent studies have shown that this adhesion can be mediated by the pericellular hyaluronan that surrounds the metastatic cells; non-metastatic cells have little pericellular hyaluronan and do not adhere to endothelial cells[123]. This interaction probably involves CD44 or RHAMM expression by endothelial cells[124–126]. Interactions between hyaluronan and LYVE1 might be involved in metastasis through lymph vessels[127].

Invasiveness. Hyaluronan is thought to be important for three different aspects of invasion. The first of these is the formation of highly hydrated, malleable matrices that facilitate changes in cell shape and tissue penetration (BOX 1); the second is the regulation of the production and cell-surface presentation of proteases; and the third is the induction of cytoskeletal rearrangements. Early studies of morphogenesis emphasized the relation between the hyaluronan-mediated hydration of extracellular matrices and cell invasiveness[5]. Moreover, hyaluronan-rich, hydrated matrices assemble around migrating cells in culture, and removal of these matrices reduces

the rate of cell movement[128]. Consistent with this, one study clearly showed that the promotion of glioblastoma-cell migration within a fibrin matrix by hyaluronan is due at least in part to increased hydration, which results in increased gel porosity[129]. However, several studies have shown that treatment with hyaluronan directly promotes migration and invasiveness of tumour cells, especially glioma cells[130,131]. Similarly, perturbation of hyaluronan interactions inhibits glioma-cell invasiveness[80]. Hyaluronan-induced signalling, especially through ERBB1 (REFS 97,132) is probably involved in this and, again, both CD44 and RHAMM are thought to have a role[97,131–133].

Invasiveness is dependent on the pericellular proteolysis of ECM barriers that prevent escape from the normal tissue architecture and penetration of blood-vessel walls. The MMPs are important for cancer-cell invasion, although it is now apparent that MMPs have several other roles in metastasis, including facilitation of tumour growth at the secondary site[134,135]. The interaction between hyaluronan and CD44 on the surfaces of glioma or lung carcinoma cells stimulates the production of MMP9 and MMP2, respectively[136,137]. In addition, the hyaluronan–CD44 interaction promotes the binding of MMP9 to the ectodomain of CD44, leading to increased invasiveness, processing of TGF-β and angiogenesis[138–140]. CD44 also targets another MMP, MT1-MMP, to lamellipodia[141], which results in increased CD44 shedding and cancer-cell migration[142,143]. Although hyaluronan seems to stimulate both the production and cell-surface presentation of MMPs, the mechanism that underlies this is not yet clear. In addition to the MMPs, hyaluronan also regulates cathepsin B activity by stimulating the interaction of CD44 with the Na^+–H^+ exchanger NHE1 and the consequent acidification of the tumour-cell milieu[144]; this effect also increases tumour-cell invasiveness.

Numerous studies have shown a close relation between interactions of hyaluronan with its receptors and cytoskeletal changes that promote migration and invasion[8,55,56]. Hyaluronan-induced signalling through SRC kinases and RAS and RHO GTPases mediates many of these changes. In addition, the cytoplasmic tail of CD44 interacts directly with ankyrin[145] and with members of the ezrin/radixin/moesin (ERM) family, which have been shown to be involved in tumorigenesis[56,146]. These proteins induce changes in the actin cytoskeleton and cell membrane that lead to cell migration. Hyaluronan also promotes the direct interaction of the cytoplasmic domain of CD44 with TGF-βRI and ankyrin in breast cancer cells, which also stimulates migration[98]. RHAMM interacts with actin filaments and microtubules[8,84,85], and hyaluronan–RHAMM interactions induce FAK phosphorylation and dephosphorylation, which are important steps in the activation of integrin-mediated cell motility[70]. Hyaluronan clearly stimulates motility through interactions with both CD44 and RHAMM, but the details of the steps in the process that are induced by cell-surface hyaluronan binding have not been fully elucidated.

Epithelial–mesenchymal transition. There are many parallels between EMTs that normally take place during embryogenesis and the progression of carcinomas to the metastatic phenotype[147]. These transitions involve the loss of intercellular junctions, escape from apoptosis and increased invasiveness — many of the attributes of metastatic cells. One of the most striking defects of *Has2*-null mice is failure to undergo an EMT that is required for cardiac development, which is due to inactivation of ERBB2/ERBB3 signalling. These defects were rescued by transgenic expression of HAS2 or by the addition of small amounts of hyaluronan to *HAS2*-null cells[93,94]. Moreover, experimentally induced stimulation of hyaluronan synthesis in normal epithelium induces mesenchymal characteristics, including anchorage-independent growth, MMP production, invasiveness and rearrangements of components of cell adhesions and the cytoskeleton[64]. In addition, disruption of hyaluronan–CD44 interactions blocks several of the effects of factors that are known to induce these events, such as HGF treatment and overexpression of β-catenin[64]. E-cadherin, which is associated with epithelial adherens junctions, is frequently down-regulated or dispersed from junctions during EMTs[147]. Consistent with this, E-cadherin can function as a suppressor of malignant cell behaviour, and increased expression of E-cadherin negatively regulates hyaluronan–CD44 interactions and the resulting processes that are triggered by this interaction[148]. This again emphasizes the roles of hyaluronan in EMT and the acquisition of malignant cell characteristics. In addition, cooperativity between the WNT signalling pathway and the dispersal of adherens junctions leads to the accumulation of β-catenin in a complex with the transcription factor TCF4/LEF and to the activation of genes that are involved in EMT[147,149]. β-catenin stimulates hyaluronan production, and its ability to induce EMT is dependent on hyaluronan[64]. An important relation between RHAMM and the WNT–β-catenin signalling pathway has also been shown, but implicates RHAMM in mesenchymal rather than epithelial transformation[150]. Clearly, hyaluronan is a key player in these transitions.

Role of hyaluronan in angiogenesis

Angiogenesis is a key step in tumour progression and an important therapeutic target. Several studies have shown that hyaluronan oligosaccharides or small polymer 'fragments' promote angiogenesis[151], indicating that hyaluronan not only stimulates malignant characteristics in cancer cells, but that its breakdown products might also promote tumour progression through the stimulation of angiogenesis.

In addition to hyaluronan levels, hyaluronidase levels are also sometimes increased in tumours. The main hyaluronidase that is expressed in tumours, HYAL1, is only active at acidic pHs. Because small fragments of hyaluronan have been found in tumour extracts[26,152], this might indicate that these fragments are derived from limited digestion by HYAL1 in the acidic conditions that are found in many tumours. Alternatively, they might

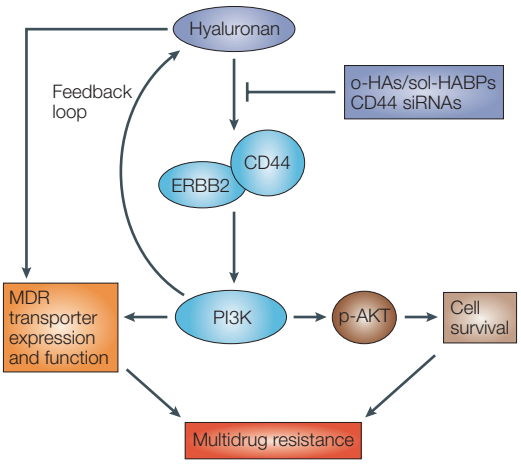


Figure 4 | **Hyaluronan and drug resistance.** Hyaluronan-dependent ERBB2–CD44 interactions regulate phosphatidylinositol 3-kinase (PI3K) activity. PI3K signalling regulates multidrug resistance (MDR) transporter expression and function[110] (S. Misra, S. Ghatak & B.P.T., unpublished observations), and also phosphorylates AKT (p-AKT) to activate cell-survival signalling. The combination of these effects promotes drug resistance. ERBB–PI3K signalling acts in a positive-feedback loop to stimulate further hyaluronan production[64,100,184], which promotes cell survival and drug resistance. These pathways are inhibited by hyaluronan oligomers (o-HAs), soluble hyaluronan-binding proteins (sol-HABPs) and small interfering RNAs (siRNAs) that are directed against CD44 (REF. 69; S. Misra, S. Ghatak & B.P.T., unpublished observations). Recent work indicates that hyaluronan also interacts directly with MDR transporters during synthesis and secretion[111].

arise by digestion with PH20, a hyaluronidase that is active at neutral pH[49]; however, the presence of PH20 in tumours is not well established[26]. Another possible means of fragmentation of hyaluronan is by reactive oxygen species[153,154].

Small fragments or oligosaccharides of hyaluronan stimulate endothelial-cell proliferation, motility and tubule formation, and induce angiogenesis in a variety of experimental systems[155–161]. These oligosaccharides are probably able to interact with CD44 and RHAMM on the surfaces of endothelial cells, although the relative contribution of each receptor is controversial[124,125,160,162]. Hyaluronan fragments also stimulate MMP production and promote migration in some cancer cells[163]. Increased hyaluronidase levels and increased hyaluronan degradation might promote tumour progression through the effects of breakdown products on angiogenesis. This would provide a rationale for the finding that increased hyaluronan plus HYAL1 is a reliable marker for several types of malignant tumour[24,32,34].

Several groups have addressed the possibility that hyaluronan breakdown products stimulate tumour progression in animal models, but the results are not clear-cut. For example, administration of hyaluronan oligosaccharides to various types of tumour xenografts inhibits rather than stimulates tumour growth[66,164]. In addition, experimental overexpression of hyaluronidase suppresses colon and breast carcinoma growth in xenografts[40,43]. However, overexpression of hyaluronidase was found to stimulate prostate tumour metastasis[46] and astrocytoma growth within the brain, but not subcutaneously[45]. Another study indicated that hyaluronidase is required for the promotion of astrocytoma growth by hyaluronan[47]. It is apparent from these studies that the relation between levels of the hyaluronan polymer, hyaluronan degradation products and the malignancy of various types of tumour cell is dependent on tissue context, and that this relationship is complex.

Future directions

An emerging theme in cancer research is the role of the microenvironment in modulating the effects of genetic alterations. Regulatory factors, ECM components and MMPs in the stroma can initiate, promote or suppress malignant changes in epithelia[9,10]. The main source of hyaluronan in many human cancers is the stroma. As is the case for several pro-malignant effectors, there are interactions that flow in both directions between the tumour cells themselves and the stroma. So, tumour cells stimulate stromal cells to produce increased levels of hyaluronan[14,15] and stromal hyaluronan acts on tumour cells to promote malignant characteristics. The same applies to MMPs[9,112] and to the TGF-β family[165]. One mechanism by which hyaluronan affects malignant cell behaviour is by inducing the formation of regulatory complexes that contain CD44 and receptor kinases that have 'classic' oncogenic functions, such as ERBB2 (FIG. 3). Integrins[9], the urokinase plasminogen activator receptor[166] and cell-surface MUCINS[167] also regulate such receptors. These signalling partners activate a network of overlapping downstream pathways that are tightly regulated in normal cells, but provide multiple means of initiating and amplifying proliferation, survival and invasive properties in cancer cells. Therefore, overexpression or disruption of one important factor — such as hyaluronan, an MMP, a growth-factor receptor or a downstream component of cell-survival pathways — can lead to the disruption of an entire network of events within cancer cells. In addition to perturbing intracellular pathways, agents that antagonize hyaluronan interactions are also likely to disrupt the organization and interactions of many other pericellular macromolecules. This in turn could influence their signalling functions and feed into other downstream cascades and networks. A challenge for the future will be to define the mechanisms by which such networks are normally regulated and how they become deregulated in different cancers, as this will help to design new means of correcting the latter, without disturbing the former in a damaging way.

Recent work has shown an important relation between hyaluronan, its receptors and the behaviour of tumour progenitor cells[18,168,169]. This is especially interesting in the light of the identification of CD44 as a marker for breast cancer cells that have stem-cell-like, tumorigenic characteristics[170]. Also of particular significance is the finding that EMMPRIN, which is an upstream regulator of hyaluronan production[113] (BOX 2),

MUCINS
Large extracellular and cell-surface glycoproteins with numerous oligosaccharide side-groups. Mucins have several physiological functions, including signal transduction. Their expression and glycosylation are altered in cancer cells.

is frequently associated with bone-marrow micrometastases[117]. These observations highlight the potentially central importance of hyaluronan in cancer and again emphasize the possibility of developing therapies that disrupt hyaluronan at these sites.

Although there are obviously complexities that still need to be clarified, it is clear that hyaluronan can promote the onset of malignant characteristics, including inappropriate cell survival, multidrug resistance, invasiveness and EMT. Similarly, perturbations of constitutive hyaluronan interactions or inhibition of hyaluronan synthesis reverses many of these characteristics. Some of these perturbations, such as treatment with hyaluronan oligomers or adenoviral delivery of hyaluronan-binding-protein decoys (FIG. 2), could be used to treat patients. EMMPRIN antagonists might also be useful in this way. A particularly attractive therapeutic strategy would be the reversal of multidrug resistance by agents that perturb hyaluronan–tumour interactions (FIG. 4), as such perturbations seem to act at several different levels that are related to malignancy and drug resistance.

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Competing interests statement

The author declares that he has no competing financial interests.

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