

Biology of Extracellular Matrix 14

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Hyaluronan

Structure, Biology and Biotechnology

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Biology of Extracellular Matrix

Volume 14

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Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

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Alberto Passi

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Preface

Hyaluronic acid (HA) is a polymer with a very simple structure but continues to reveal its wonderful and multiple properties. This book reports on some recent aspects related to the metabolism and biology of hyaluronic acid. After its discovery and the definitions of the chemical properties of the polymer that have partly explained its role in the viscoelastic properties of tissues, the discovery of HA receptors and the biological effects of their interaction have recently proposed numerous fields of study. The continuous discoveries include the role of HA in cell motility, in physiological processes such as ageing, and in disease processes including inflammation and cancer. HA was defined “a jealous molecule”, as it is jealous in making known its multiple roles in cell biology, and this reluctance to reveal its biological roles makes this study rich in new discoveries every year. Therefore, the study addressing HA is a challenging field for the scientists who deal with it.

This book is far from giving a complete picture of the roles of HA, because the process of discovering its biological roles and its uses in regenerative medicine is constantly and surprisingly evolving. Despite this, the need for a volume that summarized the latest discoveries of the field is necessary.

The book is organized in ten chapters reporting some aspects of recent interest that have appeared in the literature of this area. The authors are the scientists of the new generation who have inherited the interest in this polymer from illustrious predecessors and collect their legacy.

Chapter 1 addresses the HA synthesis and its regulation. The chapter describes particularly the HAS2 multistep regulation that includes transcriptional, translational, and epigenetic aspects. The elucidation of the molecular mechanisms that regulate HA synthesis is of great interest as alterations of HA in the extracellular matrix are common findings in pathologies.

Chapter 2 addresses the role of hyaluronan in the development. In this context, in fact, the extracellular matrix plays a key role and hyaluronic acid participates in the correct formation of organs and their orientation during development. The chapter of

Demler and Kurpios provides an important update on the latest findings in this context.

Chapter 3 describes the noncoding transcriptome in HA synthesis. Noncoding RNAs are a portion of DNA that is transcribed but not translated into proteins. Although the biological function of noncoding RNAs (ncRNAs) species needs further investigation, emerging evidences showed that they can regulate gene expression through different mechanisms, including epigenetic modifications. The authors addressed in this chapter the HAS2 regulation due to noncoding RBNAs and their potential role in human pathology.

Chapter 4 addresses an important new aspect of the HA biology and its role in the extracellular vesicles (EVs). These HA-coated EVs constitute a specific vesicle population with unique surface properties, and it is suggested that can regulate EV homing, targeting, binding, and remodelling of the ECM. The chapter by Kirsi Rilla discusses the recent advances in the biology of HA-coated filopodia and HA-coated EVs and their role as versatile nanosized communicators in health and disease, the formation mechanisms as well as their effects on target cells and their potential for clinical utilization are also discussed.

Chapter 5 presents the role of HA in a chronic disease underlining critical aspects of the latest discoveries. The chapter describes current understanding of the mechanisms controlling the upregulation of HA in progressive disease in kidney. The authors show a complete approach with potential recapitulation of embryonic events and which intracellular signalling pathways may have the potential to prevent or reverse the progression that leads to End Stage Renal Failure.

Chapter 6 presents a broad discussion of the role of inter- α -inhibitor-family proteins (IAIP), proteins that can bind HA under different conditions and whose role in human pathology is still far from being fully understood. Initially IAIP have been described in their capacity to bind and stabilize the extracellular matrix sugar hyaluronan (HA), but IAIP are now understood to exert manifold biological effects that place them at the centre of reproductive biology, immunology, organ development and cancer biology and more other areas. The themes proposed by the chapter underline how these molecules are critical in many aspects of human physiology and pathology and deserve greater attention for possible future developments for new therapeutic targets.

In Chapter 7, the topics related to the CD44v6 receptor of hyaluronic acid and its roles in the biology of tumours have been addressed. The relationship between HA and the development of cancer is based on the interaction of the polymer with its receptors whose complex biology is represented in this chapter describing the latest discoveries in the field.

In Chapter 8 was underlined the role of HA in many human pathologies is of great importance and the control with drugs of its synthesis and described in this chapter. The synthesis of HA can be controlled with a drug: 4-MU. In this chapter, the authors describe its pharmacological characteristics and underline its possible uses in human pathology.

Chapter 9 addresses the aspect of the role of HA in wound healing. HA plays an important role in regenerative medicine and this chapter describes the latest findings

regarding the role of HA in the wound healing procedure. The importance of this biological role is easily understood because many advanced dressings are in fact based on the use of HA.

The final Chapter 10 is focussed on a novel aspect of the biochemistry of HA that is showing extraordinary interest. The authors described the potential roles of sulphated hyaluronan oligosaccharides in human pathologies and in the bioengineering regenerative medicine with new scaffolds.

In conclusion the chapters of this book, far from giving conclusive answers to the multiple roles that this polymer has in cell biology and in the various pathologies as well as to the numerous uses it can have in medicine, however, it gives an update on the most recent discoveries in the field and makes it even clearer how much still exists to be discovered in this field in the future.

Varese, Italy

Alberto Passi

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Chapter 1

Biochemistry of Hyaluronan Synthesis



Ilaria Caon, Arianna Parnigoni, Evgenia Karousou, Alberto Passi,
Davide Vigetti, and Manuela Viola

Abstract Hyaluronan (HA) is a ubiquitous component of the extracellular matrix and cell microenvironment made of β D-Glucuronic acid and D-N-Acetylglucosaminedisaccharides bound by alternating glycosidic $\beta(1,3)$ and $\beta(1,4)$ linkage, respectively. Although it belongs to the family of glycosaminoglycans, it has several peculiarities. HA chains can greatly vary in length, it is not chemically modified as it does not contain sulfation and epimerization. Further, HA is synthesized by a family of three HA synthases (HAS1, 2, and 3) located on the plasma membrane. These proteins are undefined enzymes as they are never crystallized and contain all the functions required to recognize two different substrates, catalyze two different glycosidic bonds, and extrude the nascent polysaccharide through the membrane. Moreover, HASes are the key point of regulation of HA synthesis and, in Mammals, HAS2 is the most important isoenzyme. HAS2 undergoes a multistep regulation that comprises transcriptional, translational, and epigenetic modifications and integrates the metabolic status of the cell as well as the external stimuli finely adjusting the production of HA. The elucidation of the molecular mechanisms that regulate HA synthesis is of great interest as alterations of HA in the extracellular matrix are found in common pathologies and seems to have a pivotal role in the control of several aspects of cell biology as motility, survival, and proliferation. This chapter will focus on the biochemistry of HA synthesis, HASes and their regulation.

Ilaria Caon and Arianna Parnigoni contributed equally with all other contributors.

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1.1 Introduction

Over the decades, complex polysaccharides have become of fundamental importance, since those molecules of amazing diversity directly mediate a wide range of functions, from structure and storage to signalling modulation. Glycosaminoglycans (GAGs) are among the most complex polysaccharides found in the extracellular matrix that have many critical roles in tissue homeostasis and pathology (Karamanos et al. 2018; Manou et al. 2019).

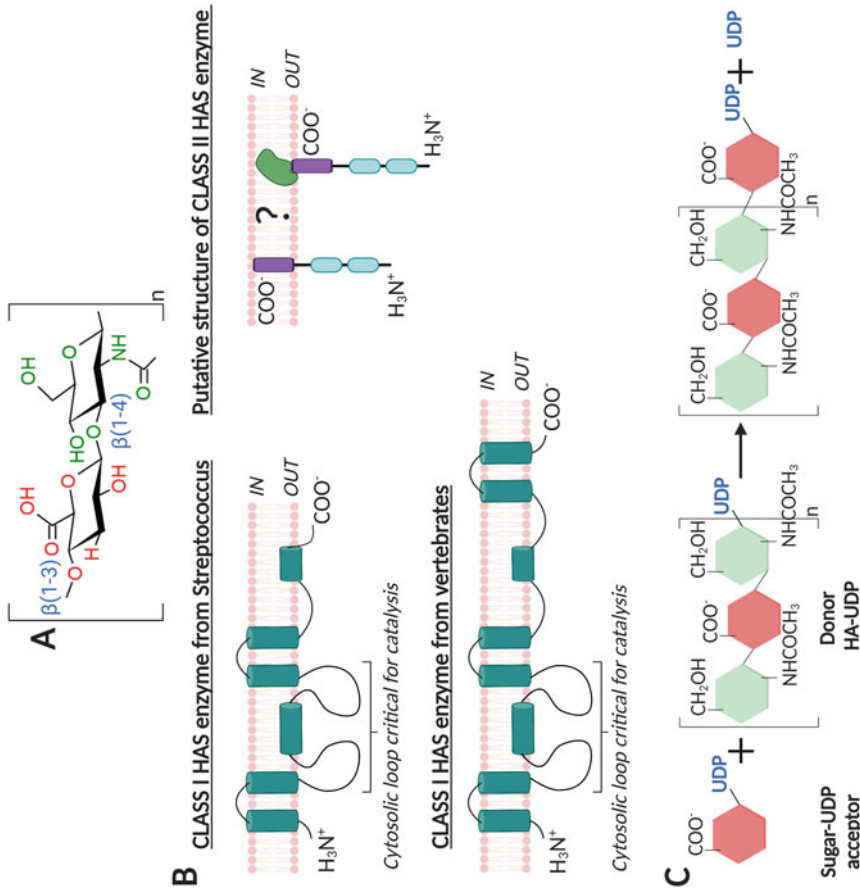
The chemical linkage of sugars in polysaccharides is carried out by enzymes of the glycosyltransferases (EC 2.4) subclass, which generally transfer glycosyl groups, including transfer to water or inorganic phosphate in case of enzymes that catalyse hydrolysis. Sub-subclasses are based on the type of sugar residue being transferred: hexosyltransferases (EC 2.4.1), pentosyltransferases (EC 2.4.2), and other glycosyl groups (EC 2.4.99).

GAGs are long linear polysaccharides consisting of repeating disaccharide units composed by an amino sugar, along with a uronic sugar or galactose (in the only case of keratan sulfate) and GAGs biosynthesis is catalysed by enzymes belonging to the family of hexosyltransferase located in the Golgi as well as on the plasma membrane. For a rapid overview of GAGs chemical structure, see (Karousou et al. 2008).

Since the first classification of glycosyltransferases recommended by the International Union of Biochemistry and Molecular Biology (IUBMB), it was evident that the biosynthesis of polysaccharides and complex carbohydrates was a huge part of the chemical reactions of the biological world counting now 1374 over a total of 1456 glycosyltransferases (Campbell et al. 1997). Among these enzymes, many of them are involved in the glycosaminoglycans synthesis in which the nascent sugar chain elongates on a pre-formed proteoglycan core protein.

1.2 Chemistry of the Polymerization

Hyaluronic acid (or hyaluronan, HA) is a peculiar GAG. It has the distinctive feature to be synthesized at the plasma membrane level as a polysaccharide chain free from linkage to any proteoglycan core protein. The HA disaccharide unit is $[-\beta(1,4)\text{-Glucuronic acid (GlcUA)}-\beta(1,3)\text{-N-Acetyl-glucosamine (GlcNAc)}-\beta 1-]_n$ in which the enzymes hyaluronan synthase (HAS) (E.C. 2.4.1.212) alternate the bond of GlcUA and GlcNAc in two different linkages, $\beta 1-3$ and $\beta 1-4$, respectively (Fig. 1.1a) (Moretto et al. 2015). Among glycosyltransferases, HASes are quite peculiar enzymes since they recognize and transfer two different sugars in an alternate and regular fashion on the growing polysaccharide chain using the appropriate UDP-sugars present in the cytoplasm. Moreover, HAS are able to extrude the huge molecule through the plasma membrane in the extracellular space. Although the synthesis of HA is a complex process, it requires only one enzyme that contains all these different functions (Kyosseff and Weigel 2007).



In human, there are three different HAS isoenzymes (HAS1, 2, and 3) encoded by three *HAS* different genes (Itano and Kimata 2002). HASes use the hexoses sugars from their precursors UDP-GlcUA and UDP-GlcNAc in the presence of Mg^{2+} or Mn^{2+} and the synthesis leads to the construction of a very long unbranched polysaccharide chain, up to 10^4 or more disaccharide units, resulting in a molecule up to a molecular mass of ~four million Daltons (Tavianatou et al. 2019a).

From an evolutionary point of view, the first evidence of HA was around 500 million years ago in chordates (Weigel and DeAngelis 2007). Although chordates are the first animal able to produce HA, they possess three to four *HAS* genes and in all the animal kingdom there is no species with only one *HAS* gene. Genomic analyses highlighted a large conserved vertebrate *HAS* gene family evolved by sequential gene duplication and divergence (Spicer and McDonald 1998). Interestingly, some microorganisms have the ability to synthesize HA as some bacterial human pathogens such as Gram-positive streptococci (e.g. *S. pyogenes*, *S. equisimilis*, *Streptococcus uberis*, and *Streptococcus zooepidemicus*), and Gram-negative *Pasteurella multocida*, as well as one algal virus (*Paramecium bursaria Chlorella* virus (PBCV-1) (DeAngelis 1999; Csoka and Stern 2013). Intriguingly, in all these microorganisms there is only one gene coding for HAS, and the origins of these genes are still difficult to unravel.

HASes protein can be divided in 2 families. Class I family includes almost all HAS enzymes (i.e. Streptococcus, amphibian, mammalian) while in Class II there is only the *Pasteurella multocida* enzyme which possesses very peculiar properties such as the membrane topology (Fig. 1.1b) and the mechanism of hyaluronan synthesis (Mandawe et al. 2018). Subsequent analysis of the catalytic mechanism suggests a further division of class I enzymes into two subfamilies, in fact, multiple studies confirmed the reducing end chain elongation for streptococcal, mouse, and human HASes, as well as the presence of UDP at the reducing end of growing chains, while amphibian *Xenopus laevis* HAS utilizes the non-reducing end, like the *Pasteurella multocida* hyaluronan synthase (Siiskonen et al. 2015). When the elongation is performed at the reducing end, the UDP-sugars are acceptors that receive the growing HA chain from an HA-UDP donor (Fig. 1.1c) (Vigetti et al. 2006; Viola et al. 2016; Weigel et al. 2017).

Excluding the Class II *Pasteurella multocida* HAS that is anchored to the membrane via a not yet defined mechanism involving the C-terminal of the proteins, Class I HASes are multi-pass transmembrane enzymes. The Streptococcal HASes possess 4 transmembrane domains, whereas mammalian proteins contain 2 extra transmembrane helices in the C-terminal (Vigetti et al. 2014b) (Fig. 1.1b). Regarding sequence homology, the streptococcal HASes are about 70% identical to each other and about 25% identical to the vertebrate HASes, while *Pasteurella multocida* (PmHAS) is totally different from all other HASes (Weigel and DeAngelis 2007).

Fig. 1.1 (continued) (bottom left). On the right, prediction of Class II hyaluronan synthase way of association to plasma membrane: direct binding through a membrane-associated domain at the C-terminus, or indirect via a binding interaction between its C-terminal domain and an unidentified integral membrane component. (c) Mechanism of hyaluronan synthesis at the reducing end

The initiation of the HA polymerization is a chemical puzzle that was hard to investigate and matter of speculation for several years; recently, a group of researchers proposed a new point of view to this old problem. From an evolutionary point of view, chitin can be considered the precursor of HA [no carried by proteins, no sulfation, connected exclusively by β bonds], which is prominent in invertebrates, from fungi to arthropods, but that has now been documented in vertebrates too (Ziatabar et al. 2018). The structure of chitin is very similar to that of HA, being an unbranched chain composed by β -(1,4)-poly-N-acetyl-glucosamine. Moreover, both chitin synthases (CHS) and hyaluronan synthases (HAS) are classified in the CAZY database as belonging to the GT-2 family (Coutinho et al. 2003), and the CHS chain synthesis is reported to occur to the non-reducing end of the molecules as for the *Xenopus laevis* HAS1 (Bodevin-Authelet et al. 2005). This suggests an evolution of the biochemistry of HA that is still visible in the distinct mechanisms of its polymerization through the “different kingdoms of life”(Blackburn et al. 2018).

Based on these considerations, Weigel et al. described that, despite the “usual” use of 2 different UDP-substrates (UDP-GlcUA and UDP-GlcNAc), class I HASes can work in the presence of GlcNAc(α 1 \rightarrow)UDP only, polymerizing a short chain, identified as (GlcNAc- β 1,4) $_n$ -GlcNAc(α 1 \rightarrow)UDP (Weigel et al. 2014), structurally similar to chitin chain. Moreover, in a further study, the same group concluded that “chitin-UDP functions in vitro and in live cells as a primer to initiate synthesis of all HA chains and these primers remain at the NR-ends of HA chains as residual chitin caps” [(GlcNAc- β 1,4)3–4] (Weigel et al. 2017). The most recent model of class I HAS mechanism of catalysis was the “pendulum model” proposed by Paul Weigel in 2015 (Weigel 2015). Recently the structure of a viral HAS has been resolved by cryo-electron microscopy supporting this pendulum model (Maloney et al. 2022)

1.3 Enzymes Involved in HA Synthesis

In addition to HASes that polymerize HA, several other enzymes are necessary to allow the proper biosynthesis of the substrates as UDP-glucose pyrophosphorylase (UGPP), UDP-glucose 6-dehydrogenase (UGDH), and the hexosamine biosynthetic pathway enzymes (Figure2). Although HA synthesis does not directly consume ATP, the production of the UDP-sugar substrates depends not only on energy availability, but also by several other pathways that integrate metabolism and catabolism (Tammi et al. 2019; Caon et al. 2020b).

1.3.1 HASes

As mentioned above, vertebrates have three isoenzymes responsible for cellular HA synthesis that possess different affinity for the substrates and kinetic properties. Among HASes, HAS1 shows the lowest affinity for UDP-GlcUA and UDP-GlcNAc, allowing an efficient synthesis of HA only in condition of abundance

of substrates. On the other hand, HAS2 and 3 have high affinity for the substrates and are the most processive enzymes in physiological conditions. Interestingly, HAS3 has the capability of synthesizing shorter HA polymer compared to HAS1 and 2. How the enzymes regulate the length of the growing HA polymer is another unclear point. UDP-sugars availability has a critical role in determining HA lengths. When substrates became limiting the molecular mass of the polysaccharide decreases. By using different stoichiometry (i.e., UDP-GlcUA and UDP-GlcNAc ratio) biotechnology companies are able to produce monodispersed HA (i.e., polymer of defined lengths). Recently it was discovered that also the C-terminal part of HASEs has a role in the regulation of HA molecular mass (Baggenstoss et al. 2017; Yu et al. 2017), by testing the effects of several mutations and C-terminal truncations.

Human adult tissues and cell lines express HAS2 and HAS3 as main isoforms, while it is still unclear the role of HAS1, whose expression seems to be restricted in specific tissues and cells. Interestingly, HAS1, HAS2, and HAS3 form homo- and heteromeric complexes, which represent the active forms of HASEs in plasma membranes.

HAS1 seems to be the less important HAS isoform contributing to HA biosynthesis and it remains a mysterious enzyme. Has1 knockout mice are apparently normal (Spicer and Nguyen 1999), however, phenotypes can be observed in HAS1-HAS3 double knockout animals that show alteration in skin inflammation and wound closure (MacK et al. 2012). HAS1 is the main HA synthase in keratinocytes (Malaisse et al. 2014) and seems that has a role in determining the HA rich niche that maintains the stemness of the cells (Chanmee et al. 2015). Moreover, together with HAS2, it is involved in determining the survival and functions of myenteric neurons during ischemia/reperfusion and in a model of experimentally induced colitis (Filpa et al. 2017; Bistoletti et al. 2020).

HAS2 is the most important HA-synthesizing enzyme and it is involved in several pathophysiological processes, such as carcinogenesis (Caon et al. 2019; Tavianatou et al. 2019b), cardiovascular diseases (Caon et al. 2020a), atherosclerosis and restenosis (Chai et al. 2005), fibrosis (Meran and Steadman 2011; Yang et al. 2019) and longevity (Tian et al. 2013). Interestingly, HAS2 is also critical in heart formation during embryogenesis and, therefore, it is essential for life. As the critical role of HAS2, it is not surprising that such enzyme has a multi-level regulation from transcriptional to post-translational level as well as epigenetics.

In contrast to HAS1 and 2 that synthesize HA polymers of large size (about 2×10^6 Da), HAS3 produces HA polymers of lower molecular mass ranging from 1×10^5 to 1×10^6 Dalton (Itano et al. 1999). Considering that the length of HA is crucial in determining its pro- and anti-inflammatory properties (Cyphert et al. 2015; Tavianatou et al. 2019a), HAS3 seems to be associated to processes that modulate immune response (Kessler et al. 2015; Homann et al. 2018). Recently HAS3 was associated to age-related ovary stiffness and fibrosis (Amargant et al. 2020). HAS3 is clearly involved in favouring the formation of cellular protrusions as filopodia which have critical function in motility and cancer invasion (Kultti et al. 2006; Koistinen et al. 2015). Nevertheless, there is little literature about specific HAS3 regulation (Rilla et al. 2012; Bai et al. 2018; Czyrnik et al. 2020).

1.3.2 *UGPP and UGDH*

UGPP is required to convert the glycolytic metabolite glucose 1 phosphate to UDP-glucose through an irreversible reaction. UDP-glucose is an essential precursor of glycogen in the cytoplasm and for the synthesis of glycoconjugates in the ER and Golgi. Although the crucial function of UGPP in the literature, studies of the properties and regulation of the animal enzyme are very limited.

UGDH is required to oxidize UDP-glucose to UDP-GlcUA by producing 2 molecules of NADH. Besides the synthesis of HA, UDP-GlcUA is involved in other cellular pathways such as the synthesis of proteoglycans and glycosaminoglycans via its conversion in UDP-xylose. Moreover, it is the substrate of glucuronidation and detoxification reactions (Vitale et al. 2021; Zimmer et al. 2020). Therefore, the amount of UDP-GlcUA available for HAS enzymes (i.e., UDP-GlcUA in the cytosol) is variable and depends on the activity of several other pathways. Moreover, the cytosolic availability of UDP-GlcUA depends on the activity of transporters (i.e., the sugar nucleotide transporter SLC35D1) that are able to shift UDP-GlcUA in the ER and Golgi (Vigetti et al. 2014b; Viola et al. 2017), connecting the HA metabolism to other GAGs synthesis. Interestingly, UGDH is also able to affect NAD: NADH ratio, which, in turn, regulates different cellular functions as sirtuins (NAD dependent deacetylases), that are recently shown to regulate HA synthesis (Caon et al. 2020a). UDP-GlcUA is crucial for HA synthesis, in fact, 4-methylumbelliferone (4-MU), the only well-known HAS inhibitor, reduces HA production limiting UDP-GlcUA availability via 4-MU glucuronidation (Vigetti et al. 2009b; Caon et al. 2020b).

1.3.3 *The Hexosamine Biosynthetic Pathway*

The hexosamine biosynthetic pathway is responsible for the production of UDP-GlcNAc (Fig. 1.2). Only about 2 to 5% of the intracellular glucose is produced through the hexosamine biosynthetic pathway (McClain and Crook 1996), but in condition of abundance of glucose (i.e. diabetes) this flux increases. The critical enzyme of the hexosamine biosynthetic pathway is the glutamine fructose-6-phosphate amidotransferase (GFAT), which regulates HA biosynthesis by modulating UDP-GlcNAc availability (Oikari et al. 2016). Mannose is another inhibitor of HA synthesis in cultured cells. Despite the molecular mechanism that leads to a reduction of HA biosynthesis is not fully understood it seems that mannose could alter the hexosamine biosynthetic pathway and GFAT (Jokela et al. 2008). Interestingly, hexosamine biosynthetic pathway integrates critical metabolites coming from amino acids (glutamine), nucleotides (uridine), carbohydrates (glucose) and fatty acids (Acetyl-CoA) metabolisms. Therefore, the cytosolic concentration of UDP-GlcNAc reflects the metabolic status of the cells and is considered a “sensing molecule”. When UDP-GlcNAc increases the enzyme O-GlcNAc transferase (OGT) rapidly modifies the hydroxyl group of serine and threonine by adding a single

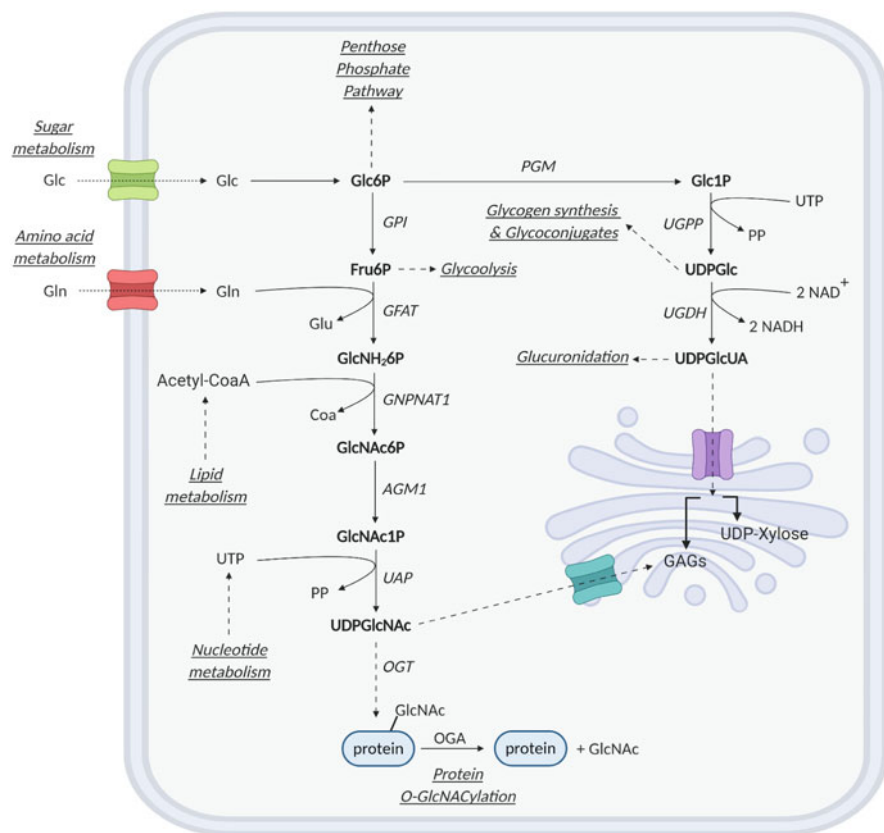


Fig. 1.2 Schematic representation of UDP-GlcUA and UDP-GlcNAc biosynthetic pathways. Connections with the main biochemical pathways are indicated with dotted lines. Abbreviation: *Glc* glucose, *Gln* glucosamine, *GlcNH₂* glucosamine, *Glc6P* glucose-6-phosphate, *Glc1P* glucose-1-phosphate, *UDPGlc* UDP-glucose, *UDPGlcUA* UDP-glucuronic acid, *Fru6P* Fructose-6-phosphate, *GlcNH₂6P* Glucosamine-6 phosphate, *GlcNAc6P* N-acetyl-glucosamine-6-phosphate, *GlcNAc1P* N-acetyl-glucosamine-1-phosphate, *UDPGlcNAc* UDP-N-acetyl-glucosamine, *Glu* glutamate, *Acetyl-CoA* acetyl-coenzyme A, *PP* pyrophosphate, *HK* hexokinase, *GK* glucokinase, *GPI* glucose-6-phosphate isomerase, *PGM* phosphoglucomutase, *UGPP* UDP-Glucose pyrophosphorylase, *UGDH* UDP-Glucose dehydrogenase, *GFAT* glutamine: fructose-6-phosphate amidotransferase, *GNK* glucosamine kinase, *GNPNAT1* GlcNH 2-6-phosphate N-acetyltransferase, *AGM1* phospho-GlcNAc mutase, *UAP* UDP-GlcNAc pyrophosphorylase, *OGT* O-GlcNAc transferase, *OGA* O-GlcNAcase

GlcNAc sugar leading to the cytosolic glycosylation named O-GlcNAcylation that can trigger several cellular responses.

1.3.4 Regulation of the HAS2 Enzyme

Although there are three synthetic enzymes responsible for HA production, in Mammals the most known protein is HAS2. The amount of HAS2 on the cell surface

Table 1.1 List of known HAS2enzymes post-translational modifications and modulator molecules

Modulator	Site	Modification	Effect	Reference
AMPK	T110	Phosphorylation	Inhibition Destabilization	(Vigetti et al. 2011) (Melero-Fernandez de Mera et al. 2018)
Casein kinase 1	T328	Phosphorylation	Increase activity	(Kasai et al. 2020)
OGT	S221	O-GlcNAcylation	Stabilization Localization	(Vigetti et al. 2012) (Melero-Fernandez de Mera et al. 2018)
Ubiquitin	K190	Ubiquitination	Increased activity Dimerization Dominant negative	(Karousou et al. 2010)
Cholesterol	Lipid microenvironment	Not determined	Increase activity	(Ontong et al. 2014)
Autophagy	Not determined	Interaction with ATG9A	Degradation	(Chen et al. 2020)

is finely tuned through a multi-level regulation. In humans the *Has2* gene is located on chromosome 8 and several transcription factors are known to sustain a basal HAS2 mRNA transcription (Tammi et al. 2011). In the nucleus, epigenetics has a crucial role in controlling *Has2* gene transcription via the long non-coding RNA HAS2-AS1 (Parnigoni et al. 2021). This is an antisense RNA as it is transcribed on the opposite strand of DNA in the *Has2* locus. HAS2-AS1 modulates chromatin structure around HAS2 promoter, controlling accessibility of transcription factors in case of nutrients abundance or deprivation (Vigetti et al. 2014a). In both these cases HAS2-AS1 is strictly controlled by NF- κ B that is activated via protein O-GlcNAcylation in nutrient-rich conditions or inhibited by deacetylation due to sirtuin activation in condition of starvation (Caon et al. 2020a). Moreover, HAS2-AS1 has been shown to interact and stabilize HAS2 transcript favouring HAS2 translation in particular cancers (Michael et al. 2011). In different tumor cells, HAS2 mRNA stability is also influenced by several miRNA that can interact with 3' UTR influencing cell behaviour (Lagendijk et al. 2011; Röck et al. 2015; Pan et al. 2017). Interestingly, it is recently reported that in triple negative breast cancer cells HAS2-AS1 controls other cellular pathways as epithelial to mesenchymal transition (Parnigoni et al. 2022).

Once translated in the secretory pathway, HAS2 protein trafficking is finely regulated by glycosylation and phosphorylation before reaching the plasma membrane (Vigetti et al. 2009a; Hascall et al. 2014; Melero-Fernandez de Mera et al. 2018). In plasma membrane HAS2 activity is influenced by lipid microenvironment (Ontong et al. 2014). Moreover, several post-translational modifications as ubiquitination (Karousou et al. 2010), phosphorylation (Vigetti et al. 2011; Yamane et al. 2011; Kasai et al. 2020) and O-GlcNAcylation (Vigetti et al. 2012) are able to

alter enzymatic activity and stability as summarized in Table 1.1 [for a detailed review of such aspects see (Caon et al. 2020b)]. The degradation of HAS2 is also finely tuned and depends on proteasome activity, lysosomes and autophagy (Vigetti et al. 2012; Melero-Fernandez de Mera et al. 2018; Chen et al. 2020; Chen and Iozzo 2020). Recently, it is reported that the secreted protein c10orf118 present in tumor microenvironment is able to modulate HAS2 expression (Caon et al. 2021).

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References

- Amargant F, Manuel SL, Tu Q et al (2020) Ovarian stiffness increases with age in the mammalian ovary and depends on collagen and hyaluronan matrices. *Aging Cell* 19:e13259. <https://doi.org/10.1111/ace1.13259>
- Baggenstoss BA, Harris EN, Washburn JL et al (2017) Hyaluronan synthase control of synthesis rate and hyaluronan product size are independent functions differentially affected by mutations in a conserved tandem B-X7-B motif. *Glycobiology* 27:154–164. <https://doi.org/10.1093/glycob/cwx089>
- Bai F, Jiu M, You Y et al (2018) MiR-29a-3p represses proliferation and metastasis of gastric cancer cells via attenuating HAS3 levels. *Mol Med Rep* 17:8145–8152. <https://doi.org/10.3892/mmr.2018.8896>
- Bistoletti M, Bosi A, Caon I et al (2020) Involvement of hyaluronan in the adaptive changes of the rat small intestine neuromuscular function after ischemia/reperfusion injury. *Sci Rep* 10:11521. <https://doi.org/10.1038/s41598-020-67876-9>
- Blackburn MR, Hubbard C, Kiessling V et al (2018) Distinct reaction mechanisms for hyaluronan biosynthesis in different kingdoms of life. *Glycobiology* 28:108–121. <https://doi.org/10.1093/glycob/cwx096>
- Bodevin-Authelet S, Kusche-Gullberg M, Pummil PE et al (2005) Biosynthesis of Hyaluronan: direction of chain elongation. *J Biol Chem* 280:8813–8818. <https://doi.org/10.1074/jbc.M412803200>
- Campbell JA, Davies GJ, Bulone V, Henrissat B (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities [1]. *Biochem J* 326:929–939
- Caon I, D’Angelo ML, Bartolini B, Caravà E, Parnigoni A, Contino F, Cancemi P, Moretto P, Karamanos NK, Passi A, Vigetti D, Karousou E, Viola M (2021) The secreted protein C10orf118 is a new regulator of hyaluronan synthesis involved in tumour-stroma cross-talk. *Cancers (Basel)* 13(5):1105. <https://doi.org/10.3390/cancers13051105>
- Caon I, Bartolini B, Moretto P et al (2020a) Sirtuin 1 reduces hyaluronan synthase 2 expression by inhibiting nuclear translocation of NF-κB and expression of the long-noncoding RNA HAS2-AS1. *J Biol Chem* 295:3485–3496. <https://doi.org/10.1074/jbc.RA119.011982>
- Caon I, Bartolini B, Parnigoni A et al (2019) Revisiting the hallmarks of cancer: the role of hyaluronan. *Semin Cancer Biol* 62:9–19
- Caon I, Parnigoni A, Viola M et al (2020b) Cell energy metabolism and Hyaluronan synthesis. *J Histochem Cytochem* First Published July 6(2020):35–47

- Chai S, Chai Q, Danielsen CC et al (2005) Overexpression of hyaluronan in the tunica media promotes the development of atherosclerosis. *Circ Res* 96:583–591. <https://doi.org/10.1161/01.RES.0000158963.37132.8b>
- Chanmee T, Ontong P, Kimata K, Itano N (2015) Key roles of hyaluronan and its CD44 receptor in the stemness and survival of cancer stem cells. *Front Oncol* 5:1
- Chen CG, Gubbiotti MA, Kapoor A et al (2020) Autophagic degradation of HAS2 in endothelial cells: a novel mechanism to regulate angiogenesis. *Matrix Biol* 90:1. <https://doi.org/10.1016/j.matbio.2020.02.001>
- Chen CG, Iozzo RV (2020) Angiostatic cues from the matrix: endothelial cell autophagy meets hyaluronan biology. *J Biol Chem jbc.REV120.014391:16797*. <https://doi.org/10.1074/jbc.rev120.014391>
- Coutinho PM, Deleury E, Davies GJ, Henrissat B (2003) An evolving hierarchical family classification for glycosyltransferases. *J Mol Biol* 328:307–317. [https://doi.org/10.1016/S0022-2836\(03\)00307-3](https://doi.org/10.1016/S0022-2836(03)00307-3)
- Csoka AB, Stern R (2013) Hypotheses on the evolution of hyaluronan: a highly ironic acid. *Glycobiology* 23:398–411. <https://doi.org/10.1093/glycob/cws218>
- Cyphert JM, Trempus CS, Garantzios S (2015) Size matters: molecular weight specificity of Hyaluronan effects in cell biology. *Int J Cell Biol* 2015:1–8. <https://doi.org/10.1155/2015/563818>
- Czyrnik ED, Wiesehöfer M, Dankert JT, Wennemuth G (2020) The regulation of HAS3 by miR-10b and miR-29a in neuroendocrine transdifferentiated LNCaP prostate cancer cells. *Biochem Biophys Res Commun* 523:713–718. <https://doi.org/10.1016/j.bbrc.2020.01.026>
- DeAngelis PL (1999) Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. *Cell Mol Life Sci* 56:670–682
- Filpa V, Bistoletti M, Caon I et al (2017) Changes in hyaluronan deposition in the rat myenteric plexus after experimentally-induced colitis. *Sci Rep* 7:17644. <https://doi.org/10.1038/s41598-017-18020-7>
- Hascall VC, Wang A, Tammi M et al (2014) The dynamic metabolism of hyaluronan regulates the cytosolic concentration of UDP-GlcNAc. *Matrix Biol* 35:14–17. <https://doi.org/10.1016/j.matbio.2014.01.014>
- Homann S, Grandoch M, Kiene LS et al (2018) Hyaluronan synthase 3 promotes plaque inflammation and atheroprogession. *Matrix Biol* 66:67–80. <https://doi.org/10.1016/j.matbio.2017.09.005>
- Itano N, Kimata K (2002) Mammalian Hyaluronan Synthases IUBMB Life (International Union Biochem Mol Biol Life) 54:195–199. <https://doi.org/10.1080/15216540214929>
- Itano N, Sawai T, Yoshida M et al (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274:25085–25092. <https://doi.org/10.1074/JBC.274.35.25085>
- Jokela TA, Jauhainen M, Auriola S et al (2008) Mannose inhibits hyaluronan synthesis by down-regulation of the cellular pool of UDP-N-acetylhexosamines. *J Biol Chem* 283:7666–7673. <https://doi.org/10.1074/jbc.M706001200>
- Karamanos NK, Piperigkou Z, Theocharis AD et al (2018) Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem Rev* 118:9152–9232
- Karousou E, Kamiryo M, Skandalis SS et al (2010) The activity of hyaluronan synthase 2 is regulated by dimerization and ubiquitination. *J Biol Chem* 285:23647–23654. <https://doi.org/10.1074/jbc.M110.127050>
- Karousou E, Viola M, Vigetti D et al (2008) Analysis of Glycosaminoglycans by electrophoretic approach. *Curr Pharm Anal* 4:78–89. <https://doi.org/10.2174/157341208784246260>
- Kasai K, Kuroda Y, Takabuchi Y et al (2020) Phosphorylation of Thr328 in hyaluronan synthase 2 is essential for hyaluronan synthesis. *Biochem Biophys Res Commun* 533:732. <https://doi.org/10.1016/j.bbrc.2020.08.093>

- Kessler SP, Obery DR, De La Motte C (2015) Hyaluronan synthase 3 null mice exhibit decreased intestinal inflammation and tissue damage in the DSS-induced colitis model. *Int J Cell Biol* 2015:1. <https://doi.org/10.1155/2015/745237>
- Koistinen V, Kärnä R, Koistinen A et al (2015) Cell protrusions induced by hyaluronan synthase 3 (HAS3) resemble mesothelial microvilli and share cytoskeletal features of filopodia. *Exp Cell Res* 337:179–191. <https://doi.org/10.1016/j.yexcr.2015.06.016>
- Kultti A, Rilla K, Tiitonen R et al (2006) Hyaluronan synthesis induces microvillus-like cell surface protrusions. *J Biol Chem* 281:15821–15828. <https://doi.org/10.1074/jbc.M512840200>
- Kyossev Z, Weigel PH (2007) An enzyme capture assay for analysis of active hyaluronan synthases. *Anal Biochem* 371:62–70. <https://doi.org/10.1016/j.ab.2007.08.025>
- Legendijk AK, Goumans MJ, Burkhard SB, Bakkers J (2011) MicroRNA-23 restricts cardiac valve formation by inhibiting has2 and extracellular hyaluronic acid production. *Circ Res* 109:649–657. <https://doi.org/10.1161/CIRCRESAHA.111.247635>
- MacK JA, Feldman RJ, Itano N et al (2012) Enhanced inflammation and accelerated wound closure following tetraphorbol ester application or full-thickness wounding in mice lacking hyaluronan synthases Has1 and Has3. *J Invest Dermatol* 132:198–207. <https://doi.org/10.1038/jid.2011.248>
- Malaisse J, Bourguignon V, De Vuyst E et al (2014) Hyaluronan metabolism in human keratinocytes and atopic dermatitis skin is driven by a balance of hyaluronan synthases 1 and 3. *J Invest Dermatol* 134:2174–2182. <https://doi.org/10.1038/jid.2014.147>
- Maloney FP, Kuklewicz J, Corey RA, Bi Y, Ho R, Mateusiak L, Pardon E, Steyaert J, Stansfeld PJ, Zimmer J (2022) Structure, substrate recognition and initiation of hyaluronan synthase. *Nature* 604(7904):195–201. <https://doi.org/10.1038/s41586-022-04534-2>
- Mandawe J, Infanzon B, Eisele A et al (2018) Directed evolution of hyaluronic acid synthase from *Pasteurella multocida* towards high-molecular-weight hyaluronic acid. *Chembiochem* 19:1414–1423. <https://doi.org/10.1002/cbic.201800093>
- Manou D, Caon I, Bouris P et al (2019) The complex interplay between extracellular matrix and cells in tissues. *Methods Mol Biol* 1952:1–20
- McClain DA, Crook ED (1996) Hexosamines and insulin resistance. *Diabetes* 45:1003–1009
- Melero-Fernandez de Mera RM, Arasu UT, Kärnä R et al (2018) Effects of mutations in the post-translational modification sites on the trafficking of hyaluronan synthase 2 (HAS2). *Matrix Biol* 80:85. <https://doi.org/10.1016/j.matbio.2018.10.004>
- Meran S, Steadman R (2011) Fibroblasts and myofibroblasts in renal fibrosis. *Int J Exp Pathol* 92:158–167
- Michael DR, Phillips AO, Krupa A et al (2011) The human hyaluronan synthase 2 (HAS2) gene and its natural antisense RNA exhibit coordinated expression in the renal proximal tubular epithelial cell. *J Biol Chem* 286:19523–19532. <https://doi.org/10.1074/jbc.M111.233916>
- Moretto P, Karousou E, Viola M et al (2015) Regulation of hyaluronan synthesis in vascular diseases and diabetes. *J Diabetes Res* 2015:1. <https://doi.org/10.1155/2015/167283>
- Oikari S, Makkonen K, Deen AJ et al (2016) Hexosamine biosynthesis in keratinocytes: roles of GFAT and GNPDA enzymes in the maintenance of UDP-GlcNAc content and hyaluronan synthesis. *Glycobiology* 26:710–722. <https://doi.org/10.1093/glycob/cww019>
- Ontong P, Hatada Y, Taniguchi S et al (2014) Effect of a cholesterol-rich lipid environment on the enzymatic activity of reconstituted hyaluronan synthase. *Biochem Biophys Res Commun* 443:666–671. <https://doi.org/10.1016/j.bbrc.2013.12.028>
- Pan B, Toms D, Li J (2017) MicroRNA-574 suppresses oocyte maturation via targeting hyaluronan synthase 2 in porcine cumulus cells. *Am J Physiol Physiol* ajpcell.00065.2:C268. <https://doi.org/10.1152/ajpcell.00065.2017>
- Parnigoni A, Caon I, Moretto P, Viola M, Karousou E, Passi A, Vigetti D (2021) The role of the multifaceted long non-coding RNAs: a nuclear-cytosolic interplay to regulate hyaluronan metabolism. *Matrix Biol Plus* 11:100060. <https://doi.org/10.1016/j.mbplus.2021.100060>
- Parnigoni A, Caon I, Teo WX, Hua SH, Moretto P, Bartolini B, Viola M, Karousou E, Yip GW, Götte M, Heldin P, Passi A, Vigetti D (2022) The natural antisense transcript HAS2-AS1

- regulates breast cancer cells aggressiveness independently from hyaluronan metabolism. *Matrix Biol* 109:140–161. <https://doi.org/10.1016/j.matbio.2022.03.009>
- Rilla K, Pasonen-Seppänen S, Kärnä R et al (2012) HAS3-induced accumulation of hyaluronan in 3D MDCK cultures results in mitotic spindle misorientation and disturbed organization of epithelium. *Histochem Cell Biol* 137:153–164. <https://doi.org/10.1007/s00418-011-0896-x>
- Röck K, Tigges J, Sass S et al (2015) miR-23a-3p causes cellular senescence by targeting Hyaluronan synthase 2: possible implication for skin aging. *J Invest Dermatol* 135:369–377. <https://doi.org/10.1038/jid.2014.422>
- Siiskonen H, Oikari S, Pasonen-Seppänen S, Rilla K (2015) Hyaluronan synthase 1: a mysterious enzyme with unexpected functions. *Front Immunol* 6:43. <https://doi.org/10.3389/fimmu.2015.00043>
- Spicer AP, McDonald JA (1998) Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem* 273:1923–1932. <https://doi.org/10.1074/JBC.273.4.1923>
- Spicer AP, Nguyen TK (1999) Mammalian hyaluronan synthases: investigation of functional relationships in vivo. In: *Biochemical Society transactions*. Portland Press Ltd, pp 109–115
- Tammi MI, Oikari S, Pasonen-Seppänen S et al (2019) Activated hyaluronan metabolism in the tumor matrix — causes and consequences. *Matrix Biol* 78–79:147–164
- Tammi RH, Passi AG, Rilla K et al (2011) Transcriptional and post-translational regulation of hyaluronan synthesis. *FEBS J* 278:1419–1428. <https://doi.org/10.1111/j.1742-4658.2011.08070.x>
- Tavianatou AG, Caon I, Franchi M et al (2019a) Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. *FEBS J* febs.14777:2883. <https://doi.org/10.1111/febs.14777>
- Tavianatou AG, Piperigkou Z, Barbera C et al (2019b) Molecular size-dependent specificity of hyaluronan on functional properties, morphology and matrix composition of mammary cancer cells. *Matrix Biol Plus* 3:100008. <https://doi.org/10.1016/j.mbplus.2019.100008>
- Tian X, Azpurua J, Hine C et al (2013) High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. *Nature* 499:346–349. <https://doi.org/10.1038/nature12234>
- Vigetti D, Clerici M, Deleonibus S et al (2011) Hyaluronan synthesis is inhibited by adenosine monophosphate-activated protein kinase through the regulation of HAS2 activity in human aortic smooth muscle cells. *J Biol Chem* 286:7917–7924. <https://doi.org/10.1074/jbc.M110.193656>
- Vigetti D, Deleonibus S, Moretto P et al (2014a) Natural antisense transcript for hyaluronan synthase 2 (HAS2-AS1) induces transcription of HAS2 via protein O-GlcNAcylation. *J Biol Chem* 289:28816–28826. <https://doi.org/10.1074/jbc.M114.597401>
- Vigetti D, Deleonibus S, Moretto P et al (2012) Role of UDP-N-acetylglucosamine (GlcNAc) and O-GlcNAcylation of hyaluronan synthase 2 in the control of chondroitin sulfate and hyaluronan synthesis. *J Biol Chem* 287:35544–35555. <https://doi.org/10.1074/jbc.M112.402347>
- Vigetti D, Genasetti A, Karousou E et al (2009a) Modulation of hyaluronan synthase activity in cellular membrane fractions. *J Biol Chem* 284:30684–30694. <https://doi.org/10.1074/jbc.M109.040386>
- Vigetti D, Ori M, Viola M et al (2006) Molecular cloning and characterization of UDP-glucose dehydrogenase from the amphibian *Xenopus laevis* and its involvement in hyaluronan synthesis. *J Biol Chem* 281:8254–8263. <https://doi.org/10.1074/jbc.M508516200>
- Vigetti D, Rizzi M, Viola M et al (2009b) The effects of 4-methylumbelliferone on hyaluronan synthesis, MMP2 activity, proliferation, and motility of human aortic smooth muscle cells. *Glycobiology* 19:537–546. <https://doi.org/10.1093/glycob/cwp022>
- Vigetti D, Viola M, Karousou E et al (2014b) Metabolic control of hyaluronan synthases. *Matrix Biol* 35:8–13. <https://doi.org/10.1016/J.MATBIO.2013.10.002>
- Viola M, Brüggemann K, Karousou E et al (2017) MDA-MB-231 breast cancer cell viability, motility and matrix adhesion are regulated by a complex interplay of heparan sulfate,

- chondroitin-/dermatan sulfate and hyaluronan biosynthesis. *Glycoconj J* 34:411. <https://doi.org/10.1007/s10719-016-9735-6>
- Viola M, Karousou E, D'Angelo ML et al (2016) Extracellular matrix in atherosclerosis: Hyaluronan and proteoglycans insights. *Curr Med Chem* 23:2958–2971. <https://doi.org/10.2174/0929867323666160607104602>
- Vitale DL, Caon I, Parnigoni A, Sevic I, Spinelli FM, Icardi A, Passi A, Vigetti D, Alaniz L (2021) Initial identification of UDP-glucose dehydrogenase as a prognostic marker in breast cancer patients, which facilitates epirubicin resistance and regulates hyaluronan synthesis in MDA-MB-231 cells. *Biomolecules* 11(2):246. <https://doi.org/10.3390/biom11020246>
- Weigel PH (2015) Hyaluronan synthase: the mechanism of initiation at the reducing end and a pendulum model for polysaccharide translocation to the cell exterior. *Int J Cell Biol* 2015: 367579
- Weigel PH, Baggenstoss BA, Washburn JL (2017) Hyaluronan synthase assembles hyaluronan on a [GlcNAc(β 1,4)]_n-GlcNAc(α 1 \rightarrow)UDP primer and hyaluronan retains this residual chitin oligomer as a cap at the nonreducing end. *Glycobiology* 27:536–554. <https://doi.org/10.1093/glycob/cwx012>
- Weigel PH, DeAngelis PL (2007) Hyaluronan synthases: a decade-plus of novel glycosyltransferases. *J Biol Chem* 282:36777–36781
- Weigel PH, West CM, Zhao P et al (2014) Hyaluronan synthase assembles chitin oligomers with -GlcNAc(α 1 \rightarrow)UDP at the reducing end. *Glycobiology* 25:632–643. <https://doi.org/10.1093/glycob/cwv006>
- Yamane T, Kobayashi-Hattori K, Oishi Y (2011) Adiponectin promotes hyaluronan synthesis along with increases in hyaluronan synthase 2 transcripts through an AMP-activated protein kinase/peroxisome proliferator-activated receptor- α -dependent pathway in human dermal fibroblasts. *Biochem Biophys Res Commun* 415:235–238. <https://doi.org/10.1016/j.bbrc.2011.09.151>
- Yang YM, Nouredin M, Liu C et al (2019) Hyaluronan synthase 2-mediated hyaluronan production mediates Notch1 activation and liver fibrosis. *Sci Transl Med* 11. <https://doi.org/10.1126/scitranslmed.aat9284>
- Yu H, Cheng F, Yu H et al (2017) Key role of the carboxyl terminus of Hyaluronan synthase in Processive synthesis and size control of hyaluronic acid polymers. *Biomacromolecules* 18: 1064–1073. <https://doi.org/10.1021/acs.biomac.6b01239>
- Ziatabar S, Zepf J, Rich S et al (2018) Chitin, chitinases, and chitin lectins: emerging roles in human pathophysiology. *Pathophysiology* 25:253–262
- Zimmer BM, Barycki JJ, Simpson MA (2020) Integration of sugar metabolism and proteoglycan synthesis by UDP-glucose dehydrogenase. *J Histochem Cytochem* 69:13

Chapter 2

Update on Hyaluronan in Development



Cora M. Demler and Natasza A. Kurpios

Abstract The development of an embryo is a carefully orchestrated ballet of cell migration, specification, proliferation, and death. These cells are surrounded by a dynamic extracellular matrix (ECM) that they respond to and use for movement, communication with other cells, and decision-making about critical cell behaviors. A key component of embryonic ECM is the glycosaminoglycan hyaluronic acid (HA). HA is required throughout every stage of development from ovulation to organogenesis to the neonatal assimilation to a world outside the mother's womb. In this chapter, we discuss the ECM components that make, break, interact with, and modify HA during some of these critical developmental milestones, with particular focus on HA in fertility, heart morphogenesis, intestinal morphogenesis, blood vascular development, and neonatal gut and lung development. We emphasize research advances that have been made in the last eight years.

2.1 Introduction

Our environment—the space around us in which we live and work—has an enormous influence on our thoughts, decisions, and actions. Similarly, the environment of a cell helps it determine where to move, when to divide, what fate it should adopt, and when a particular cellular response is needed, among other actions. This environment is largely determined by the composition of the extracellular matrix (ECM)—an array of proteins (structural, signaling, and enzymatic), glycosaminoglycans, proteoglycans, and glycoproteins interacting with the cells in different ways via surface receptors (Lu et al. 2012). The components of the ECM physically interact with the cells but also affect the movement or sequestration of extracellular signaling molecules (Rozario and DeSimone 2010; Bonnans et al. 2014; Dzamba and DeSimone 2018). Thus, the ECM influences cells both through biomechanics

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and signaling. Cells sense their environment, respond to it, and remodel the ECM creating a dynamic, two-way flow of information. The ECM in an embryo can be particularly dynamic, given the rapid growth and remodeling that occurs in embryonic tissues (Daley et al. 2008). ECM in an embryo is often very soft, which helps maintain an undifferentiated state for stem cells until the appropriate time and place for differentiation (Smith et al. 2018). Hyaluronic acid (HA) is a major player in this soft, dynamic ECM, and plays a critical role in development from the union of egg and sperm through postnatal development.

2.2 HA: A Versatile Component of ECM

HA is a non-sulfated glycosaminoglycan (GAG) made up of disaccharides of glucuronic acid and N-acetyl-glucosamine (Fig. 2.1a) (Hascall and Esko 2017). It is well-established that the size of an HA molecule has enormous impact on its function (Cyphert et al. 2015). For example, high molecular weight (HMW) HA is known to be anti-angiogenic and anti-inflammatory and a component of healthy adult tissues like the skin and joints (Cowman et al. 2015; Slevin et al. 2007; Petrey and de la Motte 2014). In contrast, low molecular weight (LMW) HA is pro-angiogenic and associated with disease states such as cancer and inflammation (Slevin et al. 2007; Petrey and de la Motte 2014). Oligo-HA (o-HA—fragments of 20 monosaccharides or fewer) has been shown to have both stimulatory and inhibitory effects on inflammation in different systems (Cyphert et al. 2015; Petrey and de la Motte 2014) and is highly pro-angiogenic (Slevin et al. 2007).

The size of HA is determined by hyaluronan synthase enzymes, with three (Has1, Has2, Has3) identified to date, and by hyaluronidases that break HA into fragments, eventually small enough to be internalized by a cell and degraded completely (Hascall and Esko 2017). As a result, hyaluronidases can affect cellular processes both by clearing whole HA from the system, or by introducing smaller fragments of HA into the system (Cyphert et al. 2015). Six genes have been identified in the canonical hyaluronidase (Hyal) family, although only four of these are true hyaluronidases: Hyal1, Hyal2, and Hyal3 catalyze HA in somatic tissues and SPAM1 has hyaluronidase activity limited to the testes. Also within this family, Hyal4 is a chondroitinase but not a hyaluronidase and PHYAL1 is a pseudogene (Hascall and Esko 2017). Other hyaluronidases lie outside of the Hyal family, including transmembrane protein 2 (TMEM2), and cell migration inducing hyaluronidase 1 (CEMIP1/KIAA1199). CEMIP1 (KIAA1199) is secreted and is important for HA degradation, although whether it has direct or indirect hyaluronidase activity remains unknown (Yoshida et al. 2013; Fink et al. 2015). TMEM2 is currently being investigated across the field and will be discussed further in this chapter.

Multiple HA receptors function in the body, transmitting signals from the ECM into the cell. Cluster of differentiation 44 (CD44) is a widely distributed cell-surface HA receptor which stimulates tyrosine kinases and Rho-like GTPase pathways (Turley et al. 2002). Receptor for HA-mediated motility (RHAMM) localizes to

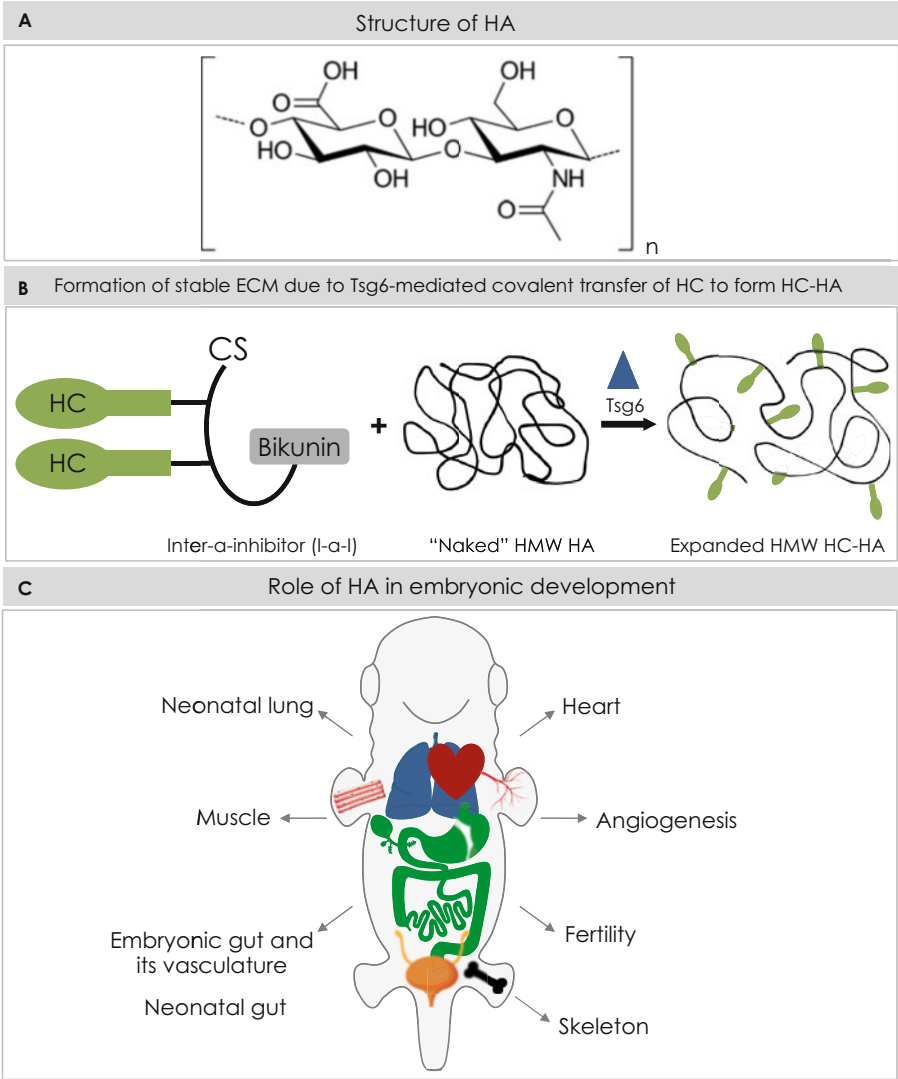


Fig. 2.1 HA plays many roles in embryonic development. **(a)** HA is a non-sulfated glycosaminoglycan (GAG) made up of disaccharide repeats of glucuronic acid and N-acetyl-glucosamine. **(b)** HA can be covalently modified by the enzyme TSG6; when the heavy chain (HC) donor IαI is available, TSG6 catalyzes the addition of HC proteins to HA, creating a stable, expanded matrix. **(c)** Summary of the roles of HA in embryogenesis

the cell surface, nucleus, and cytoplasm of diverse cell types, affecting a variety of downstream pathways (Turley et al. 2002). Lymphatic vessel endothelial receptor-1 (LYVE1) is specific to the lymphatic system (Banerji et al. 1999), where it serves to dock leukocytes to pull them into lymph vessels and lies upstream of signaling

pathways (Jackson 2019). Other receptors that bind HA directly are HA receptor for endocytosis (HARE) and layilin (Cyphert et al. 2015). Finally, toll-like receptor 2 and 4 are involved in HA-signaling pathways, although these bind HA indirectly (Ebid et al. 2014).

The possibilities of HA interactions continue to expand when considering hyaladherins, a family of proteins that can bind HA. The proteoglycans aggrecan, versican, brevican, and neurocan are included in this group, each with a link module for direct HA binding (Day and Prestwich 2002). These molecules work with HA to define the structural and signaling landscape of a particular ECM. They are also subject to cleavage by A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTSs) or Matrix Metalloproteinases (MMPs) (Apte 2013; Howell and Gottschall 2012).

A significant body of work has investigated the roles of these proteoglycans in the developing and adult brain (reviewed by Howell and Gottschall, 2012) (Howell and Gottschall 2012). During late embryonic development into postnatal development, large versican variants and neurocan levels increase in the brain. In contrast, aggrecan, brevican, and versican isoform V2 are found at higher levels in the adult brain (Milev et al. 1998).

Aggrecan has also been studied for its essential role in skeletal development: aggrecan-null mice (cartilage matrix deficiency model, or *cmd*) exhibit dwarfism, craniofacial defects including cleft palate, and early postnatal death as a consequence of respiratory failure (Watanabe et al. 1994). Evidence for the partnership of HA with aggrecan in skeletal development was shown using a limb mesenchyme-specific *Has2* knockout mouse model (*Prx-1 Cre, Has2 fl/fl*). The limbs of these mutants had defects similar to the *cmd* mutants: mutant limbs had shorter bones than wild-type limbs, defects in joint formation, mispatterning of the phalanges, and fewer hypertrophic chondrocytes at the growth plate that would give rise to bone (Matsumoto et al. 2009).

Of these hyaladherins, versican has the most diverse roles in development. Versican is a highly versatile molecule, existing in multiple isoforms and subject to cleavage by several ADAMTS (a Disintegrin and Metalloproteinase with Thrombospondin Motifs) family members (ADAMTS1, 4, 5, 9, 15, and 20), creating a bioactive “versikine” fragment (Nandadasa et al. 2014). Some of these ADAMTS family members are essential for embryo survival (ADAMTS9) (Silver et al. 2008; Kern et al. 2010; Enomoto et al. 2010), full fertility (ADAMTS1) (Russell et al. 2015), and normal patterning in development. For example, McCulloch and colleagues showed that ADAMTS5, 9, and 20 work together in the interdigital tissue (IDT) to cleave versican, and that the cleaved versican is necessary to cause apoptosis of the IDT. Loss of these ADAMTS genes in mice (particularly when more than one gene is null or haploinsufficient) leads to soft-tissue syndactyly (McCulloch et al. 2009). Other examples demonstrating the necessity of versican and ADAMTS proteins in development will be discussed later in this chapter.

Finally, HA can be covalently modified by the enzyme tumor necrosis factor stimulated gene 6 (TSG6)—this is the only known covalent modifier of HA. TSG6 adds heavy chain (HC) proteins from the donor molecule inter- α -inhibitor ($I\alpha I$)

directly to the HA molecule (Lauer et al. 2013), stabilizing the matrix in an expanded state (Fig. 2.1b) (Day and Milner 2019). In the absence of IαI, TSG6 can crosslink HA molecules, condensing the matrix (Day and Milner 2019). Thus, the density and stiffness of an HA-rich ECM is “tunable” by TSG6, thereby affecting mechanotransduction signaling pathways (Baranova et al. 2011; Humphrey et al. 2014). TSG6/HA association can also enhance the interactions of HA with its receptors CD44 and LYVE1 (Day and Milner 2019). The formation of HC-HA by TSG6 has been shown to be important in fertilization, adult tissues, disease, and as a therapeutic (reviewed by Day and Milner, 2018) (Day and Milner 2019). More recent data show that TSG6-catalyzed HA modification is also essential for proper gut morphogenesis and gut vascular development (Sivakumar et al. 2018), discussed below.

In summary, there are many different “flavors” of HA, determined by its size, binding partners, modifiers, and available receptors. This makes HA a fascinating, albeit challenging, molecule to study. The role of HA has been and continues to be studied extensively in adult disease states, but studies on its influence in embryonic development have historically been limited to fertilization and heart morphogenesis. Excitingly, HA is being increasingly recognized as an essential player in embryogenesis (Fig. 2.1c). Here we discuss some of the recent key works exploring the roles of HA in development, starting at the very beginning—fertilization.

2.3 HA in Fertility

The soft, HA-rich ECM surrounding the oocyte (the cumulus cell-oocyte complex (COC)) has been well characterized, starting with work by Salustri et al. in 1992 (Salustri et al. 1992). The COC is necessary for oocyte maturation and ovulation (Sato et al. 2001). HA and TSG6 are critical for the expansion and organization of the COC (Fülöp et al. 1997; Carrette et al. 2001; Ochsner et al. 2003), as is the protein pentraxin3 (PTX3) that binds TSG6 directly (Salustri et al. 2004; Baranova et al. 2014). Consequently, disruptions to the COC in mice by mutating *Tsg6* (Fülöp et al. 2003), IαI (Sato et al. 2001; Zhuo et al. 2001), or PTX3 (Varani et al. 2002; Garianda et al. 2002) result in decreased female fertility. In 2016, Chen and colleagues used atomic force microscopy (AFM) to demonstrate that there is an additional layer of ECM outside of the cumulus cells that is highly elastic and extremely soft; indeed, the COC is the softest mammalian tissue described to date (Chen et al. 2016).

In order for fertilization to occur, sperm must be equipped to move through the COC to fuse with the oocyte. Rodent and bovine sperm use two sperm hyaluronidases: Hyal5 and sperm adhesion protein 1 (SPAM1) to do so, while humans and most other mammals have only one sperm-associated hyaluronidase (Park et al. 2019). In mice, a single knockout of either Hyal5 or SPAM1 has no impact on male fertility, presumably because one can compensate for the loss of the other (Baba et al. 2002; Kimura et al. 2009). Recently, a double knockout of these two genes was

made for the first time. These double mutants (Hyal5 null, SPAM1 null) have impaired male fertility (Park et al. 2019). While sperm are healthy, produced in normal numbers, and capable of fertilizing a cumulus-free egg, they are largely unable to penetrate the HA-rich COC to fuse with the oocyte (Park et al. 2019). As a result, a pair of double knockout mice often fail to reproduce (Park et al. 2019). The COC presents a challenge to sperm, offering oocytes the ability to select mature, healthy sperm by their ability to bind to HA, increasing chances of fertilization (Parmegiani et al. 2010; Huszar et al. 2003).

Together, these data show that Tsg6-modified HA is critical for female fertility, whereas hyaluronidases are essential for male fertility, exemplifying the ways in which a simple GAG can impact complex processes.

2.4 HA in Organogenesis: The Heart

We have known for two decades that HA synthesis by Has2 is critical for heart and vascular development, due to significant work from Todd Camenisch and colleagues (Camenisch et al. 2000; Rodgers et al. 2006). Has2 is needed both for expansion of the cardiac cushions and for giving cardiac endothelial cells migratory abilities so they can move into the tissue and become mesenchymal cells (Camenisch et al. 2000). Has2 null mouse embryos lack cardiac jelly and have a condensed mesenchyme. This is in contrast to the GAG-rich cardiac jelly and dramatic expansion of the cardiac cushions in wild-type hearts on embryonic day (E)9.5 (Camenisch et al. 2000). Has2 mutants also lack an organized vascular network in their yolk sacs and bodies and have overall growth retardation (Camenisch et al. 2000). Consequently, Has2 null mouse embryos die between E9.5 and E10.0 (Camenisch et al. 2000). Interestingly, Corey Mjaatvedt and colleagues established that versican null mice (heart defect, or *hdf* mutant) have very similar phenotypes, most notably the lack of cardiac cushions leading to embryonic lethality (Yamamura et al. 1997; Mjaatvedt et al. 1998). Medaka fish also require versican for proper deposition of cardiac jelly (Mittal et al. 2019), indicating an evolutionarily conserved role of versican and HA in cardiac morphogenesis. Once the cardiac jelly is deposited in the fish, it expands asymmetrically—a process that is critical for later events in heart morphogenesis like septum formation and inflow and outflow tract development. Although HA appears to be deposited symmetrically in the cardiac jelly, data show that HA and proteoglycan link protein 1a (HAPLN1a), which crosslinks HA and proteoglycans, is critical for this asymmetric expansion in zebrafish (Derrick et al. 2021). Finally, work led by Christine Kern and other groups has shown that ADAMTS1, 5, and 9 are also essential for proper heart morphogenesis by cleaving versican and aggrecan (Kern et al. 2010; Kern et al. 2006; Kern et al. 2007; Dupuis et al. 2011; Dupuis et al. 2013; Dupuis et al. 2019; Cooley et al. 2012; Stankunas et al. 2008).

More recently, an important role for Hyal2 has also been illustrated in heart development. One third of Hyal2 null mice die during postnatal day 1 as a result of cleft lip/palate hindering their ability to feed (Muggenthaler et al. 2017). Another

third of embryos die before weaning, likely from a combination of cleft lip/palate and congenital heart defects (Muggenthaler et al. 2017). All of the *Hyal2* mutants have expanded heart valves and a lack of organization in the ECM, and half display acute severe cardiac defects (Chowdhury et al. 2013; Chowdhury et al. 2017). Of these, some have one of their atria grossly dilated (Chowdhury et al. 2013). Further, independent of atrial dilation, about half of *Hyal2* mutants exhibit cor triatriatum (triatrial heart), a rare congenital condition of varying severity where one atrium (usually the left) is divided into two by a thin membrane (Chowdhury et al. 2016). Two human families have been described carrying *Hyal2* mutations, with family members affected by cleft lip/palate, congenital heart defects including cor triatriatum, hearing problems, poor eyesight, and soft-tissue syndactyly, with many of these conditions reflected in the *Hyal2*-null mouse model (Muggenthaler et al. 2017). Importantly, this is the first time that a molecular basis for cor triatriatum in humans has been described. This necessity of both an HA synthase and a hyaluronidase in heart development demonstrates the importance of tight regulation of HA homeostasis for proper organogenesis.

A more recently characterized hyaluronidase, transmembrane protein 2 (TMEM2) has been shown to be important in embryonic development. TMEM2 is a single-pass transmembrane protein with its HA-cleaving domain existing outside the cell, and is believed to be the first membrane-bound hyaluronidase (Yamamoto et al. 2017). A pair of papers in 2011 demonstrated that TMEM2 is essential for proper cardiac morphogenesis (Totong et al. 2011; Smith et al. 2011). In two different zebrafish mutants obtained from forward genetics screens, disfunction of TMEM2 led to an expansion of the atrioventricular canal, the structure in the developing heart that is important for making the septa and valves (Totong et al. 2011; Smith et al. 2011). Further, when maternally-contributed TMEM2 was also removed, mutants lacked proper cardiac fusion. Normally, cardiomyocytes and endocardial cells move medially in the developing heart until they meet in the middle, fusing to form a closed tube—this does not happen in maternal-zygotic TMEM2 mutants (Totong et al. 2011). A follow-up paper in 2016 proposed an explanation for the cardiac fusion migration defect: in both the skeletal and cardiac muscle of TMEM2 mutants, the ECM showed disorganization, which could impede cell migration (Ryckebüsch et al. 2016). Because inhibition of RHAMM or CD44 results in reduced myoblast migration and proliferation, respectively, in cultures from embryonic mouse forelimbs (E10.5-E12.5) (Leng et al. 2019), it is possible that HA signaling via these receptors is affected in TMEM2 mutants. However, the mechanisms by which TMEM2 affects organogenesis remain to be seen.

2.5 HA in Organogenesis: The Gut and Its Vasculature

In 2018, Sivakumar and colleagues demonstrated for the first time the role of hyaluronan in gut development in a strikingly binary system, the dorsal mesentery (DM) (Sivakumar et al. 2018). This organ, which has left-right asymmetry, connects

the gut tube to the rest of the body. The DM is responsible for steering the early gut so it may go from a straight, centrally positioned tube to highly organized loops and coils in the adult (Kurpios et al. 2008; Davis et al. 2008) (Fig. 2.2a)—a process that is highly reproducible within a species (Savin et al. 2011). The symmetry-breaking event, termed “gut tilting,” is characterized by an expansion of the right side of the DM and a condensation of the left (Davis et al. 2008), with accompanying changes in cell shape and adhesion within the DM (Fig. 2.2b) (Kurpios et al. 2008). This event is critical for setting up proper gut morphogenesis and avoiding intestinal malrotation, a congenital birth defect that predisposes to catastrophic volvulus (Applegate 2009). Initially, it was believed that the left side of the DM, which has high expression of the major left-sided transcription factor *Pitx2* (Fig. 2.2c), began its condensation before the right initiated expansion (Kurpios et al. 2008; Davis et al. 2008; Welsh et al. 2013). Sivakumar and colleagues demonstrated that it is in fact the right side that breaks symmetry, and expansion of the right DM is due to an enrichment of HA in the ECM (Fig. 2.2c). The right-sided HA is necessarily modified by Tsg6 expressed on the right side of the DM (Fig. 2.2c)—when either HA or Tsg6 is specifically removed from the right DM, that side does not expand and gut tilting does not occur (Fig. 2.3a, b) (Sivakumar et al. 2018). Recent work has revealed that the right-sided Tsg6 and HA is downstream of BMP4 signaling, which is inhibited on the left side (Sanketi et al. 2022). Further, inspection of gross gut morphology of Tsg6 mutant mice revealed significant malrotation and looping defects, which would predispose these mice to volvulus (Sivakumar et al. 2018). Besides COC expansion, this is the only other known function of TSG6 in development.

Interestingly, the condensed left side of the DM also has HA in its ECM (Fig. 2.2c), but it is not sufficient to drive expansion. It does play another essential role, however. The DM is the key facilitator of the development of the enteric blood vascular system. When the DM is still symmetrical, there are endothelial progenitors on both the right and left sides (Sivakumar et al. 2018). As the right side begins its expansion, these progenitors are excluded from the right. The endothelial progenitors on the left are maintained, and go on to form the artery that will eventually branch and become the ileocolic and middle colic arteries (Fig. 2.2b) (Sivakumar et al. 2018). Sivakumar and colleagues showed that whereas the HA on the right side is necessary for this vascular exclusion (anti-angiogenic), the HA on the left is necessary for arteriogenesis (pro-angiogenic). How can the same molecule, HA, have opposite effects in adjacent tissues? (summarized in Fig. 2.3d). This remains an outstanding question, possibly involving additional binding partners or modulators of HA signaling, different receptors, or different sizes of HA molecules on each side. It is known, however, that HA negatively regulates the chemokine Cxcl12, ligand for the G protein-coupled chemokine receptor Cxcr4, at the transcriptional level (Sivakumar et al. 2018; Mueller et al. 2014). Expression of Cxcl12 begins bilaterally and then is limited to the left DM later in development. When HA is depleted from the right, Cxcl12 expression becomes bilateral even at those later stages, and overexpression of Cxcl12 on the left perturbs vessel formation (Sivakumar et al. 2018). This is consistent with previous work showing that the Cxcl12/Cxcr4

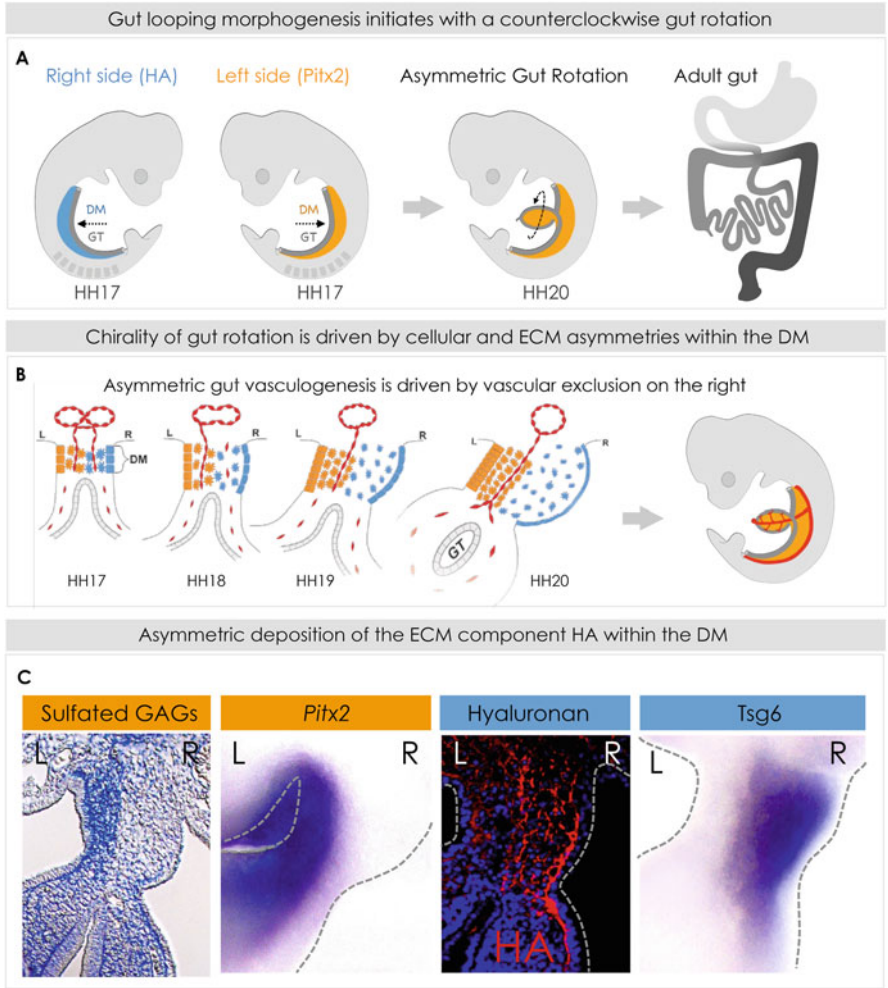


Fig. 2.2 HA in gut development. **(a)** The dorsal mesentery (DM) connects the gut tube (GT) to the rest of the body, steering the gut so it forms highly conserved looping patterns. **(b)** The DM architecture has left-right asymmetry. The left ECM condenses while the right expands, creating asymmetric forces to push the gut tube left. Concurrently, the right side excludes vascular progenitor cells and the left is permissive of vasculogenesis. **(c)** Asymmetric ECM of the DM. The left DM, first characterized as being *Pitx2*+, is enriched with sulfated GAGs as shown by Alcian Blue staining. The right DM is enriched with HA as visualized by HA-binding protein (HABP) localization. *Tsg6* expression, shown by RNA in situ hybridization, is exclusively right-sided. HH=Hamburger Hamilton staging system for chick embryos

pathway is critical for normal vascular patterning (Mahadevan et al. 2014; Tachibana et al. 1998). In all, the binary system of the DM is a beautiful example of the versatility and complexity of HA.

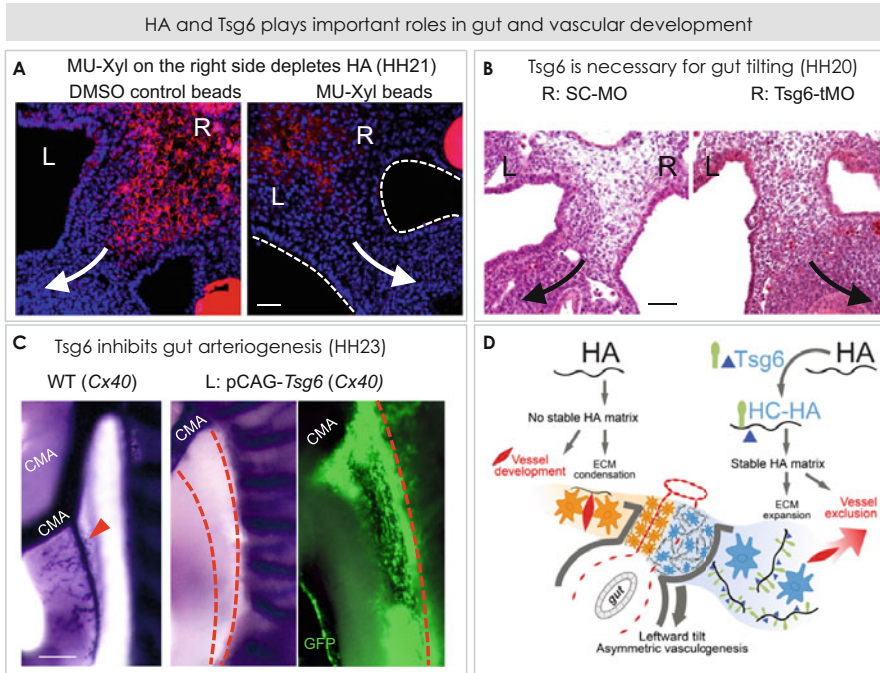


Fig. 2.3 Role of HA and Tsg6 in asymmetric gut development. **(a)** HA is necessary for gut tilting. Depletion of HA in MU-Xyl-treated embryos results in the loss of right-sided DM expansion. Black arrows. **(b)** Tsg6 is necessary for gut tilting. When a translation-blocking morpholino (tMO) against Tsg6 is electroporated on the right side, the right does not expand and gut tilting does not occur like it does in the scrambled morpholino (SC-MO) control. **(c)** Tsg6 inhibits gut arteriogenesis. Electroporating a plasmid encoding Tsg6 to the left DM (targeting shown with GFP), the key artery that branches from the cranial mesenteric artery (CMA) does not form, as shown by RNA in situ hybridization for *Cx40/Gja5*. **(d)** Model of the roles of HA and Tsg6 in the developing DM and its vasculature. HA production and Tsg6 catalysis on the right makes stable HC-HA and initiates rotation. The HC-HA matrix negatively regulates *Cxcl12* in right DM leading to vascular exclusion and left-sided restricted arteriogenesis. On the left, nascent HA deposition on the left (unmodified) is required for vascular development. Scale bars: 30 μ m (**a**); 50 μ m (**b**); 100 μ m (**c**) Figure modified from Sivakumar et al. 2018

2.6 HA in Angiogenesis

There are multiple pathways involved in vascular development, including vascular endothelial growth factor (VEGF), angiopoietin, platelet-derived growth factor, transforming growth factor β , bone morphogenetic protein, Notch, and other signaling pathways (Grant and Coultas 2019). The first connection between HA and vascular development was made by Richard Feinberg and David Beebe in 1983 (Feinberg and Beebe 1983). They showed that HA taken from human umbilical cords (now known to be HMW HA) (Kanayama et al. 1999) had an anti-vascular

effect on developing chick limb buds (Feinberg and Beebe 1983). Since then, a collection of work has demonstrated that HA can work through CD44 and RHAMM to regulate angiogenesis, particularly by affecting endothelial cell proliferation and migration (Pardue et al. 2008). This is true for development and also disease, especially cancer, where tumor size is limited by the ability of oxygen and nutrients to reach the cells. O-HA stimulates angiogenesis to vascularize tumors, allowing them to grow larger and providing an escape route for metastasizing cells (Pardue et al. 2008).

A new contribution to this area was made by De Angelis and colleagues in 2017, demonstrating the importance of TMEM2 for vascular development through VEGF signaling (De Angelis et al. 2017). In zebrafish, the secreted VEGF ligand binds to the VEGFR2/KDR receptor and activates multiple downstream pathways, with pERK1/2 signaling being the most important for zebrafish angiogenesis (Fig. 2.4a). TMEM2 zebrafish mutants have a significant reduction in the number of intersegmental vessels (ISVs) that grow in the trunk of the embryo—the vessels would sprout, but angiogenesis was unable to continue past that point (Fig. 2.4b). There was aberrant accumulation of HA in the embryo, contrasting the specific patterns of HA around the larger blood vessels in the wild-type embryo. The vascular defects could be rescued by injecting hyaluronidase into the region of the ISVs, but also by injection of o-HA. The authors showed that TMEM2 and the o-HA it produces are working with the VEGF pathway, though the nature of this interaction remains unclear. There is conflicting evidence (Rodgers et al. 2006; De Angelis et al. 2017) about whether TMEM2 can affect the VEGF pathway at the transcriptional level, but it is also possible that o-HA interacts with the ligand itself in the ECM to make the ligand available or to facilitate ligand/receptor binding (De Angelis et al. 2017).

VEGF signaling is hypothesized to also be at play in the asymmetric vascular development of the DM. Unpublished data from Natasza Kurpios's group show that there is VEGFA expression in the mesenchyme of the left DM, and VEGFR2 is expressed by the left-sided endothelial cells. It is possible that the left DM has LMW HA or o-HA that would stimulate this left-sided vascular development (De Angelis et al. 2017), but this remains to be seen. Further, the Kurpios group is currently investigating whether VEGF signaling on the right is downregulated at the transcriptional level (HA negatively regulates Cxcl12, Cxcl12 is upstream of VEGF) or whether the right side has HMW HA that sequesters VEGF ligands from their receptors. This question runs in parallel with that posed by De Angelis et al., demonstrating a need for careful analysis of the impact of HA on the VEGF signaling pathway (De Angelis et al. 2017).

HA binding partners and modifiers can also have an influence on vascular development. For example, Sivakumar et al. demonstrated that TSG6 is necessary for vascular exclusion from the right side of the DM, and is sufficient to prevent the formation of the normally occurring left-sided artery (Fig. 2.3c) (Sivakumar et al. 2018). Mittal and colleagues recently showed that in medaka fish, versican is necessary both for cardiac morphogenesis after the linear heart tube forms, and for proper lumen formation in the vasculature (Mittal et al. 2019). The linear heart tube

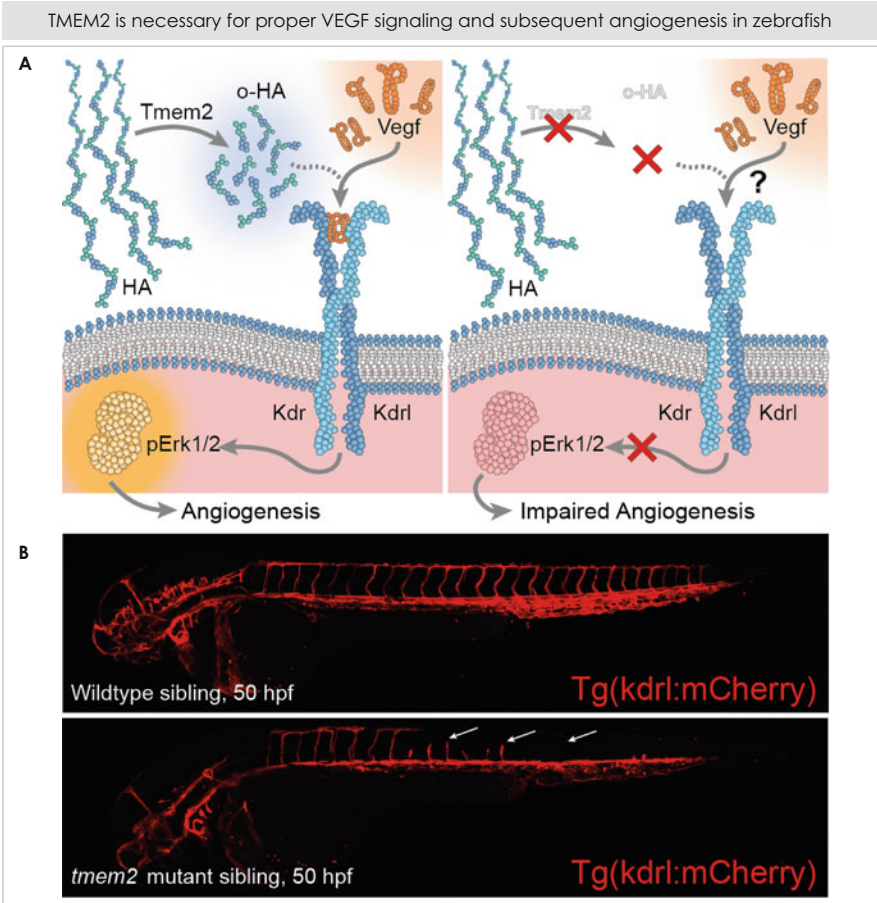


Fig. 2.4 TMEM2 is necessary for proper VEGF signaling and subsequent angiogenesis in zebrafish. **(a)** Model for the involvement of TMEM2 in VEGF signaling to promote angiogenesis. TMEM2 processes HA into o-HA. Though the mechanism remains unknown, this o-HA allows VEGF signaling through the Kdr/Kdrl receptor. **(b)** Zebrafish embryos at 50 hours post-fertilization (hpf) with mCherry-labeled Kdrl to visualize blood vasculature. TMEM2 mutants (bottom) display incomplete or absent intersegmental vessels (ISVs, white arrows) compared to wild-type sibling controls (top). Figure modified from De Angelis et al. 2017

mutant (*lht*) has a mutation in the 3'UTR of versican, potentially creating a novel microRNA binding site to repress versican synthesis. In these mutants, both vasculogenesis and angiogenesis are impaired so nascent blood vessels do not have a continuous lumen, thus preventing blood flow (Mittal et al. 2019). Versican and HA are also required for vascular development in the mouse yolk sac. De novo vessel development to vascularize the yolk sac cannot occur in versican-null or Has1/2/3 triple knockout embryos (Nandadasa et al. 2021).

In summary, it is clear that careful regulation of HA through its synthesis, breakdown, modification, and binding is essential for the development of healthy vasculature and that this role is well-conserved among vertebrates.

2.7 HA in Neonatal Development

The role of HA becomes no less important after birth, as newborn animals must adjust to eating and breathing themselves. For example, regulation of HA in the neonatal lung is extremely important. It has been shown in humans (Johnsson et al. 2003) and other mammalian models (Allen et al. 1991) that HA levels in the lung decrease as gestation proceeds, which regulates the amount of water held in the lung tissue. Babies born prematurely often have respiratory syndromes (Fraser et al. 2004), in part because less water has been cleared from the lung tissue (Johnsson et al. 1998). A recent study examining lung tissue from deceased newborn patients with respiratory conditions found a negative correlation between air space in the lung and RHAMM levels (Markasz et al. 2018). Normally RHAMM levels are low in both adult and neonatal lungs, but this work demonstrates that in some pathologies, RHAMM may be impacting development or inflammation.

Recent publications from Carol de la Motte's group show the importance of HA in breast milk for establishing the intestinal epithelial barrier (Hill et al. 2012; Gunasekaran et al. 2019; Yuan et al. 2015). This barrier forms a tight seal to keep the contents of the gut, particularly bacteria, apart from the rest of the body and the immune system (Moore et al. 2016). Consequences of compromising this barrier can be severe: the intestine launches an inflammatory response, intestinal cells may die, and bacteria may escape the intestines into the peritoneal cavity (Moore et al. 2016). Many gastrointestinal disease states are associated with compromised intestinal barrier function, including necrotizing enterocolitis (NEC), a gastrointestinal disease seen in 7–12% of prematurely born babies (Gunasekaran et al. 2019). Although the pathogenesis is not well understood, it has been noted that a diet of mother's milk has a protective effect on premature babies against getting NEC (Sullivan et al. 2010; Maffei and Schanler 2017). Breast milk is rich in nonnutritive bioactive components that help build the neonatal immune system (Ballard et al. 2013), including HA. A 2015 publication by Yuan and colleagues characterized the size distribution of HA in human milk (Yuan et al. 2015). This work demonstrated that HA isolated from milk and administered to intestinal epithelial cells and *in vivo* at physiological levels is sufficient to increase production of human beta defensin 2 (HBD2), an antimicrobial peptide that is used as a measure of immune response. Since 35 kDa HA (HA35) is known to trigger HBD2 production in intestinal epithelial cells (Hill et al. 2012), Hill and colleagues hypothesized that HA35 may be present in breast milk to maintain intestinal barrier function. Using a novel method of size-dependent fractionation by anion exchange on a spin column and competitive ELSA to isolate and analyze HA from the milk samples, the authors found that while the majority of milk HA is greater than 110 kDa, 5% is classified as LMW (Yuan et al. 2015). Interestingly,

total milk HA more strongly induces HBD2 than HA35 alone, even though HMW HA alone cannot induce HBD2 (Fig. 2.5). HA35-mediated induction of HBD2 is TLR-dependent and CD44 independent, while HBD2 induction by total milk involves both TLR4 and CD44 (Hill et al. 2012; Hill et al. 2013). The authors hypothesize that HMW HA in milk interacts with CD44 to somehow enhance the HBD2 induction caused by LMW HA (Yuan et al. 2015).

Whether HA could be used as a therapeutic to bolster neonatal intestinal barrier function is explored in a 2019 *Pediatric Research* paper by Gunasekaran and colleagues (Gunasekaran et al. 2019). NEC can be induced by intraperitoneal injection of Dithizone, followed by gavage of bacteria. If HA35 is administered by gavage once a day for 3 days prior to the Dithizone injection, epithelial barrier function is retained and pups do not suffer from NEC. While untreated NEC pups have only a 50% survival rate, treating with high concentration of HA35 increases this survival to 90%. Multiple measures of epithelial barrier function such as villi histology, pro-inflammatory cytokine levels, and permeability measured by dextran levels in the serum following dextran gavage demonstrate the marked protection afforded to HA35-treated pups (Gunasekaran et al. 2019). HA35 has already been used in a clinical trial to consider whether it may be a useful therapeutic for adults with gastrointestinal diseases such as IBD (De La Motte 2019), and the exciting research described here may position HA as a candidate for neonatal therapeutics as well (Fig. 2.5).

2.8 Conclusions

Embryogenesis is a critical, fragile time in the life of an organism. Coordinated changes take place rapidly and precisely, with tight regulation of important processes to promote their success. HA may appear to be a simple sugar, but the research described here illustrates its immense importance for proper development. This versatile molecule is a powerful component of the ECM, influencing its structure, affecting movement of ligands, and participating in signaling pathways itself. The work presented in this chapter helps us understand the complex role that HA plays as humans and other vertebrates develop from a single cell into a fully patterned adult organism, and offers exciting potential for therapeutic applications of HA.

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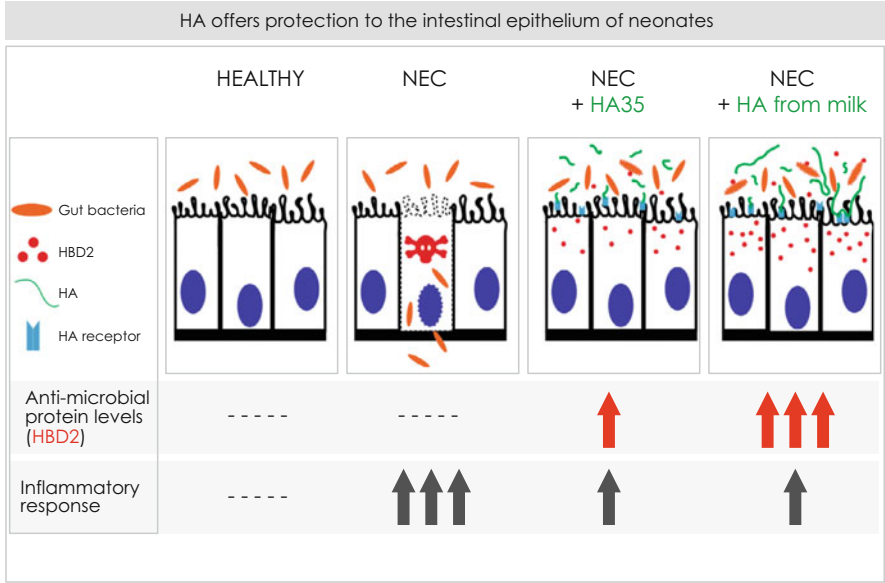


Fig. 2.5 HA offers protection to the intestinal epithelium of neonates. NEC is induced by Dithizone injection, resulting in damage and death of intestinal epithelial cells and breaching of the intestinal barrier by gut bacteria, triggering an inflammatory response. Treatment with HA35 before Dithizone injection increases HBD2 levels and offers some protection against HBD2. Whole HA from breast milk causes an even stronger upregulation of HBD2

References

Allen SJ, Sedin EG, Jonzon A, Wells AF, Laurent TC (1991) Lung hyaluronan during development: a quantitative and morphological study. *Am J Physiol Heart Circ Physiol* 260:H1449–H1454

Applegate KE (2009) Evidence-based diagnosis of malrotation and volvulus. *Pediatr Radiol* 39: 161–163

Apte S (2013) Chapter 259 the ADAMTS endopeptidases. In: *Handbook of proteolytic enzymes*, vol 1

Baba D et al (2002) Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J Biol Chem* 277:30310–30314

Ballard O, Morrow AL, Composition HM (2013) Nutrients and bioactive factors. *Pediatr Clin N Am* 60:49–74

Banerji S et al (1999) LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144:789–801

Baranova NS et al (2011) The inflammation-associated protein TSG-6 cross-links hyaluronan via hyaluronan-induced TSG-6 oligomers. *J Biol Chem* 286:25675–25686

Baranova NS et al (2014) Incorporation of pentraxin 3 into hyaluronan matrices is tightly regulated and promotes matrix cross-linking. *J Biol Chem* 289:30481–30498

Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol* 15:786–801

- Camenisch TD et al (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 106:349–360
- Carrette O, Nemade RV, Day AJ, Brickner A, Larsen WJ (2001) TSG-6 is concentrated in the extracellular matrix of mouse cumulus oocyte complexes through Hyaluronan and inter-alpha-inhibitor Binding1. *Biol Reprod* 65:301–308
- Chen X et al (2016) Micromechanical analysis of the Hyaluronan-rich matrix surrounding the oocyte reveals a uniquely soft and elastic composition. *Biophys J* 110:2779–2789
- Chowdhury B, Hemming R, Hombach-Klonisch S, Flamion B, Triggs-Raine B (2013) Murine hyaluronidase 2 deficiency results in extracellular hyaluronan accumulation and severe cardiopulmonary dysfunction. *J Biol Chem* 288:520–528
- Chowdhury B, Xiang B, Muggenthaler M, Dolinsky VW, Triggs-Raine B (2016) Hyaluronidase 2 deficiency is a molecular cause of cor triatriatum sinister in mice. *Int J Cardiol* 209:281–283
- Chowdhury B et al (2017) Hyaluronidase 2 deficiency causes increased mesenchymal cells, congenital heart defects, and heart failure. *Circ Cardiovasc Genet* 10:e001598
- Cooley MA et al (2012) Fibulin-1 is required during cardiac ventricular morphogenesis for versican cleavage, suppression of ErbB2 and Erk1/2 activation, and to attenuate trabecular cardiomyocyte proliferation. *Dev Dyn* 241:303–314
- Cowman MK, Lee HG, Schwertfeger KL, McCarthy JB, Turley EA (2015) The content and size of hyaluronan in biological fluids and tissues. *Front Immunol* 6:261
- Cyphert JM, Trempus CS, Garantzios S (2015) Size matters: molecular weight specificity of hyaluronan effects in cell biology. *Int J Cell Biol* 2015:1–8
- Daley WP, Peters SB, Larsen M (2008) Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci* 121:255–264
- Davis NM et al (2008) The chirality of gut rotation derives from left-right asymmetric changes in the architecture of the dorsal mesentery. *Dev Cell* 15:134–145
- Day AJ, Milner CM (2019) TSG-6: a multifunctional protein with anti-inflammatory and tissue-protective properties. *Matrix Biol* 78–79:60–83
- Day AJ, Prestwich GD (2002) Hyaluronan-binding proteins: tying up the giant. *J Biol Chem* 277:4585–4588
- De Angelis JE et al (2017) Tmem2 regulates embryonic Vegf signaling by controlling hyaluronic acid turnover. *Dev Cell* 40:123–136
- De La Motte C (2019) Human pilot study - HA35 (Hyaluronan molecular weight 35) dietary supplement for promoting intestinal health.
- Derrick CJ, Sánchez-Posada J, Hussein F, Tessadori F, Pollitt EJJ, Savage AM, Wilkinson RN et al (2021) Asymmetric Hapln1a drives regionalised cardiac ECM expansion and promotes heart morphogenesis in Zebrafish development. *Cardiovasc Res*. <https://doi.org/10.1093/cvr/cvab004>
- Dupuis LE, Osinska H, Weinstein MB, Hinton RB, Kern CB (2013) Insufficient versican cleavage and Smad2 phosphorylation results in bicuspid aortic and pulmonary valves. *J Mol Cell Cardiol* 60:50–59
- Dupuis LE et al (2011) Altered versican cleavage in ADAMTS5 deficient mice; a novel etiology of myxomatous valve disease. *Dev Biol* 357:152–164
- Dupuis LE et al (2019) Adamts5^{−/−} mice exhibit altered Aggrecan proteolytic profiles that correlate with ascending aortic anomalies. *Arterioscler Thromb Vasc Biol* 39:2067–2081
- Dzamba BJ, DeSimone DW (2018) Extracellular matrix (ECM) and the sculpting of embryonic tissues. *Curr Topics Develop Biol*:245–274. <https://doi.org/10.1016/bs.ctdb.2018.03.006>
- Ebid R, Lichtnekert J, Anders H-J (2014) Hyaluronan is not a ligand but a regulator of toll-like receptor signaling in mesangial cells: role of extracellular matrix in innate immunity. *ISRN Nephrol* 2014:1. <https://doi.org/10.1155/2014/714081>
- Enomoto H et al (2010) Cooperation of two ADAMTS metalloproteases in closure of the mouse palate identifies a requirement for versican proteolysis in regulating palatal mesenchyme proliferation. *Development* 137:4029–4038
- Feinberg RN, Beebe DC (1983) Hyaluronate in vasculogenesis. *Science*(80-) 220:1177–1179

- Fink SP et al (2015) Induction of KIAA1199/CEMIP is associated with colon cancer phenotype and poor patient survival. *Oncotarget* 6:30500–30515
- Fraser J, Walls M, McGuire W (2004) ABC of preterm birth: respiratory complications of preterm birth. *Br Med J* 329:962–965
- Fülöp C et al (1997) Coding sequence, exon-intron structure and chromosomal localization of murine TNF-stimulated gene 6 that is specifically expressed by expanding cumulus cell-oocyte complexes. *Gene* 202:95–102
- Fülöp C et al (2003) Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 130:2253–2261
- Garianda C et al (2002) Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* 420:182–186
- Grant ZL, Coultas L (2019) Growth factor signaling pathways in vascular development and disease. *Growth Factors* 37:53–67
- Gunasekaran A et al (2019) Hyaluronan 35 kDa enhances epithelial barrier function and protects against the development of murine necrotizing enterocolitis. *Pediatr Res* 87:1177. <https://doi.org/10.1038/s41390-019-0563-9>
- Hascall V, Esko JD (2017) Hyaluronan. In: Varki A, Cummings R, Esko J (eds) *Essentials of glycobiology*. Cold Spring Harbor Laboratory Press
- Hill DR, Kessler SP, Rho HK, Cowman MK, De La Motte CA (2012) Specific-sized hyaluronan fragments promote expression of human β -defensin 2 in intestinal epithelium. *J Biol Chem* 287:30610–30624
- Hill DR et al (2013) Human milk hyaluronan enhances innate defense of the intestinal epithelium. *J Biol Chem* 288:29090–29104
- Howell MD, Gottschall PE (2012) Lectican proteoglycans, their cleaving metalloproteinases, and plasticity in the central nervous system extracellular microenvironment. *Neuroscience* 217:6–18
- Humphrey JD, Dufresne ER, Schwartz MA (2014) Mechanotransduction and extracellular matrix homeostasis. *Nat Rev Mol Cell Biol* 15:802–812
- Huszar G et al (2003) Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril* 73:1616–1624
- Jackson DG (2019) Hyaluronan in the lymphatics: the key role of the hyaluronan receptor LYVE-1 in leucocyte trafficking. *Matrix Biol* 78–79:219–235
- Johnsson H, Eriksson L, Gerdin B, Hällgren R, Sedin G (2003) Hyaluronan in the human neonatal lung: association with gestational age and other perinatal factors. *Biol Neonate* 84:194–201
- Johnsson H, Eriksson L, Jonzon A, Laurent TC, Sedin G (1998) Lung hyaluronan and water content in preterm and term rabbit pups exposed to oxygen or air. *Pediatr Res* 44:716–722
- Kanayama N, Goto J, Terao T (1999) The role of low molecular weight hyaluronic acid contained in Wharton's jelly in necrotizing funisitis. *Pediatr Res* 45:510–514
- Kern CB et al (2006) Proteolytic cleavage of versican during cardiac cushion morphogenesis. *Dev Dyn* 235:2238–2247
- Kern CB et al (2007) Versican proteolysis mediates myocardial regression during outflow tract development. *Dev Dyn* 236:671–683
- Kern CB et al (2010) Reduced versican cleavage due to Adamts9 haploinsufficiency is associated with cardiac and aortic anomalies. *Matrix Biol* 29:304–316
- Kimura M et al (2009) Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in Fertilization1. *Biol Reprod* 81:939–947
- Kurpios NA et al (2008) The direction of gut looping is established by changes in the extracellular matrix and in cell: cell adhesion. *Proc Natl Acad Sci USA* 105:8499–8506
- Lauer ME et al (2013) Irreversible heavy chain transfer to hyaluronan oligosaccharides by tumor necrosis factor-stimulated gene-6. *J Biol Chem* 288:205–214
- Leng Y, Abdullah A, Wendt MK, Calve S (2019) Hyaluronic acid, CD44 and RHAMM regulate myoblast behavior during embryogenesis. *Matrix Biol* 78–79:236–254

- Lu P, Weaver VM, Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 196:395–406
- Maffei D, Schanler RJ (2017) Human milk is the feeding strategy to prevent necrotizing enterocolitis! *Semin Perinatol* 41:36–40
- Mahadevan A et al (2014) The left-right Pitx2 pathway drives organ-specific arterial and lymphatic development in the intestine. *Dev Cell* 31:690–706
- Markasz L, Savani RC, Sedin G, Sindelar R (2018) The receptor for hyaluronan-mediated motility (RHAMM) expression in neonatal bronchiolar epithelium correlates negatively with lung air content. *Early Hum Dev* 127:58–68
- Matsumoto K et al (2009) Conditional inactivation of Has2 reveals a crucial role for hyaluronan in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb. *Development* 136:2825–2835
- McCulloch DR et al (2009) ADAMTS metalloproteases generate active Versican fragments that regulate interdigital web regression. *Dev Cell* 17:687–698
- Milev P et al (1998) Differential regulation of expression of hyaluronan-binding proteoglycans in developing brain: Aggrecan, versican, neurocan, and brevican. *Biochem Biophys Res Commun* 247:207–212
- Mittal N et al (2019) Versican is crucial for the initiation of cardiovascular lumen development in medaka (*Oryzias latipes*). *Sci Rep* 9:9475
- Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* 202:56–66
- Moore SA et al (2016) Intestinal barrier dysfunction in human necrotizing enterocolitis. *J Pediatr Surg* 51:1907–1913
- Mueller AM, Yoon BH, Sadiq SA (2014) Inhibition of hyaluronan synthesis protects against central nervous system (CNS) autoimmunity and increases CXCL12 expression in the inflamed CNS. *J Biol Chem* 289:22888–22899
- Muggenthaler MMA et al (2017) Mutations in HYAL2, encoding hyaluronidase 2, cause a syndrome of orofacial Clefting and Cor Triatriatum sinister in humans and mice. *PLoS Genet* 13:e1006470
- Nandadasa S, Foulcer S, Apte SS (2014) The multiple, complex roles of versican and its proteolytic turnover by ADAMTS proteases during embryogenesis. *Matrix Biol* 35:34–41
- Ochsner SA et al (2003) Disrupted function of tumor necrosis factor- α -stimulated gene 6 blocks cumulus cell-oocyte complex expansion. *Endocrinology* 144:4376–4384
- Pardue EL, Ibrahim S, Ramamurthi A (2008) Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering. *Organogenesis* 4:203–214
- Park S et al (2019) SPAM1/HYAL5 double deficiency in male mice leads to severe male subfertility caused by a cumulus-oocyte complex penetration defect. *FASEB J* 33:14440–14449
- Parmegiani L et al (2010) ‘Physiologic ICSI’: hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril* 93:598–604
- Petrey AC, de la Motte CA (2014) Hyaluronan, a crucial regulator of inflammation. *Front Immunol* 5:101
- Rodgers LS et al (2006) Depolymerized hyaluronan induces vascular endothelial growth factor, a negative regulator of developmental epithelial-to-mesenchymal transformation. *Circ Res* 99: 583–589
- Rozario T, DeSimone DW (2010) The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol* 341:126–140
- Russell DL, Brown HM, Dunning KR (2015) ADAMTS proteases in fertility. *Matrix Biol* 44–46: 54–63
- Ryckebüsch L, Hernandez L, Wang C, Phan J, Yelon D (2016) Tmem2 regulates cell-matrix interactions that are essential for muscle fiber attachment. *Development* 143:2965–2972

- Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC (1992) Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev Biol* 151:541–551
- Salustri A et al (2004) PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. *Development* 131:1577–1586
- Sanketi BD, Zuela-Sopilniak N, Bundschuh E, Gopal S, Hu S, Long J, Lammerding J, Hopyan S, Kurpios NA (2022) Pitx2 patterns an accelerator-brake mechanical feedback through latent TGF β to rotate the gut. *Science* 377
- Sato H et al (2001) Impaired fertility in female mice lacking urinary trypsin inhibitor. *Biochem Biophys Res Commun* 281:1154. <https://doi.org/10.1006/bbrc.2001.4475>
- Savin T et al (2011) On the growth and form of the gut. *Nature* 476:57–62
- Silver DL et al (2008) The secreted metalloprotease ADAMTS20 is required for melanoblast survival. *PLoS Genet* 4:e1000003
- Sivakumar A et al (2018) Midgut laterality is driven by Hyaluronan on the right. *Dev Cell* 46:533–551.e5
- Slevin M et al (2007) Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol* 26:58–68
- Smith KA et al (2011) Transmembrane protein 2 (Tmem2) is required to regionally restrict atrioventricular canal boundary and endocardial cushion development. *Development* 138:4193–4198
- Smith LR, Cho S, Discher DE (2018) Stem cell differentiation is regulated by extracellular matrix mechanics. *Physiology* 33:16–25
- Stankunas K et al (2008) Endocardial Brg1 represses ADAMTS1 to maintain the microenvironment for myocardial morphogenesis. *Dev Cell* 14:298–311
- Sullivan S et al (2010) An exclusively human Milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human Milk and bovine Milk-based products. *J Pediatr* 156:562–567
- Sumeda N, O'Donnell A, Murao A, Yamaguchi Y, Midura RJ, Olson L, Apte SS (2021) The versican-hyaluronan complex provides an essential extracellular matrix niche for Flk1+ hematopoietic progenitors: versican and hyaluronan in vasculogenesis. *Matrix Biol*
- Tachibana K et al (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 393:591–594
- Totong R et al (2011) The novel transmembrane protein tmem2 is essential for coordination of myocardial and endocardial morphogenesis. *Development* 138:4199–4205
- Turley EA, Noble PW, Bourguignon LYW (2002) Signaling properties of hyaluronan receptors. *J Biol Chem* 277:4589–4592
- Varani S et al (2002) Knockout of Pentraxin 3, a downstream target of growth differentiation Factor-9, causes female subfertility. *Mol Endocrinol* 16:1154–1167
- Watanabe H et al (1994) Mouse cartilage matrix deficiency (cmd) caused by a 7 bp deletion in the aggrecan gene. *Nat Genet* 7:154–157
- Welsh IC et al (2013) Integration of left-right Pitx2 transcription and Wnt signaling drives asymmetric gut morphogenesis via Daam2. *Dev Cell* 26:629–644
- Yamamoto H et al (2017) A mammalian homolog of the zebrafish transmembrane protein 2 (TMEM2) is the long-sought-after cell-surface hyaluronidase. *J Biol Chem* 292:7304–7313
- Yamamura H, Zhang M, Markwald RR, Mjaatvedt CH (1997) A heart segmental defect in the anterior-posterior axis of a transgenic mutant mouse. *Dev Biol* 186:58–72
- Yoshida H et al (2013) KIAA1199, a deafness gene of unknown function, is a new hyaluronan binding protein involved in hyaluronan depolymerization. *Proc Natl Acad Sci* 110:5612–5617
- Yuan H, Amin R, Ye X, de la Motte CA, Cowman MK (2015) Determination of hyaluronan molecular mass distribution in human breast milk. *Anal Biochem* 474:78–88
- Zhuo L et al (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. *J Biol Chem* 276:7693–7696

Chapter 3

Long Noncoding RNAs and Epigenetic Regulation of Hyaluronan Synthesis



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Abstract Hyaluronan (HA) is a ubiquitous glycosaminoglycan of extracellular matrix, whose synthesis is due to the activity of three transmembrane isoenzymes named hyaluronan synthases 1, 2, and 3 (HAS1–3). The regulation of hyaluronan synthesis is a multistep process and it is mainly attributed to HAS2, which undergoes transcriptional and post-transcriptional modifications as well as an epigenetic control. In the last decade, the sequencing of the whole human genome contributed to the characterization of a noncoding transcriptome, that is a portion of our DNA that is transcribed into RNA but not translated into proteins. Although the biological function of noncoding RNAs (ncRNAs) species needs further investigation, emerging evidences showed that they can regulate gene expression through different mechanisms, including epigenetic modifications. According to their length, these RNA species can be classified and divided into short noncoding RNAs (i.e., microRNAs and siRNAs) and long noncoding RNAs (lncRNAs). In this chapter, we will give a general overview of lncRNAs biogenesis and functions, focusing on the role of hyaluronan synthase 2 antisense 1 (HAS2-AS1) in the epigenetic regulation of HAS2 expression. Moreover, we will describe recent findings linking HAS2-AS1 in pathologic processes such as cardiovascular diseases and cancer.

3.1 Introduction

Our modern view of eukaryotic transcriptome is the result of comprehensive studies of genomic DNA sequencing (Lander et al. 2001; Craig Venter et al. 2001). One of the most surprising results of the genomic sequencing effort was the finding that, in

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addition to protein-coding sequences and regulatory elements essential for the transcription, the majority of the genome contains sequences that are transcribed but not translated into proteins (~98% in humans). Indeed, according to the ENCODE (Encyclopedia of DNA Elements) consortium, the 80% of the human genome possess a biochemical function, in particular outside of the well-studied protein-coding regions (Birney et al. 2007). Interestingly, in eukaryotes the abundance of DNA encoding for noncoding RNAs (ncRNAs) correlates with the developmental complexity of the organism (Mattick 2011), giving a new regulatory meaning to RNA molecules and creating the additional layers of developmental complexity required for the evolution of eukaryotes. However, the discovery of the noncoding genome and the respective noncoding transcriptome gave rise to great debates among scientists concerning the biological significance of such sequences, going beyond the former definition of gene and the Central Dogma of biology. Little is known about the origin, conservation, and diversification of ncRNAs species across evolution. It has been proposed that some could derive from mechanisms of DNA-based or RNA-based duplication of existing genomic sequences, the loss of protein-coding potential or transposable element exaptation (Marques and Ponting 2014).

In the last years, a growing number of papers is describing that the action of ncRNAs may influence the synthesis/degradation of many extracellular matrix (ECM) components (Piccinini and Midwood 2014; Liu et al. 2014; D'Angelo and Agostini 2018; Biswas and Chakrabarti 2019; Wang et al. 2019). ECM is a highly dynamic and heterogenous structure present within all the tissues and organs which provides not only the mechanical scaffold for cellular constituents, but actively initiates biochemical cues fundamental for tissue homeostasis. It is mainly composed by water and proteins like collagen, elastin, and integrins but also by proteoglycans and glycosaminoglycans. In physiological conditions, ECM is constantly remodeled and allows the diffusion of signaling molecules and growth factors. Therefore, alterations in the structure of ECM or modifications to its elements are often associated to pathological conditions (Korpos et al. 2009; Frantz et al. 2010; Viola et al. 2016; Caon et al. 2019; Bartolini et al. 2020).

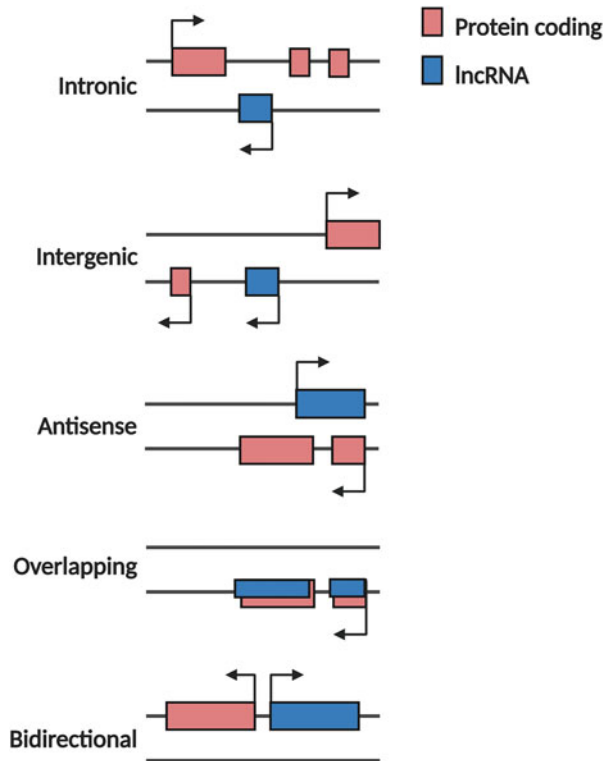
One of the most represented components of ECM is hyaluronan (HA), a linear and unsulfated glycosaminoglycan composed by repeating units of glucuronic acid and N-acetylglucosamine with a demonstrated active function in several physiological and pathological processes like aging, cancer, fibrosis, inflammation, diabetes, gut microbiota, gastrointestinal and vascular diseases (Vigetti et al. 2008; Liang et al. 2016; Filpa et al. 2017; Karamanos et al. 2018; Manou et al. 2019; Caon et al. 2019; Garantziotis and Savani 2019; Marozzi et al. 2021; Bistoletti et al. 2020; Bosi et al. 2022; Parnigoni et al. 2022a; Karousou et al. 2023; Parnigoni et al. 2021). HA is also used in several clinical applications as intra-articular injections for the treatment of osteoarthritis, eye surgery, and dermal filler (Passi and Vigetti 2019). In mammals, the production of HA is mediated by the action of three transmembrane enzymes called hyaluronan synthases 1, 2, and 3 (HAS1, HAS2, and HAS3) (Viola et al. 2015b). Among these enzymes HAS2 plays a leading role as its catalytic properties allow a very efficient HA synthesis (Itano et al. 1999). Moreover, HAS2 is critical

for animal survival (Camenisch et al. 2000), is highly expressed in several tissues and cell lines, and undergoes a tight regulation (Viola et al. 2015a). HAS2, indeed, displays a multistep regulation that involves the action of different growth factors and cytokines such as TGF- β , IGF, FGF, and prostaglandins (Porsch et al. 2013; Moretto et al. 2015). HAS2 expression is also stimulated by pro-inflammatory stimuli such as oxidized LDL (ox LDL) and TNF α (oxLDL) (Caravà et al. 2021; Viola et al. 2013; Caon et al. 2020a). In addition, HAS2 and HA deposition are influenced by nutrients availability and the amount of cytosolic substrates (i.e., UDP-N-acetylglucosamine and UDP-glucuronic acid) (Vigetti et al. 2006, 2014b; Caon et al. 2020b; Parnigoni et al. 2021), as well as by 4-methylumbelliferone (4-MU), a fluorescent derivative of coumarine used in experimental conditions to specifically inhibit HA synthesis by competing with UDP-GlcUA (Kakizaki et al. 2004; Kultti et al. 2009; Vitale et al. 2021). Lastly, the enzyme undergoes some post-translational modifications that influence its enzymatic activity and proteasomal degradation (Vigetti et al. 2011, 2012), the dimerization and the ubiquitination (Karousou et al. 2010; Mehić et al. 2017), and the subcellular localization (Melero-Fernandez de Mera et al. 2018). This chapter will deal with new and intriguing regulatory mechanisms of HAS2, focusing on epigenetics and the effects of ncRNAs on HA synthesis.

3.2 lncRNAs Classification and Functions

In general, cellular ncRNAs include some of the classical housekeeping RNAs, like the most represented ribosomal RNA (rRNA) and transfer RNA (tRNA); however, relying on transcript size, new classes of ncRNAs with a regulative function have been described: short ncRNAs with less than 200 nucleotides (e.g., microRNAs) and long ncRNAs (lncRNAs) composed by more than 200 nucleotides (Parnigoni et al. 2021). lncRNAs are present in all species including viruses, fungi, plants, prokaryotes, and animals sharing many common features with mRNA. They are synthesized by RNA polymerase II and require the canonical factors of the transcription machinery, such as the pre-initiation complex, the mediator, the transcription elongation complex, and specific transcription factors. Moreover, they are capped at 5' end and may have 3' end polyadenylation signals, they can have a multi-exonic composition and can undergo alternative splicing (although they contain fewer exons compared to mRNA). However, differently from mRNA, lncRNAs are less abundant and evolutionary conserved, their sequence conservation is poor and can display a biological function both at nuclear and cytoplasmic level. Multiple factors, such as specific RNA-protein complexes, RNA motif or even environmental changes and infections may determine their subcellular localization (Chen 2016; Gudenäs and Wang 2018). Some lncRNAs can be transmitted to adjacent cells and circulate in the serum through exosomes trafficking (Dragomir et al. 2018; Parnigoni et al. 2021). As for other RNA species, lncRNA can adopt a secondary and tertiary structure,

Fig. 3.1 Schematic representation of lncRNAs classification according to their genomic position relative to a protein-coding gene. Arrows indicate transcription direction



which strictly determines their function and their ability to bind specific interactors (Zampetaki et al. 2018).

Compared to the other classes of ncRNAs, lncRNAs exhibit a widespread range of sizes, functions, and shapes and their classification can be mainly based according to their location with respect to protein-coding genes and function.

Depending on their orientation with reference to protein-coding genes, lncRNAs can be classified into five groups: intronic, intergenic, antisense, overlapping, and bidirectional (Jiang et al. 2016; Parnigoni et al. 2021). Intronic lncRNAs are molecules that overlap within the intron of annotated coding genes, while intergenic lncRNAs (lincRNAs) are located between two protein-coding genes 1 kb far from any other coding loci. Antisense lncRNAs are transcribed from the opposite DNA strand and partially overlap with the exon of the sense RNA, overlapping lncRNAs overlap between lncRNAs and exons of a protein-coding gene, and bidirectional lncRNAs are located on the opposite strand from a protein-coding gene whose transcription is initiated less than 1 kb away (Fig. 3.1). A wide number of studies demonstrate that lncRNAs are able to regulate gene expression at multiple stages, from epigenetic and chromatin structure to transcriptional modifications, but also at translational and post-translational levels (Zhang et al. 2019; Parnigoni et al. 2021). A brilliant example of chromatin architecture regulation is the inactivation of

X-chromosome mediated by the lncRNA Xist (X-inactive-specific transcript). In female mammals, X-chromosome inactivation (XCI) is initiated by a unique locus and occurs during early embryonic development to achieve dosage compensation between females and males (Lyon 1961). Xist is transcribed from the future inactive X-chromosome (Xi) and is retained in the nucleus, where it physically spreads across Xi, wrapping the entire chromosome and recruiting multiple factors, including the polycomb repressive complexes 1 and 2 (PRC1 and 2). This triggers chromatin re-organization and the stable repression of Xi for development and adult life (Gendrel and Heard 2014). Many other nuclear lncRNAs are associated with chromatin remodeling either in *cis* (near their transcription site) or in *trans* (at sites distant from their transcription site) mediating the recruitment/inhibition of specific chromatin modifiers. For example, HOTAIR (Hox antisense intergenic RNA) is able to suppress HoxD gene expression *in trans* stimulating the recruitment of PRC2 to the HoxD locus (Rinn et al. 2007) and MALAT1 (metastasis associated lung adenocarcinoma transcript1) binds the Chromatin Remodeling Subunit BRG1 promoting the expression of inflammation-related genes (Huang et al. 2019). On the contrary, other lncRNAs can act as decoy, to prevent the interaction of histone or chromatin modifiers to specific DNA regions (Yao et al. 2019). In addition to epigenetic modifications, lncRNAs can directly regulate transcription through the formation of R-loop structures (RNA hybridized to duplex DNA) mediating the recruitment of transcription factors around the promoter regions (Postepska-Igielska et al. 2015; Parnigoni et al. 2021) or can interfere with the Pol II machinery both during the initiation and elongation process (Yao et al. 2019). Moreover, they can alter gene expression also functioning as scaffolds or architectural RNAs providing a central platform for the transient assembly of multi enzymatic complexes or cofactors (Chujo et al. 2016; Dykes and Emanuelli 2017). An intriguing post-transcriptional function of lncRNAs is their ability to regulate mRNA turnover in several ways. For instance, they can indirectly influence mRNA stability by competing for miRNAs interaction, thereby derepressing miRNAs targets (Wang et al. 2014b; Deng et al. 2015; Shan et al. 2018). Such class of lncRNA is known as competitive endogenous RNA (ceRNAs) and their function has been described as sponge effect. Other lncRNAs are able to regulate post-transcriptionally gene expression via recruitment of RNA degrading proteins, as demonstrated for a group of lncRNAs containing *Alu* elements (Gong and Maquat 2011).

Ribosome profiling analysis has identified ribosome-associated lncRNAs (Zeng and Hamada 2018). This group of lncRNAs is considered to have a function in the regulation of translation or to be used as source of new peptides. This is consistent with the notion that some lncRNAs encode for small peptides (Ruiz-Orera et al. 2014). However, due to the limited number of ribosome-associated lncRNAs, it is difficult to understand in depth their regulatory function and the essential features (or regulatory elements) that control their association with the ribosome.

Lastly, lncRNAs are able to modulate gene expression at post-translational level masking or exposing amino acid residues that are target of specific enzymatic modifications. For example, the lncRNA lnc-DC promotes the phosphorylation of STAT3 on Tyr705 by preventing the binding of the phosphatase SHP1 in dendritic

cells (Wang et al. 2014a), HOTAIR is involved in protein ubiquitination and in the degradation of Snurportin-1 and Ataxin-1 (Yoon et al. 2013) and MALAT in protein acetylation, as it interacts with Depleted in Breast Cancer 1 (DBC1) to regulate p53 acetylation (Chen et al. 2017) (Figs. 3.2 and 3.3).

3.3 HAS2-AS1 and Epigenetics

The identification of the lncRNA hyaluronan synthase 2 antisense 1 (HAS2-AS1) goes back to 2005, when Chao and Spicer characterized for the first time a natural antisense RNA transcribed from the opposite strand of *HAS2* gene locus on chromosome 8. They originally called it HASN'T (for HA synthase 2 antisense) (Chao and Spicer 2005; Parnigoni et al. 2021). The group demonstrated that in humans HAS2-AS1 shares sequence identity with the 5' untranslated region (5' UTR) of human HAS2 and that the transcript consists of four exons flanked by consensus splice acceptor and donor sequences. According to the same paper the four exons are distributed as follows with respect to HAS2 gene structure. Exon 1 is encoded by sequences located within intron 1 of HAS2, exon 2 is complementary to a portion of HAS2 exon 1, and exons 3 and 4 are encoded by sequences within HAS2 proximal promoter (Fig. 3.4). The group also reported an alternative splicing site within exon 2, that generates two isoforms of different length named short (174 nucleotides) and long (257 nucleotides) HAS2-AS1. A similar genetic organization has been demonstrated in mouse, where the transcript consists at least of 6 exons with short and long variants derived from an alternative splicing site located in an identical position with respect to human HAS2-AS1. Moreover, an equivalent location of poly A sequence is described in human and mouse (Chao and Spicer 2005; Parnigoni et al. 2021). From a functional point of view, Chao et al. demonstrated that the overexpression of both short and long variants in osteosarcoma cells reduced HAS2 mRNA levels and HA production, as well as cell proliferation. However, the authors did not explain how HAS2-AS1 could regulate HAS2 expression. Some years later, in 2011, a study of Michael and colleagues showed that HAS2 and HAS2-AS1 exhibited a coordinated expression after the stimulation with interleukine-1 beta (IL-1 β) and transforming growth factor-beta 1 (TGF- β 1) in proximal tubular epithelial cells. In silico and in vitro experiments demonstrated that HAS2 and HAS2-AS1 RNA interact by heteroduplex formation at cytoplasmic level, indicating for the first time a physical interaction between the two RNA species that could be responsible for HAS2 mRNA stabilization and expression (Michael et al. 2011). These evidences suggested a possible epigenetic regulation of HAS2.

The study demonstrating such hypothesis was conducted by Vigetti et al. in aortic smooth muscle cells. According to this paper, HAS2-AS1 was necessary to induce HAS2 expression acting *in cis* and mediating chromatin opening around HAS2 promoter. This regulatory step was triggered by the addition of a single N-acetylglucosamine residue (*O*-GlcNAcylation) to the NF- κ B subunit p65 that, in turn, modulate HAS2-AS1 promoter (Vigetti et al. 2014a, c; Parnigoni et al. 2021).

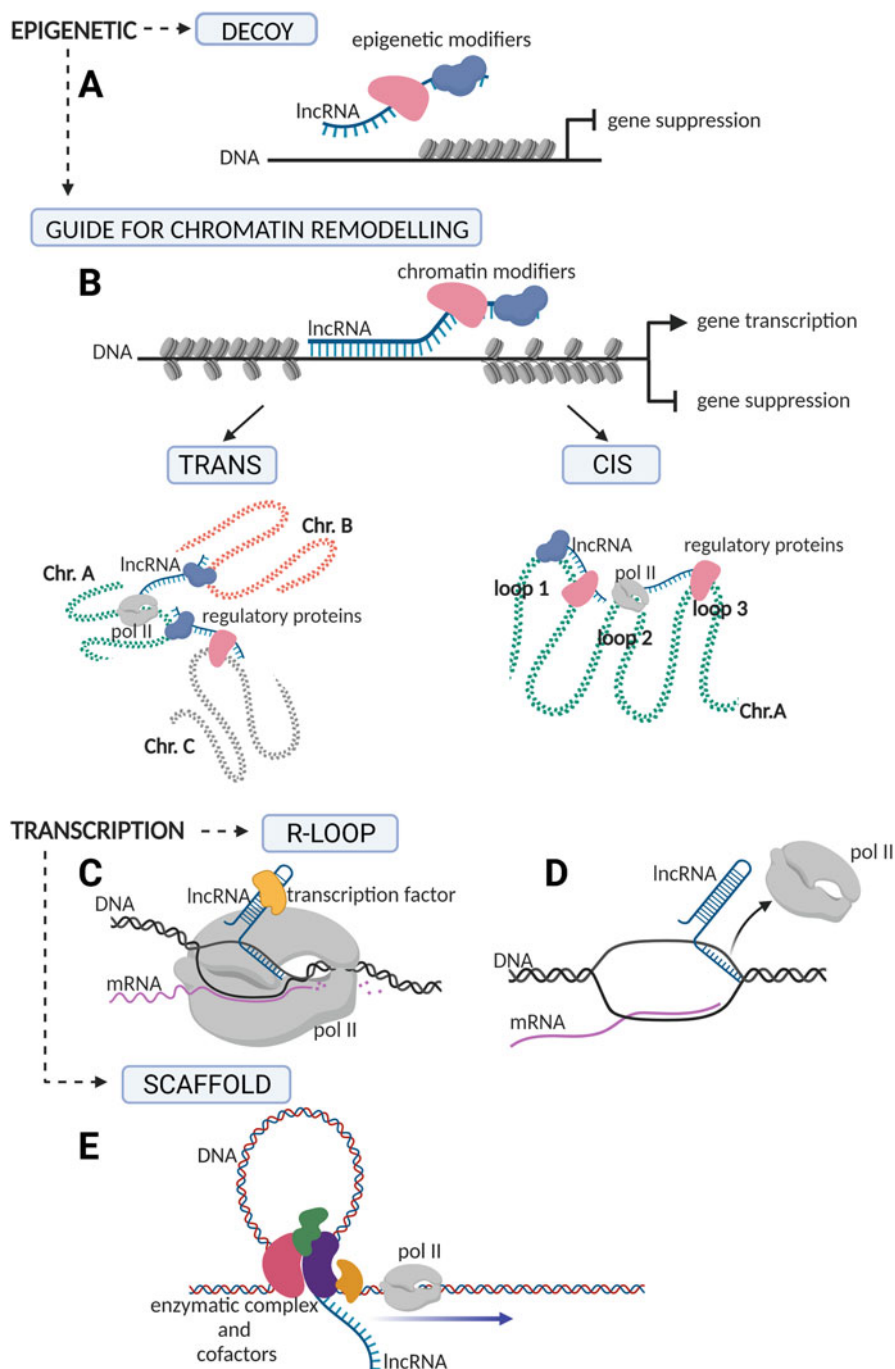


Fig. 3.2 Regulatory mechanisms associated to lncRNAs in the nucleus. lncRNAs can regulate gene expression via epigenetic mechanisms, interacting with histone-modifying enzymes to activate or repress gene transcription. They can act as decoy (a), preventing epigenetic modifiers to interact with histones; as guides for chromatin modifiers (b), allowing chromatin remodeling (either

Interestingly, the axis NF- κ B/HAS2-AS1/HAS2 is involved also in the response to sirtuin 1 (SIRT1) activity and inflammation (Vigetti et al. 2010; Caon et al. 2020a). SIRT1 is a NAD⁺ dependent deacetylase belonging to the family of sirtuins, a highly conserved class of proteins with anti-inflammatory, anti-aging, and metabolic functions. Its activity directly associates the cellular energetic status (via NAD⁺ intracellular increment, typical of caloric restriction and starvation) to the chromatin structure influencing the deacetylation of histones, transcription factors, and other cytoplasmic targets. SIRT1 can control both glucose and lipid metabolism in the liver, insulin secretion in the pancreas, senses nutrient availability in the hypothalamus and influences obesity-induced inflammation in macrophages (Li 2013). SIRT1 is also an essential factor in the protection from cellular senescence, promoting DNA repair and maintaining the normal chromatin structure and condensation state (Lee et al. 2019). Moreover, it extends organismal lifespan in several animal models including *S. cerevisiae* (Kaeberlein et al. 1999), *C. elegans* (Tissenbaum and Guarente 2001), *D. melanogaster* (Rogina and Helfand 2004), and mice (Minor et al. 2011). These longevity effects are mainly due to the interaction of SIRT1 with all the major conserved longevity pathways, such as AMP-activated protein kinase (AMPK), insulin/IGF-1 signaling (IIS), target of rapamycin (TOR), and forkhead box O (FOXO). Indeed, SIRT1 exerts a protective role in age-related diseases, including cardiovascular pathologies (D'Onofrio et al. 2015), neurodegenerative disorders (Donmez and Outeiro 2013), diabetes and other metabolic diseases (Elilob and Kilic 2018; Kitada et al. 2019).

3.4 HAS2-AS1 in Pathologies

Although few data are available about the role of HAS2-AS1 in pathologic conditions, the majority of the studies in the literature describes that HAS2-AS1 has a role in cancer and cardiovascular diseases (Fig. 3.5). lncRNAs are widely expressed and associated with several types of cancer such as prostate, lung, breast, colorectal, liver, and many other hematological malignancies (Bhan et al. 2017). As demonstrated with proteins, they may mediate oncogenic and tumor-suppressive functions; for instance, HOTAIR is able to silence tumor suppressor genes favoring metastasis formation and malignancy in breast cancer (Gupta et al. 2010), while lincRNAp21 mediates global gene repression in p53 pathway (Huarte et al. 2010). Genome wide association studies on cancer revealed that more than 80% of cancer associated-

Fig. 3.2 (continued) in *cis* or *trans*) and therefore inducing gene transcription or suppression. lncRNAs can also directly regulate gene transcription, generating R-loop structures with DNA (c) which mediate the recruitment of transcription factors; interfering with Pol II binding during initiation (d); functioning as scaffolds (e) for the assembly of the multi enzymatic complex and cofactors

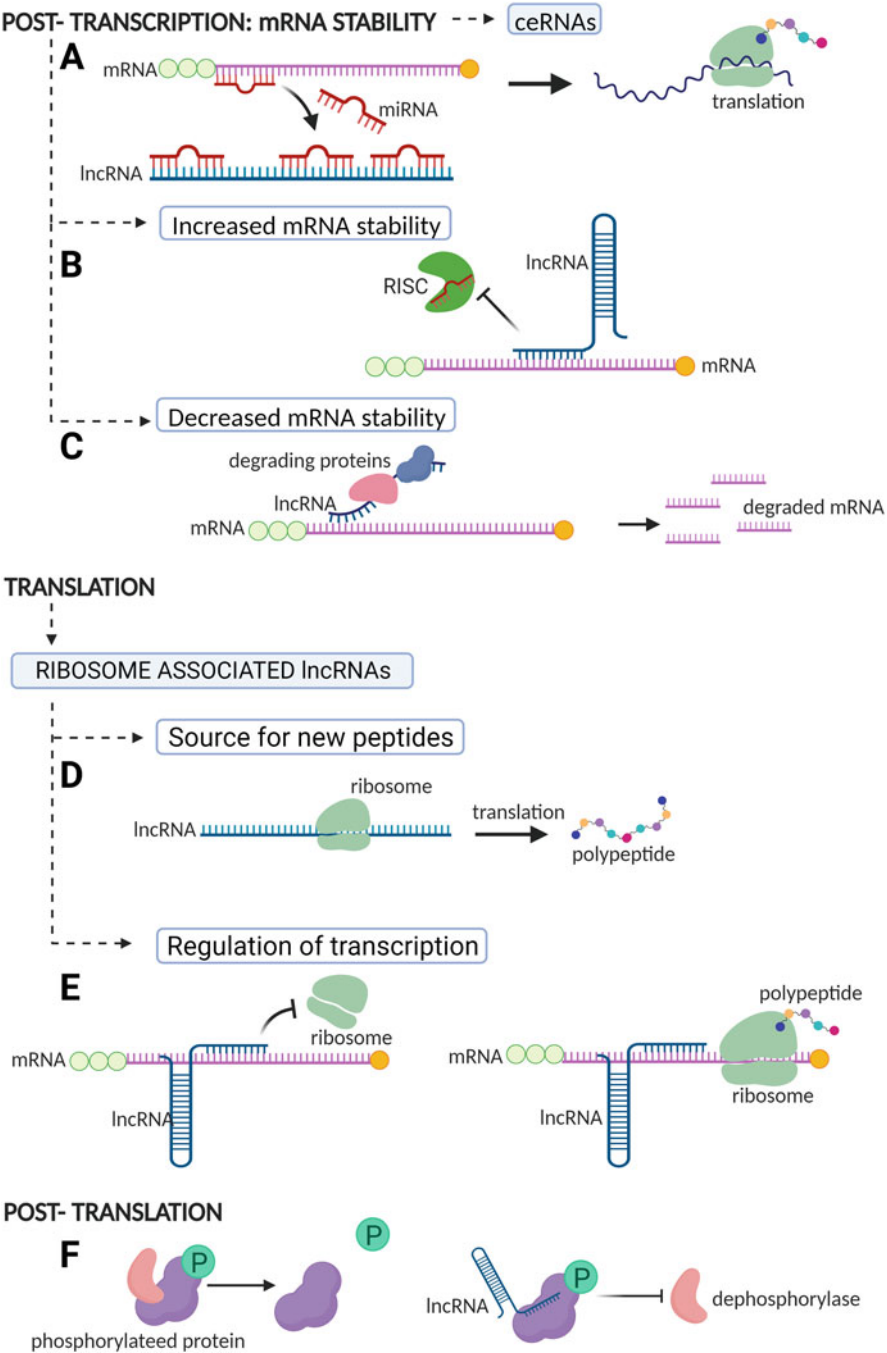


Fig. 3.3 Regulatory mechanisms associated to lncRNAs in the cytoplasm. lncRNAs can influence mRNA stability at post-transcriptional level. They act as miRNA sponges (a) regulating target gene

polymorphisms occur in noncoding regions, indicating that a significant fraction of genetic etiology of cancer is related to lncRNAs (Cheetham et al. 2013; Parnigoni et al. 2021). Interestingly, lncRNAs show specific patterns of expression in certain types and subtypes of tumors and they can be detected in different body fluids samples (Shi et al. 2016; Sarfi et al. 2019; Parnigoni et al. 2021). These features make lncRNAs potential biomarkers and therapeutic targets for the treatment of cancer.

Most of the literature about HAS2-AS1 focuses on cancer. This observation is not surprising if we consider that HA and HAS2 play a pivotal role in the development and progression of several malignancies (Rankin and Frankel 2016).

Recent experiments demonstrated that HAS2-AS1 may be considered as a predictor for clinical outcome and a potential target in cerebral tumors. Indeed, HAS2-AS1 expression levels were elevated in patients with advanced glioma or with a large tumor size and correlated with shorter free-disease survival and overall survival time. Moreover, the knockdown of HAS2-AS1 in glioma cell lines, inhibited cell viability, migration and invasion through the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway (Zhao et al. 2019; Parnigoni et al. 2021). A similar study reported that HAS2-AS1 promoted tumor progression in glioblastoma functioning as ceRNA and sponging miRNA-608 (Zhang et al. 2020). The group of Sun et al. gave another example of HAS2-AS1 sponge function, as they demonstrated that the interaction with miRNA-466 increased ovarian cancer cell proliferation and invasion (Tong et al. 2019).

Epithelial to mesenchymal transition (EMT) is a physiological process that occurs during embryonic development, wound healing, and tissue regeneration, characterized by the conversion of epithelial cells in a mesenchymal phenotype. This process is also adopted by cancer cells, sustaining tumor progression, metastatic expansion, and the acquisition of stemness. Previously experiments conducted in mouse breast cancer cells demonstrated that HAS2 and HAS2-AS1 showed a coordinated expression and are required for an efficient transforming-growth factor β (TGF β)-mediated EMT (Porsch et al. 2013; Kolliopoulos et al. 2019). In particular, the knockdown of HAS2-AS1 in mouse mammary epithelial cells inhibited the EMT process and suppressed the mesenchymal phenotype, reducing cell migration and the acquisition of stemness (Kolliopoulos et al. 2019). Recently the role of HAS2-AS1 in EMT has been shown also in human triple negative breast cancer cells (Parnigoni et al. 2022b).

Besides EMT, HAS2-AS1 plays another important role during hypoxia, a phenomenon common in the majority of malignant solid tumors. An hypoxic tumor microenvironment generally contributes to EMT, cell mobility, metastasis, and chemoresistance (Muz et al. 2015). Microarray analysis conducted in oral squamous

Fig. 3.3 (continued) expression. lncRNAs also directly bind to mRNAs, influencing their stability (b, c). lncRNAs can associate with ribosomes, being a source for new peptides (d) or helping in tuning transcription (e). lncRNAs can finally interact with proteins to control their phosphorylation, acetylation, and ubiquitination at the post-translation level (f)

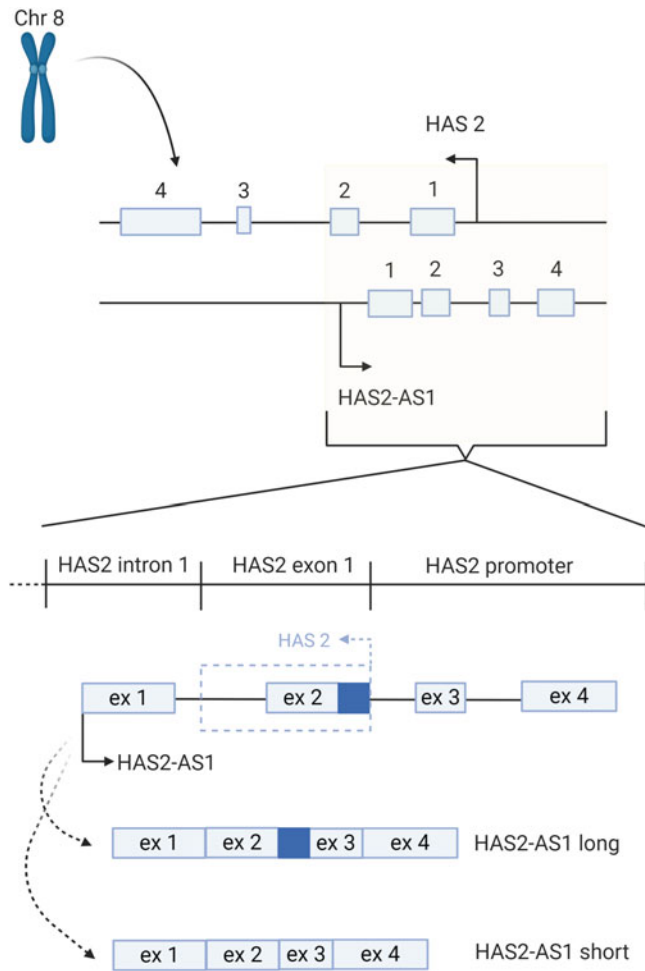


Fig. 3.4 Schematic representation of the genomic organization of human HAS2 and HAS2-AS1 loci on chromosome 8. The scheme shows the position of HAS2 and HAS2-AS1 exons, in relation to one another (up). Arrows indicate the transcription direction, and boxes are exons. Highlight of the first exon and intron of HAS2 and all HAS2-AS1 gene (middle). Dashed light blue box corresponds to HAS2 exon 1. Dark blue box indicates splicing region. Two splicing isoforms of HAS2-AS1 natural antisense of HAS2, long and short (low)

cell carcinoma (OSCC) specimens showed an aberrant expression of HAS2-AS1 compared with the normal oral mucosa. The same result was obtained comparing OSCC cell lines in normoxic vs hypoxic conditions, suggesting a connection between HAS2-AS1 expression and hypoxia (Zhu et al. 2017). In effect, HAS2-AS1 promoter contains a hypoxia responsive element (HRE), which responds to HIF-1 α stimulation. This stimulus is able to induce HAS2 expression and HA

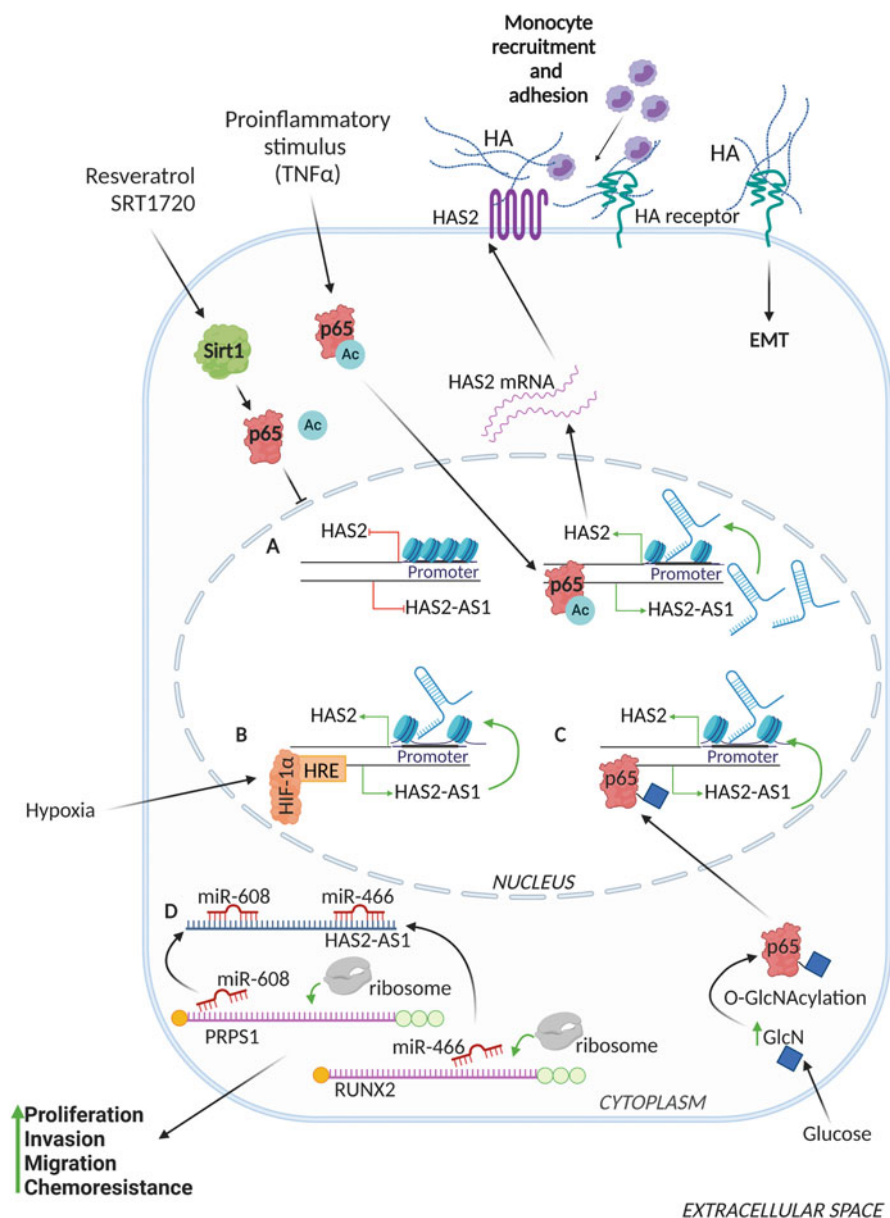


Fig. 3.5 Schematic illustration of HAS2-AS1 functions. (a) In aortic smooth muscle cells (AoSMCs) TNF α stimulation leads the acetylation of p65, which translocating into the nucleus, enhances HAS2-AS1 gene transcription. The result is the deposition of a monocyte adhesive pericellular HA matrix. Upon activation of SIRT1 (by SRT1720 or resveratrol), p65 is retained in the cytoplasm, thus preventing activation of HAS2-AS1 promoter. The resulting inhibition of HAS2 protein expression protects AoSMCs from TNF α -induced inflammation. (b) Under hypoxia condition, HIF-1 α can bind to HRE in HAS2-AS1 promoter, leading to an increase in HAS2 mRNA

production, contributing to EMT and proposing HAS2-AS1 as a potential target for anti-cancer therapy (Zhu et al. 2017).

In addition to cancer, emerging studies describe a function of HAS2-AS1 also in cardiovascular diseases, including atherosclerosis. LncRNAs can affect several processes associated to atherosclerosis, such as vascular smooth muscle cells proliferation, migration, lipid metabolism, inflammation, and endothelial cells function (Aryal et al. 2014). ECM remodeling and neointima formation are fundamental features of vascular diseases. Increased HA deposition and HAS2 expression are key players during the first stages of atherosclerosis, characterized by vascular smooth muscle cells migration, ECM deposition, and vessel wall thickening. At this stage, HA can also modulate the immune system stimulating macrophages retention and polarization and therefore sustaining inflammation. Moreover, the activation of macrophages produces reactive oxygen species contributing to LDL oxidation and foam cells formation (Viola et al. 2015a). Our group showed an increased expression of HAS2-AS1 and HAS2 in human atheromatous plaques with different grade of severity and in ApoE KO mice fed with a western diet (Vigetti et al. 2014a). Cardiovascular pathologies and diabetes are often associated to high protein *O*-GlcNAcylation, as this glycosylation is involved in vasoconstriction, vasodilation, calcification, and vascular remodeling (Wright et al. 2017). Interestingly, HAS2-AS1 was found overexpressed in mice with high levels of *O*-GlcNAcylation with respect to wild-type or heterozygous animals (Vigetti et al. 2014a), confirming the role of such glycosylation to control HAS2-AS1.

Atherosclerosis is critically fuelled by vascular inflammation through the action of cytokines like TNF α . For instance, in vitro experiments demonstrated that the treatment with TNF α increased vascular cells proliferation (Rastogi et al. 2012) and migration (Goetze et al. 1999), as well as HAS2 and HAS2-AS1 expression (Caon et al. 2020a). Interestingly, the activity of SIRT1 is able to reduce the pro-inflammatory effects triggered by TNF α , reducing HAS2-AS1 and HAS2 levels, vascular cells migration, and monocytes recruitment (Caon et al. 2020a).

A recent paper demonstrated that HAS2 expression can be driven by another lncRNA. As mentioned above, lncRNAs can modulate the expression of nearby genes. This is the case of Smooth Muscle Enriched Long Noncoding RNA (SMILR), that is found up-regulated together with HAS2-AS1 in human smooth muscle cells treated with interleukin-1 α (IL-1 α) or platelet-derived growth factor (PDGF). Moreover, SMILR levels were increased in human unstable atherosclerotic plaques

and protein level. Thus, synthesized HA binds to specific HA receptors, contributing to EMT induction in OSCC. (c) At basal conditions, the accessibility of both HAS2 and HAS2-AS1 promoters is low. After induction of *O*-GlcNAcylation (under hyperglycemic conditions) of p65, p65 binds to the HAS2-AS1 promoter increasing HAS2-AS1 accumulation, which in turn, induces chromatin remodeling around HAS2 HAS2 promoter This epigenetic modification stimulates HAS2 transcription. (d) HAS2-AS1 can act as a ceRNA for different miRNAs. Among all, HAS2-AS1 can bind miR608 and miR466, which, respectively, target PRPS1 and RUNX2 mRNAs. Concluding, HAS2-AS1 allows the expression of PRPS1 in glioblastoma cells and of RUNX2 in ovarian cancer cells, thus contributing to cancer progression

and in the plasma from patients with high plasma C-reactive protein. SMILR is located -750 kbp downstream of HAS2 gene on the same (reverse) strand and -750 kbp from HAS2-AS1 on the opposite strand of chromosome 8. SMILR knockdown significantly decreased the levels of HAS2 mRNA but not those of HAS2-AS1. Similarly, HAS2-AS1 silencing decreased HAS2 expression but not SMILR levels, indicating that HAS2-AS1 and SMILR can independently regulate HAS2. However, no molecular mechanism of HAS2 regulation by SMILR has been proposed (Ballantyne et al. 2016).

3.5 Concluding Remarks

The action of lncRNAs can be considered a further step of gene expression regulation and may help to shed light on the molecular mechanisms of physiologic and pathologic processes. The functions of lncRNAs are versatile and depend on their subcellular localization and the interaction with other specific partners (e.g., proteins or other RNA molecules). The tissue specificity of these RNA species may be used as a diagnostic tool for different diseases including cancer and could be helpful to develop novel therapeutic strategies. Moreover, the possibility to be transported through extracellular vesicles can influence the regulation of critical enzymes and signaling pathways also in distant body district. Since a dysregulation of HA synthesis and HAS2 expression are often associated to pathologic conditions, the study of HAS2-AS1 may provide new mechanisms at the base of these diseases. HAS2-AS1 is not the only ncRNA associated to hyaluronan metabolizing genes. For instance, HAS2 expression is influenced by the lncRNA SMILR (Ballantyne et al. 2016), besides a wide number of microRNAs. Moreover, *in silico* analysis show that a specific microRNA could target several genes involved in HA metabolism. For example, microRNA-145 is predicted to target HAS2-AS1, the receptor for HA CD44 and hyaluronidase 2, whereas microRNA-186 shows a prediction for HAS2 and HAS2-AS1 (www.microrna.org). Interestingly, the enzyme UDP-glucose dehydrogenase (UGDH), which catalyzes the formation of UDP-glucuronic acid, also possesses a natural antisense transcript (www.tanric.org), although its function has never been investigated.

In conclusion, ncRNAs exhibit a surprisingly wide range of functions and connections and our knowledge is still in fancy. Therefore, further studies need to be accomplished to explore their potential therapeutic application.

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Competing Interests

The authors declare they have no competing interests.

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References

- Aryal B, Rotllan N, Fernández-Hernando C (2014) Noncoding RNAs and atherosclerosis. *Curr Atheroscler Rep* 16:407. <https://doi.org/10.1007/s11883-014-0407-3>
- Ballantyne MD, Pinel K, Dakin R et al (2016) Smooth muscle enriched long noncoding RNA (SMILR) regulates cell proliferation. *Circulation* 133:2050–2065. <https://doi.org/10.1161/CIRCULATIONAHA.115.021019>
- Bartolini B, Caravà E, Caon I et al (2020) Heparan sulfate in the tumor microenvironment. *Adv Exp Med Biol* 1245:147–161. https://doi.org/10.1007/978-3-030-40146-7_7
- Bhan A, Soleimani M, Mandal SS (2017) Long noncoding RNA and cancer: a new paradigm. *Cancer Res* 77:3965–3981
- Birney E, Stamatoyannopoulos JA, Dutta A et al (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447:799–816. <https://doi.org/10.1038/nature05874>
- Bistoletti M, Bosi A, Caon I et al (2020) Involvement of hyaluronan in the adaptive changes of the rat small intestine neuromuscular function after ischemia/reperfusion injury. *Sci Rep* 10:11521. <https://doi.org/10.1038/s41598-020-67876-9>
- Biswas S, Chakrabarti S (2019) Increased extracellular matrix protein production in chronic diabetic complications: implications of non-coding RNAs. *Non-coding RNA* 5:30
- Bosi A, Banfi D, Bistoletti M, Catizzone LM, Chiaravalli AM, Moretto P, Moro E, Karousou E, Viola M, Giron MC, Crema F, Rossetti C, Binelli G, Passi A, Vigetti D, Giaroni C, Baj A (2022) Hyaluronan regulates neuronal and immune function in the rat small intestine and colonic microbiota after ischemic/reperfusion injury. *Cells* 11(21):3370. <https://doi.org/10.3390/cells11213370>
- Camenisch TD, Spicer AP, Brehm-Gibson T et al (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 106:349–360. <https://doi.org/10.1172/JCI10272>
- Caon I, Bartolini B, Moretto P et al (2020a) Sirtuin 1 reduces hyaluronan synthase 2 expression by inhibiting nuclear translocation of NF- κ B and expression of the long-noncoding RNA HAS2-AS1. *J Biol Chem* 295:3485–3496. <https://doi.org/10.1074/jbc.RA119.011982>
- Caon I, Parnigoni A, Viola M et al (2020b) Cell energy metabolism and Hyaluronan synthesis. *J Histochem Cytochem* 69(1):35–47
- Caon I, Bartolini B, Parnigoni A et al (2019) Revisiting the hallmarks of cancer: the role of hyaluronan. *Semin Cancer Biol* 62:9–19
- Caravà E, Moretto P, Caon I, Parnigoni A, Passi A, Karousou E, Vigetti D, Canino J, Canobbio I, Viola M (2021) HA and HS changes in endothelial inflammatory activation. *Biomolecules* 11(6):809. <https://doi.org/10.3390/biom11060809>
- Chao H, Spicer AP (2005) Natural antisense mRNAs to hyaluronan synthase 2 inhibit hyaluronan biosynthesis and cell proliferation. *J Biol Chem* 280:27513–27522. <https://doi.org/10.1074/jbc.M411544200>
- Cheetham SW, Gruhl F, Mattick JS, Dinger ME (2013) Long noncoding RNAs and the genetics of cancer. *Br J Cancer* 108:2419–2425. <https://doi.org/10.1038/bjc.2013.233>
- Chen LL (2016) Linking long noncoding RNA localization and function. *Trends Biochem Sci* 41:761–772

- Chen R, Liu Y, Zhuang H et al (2017) Quantitative proteomics reveals that long non-coding RNA MALAT1 interacts with DBC1 to regulate p53 acetylation. *Nucleic Acids Res* 45:9947–9959. <https://doi.org/10.1093/nar/gkx600>
- Chujo T, Yamazaki T, Hirose T (2016) Architectural RNAs (arcRNAs): a class of long noncoding RNAs that function as the scaffold of nuclear bodies. *Biochim Biophys Acta - Gene Regul Mech* 1859:139–146
- Craig Venter J, Adams MD, Myers EW et al (2001) The sequence of the human genome. *Science* (80-) 291:1304–1351. <https://doi.org/10.1126/science.1058040>
- D'Angelo E, Agostini M (2018) Long non-coding RNA and extracellular matrix: the hidden players in cancer-stroma cross-talk. *Non-coding RNA Res* 3:174–177
- D'Onofrio N, Vitiello M, Casale R et al (2015) Sirtuins in vascular diseases: emerging roles and therapeutic potential. *Biochim Biophys Acta Mol basis Dis* 1852:1311–1322
- Deng L, Yang S-B, Xu F-F, Zhang J-H (2015) Long noncoding RNA CCAT1 promotes hepatocellular carcinoma progression by functioning as let-7 sponge. *J Exp Clin Cancer Res* 34:18. <https://doi.org/10.1186/s13046-015-0136-7>
- Dommez G, Outeiro TF (2013) SIRT1 and SIRT2: emerging targets in neurodegeneration. *EMBO Mol Med* 5:344–352. <https://doi.org/10.1002/emmm.201302451>
- Dragomir M, Chen B, Calin GA (2018) Exosomal lncRNAs as new players in cell-to-cell communication. *Transl Cancer Res* 7:S243–S252
- Dykes IM, Emanuelli C (2017) Transcriptional and post-transcriptional gene regulation by long non-coding RNA. *Genomics, Proteomics Bioinforma* 15:177–186
- Elilob B, Kilic U (2018) High levels of SIRT1 expression as a protective mechanism against disease-related conditions. *Front Endocrinol (Lausanne)* 9:614
- Filpa V, Bistoletti M, Caon I et al (2017) Changes in hyaluronan deposition in the rat myenteric plexus after experimentally-induced colitis. *Sci Rep* 7:17644. <https://doi.org/10.1038/s41598-017-18020-7>
- Frantz C, Stewart KM, Weaver VM (2010) The extracellular matrix at a glance. *J Cell Sci* 123: 4195–4200
- Garantziotis S, Savani RC (2019) Hyaluronan biology: a complex balancing act of structure, function, location and context. *Matrix Biol* 78–79:1–10
- Gendrel A-V, Heard E (2014) Noncoding RNAs and epigenetic mechanisms during X-chromosome inactivation. *Annu Rev Cell Dev Biol* 30:561–580. <https://doi.org/10.1146/annurev-cellbio-101512-122415>
- Goetze S, Xi XP, Kawano Y et al (1999) TNF- α -induced migration of vascular smooth muscle cells is MAPK dependent. *Hypertension* 33:183–189. <https://doi.org/10.1161/01.HYP.33.1.183>
- Gong C, Maquat LE (2011) lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. *Nature* 470:284–290. <https://doi.org/10.1038/nature09701>
- Gudenäs BL, Wang L (2018) Prediction of lncRNA subcellular localization with deep learning from sequence features. *Sci Rep* 8:1–10. <https://doi.org/10.1038/s41598-018-34708-w>
- Gupta RA, Shah N, Wang KC et al (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464:1071–1076. <https://doi.org/10.1038/nature08975>
- Huang M, Wang H, Hu X, Cao X (2019) lncRNA MALAT1 binds chromatin remodeling subunit BRG1 to epigenetically promote inflammation-related hepatocellular carcinoma progression. *Oncoimmunology* 8. 8:e1518628. <https://doi.org/10.1080/2162402X.2018.1518628>
- Huarte M, Guttman M, Feldser D et al (2010) A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 142:409–419. <https://doi.org/10.1016/j.cell.2010.06.040>
- Itano N, Sawai T, Yoshida M et al (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274:25085–25092. <https://doi.org/10.1074/JBC.274.35.25085>
- Jiang C, Li Y, Zhao Z et al (2016) Identifying and functionally characterizing tissue-specific and ubiquitously expressed human lncRNAs. *Oncotarget* 7:7120–7133. <https://doi.org/10.18632/oncotarget.6859>

- Kaeberlein M, McVey M, Guarente L (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev* 13:2570–2580. <https://doi.org/10.1101/gad.13.19.2570>
- Kakizaki I, Kojima K, Takagaki K et al (2004) A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. *J Biol Chem* 279:33281–33289. <https://doi.org/10.1074/jbc.M405918200>
- Karamanos NK, Piperigkou Z, Theocharis AD et al (2018) Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem Rev* 118:9152–9232
- Karousou E, Kamiryo M, Skandalis SS et al (2010) The activity of hyaluronan synthase 2 is regulated by dimerization and ubiquitination. *J Biol Chem* 285:23647–23654. <https://doi.org/10.1074/jbc.M110.127050>
- Karousou E, Parnigoni A, Moretto P, Passi A, Viola M, Vigetti D (2023) Hyaluronan in the cancer cells microenvironment. *Cancers (Basel)* 15(3):798. <https://doi.org/10.3390/cancers15030798>
- Kitada M, Ogura Y, Monno I, Koya D (2019) Sirtuins and type 2 diabetes: role in inflammation, oxidative stress, and mitochondrial function. *Front Endocrinol (Lausanne)* 10:187
- Kolliopoulos C, Lin CY, Heldin CH et al (2019) Has2 natural antisense RNA and Hmga2 promote Has2 expression during TGF β -induced EMT in breast cancer. *Matrix Biol* 80:29–45. <https://doi.org/10.1016/j.matbio.2018.09.002>
- Korpos E, Wu C, Sorokin L (2009) Multiple roles of the extracellular matrix in inflammation. *Curr Pharm Des* 15:1349–1357. <https://doi.org/10.2174/138161209787846685>
- Kultti A, Pasonen-Seppänen S, Jauhainen M et al (2009) 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3. *Exp Cell Res* 315:1914–1923. <https://doi.org/10.1016/j.yexcr.2009.03.002>
- Lander ES, Linton LM, Birren B et al (2001) Initial sequencing and analysis of the human genome. *Nature* 409:860–921. <https://doi.org/10.1038/35057062>
- Lee SH, Lee JH, Lee HY, Min KJ (2019) Sirtuin signaling in cellular senescence and aging. *BMB Rep* 52:24–34
- Li X (2013) SIRT1 and energy metabolism. *Acta Biochim Biophys Sin Shanghai* 45:51. <https://doi.org/10.1093/ABBS/GMS108>
- Liang J, Jiang D, Noble PW (2016) Hyaluronan as a therapeutic target in human diseases. *Adv Drug Deliv Rev* 97:186–203
- Liu Q, Zhang X, Dai L et al (2014) Long noncoding RNA related to cartilage injury promotes chondrocyte extracellular matrix degradation in osteoarthritis. *Arthritis Rheumatol* 66:969–978. <https://doi.org/10.1002/art.38309>
- Lyon MF (1961) Gene action in the X-chromosome of the mouse (*mus musculus* L.). *Nature* 190:372–373. <https://doi.org/10.1038/190372a0>
- Manou D, Caon I, Bouris P et al (2019) The complex interplay between extracellular matrix and cells in tissues. *Methods Mol Biol* 1952:1–20. https://doi.org/10.1007/978-1-4939-9133-4_1
- Marozzi M, Parnigoni A, Negri A, Viola M, Vigetti D, Passi A, Karousou E, Rizzi F (2021) Inflammation, extracellular matrix remodeling, and proteostasis in tumor microenvironment. *Int J Mol Sci* 22(15):8102. <https://doi.org/10.3390/ijms22158102>
- Marques AC, Ponting CP (2014) Intergenic lncRNAs and the evolution of gene expression. *Curr Opin Genet Dev* 27:48–53
- Mattick JS (2011) The central role of RNA in human development and cognition. *FEBS Lett* 585:1600–1616
- Mehić M, de Sa VK, Hebestreit S et al (2017) The deubiquitinating enzymes USP4 and USP17 target hyaluronan synthase 2 and differentially affect its function. *Oncogenesis* 6:e348. <https://doi.org/10.1038/oncsis.2017.45>
- Melero-Fernandez de Mera RM, Arasu UT, Kärnä R et al (2018) Effects of mutations in the post-translational modification sites on the trafficking of hyaluronan synthase 2 (HAS2). *Matrix Biol* 80:85. <https://doi.org/10.1016/j.matbio.2018.10.004>

- Michael DR, Phillips AO, Krupa A et al (2011) The human hyaluronan synthase 2 (HAS2) gene and its natural antisense RNA exhibit coordinated expression in the renal proximal tubular epithelial cell. *J Biol Chem* 286:19523–19532. <https://doi.org/10.1074/jbc.M111.233916>
- Minor RK, Baur JA, Gomes AP et al (2011) SRT1720 improves survival and healthspan of obese mice. *Sci Rep* 1:70. <https://doi.org/10.1038/srep00070>
- Moretto P, Karousou E, Viola M et al (2015) Regulation of Hyaluronan synthesis in vascular diseases and diabetes. *J Diabetes Res* 2015:1–9. <https://doi.org/10.1155/2015/167283>
- Muz B, de la Puente P, Azab F, Azab AK (2015) The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia* 3:83. <https://doi.org/10.2147/hp.s93413>
- Parnigoni A, Caon I, Moretto P, Viola M, Karousou E, Passi A, Vigetti D (2021) The role of the multifaceted long non-coding RNAs: A nuclear-cytosolic interplay to regulate hyaluronan metabolism. *Matrix Biol Plus* 11:100060. <https://doi.org/10.1016/j.mbplus.2021.100060>. PMID: 34435179; PMCID: PMC8377009
- Parnigoni A, Viola M, Karousou E, Rovera S, Giaroni C, Passi A, Vigetti D (2022a) Hyaluronan in pathophysiology of vascular diseases: specific roles in smooth muscle cells, endothelial cells, and macrophages. *Am J Physiol Cell Physiol* 323(2):C505–C519. <https://doi.org/10.1152/ajpcell.00061.2022>
- Parnigoni A, Caon I, Teo WX, Hua SH, Moretto P, Bartolini B, Viola M, Karousou E, Yip GW, Götte M, Heldin P, Passi A, Vigetti D (2022b) The natural antisense transcript HAS2-AS1 regulates breast cancer cells aggressiveness independently from hyaluronan metabolism. *Matrix Biol* 109:140–161. <https://doi.org/10.1016/j.matbio.2022.03.009>
- Passi A, Vigetti D (2019) Hyaluronan as tunable drug delivery system. *Adv Drug Deliv Rev* 146: 83–96
- Piccinini AM, Midwood KS (2014) Illustrating the interplay between the extracellular matrix and microRNAs. *Int J Exp Pathol* 95:158–180. <https://doi.org/10.1111/iep.12079>
- Porsch H, Bernert B, Mehić M et al (2013) Efficient TGFβ-induced epithelial-mesenchymal transition depends on hyaluronan synthase HAS2. *Oncogene* 32:4355–4365. <https://doi.org/10.1038/onc.2012.475>
- Postepska-Igielska A, Giwojna A, Gasri-Plotnitsky L et al (2015) LncRNA Khps1 regulates expression of the proto-oncogene SPHK1 via triplex-mediated changes in chromatin structure. *Mol Cell* 60:626–636. <https://doi.org/10.1016/j.molcel.2015.10.001>
- Rankin KS, Frankel D (2016) Hyaluronan in cancer-from the naked mole rat to nanoparticle therapy. *Soft Matter* 12:3841–3848
- Rastogi S, Rizwani W, Joshi B et al (2012) TNF-α response of vascular endothelial and vascular smooth muscle cells involve differential utilization of ASK1 kinase and p73. *Cell Death Differ* 19:274–283. <https://doi.org/10.1038/cdd.2011.93>
- Rinn JL, Kertesz M, Wang JK et al (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129:1311–1323. <https://doi.org/10.1016/j.cell.2007.05.022>
- Rogina B, Helfand SL (2004) Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* 101:15998–16003. <https://doi.org/10.1073/pnas.0404184101>
- Ruiz-Orera J, Messegue X, Subirana JA, Alba MM (2014) Long non-coding RNAs as a source of new peptides. *elife* 3:e03523. <https://doi.org/10.7554/eLife.03523>
- Sarfi M, Abbastabar M, Khalili E (2019) Long noncoding RNAs biomarker-based cancer assessment. *J Cell Physiol* 234:16971–16986
- Shan Y, Ma J, Pan Y et al (2018) LncRNA SNHG7 sponges MIR-216b to promote proliferation and liver metastasis of colorectal cancer through upregulating GALNT1. *Cell Death Dis* 9:1–13. <https://doi.org/10.1038/s41419-018-0759-7>
- Shi T, Gao G, Cao Y (2016) Long noncoding RNAs as novel biomarkers have a promising future in cancer diagnostics. *Dis Markers* 2016:9085195. <https://doi.org/10.1155/2016/9085195>

- Tissenbaum HA, Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410:227–230. <https://doi.org/10.1038/35065638>
- Tong L, Wang Y, Ao Y, Sun X (2019) CREB1 induced lncRNA HAS2-AS1 promotes epithelial ovarian cancer proliferation and invasion via the miR-466/RUNX2 axis. *Biomed Pharmacother* 115. <https://doi.org/10.1016/j.biopha.2019.108891>
- Vigetti D, Clerici M, Deleonibus S et al (2011) Hyaluronan synthesis is inhibited by adenosine monophosphate-activated protein kinase through the regulation of HAS2 activity in human aortic smooth muscle cells. *J Biol Chem* 286:7917–7924. <https://doi.org/10.1074/jbc.M110.193656>
- Vigetti D, Deleonibus S, Moretto P et al (2012) Role of UDP-N-acetylglucosamine (GlcNAc) and O-GlcNAcylation of hyaluronan synthase 2 in the control of chondroitin sulfate and hyaluronan synthesis. *J Biol Chem* 287:35544–35555. <https://doi.org/10.1074/jbc.M112.402347>
- Vigetti D, Deleonibus S, Moretto P et al (2014a) Natural antisense transcript for hyaluronan synthase 2 (HAS2-AS1) induces transcription of HAS2 via protein O-GlcNAcylation. *J Biol Chem* 289:28816–28826. <https://doi.org/10.1074/jbc.M114.597401>
- Vigetti D, Genasetti A, Karousou E et al (2010) Proinflammatory cytokines induce hyaluronan synthesis and monocyte adhesion in human endothelial cells through hyaluronan synthase 2 (HAS2) and the nuclear factor- κ B (NF- κ B) pathway. *J Biol Chem* 285:24639–24645. <https://doi.org/10.1074/jbc.M110.134536>
- Vigetti D, Karousou E, Viola M et al (2014b) Hyaluronan: biosynthesis and signaling. *Biochim Biophys Acta - Gen Subj* 1840:2452–2459. <https://doi.org/10.1016/j.bbagen.2014.02.001>
- Vigetti D, Ori M, Viola M et al (2006) Molecular cloning and characterization of UDP-glucose dehydrogenase from the amphibian *Xenopus laevis* and its involvement in hyaluronan synthesis. *J Biol Chem* 281:8254–8263. <https://doi.org/10.1074/jbc.M508516200>
- Vigetti D, Viola M, Karousou E et al (2008) Hyaluronan-CD44-ERK1/2 regulate human aortic smooth muscle cell motility during aging. *J Biol Chem* 283:4448–4458. <https://doi.org/10.1074/jbc.M709051200>
- Vigetti D, Viola M, Karousou E et al (2014c) Epigenetics in extracellular matrix remodeling and hyaluronan metabolism. *FEBS J* 281:4980–4992. <https://doi.org/10.1111/febs.12938>
- Viola M, Bartolini B, Vigetti D et al (2013) Oxidized low density lipoprotein (LDL) affects hyaluronan synthesis in human aortic smooth muscle cells. *J Biol Chem* 288:29595–29603. <https://doi.org/10.1074/jbc.M113.508341>
- Viola M, Karousou E, D'Angelo ML et al (2015a) Regulated Hyaluronan synthesis by vascular cells. *Int J Cell Biol* 2015:208303. <https://doi.org/10.1155/2015/208303>
- Viola M, Karousou E, D'Angelo ML et al (2016) Extracellular matrix in atherosclerosis: Hyaluronan and proteoglycans insights. *Curr Med Chem* 23:2958–2971. <https://doi.org/10.2174/0929867323666160607104602>
- Viola M, Vigetti D, Karousou E et al (2015b) Biology and biotechnology of hyaluronan. *Glycoconj J* 32:93–103. <https://doi.org/10.1007/s10719-015-9586-6>
- Vitale DL, Caon I, Parnigoni A, Sevic I, Spinelli FM, Icardi A, Passi A, Vigetti D, Alaniz L (2021) Initial identification of UDP-glucose dehydrogenase as a prognostic marker in breast cancer patients, which facilitates epirubicin resistance and regulates hyaluronan synthesis in MDA-MB-231 cells. *Biomolecules* 11(2):246. <https://doi.org/10.3390/biom11020246>
- Wang P, Xue Y, Han Y et al (2014a) The STAT3-binding long noncoding RNA lnc-DC controls human dendritic cell differentiation. *Science* (80-) 344:310–313. <https://doi.org/10.1126/science.1251456>
- Wang S-H, Zhang W-J, Wu X-C et al (2014b) Long non-coding RNA Malat1 promotes gallbladder cancer development by acting as a molecular sponge to regulate miR-206. *Oncotarget* 7:37857. <https://doi.org/10.18632/oncotarget.9347>
- Wang T, Liu Y, Wang Y et al (2019) Long non-coding RNA XIST promotes extracellular matrix degradation by functioning as a competing endogenous RNA of miR-1277-5p in osteoarthritis. *Int J Mol Med* 44:630–642. <https://doi.org/10.3892/ijmm.2019.4240>

- Wright JLN, Collins HE, Wende AR, Chatham JC (2017) O-GlcNAcylation and cardiovascular disease. *Biochem Soc Trans* 45:545–553
- Yao RW, Wang Y, Chen LL (2019) Cellular functions of long noncoding RNAs. *Nat Cell Biol* 21: 542–551
- Yoon JH, Abdelmohsen K, Kim J et al (2013) Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination. *Nat Commun* 4:1–14. <https://doi.org/10.1038/ncomms3939>
- Zampetaki A, Albrecht A, Steinhofel K (2018) Long non-coding RNA structure and function: is there a link? *Front Physiol* 9:1201
- Zeng C, Hamada M (2018) Identifying sequence features that drive ribosomal association for lncRNA. *06 biological sciences 0604 genetics. BMC Genomics* 19:906. <https://doi.org/10.1186/s12864-018-5275-8>
- Zhang L, Wang H, Xu M et al (2020) Long noncoding RNA HAS2-AS1 promotes tumor progression in glioblastoma via functioning AS a competing endogenous RNA. *J Cell Biochem* 121:661–671. <https://doi.org/10.1002/jcb.29313>
- Zhang X, Wang W, Zhu W et al (2019) Mechanisms and functions of long non-coding RNAs at multiple regulatory levels. *Int J Mol Sci* 20:5573
- Zhao Z, Liang T, Feng S (2019) Silencing of HAS2-AS1 mediates PI3K/AKT signaling pathway to inhibit cell proliferation, migration, and invasion in glioma. *J Cell Biochem* 120:11510. <https://doi.org/10.1002/jcb.28430>
- Zhu G, Wang S, Chen J et al (2017) Long noncoding RNA HAS2-AS1 mediates hypoxia-induced invasiveness of oral squamous cell carcinoma. *Mol Carcinog* 1–46. <https://doi.org/10.1002/mc.22674>

Chapter 4

The Hyaluronan-Rich Zones of Plasma Membrane Protrusions and Extracellular Vesicles



Kirsi Rilla

Abstract Hyaluronan (HA) is a widespread and unique biomolecule important for hydration, lubrication, and the protection of cells and tissues. HA has been traditionally associated with vitality, development, and growth, but also with pathological conditions. The existence of HA-rich pericellular zones around many cell types has been known for decades but only recently has the role of HA as a versatile regulator of cellular activity been clarified. In cooperation with the intracellular cytoskeleton system, the presence and synthesis of HA create pericellular forces that bend the plasma membrane. Shape modulation of the plasma membrane and the regulation of this process are both determinants and indicators of cellular condition and behavior. The discovery of HA's role in the formation of different plasma membrane protrusions, and in shedding of plasma membrane-derived particles has further expanded its biochemical functions. Recent data suggest that HA is a multifunctional surface component of the extracellular vesicles (EVs). These HA-coated EVs constitute a specific vesicle population with unique surface properties, postulated to regulate EV homing, targeting, binding, and remodeling of the ECM. This chapter summarizes the recent advances made in the biology of HA-coated filopodia and HA-coated EVs and their role as versatile nanosized communicators in health and disease, their formation mechanisms as well as their effects on target cells and their potential for clinical utilization.

4.1 Hyaluronan-Rich Glycocalyx

Hyaluronan (HA) is a pivotal building material of connective tissues, a signaling molecule between cells and one of the main components of the pericellular matrix. The detailed existence of extensive HA-dependent pericellular zones has been

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described around many cell types, such as human synovial cells and fibroblasts (Clarris and Fraser 1968), chondrocytes (Knudson 1993), mesothelial cells and mesothelioma cells (Heldin and Pertoft 1993). Direct microscopic visualization of these zones has been challenging, but the indirect demonstration of the HA-rich zones as “empty” exclusion areas around cells by small particles such as fixed erythrocytes has been a widely used technique for decades.

The structural basis of the pericellular zone seems to be variable. HA can be anchored to the cell surface by one of the HA synthases or cell surface receptors such as CD44 or RHAMM and cross-linked by various HA-binding proteins, e.g. versican, aggrecan, TSG-6, and inter-alpha-trypsin inhibitor (Evanko et al. 2007). The thickness of the HA-rich pericellular zone is dependent on multiple factors, such as cell type, size of HA molecules, HAS activity, relative expression of the HAS isoforms (Brinck and Heldin 1999; Rilla et al. 2013b) and associated proteoglycans (Knudson et al. 2019). While HAS activity seems to be a major regulator of the HA coat, CD44 has also an important role in retaining HA in the pericellular matrix of many cell types such as keratinocytes (Pasonen-Seppänen et al. 2012), chondrocytes (Knudson et al. 2019), and tumor cells (Härkönen et al. 2019). In addition, in chondrocytes, the assembly of an HA coat via synthesis by HAS2 is crucial for aggrecan retention (Huang et al. 2016). In most cases, the assembly of the coat is a sum of both mechanisms, depending on the relative levels of HAS activity and the expression of HA receptors.

Since HA is a non-immunogenic polysaccharide with a conserved structure among species as well as a ubiquitous expression, its histological detection is problematic and requires more sophisticated methods than simply an incubation with antibodies (Gebauer et al. 2017). The currently used methods utilize HA-binding proteins and protein fragments modified with biotin. The specific domain in the aggrecan molecule that binds HA (HA-binding complex, HABC) has been widely exploited in the specific detection of HA in histochemistry (Tammi et al. 1988) and in liquid samples (Hiltunen et al. 2002). However, fluorescent techniques are needed for live cell detection of HA; for example, neurocan-GFP fusion protein has been utilized for HA detection (Zhang et al. 2004). The fluorescently labeled hyaluronan binding complex (fHABC) created by coupling of HABC with a fluorescent group (red in Fig. 4.1b) enables direct labeling and detecting HA in the pericellular zones of live cells (Rilla et al. 2008). Utilization of this reliable tool had a pivotal role in the discovery of HA-coated plasma membrane protrusions (Kultti et al. 2006) that provide a scaffold for the HA-rich zone (Rilla et al. 2008) and act as sources for HA-coated extracellular vesicles (Rilla et al. 2013b). This chapter will focus on these specific cellular morphological structures and discusses the dual role of HA as both an inducer and a component of filopodia and the vesicles emanating from filopodial tips (Fig. 4.1).

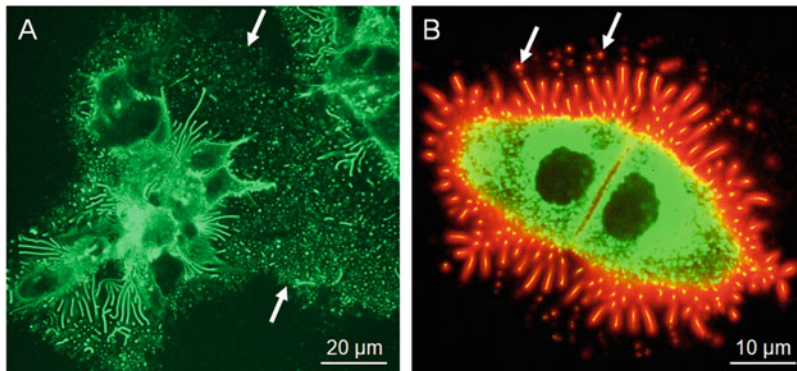


Fig. 4.1 HAS-induced filopodia, EV and HA coat. Live GFP-HAS3 expressing MCF-7 breast cancer cells grown in 3D collagen IV-rich basement membrane extract gel (Cultrex). The cells express extremely long filopodia and secrete EVs that are visualized in high numbers because they are trapped inside the gel (**a**). A live mitotic GFP-HAS3 expressing breast cancer cell stained with fHABC probe (red) shows high numbers of HA-coated filopodia and HA-coated EVs. Arrows in both panels indicate EVs

4.2 Hyaluronan-Dependent Plasma Membrane Protrusions

4.2.1 Regulation of Filopodia Formation

The shape of the plasma membrane and its dynamics are crucial for all cellular functions. Different plasma membrane extensions, with shapes varying from spheres to tubes and with sizes ranging from the nanometers to micrometers, are involved in the regulation of embryogenesis, immune reactions, and disease progression (Chhabra and Higgs 2007). Filopodia are actin-dependent finger-like plasma membrane protrusions that are often the first part of the cell to contact the extracellular cues; they act as a probe to sense the cellular microenvironment (Jacquemet et al. 2015). They are highly dynamic structures, regulated by various cytosolic actin-binding proteins that control the polymerization of the actin filaments, such as fascin, small GTPases, ERM proteins, and myosins (Mattila and Lappalainen 2008). In addition to the pushing force created by the complex actin-based machinery, deformation of the plasma membrane by membrane binding proteins containing i-BAR domains is required (Mattila et al. 2007). However, the exact mechanism by which the filopodia push themselves out of the plasma membrane is still awaiting a detailed explanation.

4.2.2 HA Induces Filopodial Growth and Maintenance

In addition to the actin cytoskeleton and plasma membrane dynamics, the third element involved in the formation of filopodia and other protrusions is the pericellular force created by the molecules covering the outer surface of the plasma membrane (Fig. 4.2). Interestingly, glycocalyx polymers located on the outer surface of the plasma membrane have been shown to be important regulators of the shape of the plasma membrane (Shurer et al. 2019). HA is one of the key players in this regulation, not just inducing and maintaining the filopodia, but also in the synthesis of the filopodial membranes (Kultti et al. 2006). Additionally, live cell experiments showed that the scaffold in an HA-dependent pericellular exclusion zone of HAS expressing cells is created by plasma membrane protrusions, rather than HA alone (Rilla et al. 2008). These protrusions exist also in chondrocytes, but their proteoglycan-rich pericellular zone is not exclusively dependent on filopodia (Rilla et al. 2008). Each HA-dependent protrusion is surrounded by an HA layer, that varies in thickness and density, depending on cell type, cell state, expression of different HAS isoenzymes and receptors. In addition to HAS overexpressing cells, HA-coated protrusions have been detected in primary human mesenchymal stem cells (hMSC) (Qu et al. 2014) and in rat mesothelial cells (Koistinen et al. 2017) without genetic manipulation, as well as in vivo in rat mesothelium (Koistinen et al. 2016) and human synovial membranes (Mustonen et al. 2016), which indicates that they are a general feature of cells with active HA synthesis.

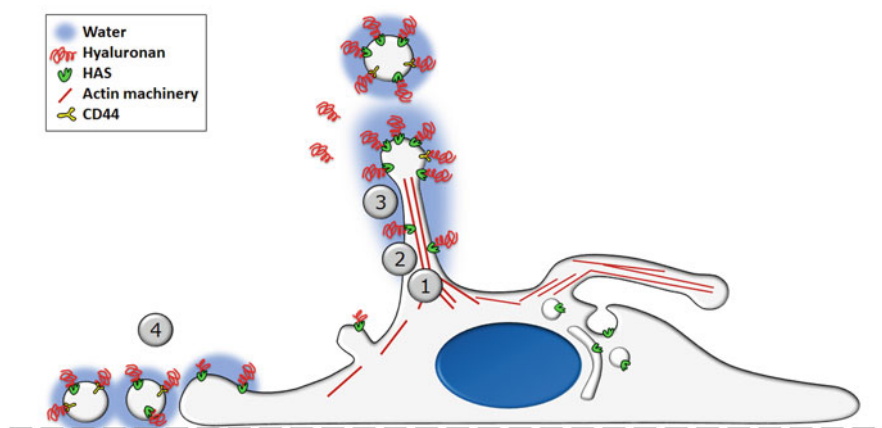


Fig. 4.2 A schematic drawing on the role of HA-coated filopodia as sources of EV shedding. Formation of filopodia and other protrusions and EV budding and shedding are highly organized systems resulting from cooperation between the forces created by three systems: (1) cytoskeleton (2) lipid membrane, and (3) pericellular glycocalyx. (4) Retraction of protrusions adhering to substratum results in vesiculation and formation of “footprints.” Modified from (Koistinen et al. 2015)

4.2.3 Role of HA as a General Structural Component of the Filopodia

Interestingly, HAS activity induces both dorsal and vertical (adhering to the plastic substratum) filopodia in monolayer cell cultures, but the structure of the adhering filopodia is less dependent on HA-coating than that of the dorsal filopodia (Koistinen et al. 2015; Rilla and Koistinen 2015), possibly because of filopodial interactions with the substratum (Fig. 4.3) via other adhesion molecules such as integrins (Arjonen et al. 2011). The strong dependence between HA synthesis and filopodia is evident by the fact that all cell types with active HA synthesis have extensive filopodia, such as cancer cells (Arjonen et al. 2011), chondrocytes (Hale and Wuthier 1987), oocytes (Makabe et al. 2006), human mesenchymal stem cells (Qu et al. 2014), dermal fibroblasts of Shar Pei dogs with cutaneous mucinosis (Docampo et al. 2011), synovial cells (Lukoschek and Addicks 2008), and mesothelial cells (Mutsaers 2004). However, the formation of filopodia is not solely dependent on HA synthesis, because cell types with very low HA synthetic activity are able to produce short filopodia (Bohil et al. 2006). Based on these findings, we can assume that without the extracellular support conferred by the HA-dependent glycocalyx, the dorsal protrusions would not be able to reach their typical length—in some cases the length of filopodia can exceed 20 μm . There is convincing evidence that HA and its interactions with other components of the glycocalyx are general regulators for filopodial growth, dynamics, and maintenance. Interestingly, HA forms the

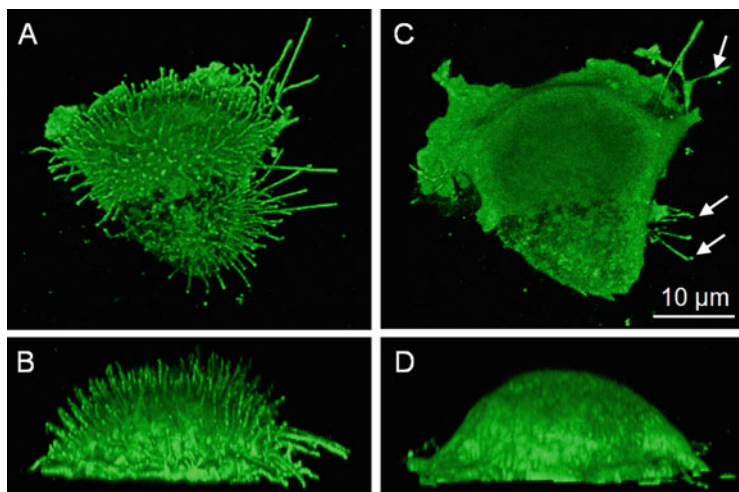


Fig. 4.3 The dependence of plasma membrane protrusions on HA coat in live GFP-HAS3 expressing breast cancer cells. The same live cell before (**a**, **b**) and 5 minutes after (**c**, **d**) digestion of the HA coat by *streptomyces* hyaluronidase treatment. The dorsal protrusions shrink or collapse after the treatment when the external support provided by HA is lost, but those protrusions adhering to the substratum do not retract (arrows). Horizontal 3D confocal projections are shown in (**a** and **c**), and vertical views in (**b** and **d**)

backbone of the perineuronal nets around neurons in the cerebral cortex and HAS2 is localized in neuronal protrusions (Fowke et al. 2017). There is also evidence that CD44 regulates the functional and structural plasticity of dendritic spines, the specific filopodial-like protrusions present in neuronal cells (Roszkowska et al. 2016).

4.2.4 Challenges in the Research Methods of HA-Rich Filopodia

The HA-rich pericellular zones are highly sensitive for fixation (Lin et al. 1997), dehydration, and other routine procedures for biological sample preparation, resulting in precipitation and leaching of pericellular HA and in the formation of HA-cables in cell cultures (Jokela et al. 2008). Furthermore, the structure of filopodia is easily damaged during fixation. Interestingly, shortly after removal of HA by hyaluronidase digestion, the filopodia seem to disappear when studied with fluorescent microscopy (Fig. 4.3). Furthermore, the morphology of hyaluronidase-treated cells and their filopodia is identical with control cells when processed for electron microscopy (Koistinen et al. 2015). This suggests that in both cases, the dehydration effect during processing for microscopy has resulted in the collapse of the filopodia. It has been also shown that the HA-positive cellular protrusions partially contribute to hyaluronan cable formation in fixed cells (Evanko et al. 2009). Because of these artifacts resulting from fixation and sample processing, live cell experiments give the most reliable results when studying the morphology and dynamics of filopodia.

4.2.5 Role of HAS Isoenzymes on the HA Coating, Filopodia, and EVs

Because most cell types producing HA express all three HAS isoenzymes, it has proved challenging to dissect the individual effect of each enzyme. Overexpression studies with cell types that have low or negligible HAS expression levels have shown that each HAS isoform has its own distinctive coat formation and that these differ significantly. While HAS2 and HAS3 induce a thick coat, HAS1 overexpression induces a very tenuous coat (Brinck and Heldin 1999; Rilla et al. 2013a) under normal conditions without specific induction of activity by increased availability of substrates for HA synthesis (Rilla et al. 2013a) or inflammatory agents such as cytokines (Siiskonen et al. 2014). Interestingly, the effect of HAS isoenzymes on the formation of filopodia is similar, being lowest in cells with HAS1 overexpression and highest in those cells with HAS3 expression (Törrönen et al. 2014). These results have been confirmed by downregulation of the HASs,

i.e. inhibition of HAS3 and to a lesser extent, HAS2, reduced the numbers of filopodia in human esophageal squamous carcinoma cells (Twarock et al. 2010).

4.2.6 HA Receptors and Filopodia

The effect of HA on the formation of filopodia can be partly mediated via the role of the actin cytoskeleton in the “inside-out” regulation and maintenance of the pericellular hyaluronan coat. Both HA receptors CD44 and RHAMM are known to be able to induce signals that regulate the cytoskeleton (Twarock et al. 2010). Despite the high positivity of filopodia for CD44, CD44 expression has not been shown to be directly associated with their formation (Kultti et al. 2006; Rilla et al. 2008; Twarock et al. 2010; Härkönen et al. 2019). For instance, CD44 overexpression induced the assembly of a tight, uniform HA layer on the surface of filopodia of CD44-negative gastric cancer cells (Härkönen et al. 2019). Blocking antibodies against RHAMM did not inhibit the formation of HAS3-induced filopodia. However, since downregulation of RHAMM by lentiviral shRNA did block filopodial growth this indicates that RHAMM has a role in this phenomenon (Twarock et al. 2010), but the possible mechanism is still unknown. These results point to a mutual connection between filopodia and HA metabolism; HA synthesis activity is associated with filopodial growth and the presence of filopodia increases the plasma membrane surface available for HA synthesis. Furthermore, HA-dependent filopodia act as specific sites for the formation of extracellular vesicles, a topic that will be addressed in detail in the next section.

4.3 Discovery of HA-Coated Extracellular Vesicles

4.3.1 Extracellular Vesicles as Novel Messengers

Extracellular vesicles (EVs) are novel extracellular messengers that exchange information between adjacent and distant cells and tissues. They comprise an extensive population of lipid-layered particles originating from the cellular membranes of all cell types (Raposo and Stoorvogel 2013). EVs are packaged with a wide variety of macromolecules, including extracellular, membrane-bound, cytosolic, and nuclear associated factors. EVs carry cytosolic components such as nucleic acids, metabolites, enzymes, and signaling molecules from the cells from which they originate and their plasma membrane lipid, protein, and carbohydrate composition also reflects that of the original cell. They transport their cargo to adjacent cells as well as distant tissues where it is transferred into the target cells. The current classification into exosomes, microvesicles, and apoptotic bodies is based on their distinctive forms of biogenesis (Mathieu et al. 2019).

EV size and molecular composition are markedly altered by oncogenic transformation and epithelial to mesenchymal transition of the cells of origin (EL Andaloussi et al. 2013). This has a substantial potential for EV utilization in diagnostics (Choi et al. 2019), allowing scoring of the patient's status without the need for a solid tissue biopsy. In addition to acting as fingerprints from original cells, EVs act as specific messengers between cells and tissues, enhancing tissue healing and regeneration (Bjørge et al. 2017) and tumor progression by regulating angiogenesis, immunosuppression, invasion, and metastasis (Kogure et al. 2019).

4.3.2 HA Accelerates its Own Accumulation on the EVs

Recent research has mainly concentrated on the nucleic acid, protein and lipid contents of EVs. Less attention has been paid to the carbohydrates carried on the surfaces of the EVs (Gerlach and Griffin 2016; Williams et al. 2018). However, in order to fully understand the biology and properties of EVs, it will be crucial to clarify the carbohydrate composition of EVs and its impact on EV biogenesis and targeting.

We have shown that the activities of both HAS3 (Rilla et al. 2013b) and HAS2 (Melero-Fernandez de Mera et al. 2019) can trigger the shedding of EVs and this process is regulated by the UDP-sugar supply (Deen et al. 2016). Additionally, enhanced expression of HAS2 and HAS3 in wounded or growth factor-treated mesothelial cells was associated with increased EV secretion (Koistinen et al. 2017). In human mesenchymal stem cells, no direct correlation was detected between HA synthesis activity and EV secretion levels, but the HA content of the EV reflected the HA synthesis rate of the original cell (Arasu et al. 2017). Since the molecular composition of the EV reflects the composition of the original cell, it is predicted that EVs budding from membranes of HA-producing cells will carry HA on their surface. Because HA synthesis accelerates EV secretion levels, and at the same time, the HA content of EV correlates with the HA content of the cells from which they originate (Arasu et al. 2017), it seems that HA has a unique capability to induce its own accumulation into EVs.

The HA-induced plasma membrane protrusions paved the way for the discovery of hyaluronan-coated EVs (Rilla et al. 2013b; Rilla et al. 2014). We have shown that HA is carried by EVs originating from cells manipulated to overexpress HAS3 (Rilla et al. 2013b) and HAS2 (Melero-Fernandez de Mera et al. 2019). In addition to genetically manipulated cells, also primary cells with high endogenous HA synthesis rates such as mesenchymal stem cells (Arasu et al. 2017) and activated mesothelial cells (Koistinen et al. 2017) secrete HA-coated EVs. Synovial fluid was the first body fluid in which HA-EVs were detected in vivo (Mustonen et al. 2016). Because levels of HA are upregulated in many disease states such as in cancer (Tammi et al. 2018) and inflammation (Misra et al. 2015) and HA is associated with stemness (Qu et al. 2014) and tissue repair (Litwiniuk et al. 2016), it can be postulated that the HA carried by EV has the potential to be exploited not only as a biomarker but also as regulator of EV targeting and immunoprotection.

4.4 EV Shedding from the Tips of Plasma Membrane Protrusions

As mentioned previously, HA is a potent regulator of the plasma membrane curvature due to its ability to create high osmotic pressure, facilitating filopodia formation (Koistinen et al. 2015) and EV budding from the tips of the buds (Rilla et al. 2013b; Rilla et al. 2014). High levels of HA on the plasma membrane increase hydrostatic pressure (Cowman et al. 2015) and provide energy for plasma membrane shaping (Richter et al. 2018). This energy creates a pulling force with which to modulate the intracellular actin-myosin-based contractile cytoskeletal machinery (Shurer et al. 2019). Interestingly, actin withdrawal from the cell periphery results in cell surface blebbing and the formation of vesicles at the tips of chondrocyte plasma membrane protrusions (Hale and Wuthier 1987). Actin is not present in the tips of HAS-induced filopodia (Koistinen et al. 2015), which may offer a putative mechanism to explain the enhanced shedding of EVs from the tips of filopodia.

It has been also suggested that the packing of HA chains in cylindrical and spherical brushes on protrusions and vesicles leads to higher energy gains than can be achieved with the planar brushes associated with plain plasma membranes (Richter et al. 2018). Osmotic changes are known to generate transient pearling and vesiculation of membrane tubes under osmotic gradients (Sanborn et al. 2013), which may partly explain the ability of HA to induce the vesiculation of filopodia to form EVs. Nevertheless, the exact mechanism whereby HA synthesis induces such a prominent and dynamic change in plasma membrane morphology, is still unresolved.

A challenging task will be to determine the relative proportion of EV originating from tips of filopodia out of the total populations of EVs. The average diameter of filopodia is 130 nm (Kultti et al. 2006), which overlaps with the typical size of exosomes (50–200 nm). Furthermore, no specific molecular markers have so far been identified. Thus, with current methods, it is challenging to reliably differentiate between exosomes generated by exocytosis and EVs originating from filopodia in EV preparations.

4.5 Hyaluronan Receptors and EVs

4.5.1 HA-Receptors as Cargos of EVs

Expression of the standard form of CD44 did not affect the numbers or size distribution of secreted EV from MKN74 gastric cancer cells (Härkönen et al. 2019). Even though the expression of CD44 did not exert any effect on the activity of EV secretion, it increased the amount of HA bound to the surface of the EVs (Härkönen et al. 2019). Recent studies suggest that the HA coat around the EV may be assembled by HAS enzymes acting on EV originating from cells with high HA

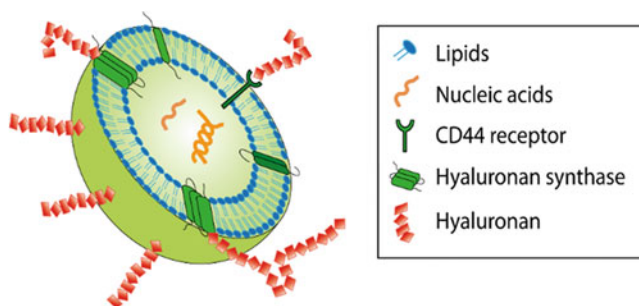


Fig. 4.4 A schematic structure of an EV carrying all macromolecules including lipids, nucleic acids, proteins, and carbohydrates. HA can be attached to the EV membrane via CD44 receptor or HA synthase. Modified from (Arasu et al. 2019b)

synthesis activity (Rilla et al. 2013b; Arasu et al. 2017; Koistinen et al. 2017) or by receptors with high affinity for HA, such as CD44 (Härkönen et al. 2019) (Fig. 4.4). As discussed in the first paragraph of this chapter, in many cases, the coat assembly is a sum of both mechanisms, and possibly also involves other HA-binding receptors present on EVs such as RHAMM (Hong et al. 2009). CD44 and its isoforms are widely expressed in different cell types, thus it is not surprising that CD44 is also a common cargo of the EV derived from many cell types such as placental mesenchymal stem cells (Salomon et al. 2013), breast cancer cells (Pokharel et al. 2016), and mesenchymal stem cells (Ramos et al. 2016; Ragni et al. 2019). Moreover, pretreatment of synovial fluid with hyaluronidase facilitated the isolation of CD44-positive EVs (Boere et al. 2016), which supports the hypothesis that CD44-positive EVs are able to bind soluble HA from the HA-rich environment of synovial fluid (Ragni et al. 2019). Even though there are rather few published works on CD44 as a cargo in EVs, there is strong evidence that CD44 is a general receptor on the surface of EVs that potentially has functional significance, e.g. allowing targeting and homing of EVs.

4.5.2 *Is CD44 a Homing Receptor for EVs?*

EVs have been described as “messages in a bottle,” but the contents inside the bottle have no relevance if they do not reach their target or cannot be opened. The surface composition of an EV is the most important feature which regulates EV adhesion and receptor–ligand interactions with their targets. CD44 is one of these receptors; in addition to HA, CD44 can bind to other ECM components including collagen, fibronectin, laminin, and osteopontin (Morath et al. 2016). CD44 is known as a “homing receptor,” because HA-CD44 interactions mediate the recruitment of activated leucocytes (McDonald and Kubes 2015), stem cells (Avigdor et al.

2004), and tumor cells (Richter et al. 2012) from the circulation. A recent study has shown that bonds between HA and CD44 are extremely strong (Bano et al. 2016), conferring resistance to shear during primary adhesion (rolling) of lymphocytes on vascular endothelial cells (Nandi et al. 2000). The same mechanism potentially regulates the homing of circulating EVs that reprogram the mesothelial cells and prepare a favorable environment for cancer cells. Furthermore, ovarian cancer cell-derived EVs can transfer CD44 to the mesothelial cells of the abdominal cavity, promoting metastasis (Nakamura et al. 2017). This is rather interesting, because our recent study showed that epithelial to mesenchymal transition is associated with CD44 expression and secretion of EVs by mesothelial cells (Koistinen et al. 2017).

There is convincing evidence for the role of CD44 in the regulation of EV binding to target cells, because blocking of CD44 decreases the numbers of EVs bound to target cells (Arasu et al. 2019b; Ragni et al. 2019). HA functionalized EVs are generated as natural vehicles to efficiently deliver doxorubicin (DOX) via CD44-mediated cancer-specific targeting (Liu et al. 2019). Additionally, CD44–HA interactions are involved in EV-induced tumor cell motility (Mu et al. 2013). These observations point to a role of HA–CD44 interactions in the regulation of EV binding to target cells and as a potential tool for cell-free therapeutic applications. However, regulation of EV homing is complicated, because in addition to HA–CD44 interactions, various molecular interactions via integrins, tetraspanins, lectins, fibronectin, and heparan sulfate proteoglycans are known to be involved in regulating cellular EV binding and uptake (Purushothaman et al. 2016; Chen and Brigstock 2016; Buzás et al. 2018).

4.6 Physiobiological Properties of HA-EVs

It is evident that the surface molecular composition is an important regulator of how EVs interact with their environment and target cells (Buzás et al. 2018). Although there are many ECM molecules carried by EVs and which can interact with them (Rilla et al. 2019), HA has a central role because of its abundance, large molecular size and its multiple functions. Since it is such a large molecule, when abundant, HA can overwhelm the smaller sugar moieties, receptors, or other active molecules on the surface of the EVs, interfering with the interactions between EVs and their targets. HA has unique features that potentially affect the physical and biological properties of the EV in several ways: Hydrophilic HA binds huge amounts of water (Cowman and Matsuoka 2005) and allows the EVs to resist osmotic changes, protects against the host immune recognition, resists mechanical stress to prolong EV lifetime, and protects their contents such as nucleic acids from degradation as well as affecting the properties and expanding the space occupied by a single EV. Additionally, HA may affect the mobility of EVs in the bloodstream or tissues, via molecular interactions or by regulating Brownian motion.

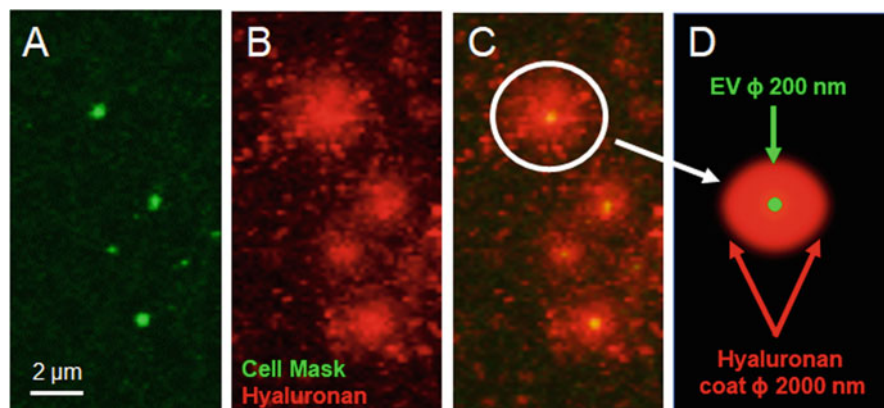


Fig. 4.5 Human mesenchymal cell-derived EVs are coated with a thick layer of HA. High-resolution confocal images of HA-coated EVs in live human MSC cultures. Lipid membranes of EV were labeled with Cell Mask (**a**, green) and HA with fHABC (**b**, red). A merged image is shown in (**c**). A figure in (**d**) demonstrates the extensions of HA layer in relation to the diameter of a single EV

Because HA chains can reach a contour length of several micrometers, this has a potentially huge effect on the physical and biological properties of the EV. Under normal physiological conditions, a single HA chain can have polymer lengths of up to 25 μm (Toole 2004). Recently, giant regenerative HA brushes up to 22 μm have been generated by immobilizing HA synthase-rich bacterial membrane fragments (Wei et al. 2019). These facts suggest that in EVs originating from HA-rich cells, an HA-rich glycocalyx may cover a major proportion of the total volume of a single EV. Fluorescence microscopy has demonstrated that the HA layer around a single EV can have a thickness of 0.5–2.0 μm (Fig. 4.5), which occupies a large space around the EV in relation to the typical nanometer scale diameter of an individual EV. Furthermore, EVs have a relatively large surface area when compared to their volume, maximizing their surface interactions with the surrounding microenvironment, and resulting in highly effective interactions between EVs with cells and extracellular molecules. Additionally, the presence of an HA brush around EVs can regulate the molecular interactions of EV, excluding or slowing down the diffusion of large macromolecules (Laurent et al. 1996). On the other hand, high levels of HA and proteoglycans could block EV access to the target cell's surface (Chang et al. 2016). Thus, HA may act both as a glue and as a barrier to regulate EV–cell interactions.

All EVs typically have a negative charge (Deregibus, et al. 2016), but interestingly, cancer cell-derived EVs have even larger negative charges, which are postulated to be induced by surface glycans that create a biologically active surface for EV (Gerlach and Griffin 2016). In addition to HA, all glycosaminoglycans (GAGs) are highly negatively charged and are the most anionic molecules produced by animal cells and tend to adopt highly extended conformations that occupy a huge volume in

relation to their mass (Cowman et al. 2015). The role of other GAGs as cargos of EVs has not been extensively investigated, but heparan sulfate proteoglycans of syndecan and glypican type have been suggested to regulate EV internalization (Christianson et al. 2013) and glypican has the potential to act as an EV biomarker in pancreatic cancer (Melo et al. 2015). Furthermore, chondroitin sulfate was shown to regulate the activity of osteoblast-derived matrix vesicles and their interactions with the ECM (Schmidt et al. 2016).

4.7 Clinical Utilization of HA Coating on EV Surfaces

EVs have a remarkable potential in numerous diagnostic and therapeutic applications (Gurunathan et al. 2019). The HA-rich glycocalyx around the EV could be utilized in many medical applications, because HA is upregulated in EVs derived from cancerous, inflammatory, and injured cells. From a biological point of view, HA has several functional impacts on the EVs. It helps to establish and regulate an EV's connections with its target cells and the surrounding microenvironment via HA–receptor interactions. It may affect an EV's mobility and change in shape, facilitate EV uptake in or fusion with the target cells, and protect the EV from immunological attack or degrading factors, prolonging EV survival in the bloodstream. Furthermore, HA-EV are potential remodulators of the ECM (Rilla et al. 2019) and help to create a favorable niche for tumor invasion and metastasis. Interestingly, HAS is activated in the plasma membrane (Rilla et al. 2005) and is enriched into the membranes of EVs (Rilla et al. 2013b). Therefore, it is even possible that EVs can continue HA synthesis on their membrane after their release from the cell. This would further enhance the role of HA-EV in ECM remodeling. Recent studies have highlighted the role of HA coat in regulating the effects of EV-mediated signaling in target cells: HA carried by tumor cell-derived EV can induce IL-10 production in monocytes (Lenart et al. 2017), and HA-coated EVs are able to induce the hedgehog signaling pathway in target cells (Arasu et al. 2019a). Next, we will discuss the potential to utilize the biological properties of HA-EV from a clinical point of view: as biomarkers, drug carriers, and tools for tissue engineering and regeneration.

4.7.1 Biomarkers

EVs are promising biomarkers for early diagnostics and liquid biopsies (Lane et al. 2018). A common feature of HA-coated EVs is that they originate from cells that have an active and ongoing HA synthesis (Fig. 4.6). Those cell types, such as stem cells, cancer cells, and fibroblasts, are typically metabolically active and have also increased migration activity and an elevated production of other ECM components.

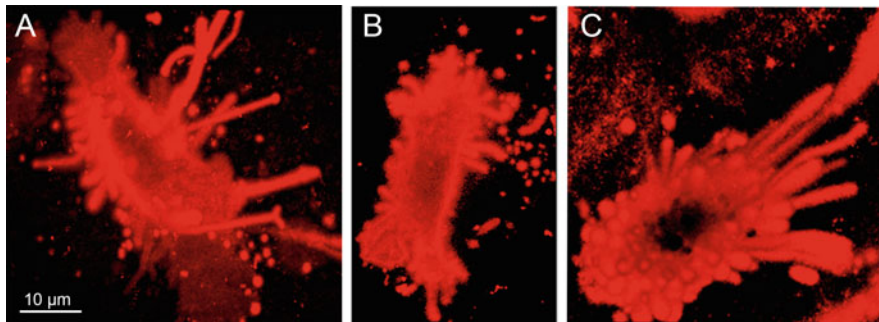


Fig. 4.6 HA-coated filopodia and shedding of HA-EVs is a common feature of cells with high levels of HA synthesis. 3D confocal projection of live EGF-treated rat mesothelial cell (a), HAS2 overexpressing breast cancer cell (b), and human bone-marrow-derived mesenchymal stem cell (c). All cells were labeled with fluorescently labeled HA-binding probe (fHABC, red)

Since HA-EV levels can be easily detected in synovial fluid with a fluorescent probe (Mustonen et al. 2016), in the future they could have potential as multipurpose biomarkers. Such a widely expressed and abundant molecule as HA may be not specific enough to act as a biomarker. However, levels of HA may fluctuate, for example, as a result of changes in patient status during inflammation, tissue injury, or malignancy, and this could be utilized for monitoring of disease progression.

4.7.2 Utilization of HA-EV as Drug Carriers

Because HA is a hydrophilic, biocompatible, non-immunogenic, and biodegradable molecule, it provides opportunities through which to engineer EVs for therapeutic purposes and utilize them in diagnostics. Due to the high affinity of HA for CD44, this phenomenon has been exploited in the development of different artificial drug nanocarriers for targeted therapy (Wickens et al. 2017). The effect of CD44 on the cellular uptake of HA-coated liposomes is dependent not only on molecular weight and grafting HA, but also on the level of CD44 expression of target tumor cells (Qhattal and Liu 2011; Oommen et al. 2016). These findings are applicable to natural cell membrane-derived lipid vesicles and support the role of HA–CD44 interactions in homing and interactions of EVs with target tissues and cells. Interestingly, it has been postulated recently that actually part of the DNA (and RNA) carried by EVs is stuck on the outer surface of the EV instead of inside (Lázaro-Ibáñez et al. 2019), but it is not known how DNA can become adhered in this manner. It is interesting to hypothesize that HA could participate in adhesion of nucleic acids on the EV surfaces, a mechanism to be utilized in the development of EVs as drug carriers.

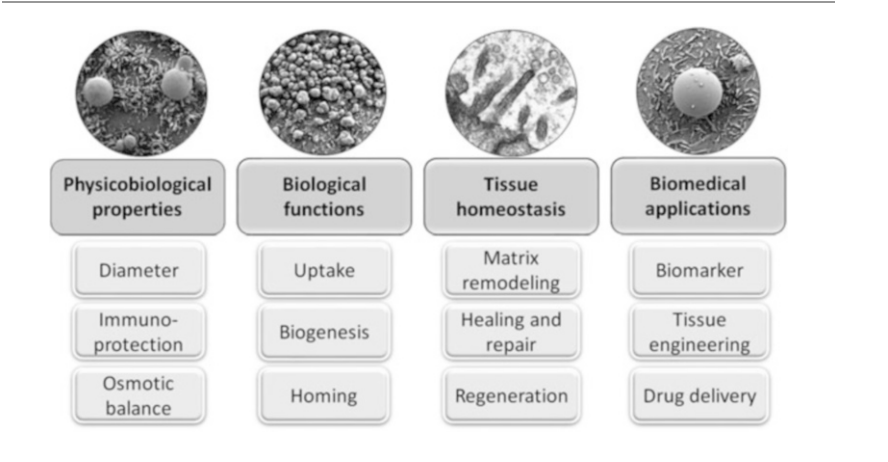
4.7.3 Tissue Engineering

Recent studies have proven the potential of EV in regenerative medicine (Bjørge et al. 2017) due to their biocompatibility, systemic distribution, safety, and regenerative properties. MSC-derived EVs appear to be involved in both MSC-induced immunomodulation, extracellular matrix remodeling and regeneration of target tissues. The HA coat of MSC-derived EVs (Arasu et al. 2017) is proposed to enhance all of the above-mentioned features. HA may be partly responsible for the low immunogenicity of stem-cell-derived EVs as well as their biodistribution and homing into target tissues. The central role of HA was highlighted in a recent study which revealed that MSC-derived EVs both deliver HA and induce its production in target tissue in a rat lung ischemia/reperfusion injury model (Lonati et al. 2019). Additionally, HA-CD44 interactions regulated the uptake of MSC-derived EV in human osteoarthritic synoviocytes, resulting in decreased expression of inflammatory cytokines and chemokines (Ragni et al. 2019). Tumor cell-derived EV may also participate in the degradation of HA, while GPI-anchored PH20 hyaluronidase carried by small EVs induced tumor HA degradation, which in turn, activated anti-cancer immune responses to inhibit tumor growth (Hong et al. 2019). EVs play a key role in the regulation of joint homeostasis and are present in synovial fluid and cartilage extracellular matrix. Both the immune-suppressive and the regenerative properties of HA-EVs are potential tools for joint repair and regeneration (Malda et al. 2016) as in joint diseases there is an involvement of both inflammation and the associated articular tissue destruction.

4.8 Conclusions

Pericellular HA supports the formation of a dynamic and continuously changing cell surface architecture. There is an increasing amount of evidence that HA has a role as both a general inducer and a cargo of EVs. The surface molecular composition of the EV is one of the most important factors regulating its release, transfer, and communication with the extracellular milieu and its target cells. The HA-rich glycocalyx is not just an important regulator for EV biogenesis and environmental interactions, but it also acts as a promising tool for tissue repair and regeneration, targeted EV-based therapy, and drug delivery. Furthermore, HA and its receptor CD44 are potential multipurpose biomarkers because they are upregulated in EVs derived from cancerous, inflammatory, and injured cells and tissues. Additionally, HA has multiple roles in mediating the functions of EVs, such as protection against immunological reactions, targeting and regulation of adhesion during homing of the EV to recipient tissues or cells, which are all crucial factors in the regulation of EV targeting and functions in both normal and diseased tissues (Table 4.1).

Table 4.1 Summary of the multiple roles of the HA-coating in EV biology and its medical applications



In addition to the role of HA-EVs in the regulation of cellular functions, these structures are potential tools with biomedical applications

References

Arasu UT, Deen AJ, Pasonen-Seppänen S et al (2019a) HAS3-induced extracellular vesicles from melanoma cells stimulate IHH mediated c-Myc upregulation via the hedgehog signaling pathway in target cells. *Cell Mol Life Sci* 77:4093. <https://doi.org/10.1007/s00018-019-03399-5>

Arasu UT, Härkönen K, Koistinen A, Rilla K (2019b) Correlative light and electron microscopy is a powerful tool to study interactions of extracellular vesicles with recipient cells. *Exp Cell Res* 376:149–158. <https://doi.org/10.1016/j.yexcr.2019.02.004>

Arasu UT, Kärnä R, Härkönen K et al (2017) Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles. *Matrix Biol* 64:54–68. <https://doi.org/10.1016/j.matbio.2017.05.001>

Arjonen A, Kaukonen R, Ivaska J (2011) Filopodia and adhesion in cancer cell motility. *Cell Adhes Migr* 5:421–430. <https://doi.org/10.4161/cam.5.5.17723>

Avigdor A, Goichberg P, Shivtiel S et al (2004) CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* 103:2981–2989. <https://doi.org/10.1182/blood-2003-10-3611>

Bano F, Banerji S, Howarth M et al (2016) A single molecule assay to probe monovalent and multivalent bonds between hyaluronan and its key leukocyte receptor CD44 under force. *Sci Rep* 6:34176. <https://doi.org/10.1038/srep34176>

Bjørge IM, Kim SY, Mano JF et al (2017) Extracellular vesicles, exosomes and shedding vesicles in regenerative medicine - a new paradigm for tissue repair. *Biomater Sci* 6:60–78. <https://doi.org/10.1039/c7bm00479f>

Boere J, van de Lest CHA, Libregts SFWM et al (2016) Synovial fluid pretreatment with hyaluronidase facilitates isolation of CD44+ extracellular vesicles. *J Extracell vesicles* 5:31751

Bohil AB, Robertson BW, Cheney RE (2006) Myosin-X is a molecular motor that functions in filopodia formation. *Proc Natl Acad Sci* 103:12411–12416. <https://doi.org/10.1073/pnas.0602443103>

- Brinck J, Heldin P (1999) Expression of recombinant Hyaluronan synthase (HAS) isoforms in CHO cells reduces cell migration and cell surface CD44. *Exp Cell Res* 252:342–351. <https://doi.org/10.1006/excr.1999.4645>
- Buzás EI, Tóth EÁ, Sódar BW, Szabó-Taylor KÉ (2018) Molecular interactions at the surface of extracellular vesicles. *Semin Immunopathol* 40:453–464. <https://doi.org/10.1007/s00281-018-0682-0>
- Chang PS, McLane LT, Fogg R et al (2016) Cell surface access is modulated by tethered bottlebrush proteoglycans. *Biophys J* 110:2739–2750. <https://doi.org/10.1016/j.bpj.2016.05.027>
- Chen L, Brigstock DR (2016) Integrins and heparan sulfate proteoglycans on hepatic stellate cells (HSC) are novel receptors for HSC-derived exosomes. *FEBS Lett* 590:4263–4274. <https://doi.org/10.1002/1873-3468.12448>
- Chhabra ES, Higgs HN (2007) The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol* 9:1110–1121. <https://doi.org/10.1038/ncb1007-1110>
- Choi D, Spinelli C, Montermini L, Rak J (2019) Oncogenic regulation of extracellular vesicle proteome and heterogeneity. *Proteomics* 19:1800169. <https://doi.org/10.1002/pmic.201800169>
- Christianson HC, Svensson KJ, van Kuppevelt TH et al (2013) Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci U S A* 110:17380–17385. <https://doi.org/10.1073/pnas.1304266110>
- Clarris BJ, Fraser JRE (1968) On the pericellular zone of some mammalian cells in vitro. *Exp Cell Res* 49:181–193. [https://doi.org/10.1016/0014-4827\(68\)90530-2](https://doi.org/10.1016/0014-4827(68)90530-2)
- Cowman MK, Matsuoka S (2005) Experimental approaches to hyaluronan structure. *Carbohydr Res* 340:791–809. <https://doi.org/10.1016/j.carres.2005.01.022>
- Cowman MK, Schmidt TA, Raghavan P, Stecco A (2015) Viscoelastic properties of Hyaluronan in physiological conditions. *F1000Research* 4:622. <https://doi.org/10.12688/f1000research.6885.1>
- Deen AJ, Arasu UT, Pasonen-Seppänen S et al (2016) UDP-sugar substrates of HAS3 regulate its O-GlcNAcylation, intracellular traffic, extracellular shedding and correlate with melanoma progression. *Cell Mol Life Sci* 73:3183–3204. <https://doi.org/10.1007/s00018-016-2158-5>
- Docampo MJ, Zanna G, Fondevila D et al (2011) Increased HAS2-driven hyaluronic acid synthesis in shar-pei dogs with hereditary cutaneous hyaluronosis (mucinosis). *Vet Dermatol* 22:535–545. <https://doi.org/10.1111/j.1365-3164.2011.00986.x>
- EL Andaloussi S, Mäger I, Breakefield XO, Wood MJA (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 12:347–357. <https://doi.org/10.1038/nrd3978>
- Evanko SP, Potter-Perigo S, Johnson PY, Wight TN (2009) Organization of hyaluronan and versican in the extracellular matrix of human fibroblasts treated with the viral mimetic poly I: C. *J Histochem Cytochem* 57:1041–1060. <https://doi.org/10.1369/jhc.2009.953802>
- Evanko SP, Tammi MI, Tammi RH, Wight TN (2007) Hyaluronan-dependent pericellular matrix. *Adv Drug Deliv Rev* 59:1351–1365
- Fowke TM, Karunasinghe RN, Bai J-Z et al (2017) Hyaluronan synthesis by developing cortical neurons in vitro. *Sci Rep* 7:44135. <https://doi.org/10.1038/srep44135>
- Gebauer F, Kemper M, Sauter G et al (2017) Is hyaluronan deposition in the stroma of pancreatic ductal adenocarcinoma of prognostic significance? *PLoS One* 12:e0178703. <https://doi.org/10.1371/journal.pone.0178703>
- Gerlach JQ, Griffin MD (2016) Getting to know the extracellular vesicle glycome. *Mol BioSyst* 12: 1071–1081. <https://doi.org/10.1039/c5mb00835b>
- Gurunathan S, Kang M-H, Jeyaraj M et al (2019) Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. *Cell* 8. <https://doi.org/10.3390/cells8040307>
- Hale JE, Wuthier RE (1987) The mechanism of matrix vesicle formation. Studies on the composition of chondrocyte microvilli and on the effects of microfilament-perturbing agents on cellular vesiculation. *J Biol Chem* 262:1916–1925

- Härkönen K, Oikari S, Kyykallio H et al (2019) CD44s assembles Hyaluronan coat on Filopodia and extracellular vesicles and induces Tumorigenicity of MKN74 gastric carcinoma cells. *Cells* 8:276. <https://doi.org/10.3390/cells8030276>
- Heldin P, Persson H (1993) Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. *Exp Cell Res* 208:422–429. <https://doi.org/10.1006/excr.1993.1264>
- Hiltunen ELJ, Anttila M, Kultti A et al (2002) Elevated hyaluronan concentration without hyaluronidase activation in malignant epithelial ovarian tumors. *Cancer Res* 62:6410–6413
- Hong B, Cho J-H, Kim H et al (2009) Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics* 10: 556. <https://doi.org/10.1186/1471-2164-10-556>
- Hong Y, Kim YK, Kim GB et al (2019) Degradation of tumour stromal hyaluronan by small extracellular vesicle-PH20 stimulates CD103⁺ dendritic cells and in combination with PD-L1 blockade boosts anti-tumour immunity. *J Extracell Vesicles* 8:1670893. <https://doi.org/10.1080/20013078.2019.1670893>
- Huang Y, Askew EB, Knudson CB, Knudson W (2016) CRISPR/Cas9 knockout of HAS2 in rat chondrosarcoma chondrocytes demonstrates the requirement of hyaluronan for aggrecan retention. *Matrix Biol* 56:74–94. <https://doi.org/10.1016/j.matbio.2016.04.002>
- Jacquemet G, Hamidi H, Ivaska J (2015) Filopodia in cell adhesion, 3D migration and cancer cell invasion. *Curr Opin Cell Biol* 36:23–31. <https://doi.org/10.1016/j.ceb.2015.06.007>
- Jokela TA, Lindgren A, Rilla K et al (2008) Induction of hyaluronan cables and monocyte adherence in epidermal keratinocytes. *Connect Tissue Res* 49:115–119. <https://doi.org/10.1080/03008200802148439>
- Knudson CB (1993) Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. *J Cell Biol* 120:825–834. <https://doi.org/10.1083/jcb.120.3.825>
- Knudson W, Ishizuka S, Terabe K et al (2019) The pericellular hyaluronan of articular chondrocytes. *Matrix Biol* 78–79:32–46. <https://doi.org/10.1016/j.matbio.2018.02.005>
- Kogure A, Kosaka N, Ochiya T (2019) Cross-talk between cancer cells and their neighbors via miRNA in extracellular vesicles: an emerging player in cancer metastasis. *J Biomed Sci* 26:7. <https://doi.org/10.1186/s12929-019-0500-6>
- Koistinen V, Härkönen K, Kärnä R et al (2017) EMT induced by EGF and wounding activates hyaluronan synthesis machinery and EV shedding in rat primary mesothelial cells. *Matrix Biol* 63:38–54. <https://doi.org/10.1016/j.matbio.2016.12.007>
- Koistinen V, Jokela T, Oikari S et al (2016) Hyaluronan-positive plasma membrane protrusions exist on mesothelial cells in vivo. *Histochem Cell Biol* 145:531. <https://doi.org/10.1007/s00418-016-1405-z>
- Koistinen V, Kärnä R, Koistinen A et al (2015) Cell protrusions induced by hyaluronan synthase 3 (HAS3) resemble mesothelial microvilli and share cytoskeletal features of filopodia. *Exp Cell Res* 337:179. <https://doi.org/10.1016/j.yexcr.2015.06.016>
- Kultti A, Rilla K, Tiihonen R et al (2006) Hyaluronan synthesis induces microvillus-like cell surface protrusions. *J Biol Chem* 281:15821–15828. <https://doi.org/10.1074/jbc.M512840200>
- Ramos TL, Sánchez-Abarca LI, Muntión S et al (2016) MSC surface markers (CD44, CD73, and CD90) can identify human MSC-derived extracellular vesicles by conventional flow cytometry. *Cell Commun Signal* 14:2. <https://doi.org/10.1186/s12964-015-0124-8>
- Lane RE, Korb D, Hill MM, Trau M (2018) Extracellular vesicles as circulating cancer biomarkers: opportunities and challenges. *Clin Transl Med* 7:14. <https://doi.org/10.1186/s40169-018-0192-7>
- Laurent TC, Laurent UB, Fraser JR (1996) The structure and function of hyaluronan: an overview. *Immunol Cell Biol* 74:A1–A7. <https://doi.org/10.1038/icb.1996.32>
- Lázaro-Ibáñez E, Lässer C, Shelke GV et al (2019) DNA analysis of low- and high-density fractions defines heterogeneous subpopulations of small extracellular vesicles based on their DNA cargo and topology. *J Extracell Vesicles* 8:1656993. <https://doi.org/10.1080/20013078.2019.1656993>

- Lenart M, Rutkowska-Zapala M, Baj-Krzyworzeka M et al (2017) Hyaluronan carried by tumor-derived microvesicles induces IL-10 production in classical (CD14++CD16-) monocytes via PI3K/Akt/mTOR-dependent signalling pathway. *Immunobiology* 222:1–10. <https://doi.org/10.1016/j.imbio.2015.06.019>
- Lin W, Shuster S, Maibach HI, Stern R (1997) Patterns of Hyaluronan staining are modified by fixation techniques. *J Histochem Cytochem* 45:1157–1163. <https://doi.org/10.1177/002215549704500813>
- Litwiniuk M, Krejner A, Speyrer MS et al (2016) Hyaluronic acid in inflammation and tissue regeneration. *Wounds a Compend Clin Res Pract* 28:78–88
- Liu J, Ye Z, Xiang M et al (2019) Functional extracellular vesicles engineered with lipid-grafted hyaluronic acid effectively reverse cancer drug resistance. *Biomaterials* 223:119475. <https://doi.org/10.1016/j.BIOMATERIALS.2019.119475>
- Lonati C, Bassani GA, Brambilla D et al (2019) Mesenchymal stem cell-derived extracellular vesicles improve the molecular phenotype of isolated rat lungs during ischemia/reperfusion injury. *J Hear Lung Transplant* 38:1306–1316. <https://doi.org/10.1016/j.healun.2019.08.016>
- Lukoschek M, Addicks K (2008) Histologische, morphometrische Untersuchung der menschlichen Synovialmembran. *Z Orthop Ihre Grenzgeb* 129:136–140. <https://doi.org/10.1055/s-2008-1040172>
- Makabe S, Naguro T, Stallone T (2006) Oocyte–follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission electron microscopy in humans. *Microsc Res Tech* 69:436–449. <https://doi.org/10.1002/jemt.20303>
- Malda J, Boere J, Van De Lest CHA et al (2016) Extracellular vesicles - new tool for joint repair and regeneration. *Nat Rev Rheumatol* 12:243–249
- Mathieu M, Martin-Jaular L, Lavie G, Théry C (2019) Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* 21:9–17
- Mattila PK, Lappalainen P (2008) Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol* 9:446–454. <https://doi.org/10.1038/nrm2406>
- Mattila PK, Pykäläinen A, Saarikangas J et al (2007) Missing-in-metastasis and IRSp53 deform PI (4,5)P2-rich membranes by an inverse BAR domain-like mechanism. *J Cell Biol* 176:953–964. <https://doi.org/10.1083/jcb.200609176>
- McDonald B, Kubes P (2015) Interactions between CD44 and Hyaluronan in leukocyte trafficking. *Front Immunol* 6:68. <https://doi.org/10.3389/fimmu.2015.00068>
- Melero-Fernandez de Mera RM, Arasu UT, Kärnä R et al (2019) Effects of mutations in the post-translational modification sites on the trafficking of hyaluronan synthase 2 (HAS2). *Matrix Biol* 80:85–103. <https://doi.org/10.1016/j.matbio.2018.10.004>
- Melo SA, Luecke LB, Kahlert C et al (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 523:177–182. <https://doi.org/10.1038/nature14581>
- Misra S, Hascall VC, Markwald RR, Ghatak S (2015) Interactions between Hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. *Front Immunol* 6:201. <https://doi.org/10.3389/fimmu.2015.00201>
- Morath I, Hartmann TN, Orian-Rousseau V (2016) CD44: more than a mere stem cell marker. *Int J Biochem Cell Biol* 81:166–173. <https://doi.org/10.1016/j.biocel.2016.09.009>
- Mu W, Rana S, Zöller M (2013) Host matrix modulation by tumor exosomes promotes motility and invasiveness. *Neoplasia* 15:875–887. <https://doi.org/10.1593/neo.13786>
- Mustonen A-M, Nieminen P, Joukainen A et al (2016) First in vivo detection and characterization of hyaluronan-coated extracellular vesicles in human synovial fluid. *J Orthop Res* 34:1960. <https://doi.org/10.1002/jor.23212>
- Mutsaers SE (2004) The mesothelial cell. *Int J Biochem Cell Biol* 36:9–16. [https://doi.org/10.1016/s1357-2725\(03\)00242-5](https://doi.org/10.1016/s1357-2725(03)00242-5)
- Nakamura K, Sawada K, Kinose Y et al (2017) Exosomes promote ovarian cancer cell invasion through transfer of CD44 to peritoneal mesothelial cells. *Mol Cancer Res* 15:78–92. <https://doi.org/10.1158/1541-7786.MCR-16-0191>

- Nandi A, Estess P, Siegelman MH (2000) Hyaluronan anchoring and regulation on the surface of vascular endothelial cells is mediated through the functionally active form of CD44. *J Biol Chem* 275:14939–14948
- Ommen OP, Duehrkop C, Nilsson B et al (2016) Multifunctional hyaluronic acid and chondroitin sulfate nanoparticles: impact of glycosaminoglycan presentation on receptor mediated cellular uptake and immune activation. *ACS Appl Mater Interfaces* 8:20614–20624. <https://doi.org/10.1021/acsami.6b06823>
- Pasonen-Seppänen S, Hyttinen JMT, Rilla K et al (2012) Role of CD44 in the organization of keratinocyte pericellular hyaluronan. *Histochem Cell Biol* 137:107–120. <https://doi.org/10.1007/s00418-011-0883-2>
- Pokharel D, Padula M, Lu J et al (2016) The role of CD44 and ERM proteins in expression and functionality of P-glycoprotein in breast cancer cells. *Molecules* 21:290. <https://doi.org/10.3390/molecules21030290>
- Purushothaman A, Bandari SK, Liu J et al (2016) Fibronectin on the surface of myeloma cell-derived exosomes mediates exosome-cell interactions. *J Biol Chem* 291:1652–1663. <https://doi.org/10.1074/jbc.M115.686295>
- Qhattal HSS, Liu X (2011) Characterization of CD44-mediated cancer cell uptake and intracellular distribution of Hyaluronan-grafted liposomes. *Mol Pharm* 8:1233–1246. <https://doi.org/10.1021/mp2000428>
- Qu C, Rilla K, Tammi R et al (2014) Extensive CD44-dependent hyaluronan coats on human bone marrow-derived mesenchymal stem cells produced by hyaluronan synthases HAS1, HAS2 and HAS3. *Int J Biochem Cell Biol* 48:45. <https://doi.org/10.1016/j.biocel.2013.12.016>
- Ragni E, Perucca Orfei C, De Luca P et al (2019) Interaction with hyaluronan matrix and miRNA cargo as contributors for in vitro potential of mesenchymal stem cell-derived extracellular vesicles in a model of human osteoarthritic synoviocytes. *Stem Cell Res Ther* 10:109. <https://doi.org/10.1186/s13287-019-1215-z>
- Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 200:373–383. <https://doi.org/10.1083/jcb.201211138>
- Richter RP, Baranova NS, Day AJ, Kwok JC (2018) Glycosaminoglycans in extracellular matrix organisation: are concepts from soft matter physics key to understanding the formation of perineuronal nets? *Curr Opin Struct Biol* 50:65–74. <https://doi.org/10.1016/j.sbi.2017.12.002>
- Richter U, Wicklein D, Geleff S, Schumacher U (2012) The interaction between CD44 on tumour cells and hyaluronan under physiologic flow conditions: implications for metastasis formation. *Histochem Cell Biol* 137:687–695. <https://doi.org/10.1007/s00418-012-0916-5>
- Rilla K, Siiskonen H, Tammi M, Tammi R (2014) Hyaluronan-coated extracellular vesicles – a novel link between hyaluronan and cancer. *Adv Cancer Res* 123:121–148
- Rilla K, Koistinen A (2015) Correlative light and electron microscopy reveals the HAS3-induced dorsal plasma membrane ruffles. *Int J Cell Biol* 2015:1. <https://doi.org/10.1155/2015/769163>
- Rilla K, Mustonen AM, Arasu UT et al (2019) Extracellular vesicles are integral and functional components of the extracellular matrix. *Matrix Biol* 75–76:201–219. <https://doi.org/10.1016/j.matbio.2017.10.003>
- Rilla K, Oikari S, Jokela TA et al (2013a) Hyaluronan synthase 1 (HAS1) requires higher cellular udp-glcna concentration than HAS2 and HAS3. *J Biol Chem* 288:5973–5983. <https://doi.org/10.1074/jbc.M112.443879>
- Rilla K, Pasonen-Seppänen S, Deen AJ et al (2013b) Hyaluronan production enhances shedding of plasma membrane-derived microvesicles. *Exp Cell Res* 319:2006. <https://doi.org/10.1016/j.yexcr.2013.05.021>
- Rilla K, Siiskonen H, Spicer AP et al (2005) Plasma membrane residence of hyaluronan synthase is coupled to its enzymatic activity. *J Biol Chem* 280:31890. <https://doi.org/10.1074/jbc.M504736200>
- Rilla K, Tiihonen R, Kultti A et al (2008) Pericellular hyaluronan coat visualized in live cells with a fluorescent probe is scaffolded by plasma membrane protrusions. *J Histochem Cytochem* 56: 901. <https://doi.org/10.1369/jhc.2008.951665>

- Roszkowska M, Skupien A, Wójtowicz T et al (2016) CD44: a novel synaptic cell adhesion molecule regulating structural and functional plasticity of dendritic spines. *Mol Biol Cell* 27: 4055–4066. <https://doi.org/10.1091/mbc.E16-06-0423>
- Salomon C, Ryan J, Sobrevia L et al (2013) Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and Vasculogenesis. *PLoS One* 8:e68451. <https://doi.org/10.1371/journal.pone.0068451>
- Sanborn J, Oglecka K, Kraut RS, Parikh AN (2013) Transient pearling and vesiculation of membrane tubes under osmotic gradients. *Faraday Discuss* 161:167–76; discussion 273–303. <https://doi.org/10.1039/c2fd20116j>
- Schmidt JR, Kliemt S, Preissler C et al (2016) Osteoblast-released matrix vesicles, regulation of activity and composition by sulfated and non-sulfated glycosaminoglycans. *Mol Cell Proteomics* 15:558–572. <https://doi.org/10.1074/mcp.M115.049718>
- Shurer CR, Kuo JC-H, Roberts LM et al (2019) Physical principles of membrane shape regulation by the Glycocalyx. *Cell* 177:1757. <https://doi.org/10.1016/j.cell.2019.04.017>
- Siiskonen H, Kärnä R, Hyttinen JM et al (2014) Hyaluronan synthase 1 (HAS1) produces a cytokine-and glucose-inducible, CD44-dependent cell surface coat. *Exp Cell Res* 320:153. <https://doi.org/10.1016/j.yexcr.2013.09.021>
- Tammi MI, Oikari S, Pasonen-Seppänen S et al (2018) Activated hyaluronan metabolism in the tumor matrix — causes and consequences. *Matrix Biol*. <https://doi.org/10.1016/J.MATBIO.2018.04.012>
- Tammi R, Ripellino JA, Margolis RU, Tammi M (1988) Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe. *J Invest Dermatol* 90:412–414. <https://doi.org/10.1111/1523-1747.ep12456530>
- Toole BP (2004) Hyaluronan: From extracellular glue to pericellular cue. *Nat Rev Cancer* 4:528–539
- Törrönen K, Nikunen K, Kärnä R et al (2014) Tissue distribution and subcellular localization of hyaluronan synthase isoenzymes. *Histochem Cell Biol* 141:17. <https://doi.org/10.1007/s00418-013-1143-4>
- Twarock S, Tammi MI, Savani RC, Fischer JW (2010) Hyaluronan stabilizes focal adhesions, filopodia, and the proliferative phenotype in esophageal squamous carcinoma cells. *J Biol Chem* 285:23276–23284. <https://doi.org/10.1074/jbc.M109.093146>
- Wei W, Faubel JL, Selvakumar H et al (2019) Self-regenerating giant hyaluronan polymer brushes. *Nat Commun* 10:5527. <https://doi.org/10.1038/s41467-019-13440-7>
- Wickens JM, Alsaab HO, Kesharwani P et al (2017) Recent advances in hyaluronic acid-decorated nanocarriers for targeted cancer therapy. *Drug Discov Today* 22:665–680. <https://doi.org/10.1016/j.drudis.2016.12.009>
- Williams C, Royo F, Aizpurua-Olaizola O et al (2018) Glycosylation of extracellular vesicles: current knowledge, tools and clinical perspectives. *J. Extracell. Vesicles*, p 7
- Zhang H, Baader SL, Sixt M et al (2004) Neurocan-GFP fusion protein: a new approach to detect hyaluronan on tissue sections and living cells. *J Histochem Cytochem* 52:915–922. <https://doi.org/10.1369/jhc.3A6221.2004>

Chapter 5

Hyaluronan in Kidney Fibrosis



Irina Grigorieva, Emma L. Woods, Robert Steadman, Timothy Bowen, and Soma Meran

Abstract The kidney is the organ responsible for the regulation of the body's fluid balance. Hyaluronan (HA), because of its unique hydration capacity, is used dynamically by the normal renal medulla to ensure whole-body fluid homeostasis. While levels of HA in the medulla fluctuate and can be very high, HA levels in the renal cortex remain static and are almost undetectable. Levels in the cortex are only upregulated in both acute and chronic renal diseases such as diabetes, ischemia-reperfusion injury, tubulointerstitial inflammation and renal transplant rejection. This chapter describes our current understanding of the mechanisms controlling the upregulation of HA in progressive disease, their potential recapitulation of embryonic events and which intracellular signalling pathways may have the potential to prevent or reverse the progression that leads to End Stage Renal Failure.

5.1 Introduction

The kidney is the organ responsible for the regulation of the body's fluid balance. Hyaluronan (HA), because of its unique hydration capacity, is used dynamically by the normal renal medulla to ensure whole-body fluid homeostasis. While levels of HA in the medulla fluctuate and can be very high, HA levels in the renal cortex remain static and are almost undetectable. Levels in the cortex are only upregulated in both acute and chronic renal diseases such as diabetes, ischemia-reperfusion injury, tubulointerstitial inflammation and renal transplant rejection. This chapter describes our current understanding of the mechanisms controlling the upregulation of HA in progressive disease, their potential recapitulation of embryonic events and which intracellular signalling pathways may have the potential to prevent or reverse the progression that leads to End Stage Renal Failure.

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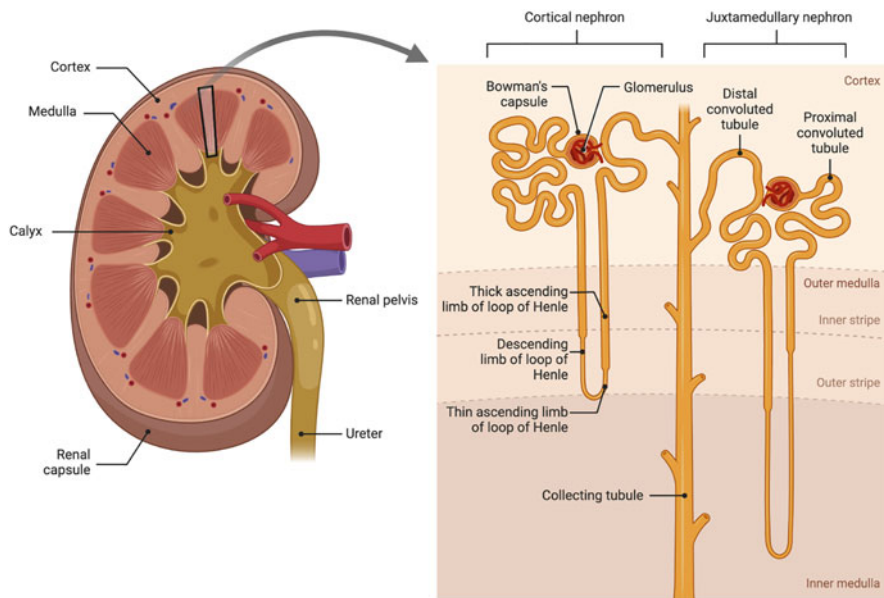


Fig. 5.1 Gross histology and functional unit of the kidney. Adapted from “Kidney Anatomy”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>

The kidneys are paired organs found in the retroperitoneal cavity on either side of the thoracolumbar vertebral column. They play a central role in tissue homeostasis, salt and water balance and elimination of biological waste products. The kidney parenchyma comprises of the outer renal cortex and the inner renal medulla. These two areas house the functional unit of the kidneys called the nephron. Each nephron comprises a glomerulus, proximal tubule, Loop of Henle, distal tubule and collecting duct. The glomeruli and tubules sit predominantly in the renal cortex with the Loop of Henle and collecting ducts sitting predominantly in the inner renal medulla. Nephrons consist of various specialised epithelial cell types including parietal epithelial cells (PECs) and podocytes. Furthermore, the nephrons are surrounded by peritubular capillary network and sit within a meshwork of connective tissue comprised of stromal cells and matrix molecules called the renal interstitium (Fig. 5.1).

5.1.1 HA in Kidney Development

HA is an important structural component of the extracellular matrix of a variety of tissues, but it also directly interacts with cells to influence their behaviour, signalling and crosstalk with other cells. HA therefore has a vital role during mammalian

organogenesis and development. HA is synthesised by three hyaluronic acid synthase genes, *Has1*, *Has2* and *Has3*. *Has2* is responsible for most of HA synthesised during murine embryonic development and mouse embryos lacking the *Has2* gene exhibit growth retardation evident by embryonic day 9 and die at mid-gestation (before metanephric kidney development and ureteric bud outgrowth) (Camenisch et al. 2000). By contrast, mice lacking one or both of the *Has1* and *Has3* genes are viable and exhibit no obvious phenotype (Bai et al. 2005; Kobayashi et al. 2010).

Metanephric kidney development begins with the outgrowth of the ureteric bud (UB) from the Wolffian duct at week 5 in human development and approximately day 10 of mouse development. The UB invades the surrounding metanephric mesenchyme (MM) and begins the process of branching morphogenesis to form the collecting duct network. MM cells coalesce around the tips of the branching UB, thereby providing factors that promote UB branching morphogenesis and maturation, while the UB releases factors that promote epithelialisation and differentiation of MM cells by a process of mesenchymal-to-epithelial transition (MET) (Little and McMahon 2012). The MM-derived nephron progenitors form an epithelial renal vesicle which elongates and segments progressing to a comma-shaped body, S-shaped body and early capillary loop stage before maturing into a nephron. A third self-renewing FOXD1+ progenitor population is found within the MM and is the source of kidney stromal cell types, whereas a distinct progenitor population gives rise to an extensive endothelial network that surrounds the branching tips. Functional evidence suggests that support of the nephron progenitors and patterning of both the nephron and collecting duct epithelium is influenced by the surrounding stromal cells and their extracellular matrix (Das et al. 2013).

While in mature kidney HA is found only within the medullary intersitium and is absent from the cortex in healthy states, in developing kidney HA is abundant in both the cortical and medullary stroma as well as in the nephrogenic niches containing progenitor populations (Fig. 5.2a). This HA is produced by the stromal FOXD1+ progenitor-derived cells and has been shown to modulate UB branching morphogenesis, induction of MET, nephron progenitor differentiation and tubular maturation (Rosines et al. 2007; Pohl et al. 2000; Stridh et al. 2012). However, the role of HA in these processes is dependent on its size, concentration and how it assembles in pericellular matrices (Rosines et al. 2007; Stridh et al. 2012; Toole 2001; Kaul et al. 2021).

The *Has2* gene knockout mouse is lethal prior to kidney development (Camenisch et al. 2000); and kidney-specific *Has2* conditional knockout models have not been developed to date; therefore, it is difficult to study the effect of HA deficiency on kidney development. To address this, Rosines et al. used in vitro metanephric kidney cultures isolated from rat embryos and applied *Streptomyces hyalurolyticus*-derived hyaluronidase (HA-ase), which is specific for HA and does not cleave chondroitin sulfates (Rosines et al. 2007). This resulted in a > 70% decrease in branching morphogenesis and kidney size as measured by tip number, and this decrease was reversed by the addition of HA to the HA-ase (Fig. 5.2b). This effect of exogenous HA was bimodal, either promoting or inhibiting branching

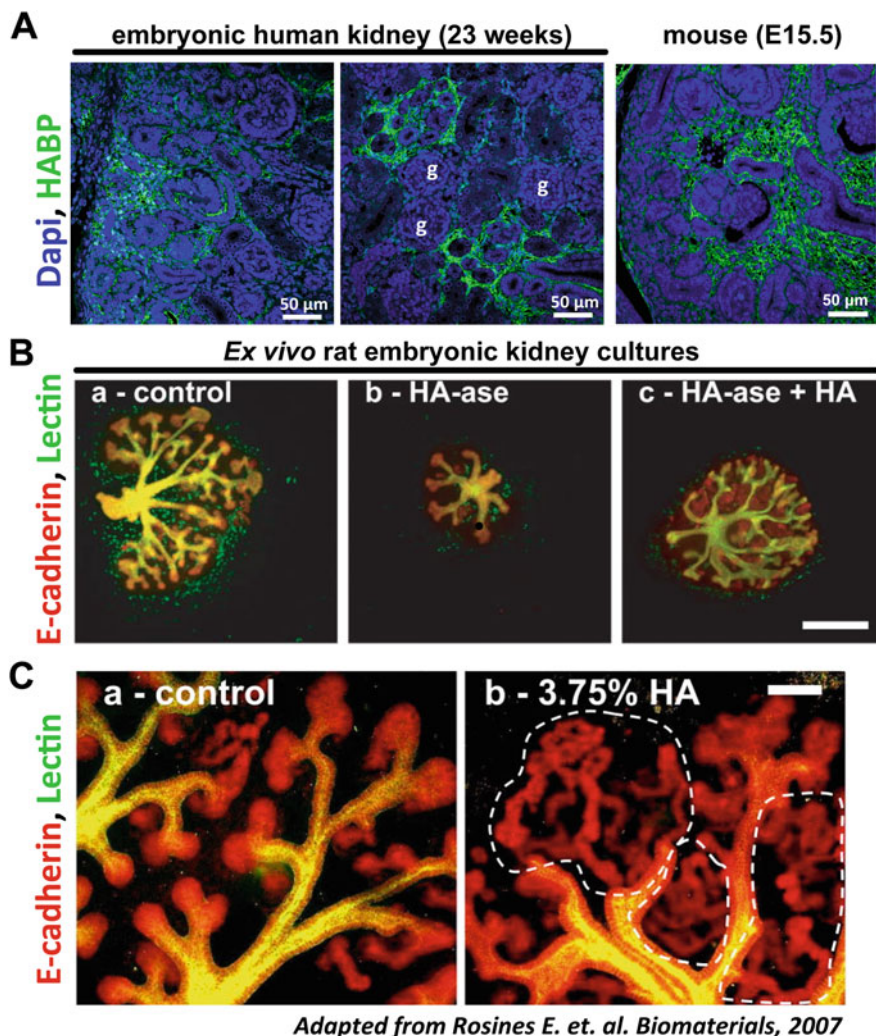


Fig. 5.2 (a) Distribution of HA in human and mouse developing kidneys showing enrichment of HA in cortical interstitium and nephrogenic niches containing renal progenitor populations. (b) Removal of HA from ex vivo rat embryonic kidney cultures disrupts branching morphogenesis. (c) Addition of HA to embryonic kidney cultures promotes MET and nephron elongation and growth. Adapted from Rosines E. et al., *Biomaterials*, 2007

morphogenesis depending on the concentration of exogenous HA added. Low concentrations of HA (0.1%) tripled the number of tips seen in the metanephric kidney culture compared to controls, while high concentrations of HA (3.75%) reduced the number of tips by 75% (Rosines et al. 2007). Moreover, the addition of high molecular weight (MW) HA (MW >230 KD) to isolated UBs strongly inhibited branching morphogenesis in a concentration-dependent manner, whereas low MW and low concentration HA did not affect branching in the isolated UB

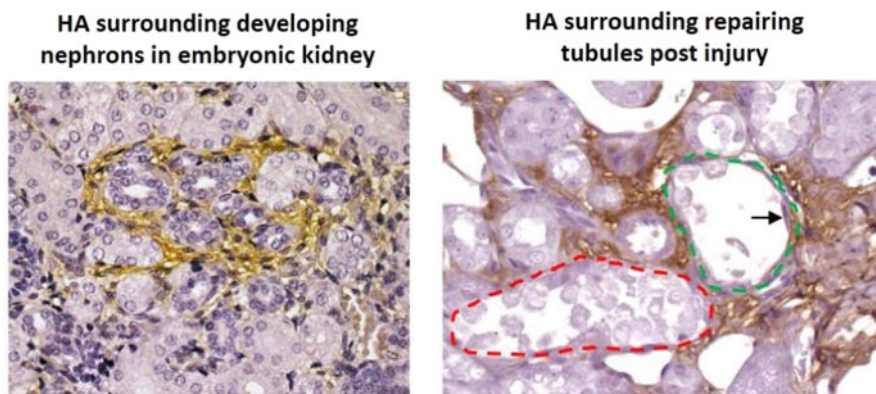


Fig. 5.3 HA in developing kidney resembles HA which is induced post kidney injury. HA surrounds proximal tubules undergoing repair (*outlined in green*) with evidence of dedifferentiated cells (*arrow*), but not those that are severely damaged (*outlined in red*)

system or in whole kidney culture (Rosines et al. 2007). The potential for HA molecular weight to be a factor in kidney development was also supported by high expression of *Hyal2* an endogenous hyaluronidase that degrades HA to 20 kDa fragments, which is threefold higher in developing compared to adult kidneys. In addition to UB branching, HA also appears to promote MET of MM and nephron development. After 7 days in culture, the mesenchyme of control kidneys formed comma and S-shaped bodies with a few extended tubules, but the mesenchyme-derived tubules of HA-treated kidneys were much longer and more convoluted (Fig. 5.2c) suggesting enhanced MET and tubular growth (Rosines et al. 2007).

This effect of HA on renal tubule development and differentiation may be “replayed” during renal tubular repair after acute kidney injury (AKI). The proximal tubule (PT) epithelium makes up the bulk of the kidney cortex and is the renal compartment most vulnerable to injury. However, PT epithelium also has a remarkable capacity for repair after AKI. Post injury, epithelial cells die but the surviving epithelia de-differentiate accompanied by inflammation. De-differentiated epithelial cells then proliferate and re-differentiate to repair the damaged nephron by a process that may be similar to developmental MET (Kusaba et al. 2014; Chang-Panesso and Humphreys 2017; Little and Kairath 2017).

Remarkably, HA which is absent from the cortical interstitium in healthy kidney is rapidly “re-activated” within 24 hours post injury in our experimental AKI models and is found in the cortical stroma surrounding proximal tubules undergoing repair and not those that are severely damaged and apoptotic/necrotic (Fig. 5.3). This HA in the cortical interstitium post-injury resembles the distribution of HA in embryonic kidneys surrounding developing cortical nephrons (Fig. 5.2a). It may be that early induction of HA post injury promotes epithelial repair by mimicking the embryonic stromal environment. In contrast, in healthy post-natal kidneys when the tubules are fully mature, HA is virtually absent from the cortical stroma or is found in small amounts typically surrounding arterioles, in juxta-glomerular niches and in

endothelial glycocalyx. On the other hand, accumulation of cortical HA is also associated with chronic kidney disease and correlates with fibrosis progression (Stridh et al. 2012; Albeiroti et al. 2015). This perplexing double-edged nature of the HA molecule, at times promoting pro-fibrotic events and at other times promoting anti-fibrotic events, has been noted in several studies (Kaul et al. 2021). Different molecular weights of HA can be attributed to these disparities, though most studies have yet to focus on this subtlety.

5.2 Chronic Kidney Disease

Chronic Kidney Disease (CKD) is defined as abnormalities in kidney structure or function, persisting for more than 3 months (KDIGO 2012). Glomerular Filtration Rate (GFR) is the best available predictor of kidney function (Levey et al. 2015) and is defined as the estimated blood flow through the glomeruli each minute. Current guidelines define CKD internationally by an estimated GFR of less than 60 mL/min per 1.73m^2 or an estimated GFR of less than 90 mL/min per 1.73m^2 with structural kidney and/or urinary abnormalities (Webster et al. 2017). Markers of structural abnormalities and urinary abnormalities include albuminuria/proteinuria, electrolyte disturbance, histological irregularities or structural issues noted via imaging (KDIGO 2012).

CKD has been categorised into five stages to allow for prognostication based on the stage of disease and clinical diagnosis (Levey et al. 2011). GFR categories from G1 to G5 are assigned based on declining renal function, with G5 signifying the worst function, also described as End-Stage Renal Failure (ESRF). Additionally, an albuminuria category of A1 to A3 is assigned based on the increasing amount of albumin detected in the urine, with A3 denoting the most severe disease. This is depicted in Table 5.1 based on the Kidney Disease Improving Global Outcomes Guidelines published in 2012.

CKD affects approximately 15% of adult populations aged 35 and over in England (Fat et al. 2016). This is similar to the prevalence across the world with both developed and low- and middle-income countries demonstrating a high prevalence of this condition (Barsoum 2006). Furthermore, the burden of CKD is expected to continue to rise, with prevalence rates in adults over the age of 30 expected to reach 16.7% by 2030, predominantly due to an ever-ageing population and increase in prevalence of co-morbidities such as obesity, hypertension, diabetes and cardiovascular disease (Hoerger et al. 2015). Identifying risk factors that are associated with developing CKD is central to developing prevention and screening strategies to target high-risk groups. In particular, identifying risk factors and biomarkers that drive the progression of CKD to ESRF is critical to developing prevention and/or reversal strategies and is the subject of considerable ongoing research. Increased age, male gender, black and ethnic minority groups, poor socioeconomic status and presence of comorbidities such as hypertension are important risk factors for developing CKD globally (Evans and Taal 2011).

Table 5.1 CKD stages and prognostication based on (KDIGO 2012)

Prognosis of CKD by GFR and Albuminuria Categories KDIGO 2012				Persistent Albuminuria Categories: Description and Range		
				A1	A2	A3
				Normal/mildly increased	Moderately increased	Severely increased
				< 30 mg/g	30-300 mg/g	> 300 mg/g
GFR categories (mL/min/ 1.73 m ²) Description and Range	G1	Normal or high	>90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney Failure	<15			
Green=low risk. Yellow=moderately increased risk. Orange=high risk. Red=severely high risk.						

5.3 Causes of CKD

CKD can be attributed to a number of different underlying pathologies, of which hypertension and diabetes are the principal causes globally. Diabetes accounts for up to half of all cases of CKD worldwide in both developed and developing countries (Webster et al. 2017). Hypertension is both a risk factor and cause of CKD and treatment of hypertension is essential for slowing the rate of CKD progression. Glomerulonephritis refers to a wide range of conditions that describe inflammation and damage to the glomeruli within the renal cortex. These inflammatory and immune-mediated groups of conditions require specialist investigation and treatment but altogether account for approximately 30% of CKD. Other causes of CKD include genetic diseases such as polycystic kidney disease and Alport’s syndrome, drugs including non-steroidal anti-inflammatory drugs and lithium, reflux nephropathy and obstructive nephropathy and infections including HIV and SARS-CoV-2 (Evans and Taal 2011). However, irrespective of the underlying cause of CKD that led to the initial damage, fibrosis within the renal tubules and interstitium is the pathological process that drives the progression of CKD to ESRF. Histologically this is observed as glomerulosclerosis, tubular atrophy and interstitial fibrosis, with areas of tubulointerstitial fibrosis correlating closely with stage 5 CKD and poor renal outcomes (Eddy 1996).

5.4 Fibrosis: Dysregulated Wound Healing

Tissue repair through scar formation is a ubiquitous process in many organs. It is most often associated with cutaneous wounds and involves a highly orchestrated cascade, leading to healing and regeneration of the tissue. Wound healing develops in four overlapping phases, which have distinct biological characteristics: inflammation, proliferation, contraction and remodelling. These phases require the interaction of multiple distinct local and systemic cell types and regulatory signalling mediators, such as cytokines and growth factors. If these phases are disrupted, the healing process may fail or be delayed. However, excessive and persistent scarring or fibrosis may also occur as a result of aberrant healing leading to keloid and hypertrophic scars (Gauglitz et al. 2011; Berman et al. 2017).

In normal healing, there is a release of inflammatory mediators that initiates a coagulation cascade (Kumar et al. 2015) triggering blood clot formation and formation of a provisional ECM. Platelets bind to this ECM and degranulate, promoting vasodilation and increased blood vessel permeability. Myofibroblasts (activated collagen secreting, α -smooth muscle actin-positive (α -SMA⁺) fibroblasts) and other resident cells produce Matrix Metalloproteinases (MMPs), which disrupt basement membranes and the normal architecture of the surrounding tissue, allowing recruitment of leukocytes across the provisional ECM. Some of these, macrophages and neutrophils, for example, remove tissue debris, dead cells and any invading organisms. Others, such as lymphocytes, become activated and secrete pro-scarring cytokines and growth factors (Wynn 2003; Li et al. 2006; Parsons et al. 2007) which further activate resident fibroblasts. These differentiate into α -SMA-expressing myofibroblasts as they migrate along the fibrin lattice into the wound. They then promote wound contraction and closure. However, chronic activation and inflammation may trigger an excessive accumulation of ECM components, leading to the formation of the permanent fibrotic scars described above. Changes to collagen turnover and ECM remodelling alter the equilibrium between the synthesis and catabolism of collagen and other ECM components (such that there is increased ECM deposition and accumulation over time).

5.5 Myofibroblasts

Myofibroblasts were first described by Gabbiani et al. in 1971 as large fibroblastic cells within granulation tissue. They have filamentous fibres incorporating α -SMA that allow the cell to provide a contractile force at the wound edge to allow wound closure (Gabbiani et al. 1971; Majno et al. 1971). This and the production of extracellular matrix (ECM) components such as types I and III collagen, fibronectin (particularly the EDA splice variant) and HA are well-described markers of myofibroblasts in pathology and fibrosis (Gabbiani 1981; Klingberg et al. 2013).

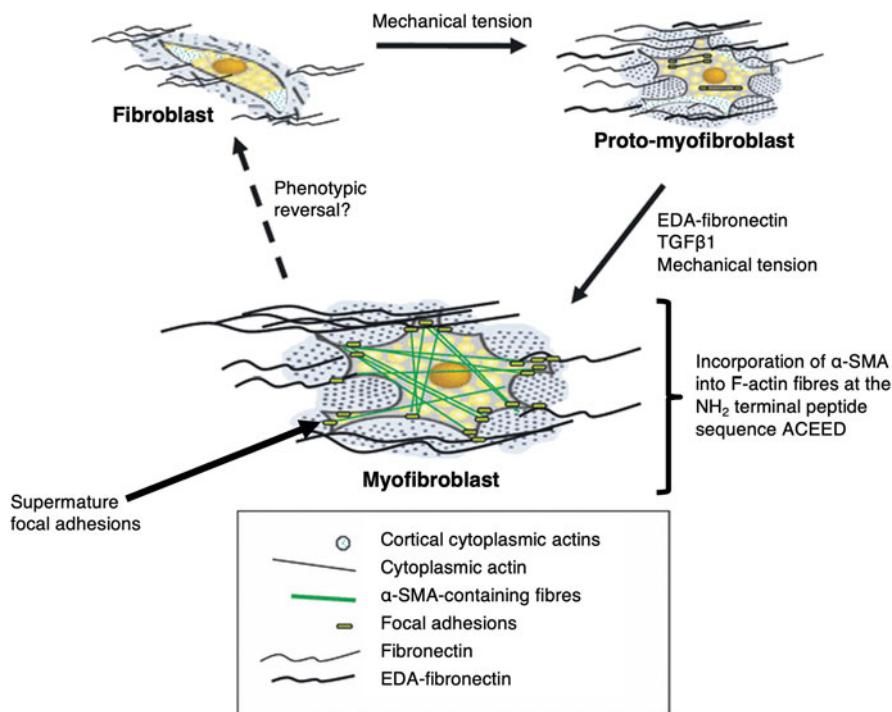


Fig. 5.4 Fibroblast to Myofibroblast Differentiation. Schematic adapted from (Tomasek et al. 2002) (Meran and Steadman 2011) illustrates the differentiation process of fibroblasts to myofibroblasts (via the intermediate stage of a proto-myofibroblast) and the altered protein expression at each stage

Many distinct cell types have been described as the source of myofibroblasts, suggesting these cells could arise in most tissues. Sources include resident fibroblasts, endothelial cells, epithelial cells and pericytes, circulating bone marrow-derived fibrocytes, mesenchymal stem cells, de-differentiated smooth muscle cells and mononuclear phagocytes (Pakshir et al. 2020; Hinz et al. 2007; Vierhout et al. 2021; Schuster et al. 2021). Regardless of the originating cell type, mature myofibroblasts are generated as a result of interactions with the ECM, particularly collagens and EDA-fibronectin and in response to Transforming Growth Factor (TGFβ1) mostly released from platelets and inflammatory cells (Fig. 5.4).

5.6 The Mechanisms Controlling TGFβ-Induced Myofibroblast Differentiation

TGFβ1 binds and induces TGFβReceptor I and II association, leading to TGFβRII phosphorylation followed by activation of the transcription factors Smad2 and Smad3, which form a trimeric complex with Smad4. These translocate to the nucleus

where they act as regulators of gene expression (Shi and Massague 2003). This is the canonical TGF signalling pathway. In 2004, however, our group reported that HA accumulated as a pericellular coat around myofibroblasts, partly as a result of reduced degradation by hyaluronidases (Jenkins et al. 2004). In 2007 we described an alternative pathway that was HA-dependent and complementary to the canonical pathway and in which the synthesis, accumulation and assembly of HA were all essential to myofibroblast differentiation (Meran et al. 2007). Subsequent investigations have begun to dissect the mechanisms underlying this HA dependence.

5.7 HA Synthesis: The Hyaluronan Synthase (HAS) Genes

Isoenzymes HAS1, HAS2 and HAS3 variants HAS3 v1 and HAS3 v2 are encoded by mRNAs at loci on human chromosomes 19q13.41, 8q24.13 and 16q22.1, respectively (Spicer et al. 1997). We carried out a comprehensive comparison of human and mouse intron/exon structures and observed extensive evolutionary conservation for all the *HAS* genes (Monslow et al. 2003). Currently, there are two National Centre for Biotechnology Information (NCBI) reference messenger (m)RNA sequences for HAS1, one for HAS2 and three for HAS3 (Midgley and Bowen 2022). There is also one NCBI reference sequence for long noncoding RNA transcript HAS2-AS1. HAS2-AS1 is transcribed from the opposite genomic DNA strand at the HAS2 locus, and partially overlaps the HAS2 sequence (Midgley and Bowen 2022; Chao and Spicer 2005; Michael et al. 2011).

5.8 Transcriptional Regulation

We used 5'-rapid amplification of complementary DNA ends to extend the 5'-untranslated regions (5'-UTRs) of the previous NCBI reference sequences of HAS1 (Chen et al. 2012) and HAS2 (Monslow et al. 2004), and thereby identify their promoters. This analysis has since been carried out for HAS3 (Wang et al. 2015).

Our in vitro analysis in human dermal fibroblasts (DFs) showed that IL-1 β HAS1 induction was mediated by Sp3 and that TGF β 1-driven HAS1 upregulation was Smad3-dependent (Chen et al. 2012). We have also shown that Sp1 and Sp3 are involved in the constitutive expression of HAS2 in human renal proximal tubular epithelial cells (PTCs) (Monslow et al. 2006) and, in collaboration, by retinoic acid receptor and NF- κ B in human keratinocytes (Saavalainen et al. 2007).

Further information on factors mediating HAS1, HAS2 and HAS3 transcriptional regulation have been reviewed expertly by Heldin and colleagues (Heldin et al. 2019). More recent reports of factors repressing HAS2 expression include the finding that inhibition of NF- κ B and HAS2-AS1 expression by sirutin 1 downregulates HAS2 in human aortic smooth muscle cells (Caon et al. 2020). Similarly, vitamin D receptor-driven inhibition of HAS2 expression and HA

synthesis has been observed in multiple breast cancer models (Narvaez et al. 2020), and transcription factor inhibitor of differentiation 3 downregulated HAS2 expression and HA synthesis in mouse epididymal adipose tissue (Misiou et al. 2021). Exposure of DFs to UVA radiation resulted in the downregulation of HA synthesis, expression of HAS2 and transcription factor STAT3, NF- κ B phosphorylation and JAK/STATs signalling (Terazawa et al. 2021).

Recent findings on factors upregulating HAS2 expression include mycosporine-like amino acid induction of HAS2 expression by activating the p38/MSK1/CREB/c-FOS/AP-1 axis in DFs (Terazawa et al. 2020). In eutopic endometrial stromal cells, EGF increases the expression of endometriosis-associated HA and HAS2, both of which mediate stromal cell migration and invasion (Zhan et al. 2020). G protein alpha subunit 14 promotes endometrial cancer cell growth via upregulation of Krüppel-like factor 7 and subsequent induction of HAS2 expression (Wang et al. 2021).

We have shown promoter activity of the sequences immediately upstream of long noncoding RNA HAS2-AS1 in PTCs (Michael et al. 2011). Vigetti and colleagues (Vigetti et al. 2014) then demonstrated NF- κ B-dependent upregulation of this transcript and provided a mechanism for epigenetic factors that regulate its expression in human aortic smooth muscle cells. Further details of the transcription factors and the role of epigenetics in the regulation of HAS2-AS1 expression are authoritatively summarised in this recent review (Parnigoni et al. 2021).

5.9 microRNA Regulation

The current NCBI reference sequences for HAS2 and two of the three HAS3 transcripts have 3'-UTRs over 1900 base pairs in length, spanning numerous putative microRNA (miRNA) binding sites. The HAS1 3'-UTR is considerably smaller, and therefore far fewer miRNA interactions are predicted. Nevertheless, indirect miRNA regulation of HAS1 expression has been posited through repression of STAT3 expression by miR-125a, thereby inhibiting migration and invasion of renal cell carcinoma cells (Sun et al. 2019).

Lagendijk et al. showed that miR-23 repression of Has2 expression in zebrafish is essential for correct cardiac development (Lagendijk et al. 2011). In primary human DFs, inhibition of HAS2 expression by miR-23a results in significantly decreased HA synthesis and cellular senescence (Rock et al. 2015). Members of the let-7 miRNA family repress HAS2 expression in human cell lines (Yang et al. 2012), while an increased abundance of specific let-7 transcripts has been observed in the vitreous and retina of rats during ageing, downregulating HAS2 expression (Akamine et al. 2021).

Mature miR-26b sequences are highly conserved between humans and pigs. This transcript targets HAS2 and increases porcine ovarian granulosa cell apoptosis via the miR-26b-HAS2-HA-CD44-Caspase-3 pathway (Liu et al. 2016). HAS2 expression is also targeted by miR-574 in porcine cumulus cells, having a negative impact

on oocyte quality during in vitro maturation, an effect that is reversed by inhibition of miR-574 (Pan et al. 2018).

Downregulation of Wnt3a by miR-410 results in upregulated expression of HAS2 and other chondrogenic markers in primary human bone marrow mesenchymal stem cells, promoting chondrogenic differentiation (Zhang et al. 2017). In an analysis of synovial fibroblasts from osteoarthritis patients, oestradiol increased the abundance of miR-101, which in turn regulated HAS2 expression (Feng et al. 2019).

Repression of HAS3 expression by miR-29a inhibits proliferation and metastasis of gastric cancer cells (Bai et al. 2018), while both miR-10b and miR-29a have been shown to regulate HAS3 expression in prostate cancer cells (Czyrnik et al. 2020).

5.10 HA Cell Surface Assembly

The HA pericellular coat is anchored to the cell surface by CD44 (Meran et al. 2007; Meran et al. 2008) and crosslinked by hyaladherins (Webber et al. 2009a; Webber et al. 2009b). All CD44 variants and the standard form (CD44s) possess HA binding motifs in their extracellular domain. Studies have shown that silencing CD44 using small interfering RNA (siRNA) inhibited the formation of HA pericellular coats and prevented the downstream signalling needed for myofibroblast differentiation (Meran et al. 2011; Midgley et al. 2013). Recently we showed that it is CD44s that is the specific variant controlling differentiation (Woods et al. 2021).

Hyaladherins including inter- α -inhibitor (I α I) heavy chains (HC) and tumour necrosis factor-stimulated gene (TSG)-6 are also important for myofibroblast formation (Selbi et al. 2004; Zhuo and Kimata 2008). In addition, to the well-described involvement of HC1, HC2 and HC3, we have identified the recently described HC5 as another hyaladherin that is essential for the formation of the myofibroblast pericellular HA matrix (Martin et al. 2016).

CD44 is present throughout the membranes of fibroblasts but is clustered into cholesterol-rich lipid rafts in myofibroblasts. Enzymatic removal of the HA pericellular coat released CD44 allowing it to move freely in the membrane and inhibited downstream signalling and myofibroblast differentiation (Webber et al. 2009a; Webber et al. 2009b). CD44 was shown to be co-localised with the epidermal growth factor receptor (EGFR) in the lipid rafts, resulting in the activation of EGFR and phosphorylation of downstream effectors such as Extracellular signal Regulated Kinases (ERK)1/2 and calcium calmodulin kinase-II (CaMKII). Inhibition of ERK1/2 or CaMKII prevented myofibroblast differentiation. For myofibroblast differentiation to occur canonical TGF- β RII phosphorylation and CD44/EGFR signalling were both essential (Midgley et al. 2013).

5.11 BMP7 and Fibrosis Prevention and Reversal

Bone morphogenetic protein-7 (BMP7) is a member of the transforming growth factor-13 (TGF- β 1) superfamily that demonstrates reduced expression in progressive renal diseases. Studies in animal models of renal disease, including ischemic reperfusion injury, unilateral urethral obstruction, nephrotoxin-induced glomerulonephritis and streptozotocin-induced diabetic nephropathy, have shown that BMP7 can both prevent and reverse renal fibrosis (Hruska et al. 2000; Zeisberg et al. 2005; Wang et al. 2003; Vukicevic et al. 1998). However, BMP7 is not currently viable as a clinical therapy because it has been implicated in the progression of bone metastasis in osteotropic cancers and in promoting ectopic bone formation (Buijs et al. 2010). Dissecting the mechanisms by which BMP7 prevents and reverses fibrosis has been investigated to yield more suitable therapeutic targets.

Studies have shown that HA can both mediate and modulate BMP7 responses in biological systems. In chondrocytes, BMP7-dependent Smad1 signalling was mediated through CD44-Smad1 interactions (Andhare et al. 2009). Other studies demonstrated that pericellular HA augments BMP7 Smad signalling (Peterson et al. 2004). In renal epithelial cells, BMP7 has also been shown to induce the formation of HA cables (Selbi et al. 2004). More recent studies have shown that alterations in HA play a critical role in BMP7-mediated antagonism of the pro-fibrotic effects of TGF- β 1 and this is key to the prevention and reversal of progressive renal disease described above (Midgley et al. 2015) (Fig. 5.5). These studies demonstrate that BMP7 prevents and reverses TGF- β 1 mediated induction of fibroblast to myofibroblast differentiation by promoting intracellular internalisation of pericellular HA matrices into catalytic endosomes. This prevents TGF- β 1-driven pericellular HA accumulation, which is critical for differentiation to and maintenance of the alpha-smooth muscle actin positive myofibroblast phenotype in the renal stroma. The weak HA catalytic enzyme, Hyaluronidase-2, was pivotal to this process of HA internalisation and its varying roles in promotion and prevention of renal fibrosis are described in further detail in the next section.

5.12 Hyaluronidase-2 (HYAL2) and the Kidney

HYAL2 is a cell-surface and acid-active HA-degrading enzyme, which is widely expressed in somatic tissues and is described as being anchored to the cell membrane via a GPI (glycosylphosphatidylinositol) link. HYAL2 only has catabolic function within a narrow acidic pH range and compared to other hyaluronidases, it has only weak HA-degrading activity (Vigdorovich et al. 2007). Many studies also indicate that a significant proportion of HYAL2 may be enzymatically inactive and its cellular function in this context has been previously unclear (Vigdorovich et al. 2007; Duterme et al. 2009). However, mouse studies demonstrated that *Hyal2*^{-/-} tissues do accumulate extracellular HA, signifying that in certain contexts

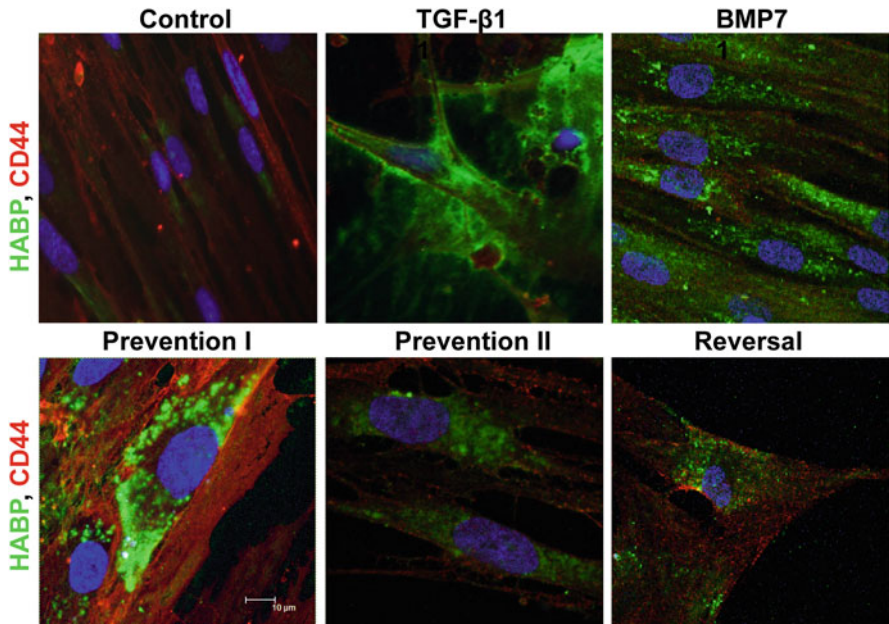


Fig. 5.5 TGF- β 1 drives formation of HA pericellular matrices while BMP7 treatments promote intracellular internalisation of pericellular HA matrices leading to prevention and reversal of myofibroblast phenotype (Midgley et al. JBC, 2015)

HYAL2 does play an important role in HA turnover (Chowdhury et al. 2013). Recent advances have demonstrated that HYAL2 has important and distinct enzymatic as well as non-enzymatic functions at the cell surface, cytoplasm and nucleus that is relevant to both promotion and prevention of pro-fibrotic cell phenotype.

Cell-surface HYAL2 is anchored to the cell membrane via a glycosylphosphatidylinositol link (Andre et al. 2011). It is thought to have weak enzymatic activity with the bulk of HA cleavage through the generation of acidic pockets generated by the Na^+/H^+ Transporter, NHE1, which catalyses the extrusion of intracellular H^+ in exchange for extracellular Na^+ (Bourguignon et al. 2004). Activated HYAL2 catalyses high molecular mass HA into intermediate size fragments of ~20 kDa (50 disaccharides), which can then be further internalised for degradation. Stromal fibroblasts that are in a quiescent, inactive and non-fibrogenic phase are identified as having abundant cell-surface HYAL2. In this context, HYAL2 colocalises with the standard isoform of the principal HA receptor, CD44s and is involved in the degradation of the pericellular HA coat, preventing its accumulation and thereby maintaining an undifferentiated fibroblast phenotype (Jenkins et al. 2004; Duterme et al. 2009; Andre et al. 2011).

Cytoplasmic HYAL2 in contrast is found to be abundant in myofibroblasts and exists in two cellular locations within this pro-fibrotic cell phenotype. Cytoplasmic endosomes and lysosomes containing HA and HYAL2 have been identified. These vesicles are known to be acidic and the HYAL2 in this context is also thought to

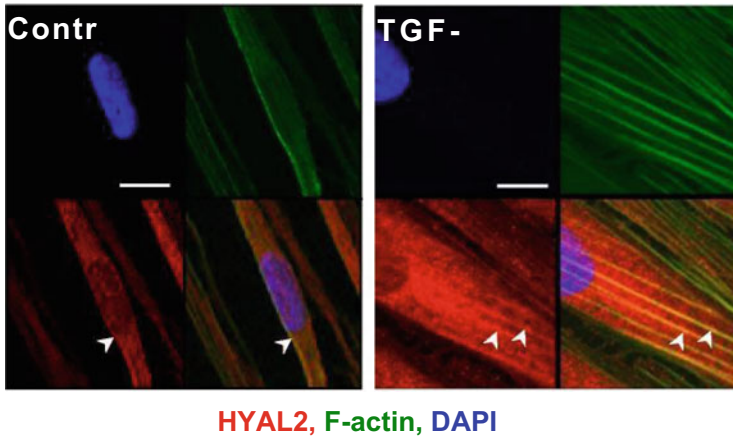


Fig. 5.6 HYAL2 is upregulated and relocated from the cell membrane to the cell cytoskeleton following TGF- β 1-driven myofibroblast differentiation (Midgley et al., 2020)

function in an enzymatic capacity promoting HA turnover and breakdown. However, cytoplasmic HYAL2 has also been identified aligned with the actin cytoskeleton in myofibroblasts (Fig. 5.6). This cytoskeletal-linked HYAL2 interacts with Rho-kinase, promoting its downstream signalling through myosin light chain kinase activity. This interaction facilitates RHOA-mediated pro-fibrotic myofibroblast functions such as myofibroblast contractility and myofibroblast migration. It also promotes downstream gene expression of other RHOA-dependent pro-fibrotic factors including CCN2 (also known as connective tissue growth factor), fibronectin, matrix metalloproteinase 2 (MMP2) and collagen I (Midgley et al. 2020). Therefore, in contrast to cell-surface HYAL2 which appears to facilitate a quiescent fibroblast phenotype, cytoskeletal-linked HYAL2 promotes the pro-fibrotic functions of the α -smooth muscle actin myofibroblast. This is also true in vivo where HYAL2 demonstrates increased expression and colocalises with α -smooth muscle actin positive myofibroblasts in the renal interstitium following experimental renal fibrosis (Fig. 5.7).

The presence of nuclear HYAL2 is classically demonstrated in the nucleus of fibroblasts and myofibroblasts stimulated with the anti-fibrotic BMP7. In these cells, the interaction of cell-surface HYAL2 with the Na^+/H^+ ion exchanger promotes HYAL2 and HA internalisation into endosomes, mediating HA breakdown (Midgley et al. 2015). Moreover, internalised HYAL2 also translocates to the nucleus and influences alternative splicing of the HA receptor, CD44. CD44 is a transmembrane glycoprotein, which exhibits considerable structural diversity due to alternative pre-mRNA splicing. Standard CD44 (CD44s) is the most abundantly expressed isoform and is critical in maintaining the TGF- β 1-driven HA coat (Midgley et al. 2013). Other CD44 variant isoforms have the same overall structure as CD44s but contain additional extracellular stem regions, encoded by combinations of variably expressed exons. BMP7-driven prevention and reversal of

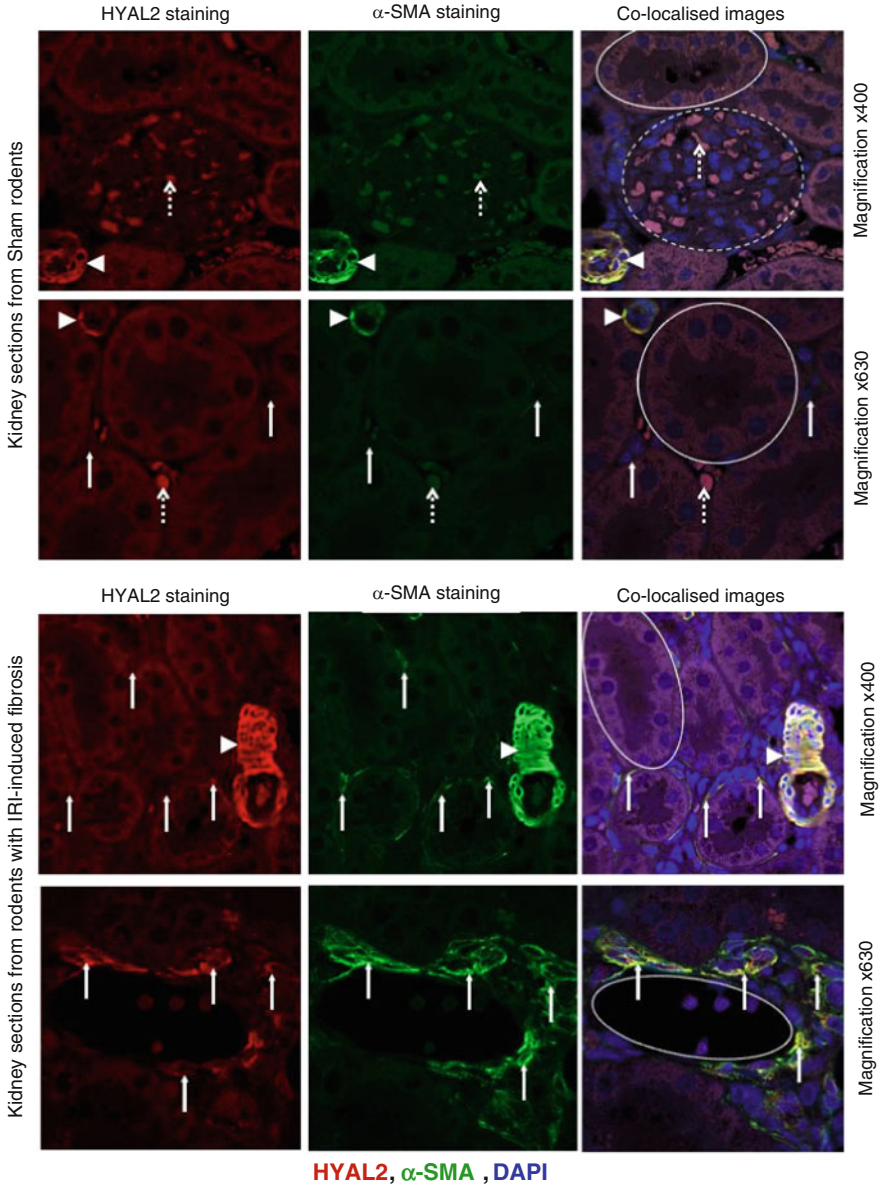


Fig. 5.7 HYAL2 demonstrates increased interstitial expression in renal fibrosis in areas of α -smooth muscle actin-positive myofibroblasts (Midgley et al., 2020)

myofibroblast phenotype is dependent on cell-surface expression of the variant isoform, CD44v7/8, which drives internalisation of the HA coat (Midgley et al. 2015). Recent advances demonstrate that nuclear HYAL2 is critical in driving alternative splicing events promoting cell-surface CD44v7/8 expression, in

preference over CD44s. Specifically, HYAL2 displaces SRSF proteins from CD44 pre-mRNA introns and spliceosome complexes, resulting in the inclusion of exons 11 and 12 (Midgley et al. 2017). In concert, these data demonstrate that HYAL2 functionality is complex and dependent not only on its interactions but its localisation within the cell. Understanding how HYAL2 may be regulated to influence its distinct and opposing functions relevant to fibrosis remains incompletely understood and is the subject of much ongoing research.

5.13 Conclusions

HA synthesis, accumulation and assembly are processes central to acute kidney injury and to the progression of fibrotic disease. The cells driving disease progression are based in the tubular interstitium and evidence suggests the early induction of HA following injury promotes epithelial repair by mimicking the stromal environment of the embryonic kidney. In contrast, in healthy post-natal kidneys, when tubules are fully mature, HA is virtually absent from the cortical stroma, found only in small amounts surrounding arterioles, juxta-glomerular niches and in the endothelial glycocalyx. On the other hand, accumulation of cortical HA is also associated with chronic kidney disease and correlates with fibrosis progression. This fascinating ambiguity has been and will continue to be the focus of many renal research groups and may provide important targets for clinical interventions in the future.

References

- Akamine PS et al (2021) Age-related increase of let-7 family microRNA in rat retina and vitreous. *Exp Eye Res* 204:108434
- Albeiroti S, Soroosh A, de la Motte CA (2015) Hyaluronan's role in fibrosis: a pathogenic factor or a passive player? *Biomed Res Int* 2015:790203
- Andhare RA et al (2009) Hyaluronan promotes the chondrocyte response to BMP-7. *Osteoarthritis Cartil* 17(7):906–916
- Andre B et al (2011) Hyal2 is a glycosylphosphatidylinositol-anchored, lipid raft-associated hyaluronidase. *Biochem Biophys Res Commun* 411(1):175–179
- Bai F et al (2018) miR29a3p represses proliferation and metastasis of gastric cancer cells via attenuating HAS3 levels. *Mol Med Rep* 17(6):8145–8152
- Bai KJ et al (2005) The role of hyaluronan synthase 3 in ventilator-induced lung injury. *Am J Respir Crit Care Med* 172(1):92–98
- Barsoum RS (2006) Chronic kidney disease in the developing world. *N Engl J Med* 354(10):997–999
- Berman B, Maderal A, Raphael B (2017) Keloids and hypertrophic scars: pathophysiology, classification, and treatment. *Dermatol Surg* 43(Suppl 1):S3–S18
- Bourguignon LY et al (2004) CD44 interaction with Na⁺-H⁺ exchanger (NHE1) creates acidic microenvironments leading to hyaluronidase-2 and cathepsin B activation and breast tumor cell invasion. *J Biol Chem* 279(26):26991–27007

- Buijs JT et al (2010) Bone morphogenetic proteins and its receptors; therapeutic targets in cancer progression and bone metastasis? *Curr Pharm Des* 16(11):1291300
- Camenisch TD et al (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 106(3):349–360
- Caon I et al (2020) Sirtuin 1 reduces hyaluronan synthase 2 expression by inhibiting nuclear translocation of NF-kappaB and expression of the long-noncoding RNA HAS2-AS1. *J Biol Chem* 295(11):3485–3496
- Chang-Panesso M, Humphreys BD (2017) Cellular plasticity in kidney injury and repair. *Nat Rev Nephrol* 13(1):39–46
- Chao H, Spicer AP (2005) Natural antisense mRNAs to hyaluronan synthase 2 inhibit hyaluronan biosynthesis and cell proliferation. *J Biol Chem* 280(30):2751322
- Chen L et al (2012) Identification and analysis of the human hyaluronan synthase 1 gene promoter reveals Smad3- and Sp3-mediated transcriptional induction. *Matrix Biol* 31(7–8):373–379
- Chowdhury B et al (2013) Murine hyaluronidase 2 deficiency results in extracellular hyaluronan accumulation and severe cardiopulmonary dysfunction. *J Biol Chem* 288(1):520–528
- Czyrnik ED et al (2020) The regulation of HAS3 by miR-10b and miR-29a in neuroendocrine transdifferentiated LNCaP prostate cancer cells. *Biochem Biophys Res Commun* 523(3):713–718
- Das A et al (2013) Stromal–epithelial crosstalk regulates kidney progenitor cell differentiation. *Nat Cell Biol* 15(9):1035–1044
- Duterme C et al (2009) Two novel functions of hyaluronidase-2 (Hyal2) are formation of the glycocalyx and control of CD44-ERM interactions. *J Biol Chem* 284(48):33495–33508
- Eddy AA (1996) Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 7(12):2495–2508
- Evans P, Taal M (2011) Epidemiology and causes of chronic kidney disease. *Medicine* 39:402–406
- Fat LN, Mindell J, Roderick P (2016) Health Survey for England 2016 (Kidney and Liver Disease). NHS Digital
- Feng C et al (2019) Estrogen-mediated MicroRNA-101-3p expression represses Hyaluronan synthase 2 in synovial fibroblasts from idiopathic condylar resorption patients. *J Oral Maxillofac Surg* 77(8):1582–1593
- Gabbiani G (1981) The myofibroblast: a key cell for wound healing and fibrocontractive diseases. *Prog Clin Biol Res* 54:183–194
- Gabbiani G, Ryan GB, Majne G (1971) Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27(5):549–550
- Gauglitz GG et al (2011) Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Mol Med* 17(1–2):113–125
- Heldin P et al (2019) Regulation of hyaluronan biosynthesis and clinical impact of excessive hyaluronan production. *Matrix Biol* 78-79:100–117
- Hinz B et al (2007) The myofibroblast: one function, multiple origins. *Am J Pathol* 170(6):1807–1816
- Hoerger TJ et al (2015) The future burden of CKD in the United States: a simulation model for the CDC CKD initiative. *Am J Kidney Dis* 65(3):403–411
- Hruska KA et al (2000) Osteogenic protein-1 prevents renal fibrogenesis associated with ureteral obstruction. *Am J Physiol Renal Physiol* 279(1):F130–F143
- Jenkins RH et al (2004) Myofibroblastic differentiation leads to hyaluronan accumulation through reduced hyaluronan turnover. *J Biol Chem* 279(40):41453–41460
- Kaul A et al (2021) Hyaluronan, a double-edged sword in kidney diseases. *Pediatr Nephrol* 37(4):735–744
- KDIGO (2012) In: C.W. Group (ed) KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney International Supplements
- Klingberg F, Hinz B, White ES (2013) The myofibroblast matrix: implications for tissue repair and fibrosis. *J Pathol* 229(2):298–309

- Kobayashi N et al (2010) Hyaluronan deficiency in tumor stroma impairs macrophage trafficking and tumor neovascularization. *Cancer Res* 70(18):7073–7083
- Kumar V, Abul KA, Jon CA (2015) *Pathologic basis of disease*. Elsevier/Saunders
- Kusaba T et al (2014) Differentiated kidney epithelial cells repair injured proximal tubule. *Proc Natl Acad Sci U S A* 111(4):1527–1532
- Legendijk AK et al (2011) MicroRNA-23 restricts cardiac valve formation by inhibiting Has2 and extracellular hyaluronic acid production. *Circ Res* 109(6):649–657
- Levey AS, Becker C, Inker LA (2015) Glomerular filtration rate and albuminuria for detection and staging of acute and chronic kidney disease in adults: a systematic review. *JAMA* 313(8):837–846
- Levey AS et al (2011) The definition, classification, and prognosis of chronic kidney disease: a KDIGO controversies conference report. *Kidney Int* 80(1):17–28
- Li MO et al (2006) Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 24:99–146
- Little MH, Kairath P (2017) Does renal repair recapitulate kidney development? *J Am Soc Nephrol* 28(1):34–46
- Little, M.H. and A.P. McMahon, *Mammalian kidney development: principles, progress, and projections*. Cold Spring Harb Perspect Biol, 2012. 4(5)
- Liu J et al (2016) Conserved miR-26b enhances ovarian granulosa cell apoptosis through HAS2-HA-CD44-Caspase-3 pathway by targeting HAS2. *Sci Rep* 6:21197
- Majno G et al (1971) Contraction of granulation tissue in vitro: similarity to smooth muscle. *Science* 173(3996):548–550
- Martin J et al (2016) Tumor necrosis factor-stimulated gene 6 (TSG-6)-mediated interactions with the inter-alpha-inhibitor heavy chain 5 facilitate tumor growth factor beta1 (TGFbeta1)-dependent fibroblast to Myofibroblast differentiation. *J Biol Chem* 291(26):13789–13801
- Meran S et al (2007) Involvement of hyaluronan in regulation of fibroblast phenotype. *J Biol Chem* 282(35):25687–25697
- Meran S et al (2008) Hyaluronan facilitates transforming growth factor-beta1-mediated fibroblast proliferation. *J Biol Chem* 283(10):6530–6545
- Meran S, Steadman R (2011) Fibroblasts and myofibroblasts in renal fibrosis. *Int J Exp Pathol* 92(3):158–167
- Meran S et al (2011) Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. *J Biol Chem* 286(20):17618–17630
- Michael DR et al (2011) The human hyaluronan synthase 2 (HAS2) gene and its natural antisense RNA exhibit coordinated expression in the renal proximal tubular epithelial cell. *J Biol Chem* 286(22):19523–19532
- Midgley AC, Bowen T (2022) Analysis of human Hyaluronan synthase gene transcriptional regulation and downstream Hyaluronan cell surface receptor mobility in Myofibroblast differentiation. *Methods Mol Biol* 2303:453–468
- Midgley AC et al (2013) Transforming growth factor-beta1 (TGF-beta1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (HA)-facilitated epidermal growth factor receptor (EGFR) and CD44 co-localization in lipid rafts. *J Biol Chem* 288(21):14824–14838
- Midgley AC et al (2015) Hyaluronan regulates bone morphogenetic protein-7-dependent prevention and reversal of myofibroblast phenotype. *J Biol Chem* 290(18):11218–11234
- Midgley AC et al (2017) Nuclear hyaluronidase 2 drives alternative splicing of CD44 pre-mRNA to determine profibrotic or antifibrotic cell phenotype. *Sci Signal* 10(506)
- Midgley AC et al (2020) Hyaluronidase-2 regulates RhoA signaling, Myofibroblast contractility, and other key Profibrotic Myofibroblast functions. *Am J Pathol* 190(6):1236–1255
- Misiou A et al (2021) Helix-loop-helix factor Id3 (inhibitor of differentiation 3): a novel regulator of Hyaluronan-mediated adipose tissue inflammation. *Arterioscler Thromb Vasc Biol* 41(2):796–807

- Monslow J et al (2003) The human hyaluronan synthase genes: genomic structures, proximal promoters and polymorphic microsatellite markers. *Int J Biochem Cell Biol* 35(8):1272–1283
- Monslow J et al (2004) Identification and analysis of the promoter region of the human hyaluronan synthase 2 gene. *J Biol Chem* 279(20):20576–20581
- Monslow J et al (2006) Sp1 and Sp3 mediate constitutive transcription of the human hyaluronan synthase 2 gene. *J Biol Chem* 281(26):18043–18050
- Narvaez CJ et al (2020) Inhibition of HAS2 and hyaluronic acid production by 1,25-Dihydroxyvitamin D3 in breast cancer. *Oncotarget* 11(30):2889–2905
- Pakshir P et al (2020) The myofibroblast at a glance. *J Cell Sci* 133(13)
- Pan B, Toms D, Li J (2018) MicroRNA-574 suppresses oocyte maturation via targeting hyaluronan synthase 2 in porcine cumulus cells. *Am J Physiol Cell Physiol* 314(3):C268–C277
- Parnigoni A et al (2021) The role of the multifaceted long non-coding RNAs: a nuclear-cytosolic interplay to regulate hyaluronan metabolism. *Matrix Biol Plus* 11:100060
- Parsons CJ, Takashima M, Rippe RA (2007) Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 22(Suppl 1):S79–S84
- Peterson RS et al (2004) CD44 modulates Smad1 activation in the BMP-7 signaling pathway. *J Cell Biol* 166(7):1081–1091
- Pohl M et al (2000) Role of hyaluronan and CD44 in in vitro branching morphogenesis of ureteric bud cells. *Dev Biol* 224(2):312–325
- Rock K et al (2015) miR-23a-3p causes cellular senescence by targeting hyaluronan synthase 2: possible implication for skin aging. *J Invest Dermatol* 135(2):369–377
- Rosines E, Schmidt HJ, Nigam SK (2007) The effect of hyaluronic acid size and concentration on branching morphogenesis and tubule differentiation in developing kidney culture systems: potential applications to engineering of renal tissues. *Biomaterials* 28(32):4806–4817
- Saavalainen K et al (2007) Integration of the activation of the human hyaluronan synthase 2 gene promoter by common cofactors of the transcription factors retinoic acid receptor and nuclear factor kappaB. *J Biol Chem* 282(15):11530–11539
- Schuster R et al (2021) The inflammatory speech of fibroblasts. *Immunol Rev* 302(1):126–146
- Selbi W et al (2004) BMP-7 modulates hyaluronan-mediated proximal tubular cell-monocyte interaction. *J Am Soc Nephrol* 15(5):1199–1211
- Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113(6):685–700
- Spicer AP et al (1997) Chromosomal localization of the human and mouse hyaluronan synthase genes. *Genomics* 41(3):493–497
- Stridh S, Palm F, Hansell P (2012) Renal interstitial hyaluronan: functional aspects during normal and pathological conditions. *Am J Physiol Regul Integr Comp Physiol* 302(11):R1235–R1249
- Sun M et al (2019) MicroRNA-125a suppresses cell migration, invasion, and regulates hyaluronic acid synthase 1 expression by targeting signal transducers and activators of transcription 3 in renal cell carcinoma cells. *J Cell Biochem* 120(2):1894–1902
- Terazawa S et al (2020) Mycosporine-like amino acids stimulate hyaluronan secretion by up-regulating hyaluronan synthase 2 via activation of the p38/MSK1/CREB/c-Fos/AP-1 axis. *J Biol Chem* 295(21):7274–7288
- Terazawa S et al (2021) The attenuated secretion of Hyaluronan by UVA-exposed human fibroblasts is associated with up- and downregulation of HYBID and HAS2 expression via activated and inactivated signaling of the p38/ATF2 and JAK2/STAT3 cascades. *Int J Mol Sci* 22(4)
- Tomasek JJ et al (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3(5):349–363
- Toole BP (2001) Hyaluronan in morphogenesis. *Semin Cell Dev Biol* 12(2):79–87
- Vierhout M et al (2021) Monocyte and macrophage derived myofibroblasts: is it fate? A review of the current evidence. *Wound Repair Regen* 29(4):548–562
- Vigdorovich V, Miller AD, Strong RK (2007) Ability of hyaluronidase 2 to degrade extracellular hyaluronan is not required for its function as a receptor for jaagsiekte sheep retrovirus. *J Virol* 81(7):3124–3129

- Vigetti D et al (2014) Natural antisense transcript for hyaluronan synthase 2 (HAS2-AS1) induces transcription of HAS2 via protein O-GlcNAcylation. *J Biol Chem* 289(42):28816–28826
- Vukicevic S et al (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. *J Clin Invest* 102(1):202–214
- Wang J et al (2021) GNA14 stimulation of KLF7 promotes malignant growth of endometrial cancer through upregulation of HAS2. *BMC Cancer* 21(1):456
- Wang S et al (2003) Bone morphogenic protein-7 (BMP-7), a novel therapy for diabetic nephropathy. *Kidney Int* 63(6):2037–2049
- Wang S et al (2015) Identification and analysis of the promoter region of the human HAS3 gene. *Biochem Biophys Res Commun* 460(4):1008–1014
- Webber J et al (2009a) Hyaluronan orchestrates transforming growth factor-beta1-dependent maintenance of myofibroblast phenotype. *J Biol Chem* 284(14):9083–9092
- Webber J et al (2009b) Modulation of TGFbeta1-dependent myofibroblast differentiation by hyaluronan. *Am J Pathol* 175(1):148–160
- Webster AC et al (2017) Chronic kidney disease. *Lancet* 389(10075):12381252
- Woods EL et al (2021) CD147 mediates the CD44s-dependent differentiation of myofibroblasts driven by transforming growth factor-beta1. *J Biol Chem* 297(3):100987
- Wynn TA (2003) IL-13 effector functions. *Annu Rev Immunol* 21:425–456
- Yang X et al (2012) An anti-let-7 sponge decoys and decays endogenous let-7 functions. *Cell Cycle* 11(16):3097–3108
- Zeisberg M, Shah AA, Kalluri R (2005) Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. *J Biol Chem* 280(9):8094–8100
- Zhan H et al (2020) Epidermal growth factor promotes stromal cells migration and invasion via up-regulation of hyaluronate synthase 2 and hyaluronan in endometriosis. *Fertil Steril* 114(4):888–898
- Zhang Y, Huang X, Yuan Y (2017) MicroRNA-410 promotes chondrogenic differentiation of human bone marrow mesenchymal stem cells through down-regulating Wnt3a. *Am J Transl Res* 9(1):136–145
- Zhuo L, Kimata K (2008) Structure and function of inter-alpha-trypsin inhibitor heavy chains. *Connect Tissue Res* 49(5):311–320

Chapter 6

Inter- α -inhibitor Proteins: A Review of Structure and Function



Stavros Garantziotis

Abstract Inter- α -inhibitor-family proteins (IAIP) are a multifaceted group of complex molecules with important roles in mammalian biology. In the past 20 years, new research has highlighted the central role of this protein family in homeostasis and disease pathogenesis. These insights suggest that IAIP may have significant utility as treatment targets in human disease, and therefore, research into IAIP should be intensified. This review summarizes the current knowledge on IAIP and highlights the remaining gaps in knowledge.

6.1 Introduction

Inter- α -inhibitor-family proteins (IAIPs), also referred to as Inter- α -trypsin inhibitors, is an extensively studied family of serum and extracellular matrix molecules that was identified over 100 years ago. IAIPs were originally described in the urine of patients with lung and kidney disease in 1909 and in healthy human urine in 1910. The trypsin inhibitory moiety (also known as urinary trypsin inhibitor [UTI] bikunin, and ulinastatin) was independently discovered in the 1950s, and IAIP was purified in serum in the 1960s, but the association of these two moieties was not evident until the late 1980s. Originally described in their capacity to bind and stabilize the extracellular matrix sugar hyaluronan (HA), IAIPs are now understood to exert manifold biological effects that place them at the center of reproductive biology, immunology, organ development, and cancer biology, just to name a few areas. Furthermore, there has recently been significant interest in the use of IAIP as a therapeutic in human disease. It is therefore important to understand IAIP biology and function in a translational context as well.

Several excellent review papers provide an introduction to IAIP (Salier et al. 1996; Blom et al. 1999; Fries and Blom 2000; Zhuo et al. 2004; Zhuo and Kimata 2008; Kobayashi 2006; Pugia et al. 2007; Lord et al. 2020). The present work will

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provide an update of the latest scientific insights into the IAIP family, the state of the art on IAIP molecular structure, and how it relates to their function and mechanism of action especially as it may affect inflammation. The document also points the reader to key developments supporting the promising role of IAIP for use in the treatment of human disease.

6.2 Molecular Structure of IAIP

Proteins of the Inter- α -inhibitor (IAIP) family were originally discovered in urine and serum due to their inhibitory activity against trypsin. The genomic and protein structures of the IAIP family molecules were largely elucidated in the 1980s and 1990s. The proteins of this family are composed of a common light chain and many closely related heavy chains (HCs). The light chain is a typical proteoglycan molecule with a single chondroitin 4-sulfate chain. The central protein, known as bikunin, contains two tandem Kunitz-type protease inhibition domains that contribute to the inhibitory activity of proteases of this family. A bikunin molecule is usually bound to 1 or 2 HCs, represented as a pre- α -trypsin inhibitor (P α I, HC3-bikunin in humans) and I α I [HC1-bikunin-HC2 in humans] via a unique ester bond between the carboxylic group of the C-terminal aspartic acid residue of the HC and the C-6 hydroxyl group of an internal N-acetyl galactosamine residue of chondroitin sulfate. This protein-glycosaminoglycan structure is typical of the IAIP family of proteins (Salier et al. 1996; Zhuo et al. 2004; Zhuo and Kimata 2008). P α I and I α I together, along with much lower concentrations of free bikunin and heavy chains, will be referred to as the IAIP in this work.

The multi-peptide structure of IAIP was initially masked by its unusual protein-glycosaminoglycan-protein structure in which the polypeptide subunits are covalently linked via a chondroitin sulfate chain (Enghild et al. 1991). In 1981, the UTI carbohydrates were identified as: (a) an O-linked, GalNAc-rich chain attached to serine at position 10; and (b) an N-linked oligosaccharide at position 45 (Hochstrasser et al. 1981). Chondroitinase and testicular hyaluronidase digestion of IAIP identified O-glycan as a low-sulfated chondroitin sulfate chain (Thuveson and Fries 1999; Heron et al. 1994) and separated HCs and bikunin (Enghild et al. 1989; Jessen et al. 1988). The binding of chondroitin sulfate to bikunin is a typical O-xylosylserine (Balduyck et al. 1986). Analyses also identified the new ester bond that binds the α -carboxyl of the C-terminal aspartates of HCs with the C-6 hydroxyl groups of GalNAc inside the chondroitin sulfate chain (Enghild et al. 1991; Enghild et al. 1993). The amino acid sequence of the chondroitin sulfate attachment site in bikunin, Glu-Gly-Ser-Gly, is well conserved among all the species examined. The chondroitin sulfate chain is relatively short (Mr approx. 8000), with 12–18 disaccharide repeats (GlcUA β 1,3-GalNAc β 1,4-) and a conventional binding region (GlcUA β 1–3Gal β 1–3-Gal_1-4Xyl β 1)-O-Ser (Toyoda et al. 1993; Enghild et al. 1999).

Approximately 30% of GalNAc, usually those close to the binding region, are sulfated to C-4 hydroxyl groups (Enghild et al. 1999). Chondroitin sulfate chains

synthesized during inflammation are longer and have less sulfation (Capon et al. 2003). Under the electron microscope, bikunin appears as a small sphere (diameter approx. 2 nm), whereas HCs have an N-terminal globule (diameter approx. 11 nm) with a thin tail (length approx. 15.5 nm) attached to the bikunin sphere (Blom et al. 1999). The two HCs of the human IAIP are located close to each other in the less sulfated region of the chondroitin sulfate chain (Enghild et al. 1999). The chondroitin sulfate chain and divalent cations appears to contribute to the stabilization of the IAIP molecules (Scavenius et al. 2016). Thus, the IAIPs are complex and intrinsically interactive proteins, both during their biosynthesis and during their life cycle, supporting the utility of full IAIP (as opposed to isolated HC or bikunin) in disease.

6.3 Heavy Chain Isoforms

The typical HC isoforms of circulating IAIP, i.e., HC 1–3, have been well characterized (Zhuo and Kimata 2008). These three isoforms resemble each other in many aspects and have a remarkably high sequence homology (40–60% amino acid sequence homology). They are all expressed in the liver and pair with bikunin before being released into the bloodstream. Under the electron microscope, they appear to have a granular head with a thin tail (Blom et al. 1999). The most obvious difference between the 3 HC isoforms is their selective combination with bikunin. For example, the predominant HC isoforms in human α 1 and α 2 are HC 1–2 and HC3, respectively. The mechanism underlying this selectivity is unclear. Furthermore, the selectivity appears to be species-dependent. Bovine α 1 comprises HC2 and HC3, whereas both human and bovine α 1 are found in the hamster (Castillo and Templeton 1993; Yamamoto et al. 1996). HC 1–3 prepropeptides (approximately 900 amino acid residues) comprise a signal peptide, a short propeptide, a mature form of HC and a C-terminal polypeptide (Chan et al. 1995). Signal peptides and propeptides are removed in the endoplasmic reticulum, probably by a Golgi enzyme in transit (Thuveson and Fries 1999). Large C-terminal polypeptides (240–280 amino acid residues) are released during the coupling of the mature form of HC with bikunin in the trans-Golgi network (Heron et al. 1994). The amino acid sequence, DPFHII, at the cleavage site, where D is the C-terminal amino acid residue of mature HCs, is well conserved in HCs 1–3. It is proposed that the coupling reaction comprises two coordinated steps: cleavage of the Asp-Pro bond followed by the formation of the ester bond (Zhuo and Kimata 2008).

Heavy chains contain a well-characterized von Willebrand factor A (vWA) domain, which contains a metal-ion-dependent adhesion site (MIDAS), a less-well characterized vault-protein domain, as well as (in HC2) a gamma-carboxyglutamic (Gla) domain (Zhuo et al. 2004). Several other HC isoforms exist, such as HC4 (encoded by ITIH4 (Jean et al. 1997)), HC5 (encoded by ITIH5 (Himmelfarb et al. 2004)) and other postulated or surmised chains. Given that different HC are expressed in different tissues, the question remains whether this organ specificity is simply a matter of genetic redundancy, or whether there are subtle differences in the activity of the HC that is tailored to a biological function in each organ or tissue. This will be explored further in this chapter.

6.4 Biosynthesis of IAIP

The primary synthetic site for circulating plasma IAIP is the liver, which is also the site of the highest IAIP expression (Salier et al. 1996; Zhuo et al. 2004). However, IAIP has been detected in several tissues, including the brain, lung, kidney, intestine, skin, muscle, cartilage, ovaries and the placenta and can be synthesized by cell types beyond hepatocytes (Kobayashi et al. 1999; Blom et al. 1997). The synthesis steps of IAIP have been largely identified (Zhuo et al. 2004). Bikunin is first synthesized in fusion with α 1-microglobulin (Lindqvist et al. 1992) and cleavage occurs in the trans-Golgi after the addition of the heavy chains (HC) of chondroitin sulfate and IAIP (Bratt et al. 1993). The connecting tetrapeptide (Arg-X1-X2-Arg) conforms to a consensus sequence recognized by the intracellular endo protease family that includes furin. However, furin does not appear to be responsible for cleavage (Bratt et al. 1994). HCs undergo several proteolytic steps during biosynthesis. The signal and subsequent N-terminal propeptides are removed in the endoplasmic reticulum, probably by a Golgi enzyme in transit (Thuveson and Fries 1999; Thogersen and Enghild 1995). The large C-terminal extension (240–280 amino acid residues starting with the conserved sequence Pro-His-Phe-Ile-Ile) is released when the rest of the HC is coupled to the chondroitin sulfate. Therefore, the assembly of an HC involves two likely coordinated steps: a cleavage of the Asp-Pro bond and then the formation of the ester bond (Thogersen and Enghild 1995). The C-terminal extension seems to be required for the formation of the ester linkage (Thuveson and Fries 1999). No protease with specificity toward the conserved sequence around the cleavage site has been described. The majority of IAIP in serum is found in two forms: $\text{I}\alpha\text{I}$ and $\text{P}\alpha\text{I}$. The main difference among these is the HC composition and the stoichiometry between HC and bikunin. In humans, $\text{I}\alpha\text{I}$ (220–250 kDa) consists of HC1–2 in a ratio 2:1 to bikunin (2 HC and 1 bikunin molecule), while $\text{P}\alpha\text{I}$ (125 kDa) consists of HC3 in 1:1 ratio with bikunin. In human plasma, the majority of IAIP is in the form of $\text{I}\alpha\text{I}$ (Enghild et al. 1989). In humans, the concentration of $\text{P}\alpha\text{I}$ is about a third of that of $\text{I}\alpha\text{I}$, whereas $\text{P}\alpha\text{I}$ is more abundant than $\text{I}\alpha\text{I}$ in rodent plasma (Odum 1990).

6.5 Regulation of IAIP Biosynthesis

There is limited information about the genetic regulation of IAIP expression. Liver-specific expression of bikunin is especially promoted by a cluster of hepatic nuclear factors (HNF1–4) that significantly enhance a weak global promoter activity (Rouet et al. 1992; Rouet et al. 1995). *ITIH1–3* genes are products of duplication, which suggests some similarity in gene structure and regulation. The *ITIH2* gene has been mapped to 10p 14–15, whereas *ITIH1* and *ITIH3* map to 3p211-p212; in particular, *ITIH1* and *ITIH3* genes are found in tandem (Diarra-Mehrpour et al. 1998; Diarra-Mehrpour et al. 1994) and are very similar in their structure. *ITIH1* and *ITIH3*

promoter regions contain binding sites for p53, the Ets-like element family (Ets-1, PuF, PEA3) AP1 site, AP2, NF- κ B, serum response element, and PPAR (Diarra-Mehrpour et al. 1998). The transcription factors Sp1, C/EBP α and C/EBP β promote, while a tripartite complex of Creb2/GKLF suppresses ITIH3 expression (Diarra-Mehrpour et al. 1998; Ruminy et al. 2003). ITIH3 is also responsive to sonic hedgehog elements (Gli1–2) at least in brain cells (Kato et al. 2001). The response to inflammation is complex. In hepatic HepG2 cells, IL-6 exposure has an inverse U-shape effect on ITIH gene expression: low- and intermediate-dose IL-6 leads to upregulation of both ITIH2 and ITIH3, while very high IL-6 levels lead to a tapering off of this induction, which in the case of ITIH2 suppresses expression below naive levels, while ITIH3 expression remains upregulated (compared to naive cells) at all IL-6 doses, although the induction is less at high IL-6 doses (Sarafan et al. 1995). In conclusion, IAIP expression appears to be regulated by inflammation and injury-related factors (p53, NF- κ B, PPAR, IL-6), which in most cases lead to induction of expression. In naïve organisms, expression is mostly confined to the liver, where the expression is driven by tissue-specific enhancers.

6.6 Metabolism and Excretion of IAIP

Investigation on the metabolism of IAIP in mice, using 125 I labeled α I, found a multiphasic clearance of the inhibitor that was not due to degradation or aggregation but was likely the result of extravascular distribution and endothelial binding (Pratt and Pizzo 1986). Studies of the plasma clearance of 125 I-labeled rat bikunin support that bikunin is cleared by the kidneys via glomerular filtration while a small portion of the bikunin was taken up in the liver, partially degraded and released into the bile. In the same set of studies, some labeled bikunin was found in the subcutaneous connective tissue (Potempa et al. 1989).

In the late 1990s, it was established that human neutrophil elastase degrades IAIP *in vitro* to liberate bikunin (or urinary trypsin inhibitor (UTI)) (Hirose et al. 1998). During inflammation, circulating IAIP (which is very sensitive to numerous proteases such as elastase and cathepsin G released by stimulated neutrophils) is degraded into lower molecular weight derivatives that are present in plasma, among which UTI which is excreted in urine (Balduyck and Mizon 1991). Because IAIPs are very sensitive to proteolysis by neutrophils that are stimulated during sepsis, it was suggested that IAIP plasma levels would be a useful marker for neutrophil proteinase activity and that the IAIP-related components characterized from plasma of the sepsis patients arise from proteolytic cleavage (Balduyck et al. 2000). Indeed, consumption of IAIP seems to be a hallmark of severe sepsis and is directly proportional to mortality in sepsis (Baek et al. 2003; Lim et al. 2003; Opal et al. 2007; Chaaban et al. 2009).

The fate of IAIP in homeostasis and as a result of IAIP role in matrix stabilization is well described. Circulating IAIP is transferred into the matrix, either because of vascular leakage during inflammation, or in the physiological process of tissue

maturation and development (e.g., in the developing cumulus oophorus) (Zhuo et al. 2004; Zhuo and Kimata 2008; Zhuo and Kimata 2001). In the matrix, IAIP heavy chains interact with pentraxin-3, and the full IAIP molecule interacts with TNF-stimulated gene-6 (TSG-6), which catalyzes a transesterification reaction whereby first one, and then the other HC is transferred onto the hyaluronan molecule. ITIH2 is apparently necessary for this reaction (Sanggaard et al. 2010a; Sanggaard et al. 2008). In this process, HC are transferred from the IAIP molecule onto a hyaluronan-pentraxin matrix (Baranova et al. 2014) and the bikunin moiety is released into the tissue, from thence into the circulation, where it is rapidly eliminated through renal filtration with a half-life time of 4–30 minutes. HC or IAIP are not found in the urine in non-nephropathic patients, because their large MW makes renal filtration almost impossible. It is very likely, that IAIP HCs play an important role not only in hyaluronan cross-stabilization but in the generation of a scaffold, interlaced with other proteins and glycoproteins such as vitronectin or aggrecan, which promotes cellular growth and resilience to injury (Adair et al. 2009; Pijuan-Galito et al. 2014; Pijuan-Galito et al. 2016).

In inflammation, the process of IAIP metabolism is enhanced, but the degree of this increase is not clearly described. IAIP is subject to increased extravasation into tissues, as a result of exacerbated vascular permeability during inflammation; furthermore, extracellular proteases induce IAIP degradation (Mizon et al. 2002) leading to bikunin release. It is likely that these mechanisms all contribute to promoting the tissue-protective effects of IAIP in injury; however, they also lead to a significant decline in circulating IAIP levels.

Recently, other IAIP interactions have been described, e.g., with complement (Garantziotis et al. 2007; Okroj et al. 2012), coagulation factors (Scavenius et al. 2011; Winkler et al. 2013), heparin-AT III complexes (McKay and Laurell 1980), and histones (Pemberton et al. 2010; Chaaban et al. 2015). These interactions are likely to lead to further intravascular consumption and degradation of IAIP in disease, but nothing is currently known about the extent or rate of this process.

6.7 IAIP Function

IAIP function is dictated by the biological actions of IAIP molecules (heavy chains, bikunin) and the tissue localization which determines IAIP molecular partners. IAIPs were initially studied extensively as protease inhibitors. Bikunin shows modest inhibitory activity against proteases, including trypsin, chymotrypsin, neutrophil elastase, and plasmin (Potempa et al. 1989). Although circulating levels of IAIP are high (200–800 µg/ml), they account for only about 5% of the total protease inhibitory activity in plasma. However, bikunin inhibits plasmin on the surface of tumor cells more efficiently than plasmin in solution (see below), unlike α 2-macroglobulin and the α 1-trypsin inhibitor, which could be related to the antimetastatic effect of bikunin (Kobayashi et al. 1995; Kobayashi et al. 1994; Kobayashi et al. 2003). Furthermore, bikunin may also inhibit granzyme K, the serine protease stored

in lymphocyte granules, which is involved in cytotoxic defense mediated by T cells and natural killer cells after recognition of target cells (Wilharm et al. 1999). Bikunin also has non-protease-inhibitory effects: it inhibits LPS-induced increase of cytoplasmic free Ca^{++} in immune and structural cells (Kanayama et al. 1997) and protects epithelia from injury after IL-1 β and TNF- α exposure (el Maradny et al. 1994) through a Ca^{++} -inhibitory effect. Ca^{++} inhibition was seen in several cell types (Kanayama et al. 1997; Kanayama et al. 1995; Kanayama et al. 1998) suggesting that this is likely a class effect on Ca^{++} channels. Bikunin also inhibits the activation of neutrophils after LPS exposure, by reducing the activity of ERK1/2, p38 (Kanayama et al. 2007).

Other various activities, in particular pharmaceutical effects, of bikunin have been described, such as mitogenic activity, inhibitory activity against hyaluronidase, prevention of premature birth, ischemic stress and renal failure, and suppression of pancreatitis, colitis, and arthritis, for which various mechanisms have been proposed (Fries and Blom 2000). These studies have focused on the bikunin portion of the IAIP family molecules. However, over 90% of circulating bikunin contains HC, whereas urinary bikunin is predominantly free-HC. Furthermore, the plasma half-life of radiolabeled bikunin injected into rats, mice, and humans is between 4 and 30 min (Sugiki et al. 1989; Jonsson-Berling and Ohlsson 1991; Sjoberg et al. 1995). In contrast, IAIP remains detectable for 10 hours when injected into mice (Zhuo et al. 2001). Thus, the full IAIP molecule has several additional functions, as well as expanded activity time compared to bikunin. It is still unclear whether the above-described bikunin effects only happen after the dissociation of bikunin/chondroitin sulfate from the heavy chains, or whether the mature IAIP can have similar functions.

Recently, biochemical and structural studies have begun to shed light on the specific functions of heavy chains. For example, heavy chain (HC) 1 was found to have an integrin-like 3D structure which may enable it to bind extracellular proteins (Briggs et al. 2020). In addition, heavy chains appear to differ in their exact functions. For example, HC1 and HC3 cannot form covalent complexes with TSG-6, while HC2 is able to do so (Sanggaard et al. 2008). These findings suggest that there are subtle structural and functional differences between IAIP heavy chains, which may reflect their specific expression in different tissues, and that IAIP heavy chains should be studied separately and not assumed to be identical.

6.8 Interactions of IAIP and its Components with Extracellular Matrix Components

In the ovarian cumulus oophorus, hyaluronan is organized into a network cross-linked by the cooperative action of IAIP, pentraxin-3, and TNF-stimulated gene-6 (TSG-6), which drives cumulus expansion and provides the cumulus matrix with the necessary viscoelastic properties. Stabilization of the matrix involves TSG-6-

mediated transfer of the heavy chains (HC) of IAIP onto hyaluronan (to form HC-HA covalent complexes that are cross-linked by pentraxin-3) and that this occurs through the formation of HC-TSG-6 covalent intermediates (Sanggaard et al. 2010a; Sanggaard et al. 2008; Sanggaard et al. 2010b; Colon et al. 2009; Milner et al. 2007). Thus, female mice that are deficient in IAIP, TSG-6, or pentraxin-3 are all infertile due to absent cumulus oophorus maturation (Zhuo et al. 2001; Fulop et al. 2003; Salustri et al. 2004). The tertiary structure of the human TSG-6, and heavy chains, and the transfer mechanism of HC from the IAIP to hyaluronan have been studied in significant detail. TSG-6 contains a calcium ion binding site and a chelating glutamic acid residue that mediates the formation of HC-TSG-6. This occurs through an initial non-covalent, metal ion-dependent interaction between TSG-6 and HCs that also requires the presence of a magnesium ion associated with the HCs (Briggs et al. 2015). Chondroitin sulfate containing 4-sulfated linkage region structures and disulfate disaccharides on IAIP are involved in the HC transfer (Lord et al. 2013). IAIP heavy chains are crosslinked through pentraxin-3 (Salustri et al. 2004). Interestingly, it appears that IAIP and pentraxin-3 may need to be pre-linked, before IAIP encounters TSG-6, for the hyaluronan crosslinking to be successful (Baranova et al. 2014). This TSG-6/hyaluronan independent interaction of IAIP is interesting, since pentraxin-3 has a role in lung injury repair (Doni et al. 2015), antimicrobial lung defense (Cunha et al. 2014; Jaillon et al. 2013), and allograft survival in lung transplantation (Diamond et al. 2012). Thus, IAIP is connected to a matrix-based process of tissue repair and regeneration after injury. Hyaluronan-IAIP rafts were detected in the apical surface of airway epithelia and are thought to contribute to epithelial defense by trapping and localizing immune cells on the luminal size (Abbadi et al. 2016).

6.9 Interactions of IAIP and its Components with Coagulation Factors and Complement

IAIP was found in considerable amounts in euglobulin fractions of human plasma since the 1970s (Kluft 1976). Prothrombin complex concentrate contains 15-fold enriched IAIP (Steinbuch et al. 1984), and the fibrin clot contains significant amounts of IAIP (Scavenius et al. 2011). IAIP is a substrate for factor XIIIa and tissue transglutaminase and is therefore incorporated to the fibrin clot by cross-linking to fibrinogen (Scavenius et al. 2011). Teleologically this would suggest a role of IAIP in the regulation of the coagulation cascade (Scavenius et al. 2011). IAIP binds strongly to coagulation factor IX (Winkler et al. 2013). Importantly, factor IX and its activator, factor XI play an important role in vascular thrombosis (Renne et al. 2005), cerebral ischemia (Choudhri et al. 1999), and disseminated intravascular coagulation (DIC) in sepsis (Tucker et al. 2008). The IAIP light chain, bikunin, is a strong factor XI inhibitor (in addition to inhibiting plasmin, kallikrein, factor VIIa-tissue factor, factor IX, factor X, and factor XII (Delaria et al. 1997)). Bikunin

uniquely inhibits both plasma, as well as cell-bound plasmin (Kobayashi et al. 1994), supporting a role of IAIP both intravascularly as well as in tissue injury. The observed activity of IAIP as a coagulation factor inhibitor supports its role in sepsis, ARDS, and cerebral ischemia. Notably, IAIP binding to factor IX was tight enough that it could not be removed through nanofiltration in varying ionic strengths, as opposed to (for example) complement C4 (Winkler et al. 2013), and the incorporation of IAIP into the fibrin clot was through covalent bonds (Scavenius et al. 2011); this suggests that free intravascular IAIP may be decreased in DIC and other conditions with activated coagulation, due to complex formation with coagulation factors, even though total plasma levels may appear unaffected.

The first study to clearly suggest that IAIP may inhibit complement activation and thus prevent complement-induced injury was published in 2007 (Garantziotis et al. 2007). Subsequently, purified human IAIP was found to inhibit classical, lectin, and alternative complement pathways *in vitro* when added in excess to human serum. The inhibitory activity was dependent on heavy chains, but not bikunin, was detected at the level of initiator molecules (MBL, properdin) in the lectin/alternative pathways or at the level of C4b in the classical pathway (Okroj et al. 2012) and complement binding was shown to be mediated via a MIDAS (Briggs et al. 2020). Furthermore, IAIP influenced the formation and assembly of the C1 complex and prevented the assembly of the C3-convertase of the classical pathway. Interactions with the extracellular matrix did not affect IAIP's ability to inhibit complement, thus implicating IAIP as a potentially important complement inhibitor once enriched in the matrix during local inflammatory processes. In support of this, IAIP were shown to inhibit complement-mediated endothelial injury in sepsis (Stober et al. 2019), and a correlation was found between IAIP-containing proteins and hemolytic activity in the synovial fluid of rheumatoid arthritis patients (Okroj et al. 2012).

6.10 Interactions of IAIP and its Components with Danger-Associated Molecular Patterns and Pathogen-Associated Molecular Patterns

IAIP can be viewed as part of the innate immune system which interacts with either endogenous ("danger-associated") or exogenous ("pathogen-associated") molecular patterns and mediates immune responses to these. Dengue infection leads to severe IAIP deficiency, which is predictive of disease severity (Koraka et al. 2010). A domain from the envelope glycoprotein of dengue virus has been shown to bind to several IAIP components (Huerta et al. 2016). The binding was putatively attributed to the ITIH heavy chain gamma-carboxyglutamic (Gla) domain, which is known to also bind other viruses such as adenovirus and HIV (Parker et al. 2006; Munch et al. 2007). Among the danger-associated molecular patterns, High-mobility group box-1 was recently found to bind to IAIP after ischemic brain injury (Hatayama et al. 2021). Furthermore, shotgun proteomic analysis of histone-spiked plasma showed

that histones bind to IAIP (Pemberton et al. 2010). Another study confirmed that IAIPs bind extracellular histones and found that this binding served to neutralize histone-induced adverse effects (Chaaban et al. 2015). Thus, IAIP may be sequestered in plasma by circulating infectious and danger-associated molecular patterns, although measured IAIP levels may appear unaffected. Given that this area of research is still in its early stages, it is to be predicted that additional interactions of IAIP will be uncovered in the future.

6.11 Effects of IAIP and its Components on Differentiated Cell and Stem Cell Growth

I α I (specifically its HC2 domain) activates the Yes/Yes-associated protein (YAP)/TEAD transcription factor pathway and induces expression of Oct4 and Nanog in mouse ES (mES) cells (Pijuan-Galito et al. 2014). Hyaluronan and integrins (particularly ones containing RGD domain) are involved in IAIP-mediated stem cell attachment (Pijuan-Galito et al. 2016). Laminin and vitronectin binding probably mediate this IAIP effect (Pijuan-Galito et al. 2016). The interaction of IAIP with vitronectin via the RGD-binding domain was shown earlier (Adair et al. 2009). Furthermore, the creation of IAIP-hyaluronan microenvironment has been shown to support the transplantation and propagation of mesenchymal stem cells (Torihashi et al. 2015). In another paper, an IAIP-hyaluronan-pentraxin-3 matrix was shown to support ex vivo expansion of stem cells (Tseng 2016). The complexes of IAIP heavy chains with hyaluronan, that support cell growth and inhibit apoptosis of differentiated cells have been characterized in detail (He et al. 2009).

6.12 Associations of IAIP Genetics and Biology with Disease

There is limited information on the genetic associations of IAIP proteins with disease susceptibility. An ITIH3 polymorphism has been strongly associated with bipolar disorder and schizophrenia (Scott et al. 2009; Hamshere et al. 2013; Psychiatric 2011; Schizophrenia Psychiatric Genome-Wide Association Study C 2011; Witt et al. 2014), and the same polymorphism in addition to an SNP in the ITIH1 area (that is in linkage disequilibrium with the ITIH3 SNP) may have pharmacogenomic effects on the response to antipsychotic medications (Brandl et al. 2016; Eum et al. 2021). ITIH3 has also been genetically linked to autism spectrum disorder (Xie et al. 2020). A separate study found that bikunin- (but not ITIH4-) deficient mice demonstrated increased anxiety behavior and fear conditioning responses on behavioral testing (Goulding et al. 2019), suggesting that the genetic associations have a biological underpinning. ITIH2 was associated in proteomic and genetic analysis with different types of dementia (Katzeff et al. 2020; Tang et al. 2020). Recent work

from the Stonestreet lab has shown significant effects of IAIP in ameliorating ischemic injury in the developing and adult brain (Hatayama et al. 2021; Bradford et al. 2020; Schuffels et al. 2020; Chen et al. 2019; Threlkeld et al. 2017). Collectively, these data support an important role of IAIP in brain development, homeostasis, and injury response, which is only now being uncovered.

IAIP heavy chain genes are downregulated in multiple human solid tumors, and thus an anti-metastatic effect of IAIP has been postulated (Hamm et al. 2008), and in some instances described (Werbowski-Ogilvie et al. 2006) but the exact mechanism remains elusive. Serum IAIP levels have been associated with female genital cancers (Obayashi et al. 2008; Yabushita et al. 2011) and ITIH2 expression with lung cancer (Wu et al. 2013). ITIH3 has been described as an early biomarker of gastric cancer in Asian populations (Chong et al. 2010; Uen et al. 2013).

There is a notable trend in recent years for proteomic analyses to identify IAIP as biomarkers of disease activity, mostly in inflammatory diseases (or diseases with an inflammatory component). For example, circulating ITIH1 was associated with osteoarthritis (Lourido et al. 2017). ITIH3 was significantly hypomethylated in an epigenome-wide analysis of CD4+ cells in patients with rheumatoid arthritis (Guo et al. 2016) and ITIH3 levels (as well as anti-ITIH3 antibodies) were identified as markers of rheumatoid arthritis and secondary Sjogren's syndrome (Liao et al. 2016). The identification of IAIP as biomarkers in inflammatory disease suggests that their role may be more than just sentinels, but rather may be involved in the pathogenesis of chronic inflammation.

There is also evidence for the involvement of IAIP in metabolic disease. ITIH1 overexpression promotes insulin resistance (Kim et al. 2019) while ITIH2 was increased in the extracellular vesicles of patients with glucose intolerance (Masi et al. 2021). In a proteomic analysis, ITIH1, ITI2, and ITI3 (notably, along with hyaluronan receptor CD44) were identified as bound to Lipoprotein(a), which consists of low-density lipoprotein bound to apolipoprotein(a) and is one of the strongest risk factors for atherosclerotic cardiovascular disease (Bourgeois et al. 2021). A functional ITIH3 SNP was associated with susceptibility to myocardial infarction in a Japanese population (Ebana et al. 2007).

More recently, IAIP proteins have been identified as having an association with COVID-19 outcomes (Völlmy et al. 2021).

To further investigate the physiological functions of IAIP in vivo, bikunin-deficient mice were generated by gene targeting (Zhuo et al. 2001). The biosynthesis of mature heavy chain proteins of the IAIP family is eliminated in these mice as well since bikunin is essential for this process. While these mice show no obvious abnormalities and appear healthy on gross inspection, they do display several pathological features when examined in more detail. Females display a severe reduction in fertility (Zhuo et al. 2001) because IAIP heavy chains that bind to hyaluronan, contribute to the microenvironment in which ovulation takes place (Zhuo et al. 2001). Furthermore, *bik*^{-/-} mice had higher lethality after systemic LPS administration compared with wild-type mice, which was reduced by the administration of exogenous bikunin, suggesting involvement of IAIP in the progression and pathogenesis of systemic inflammation and endotoxic shock

(Wakahara et al. 2005). IAIP is generally involved in the pathophysiology of inflammatory diseases, including rheumatoid arthritis and airway allergy: for example, deficiency in the IAIP-hyaluronan complex in *bik*^{-/-} mice enhances airway hyperresponsiveness in a murine model of asthma (Zhu et al. 2010). Furthermore, in an experimental naphthalene lung injury model in *bik*^{-/-} mice, a significant loss of columnar epithelium and abnormal nuclei in the large and small airways were found, whereas wild-type mice had an orderly epithelial cell pattern suggesting IAIP promotes bronchial epithelial repair after injury mediated at least in part through vitronectin binding (Adair et al. 2009). More recently, it has been reported that enhanced spontaneous cancer metastasis was found in *bik*^{-/-} mice, and administration of exogenous bikunin caused a significant reduction of lung metastasis in these IAIP-deficient mice (Chen et al. 2019). More studies using these mice, and newer developed models with specific IAIP heavy chain deficiency, will be necessary to understand the mechanism of IAP action in disease.

6.13 Conclusions and Future Directions

IAIPs are a family of related but not identical proteins with manifold functions and interactions that we are only now beginning to understand. Much more remains to be elucidated: the full extent of IAIP protein structure (particularly the heavy chains) needs to be uncovered, and the function of heavy chain protein domains elucidated; the role of the chondroitin sulfate backbone and modifications thereof remains to be understood; we do not yet know the full spectrum of molecule classes (exogenous or endogenous) that IAIPs interact with; we do not understand the effects of IAIP in solution (e.g., in plasma), in transient matrices (e.g., in fibrin clots or immature fibrotic loci) and in mature extracellular matrices (e.g., fibrotic tissue or healthy connective tissue); we also do not know whether IAIPs play a role intracellularly, as well they may. However, an abundance of associations has been published, which leads us to expect that IAIPs play a significant role in health maintenance and disease pathogenesis. Thus, it will be imperative to continue and intensify the research efforts into the function and role of IAIP in biology.

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References

- Abbadi A, Lauer M, Swaidani S, Wang A, Hascall V (2016) Hyaluronan rafts on airway epithelial cells. *J Biol Chem* 291(3):1448–1455
- Adair JE, Stober V, Sobhany M, Zhuo L, Roberts JD, Negishi M et al (2009) Inter-alpha-trypsin inhibitor promotes bronchial epithelial repair after injury through vitronectin binding. *J Biol Chem* 284(25):16922–16930

- Baek YW, Brokat S, Padbury JF, Pinar H, Hixson DC, Lim YP (2003) Inter-alpha inhibitor proteins in infants and decreased levels in neonatal sepsis. *J Pediatr* 143(1):11–15
- Balduyck M, Albani D, Jourdain M, Mizon C, Tournois A, Drobecq H et al (2000) Inflammation-induced systemic proteolysis of inter-alpha-inhibitor in plasma from patients with sepsis. *J Lab Clin Med* 135(2):188–198
- Balduyck M, Mizon C, Loutfi H, Richet C, Roussel P, Mizon J (1986) The major human urinary trypsin inhibitor is a proteoglycan. *Eur J Biochem / FEBS*. 158(2):417–422
- Balduyck M, Mizon J (1991) Inter-alpha-trypsin inhibitor and its plasma and urine derivatives. *Ann Biol Clin (Paris)* 49(5):273–281
- Baranova NS, Inforzato A, Briggs DC, Tilakaratna V, Enghild JJ, Thakar D et al (2014) Incorporation of pentraxin 3 into hyaluronan matrices is tightly regulated and promotes matrix cross-linking. *J Biol Chem* 289(44):30481–30498
- Blom AM, Morgelin M, Oyen M, Jarvet J, Fries E (1999) Structural characterization of inter-alpha-inhibitor. Evidence for an extended shape. *J Biol Chem* 274(1):298–304
- Blom AM, Thuveson M, Fries E (1997) Intracellular coupling of bikunin and the heavy chain of rat pre-alpha-inhibitor in COS-1 cells. *Biochem J* 328(Pt 1):185–191
- Bourgeois R, Girard A, Perrot N, Guertin J, Mitchell PL, Couture C et al (2021) A comparative analysis of the lipoprotein(a) and low-density lipoprotein proteomic profiles combining mass spectrometry and Mendelian randomization. *CJC Open* 3(4):450–459
- Bradford A, Hernandez M, Kearney E, Theriault L, Lim YP, Stonestreet BS et al (2020) Effects of juvenile or adolescent working memory experience and Inter-alpha inhibitor protein treatment after neonatal hypoxia-ischemia. *Brain Sci* 10(12)
- Brandl EJ, Lett TA, Chowdhury NI, Tiwari AK, Bakanidze G, Meltzer HY et al (2016) The role of the ITIH3 rs2535629 variant in antipsychotic response. *Schizophr Res* 176(2–3):131–135
- Bratt T, Cedervall T, Akerstrom B (1994) Processing and secretion of rat alpha 1-microglobulin-bikunin expressed in eukaryotic cell lines. *FEBS Lett* 354(1):57–61
- Bratt T, Olsson H, Sjoberg EM, Jergil B, Akerstrom B (1993) Cleavage of the alpha 1-microglobulin-bikunin precursor is localized to the Golgi apparatus of rat liver cells. *Biochim Biophys Acta* 1157(2):147–154
- Briggs DC, Birchenough HL, Ali T, Rugg MS, Waltho JP, Ievoli E et al (2015) Metal ion-dependent heavy chain transfer activity of TSG-6 mediates assembly of the cumulus-oocyte matrix. *J Biol Chem* 290(48):28708–28723
- Briggs DC, Langford-Smith AW, Birchenough HL, Jowitt TA, Kielty CM, Enghild JJ, Baldock C, Milner CM, Day AJ (2020) Inter- α -inhibitor heavy chain-1 has an integrin-like 3D structure mediating immune regulatory activities and matrix stabilization during ovulation. *J Biol Chem* 295(16):5278–5291
- Capon C, Mizon C, Lemoine J, Rodie-Talbere P, Mizon J (2003) In acute inflammation, the chondroitin-4 sulphate carried by bikunin is not only longer, it is also undersulphated. *Biochimie* 85(1–2):101–107
- Castillo GM, Templeton DM (1993) Subunit structure of bovine ESF (extracellular-matrix stabilizing factor(s)). A chondroitin sulfate proteoglycan with homology to human I alpha i (inter-alpha-trypsin inhibitors). *FEBS Lett* 318(3):292–296
- Chaaban H, Keshari RS, Silasi-Mansat R, Popescu NI, Mehta-D'Souza P, Lim YP et al (2015) Inter-alpha inhibitor protein and its associated glycosaminoglycans protect against histone-induced injury. *Blood* 125:2286
- Chaaban H, Singh K, Huang J, Siryaporn E, Lim YP, Padbury JF (2009) The role of inter-alpha inhibitor proteins in the diagnosis of neonatal sepsis. *J Pediatr* 154(4):620–2 e1
- Chan P, Risler JL, Raguenes G, Salier JP (1995) The three heavy-chain precursors for the inter-alpha-inhibitor family in mouse: new members of the multicopper oxidase protein group with differential transcription in liver and brain. *Biochem J* 306(Pt 2):505–512
- Chen X, Nakada S, Donahue JE, Chen RH, Tucker R, Qiu J et al (2019) Neuroprotective effects of inter-alpha inhibitor proteins after hypoxic-ischemic brain injury in neonatal rats. *Exp Neurol* 317:244–259

- Chong PK, Lee H, Zhou J, Liu SC, Loh MC, Wang TT et al (2010) ITIH3 is a potential biomarker for early detection of gastric cancer. *J Proteome Res* 9(7):3671–3679
- Choudhri TF, Hoh BL, Prestigiacomo CJ, Huang J, Kim LJ, Schmidt AM et al (1999) Targeted inhibition of intrinsic coagulation limits cerebral injury in stroke without increasing intracerebral hemorrhage. *J Exp Med* 190(1):91–99
- Colon E, Shytuhina A, Cowman MK, Band PA, Sanggaard KW, Enghild JJ et al (2009) Transfer of inter-alpha-inhibitor heavy chains to hyaluronan by surface-linked hyaluronan-TSG-6 complexes. *J Biol Chem* 284(4):2320–2331
- Cunha C, Aversa F, Lacerda JF, Busca A, Kurzai O, Grube M et al (2014) Genetic PTX3 deficiency and aspergillosis in stem-cell transplantation. *N Engl J Med* 370(5):421–432
- Delaria KA, Muller DK, Marlor CW, Brown JE, Das RC, Rocznik SO et al (1997) Characterization of placental bikunin, a novel human serine protease inhibitor. *J Biol Chem* 272(18):12209–12214
- Diamond JM, Meyer NJ, Feng R, Rushefski M, Lederer DJ, Kawut SM et al (2012) Variation in PTX3 is associated with primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 186(6):546–552
- Diarra-Mehrpour M, Bourguignon J, Sarafan N, Bost F, Sesboue R, Muschio-Bonnet F et al (1994) Tandem orientation of the inter-alpha-trypsin inhibitor heavy chain H1 and H3 genes. *Biochim Biophys Acta* 1219(2):551–554
- Diarra-Mehrpour M, Sarafan N, Bourguignon J, Bonnet F, Bost F, Martin JP (1998) Human inter-alpha-trypsin inhibitor heavy chain H3 gene. Genomic organization, promoter analysis, and gene linkage. *J Biol Chem* 273(41):26809–26819
- Doni A, Musso T, Morone D, Bastone A, Zambelli V, Sironi M et al (2015) An acidic microenvironment sets the humoral pattern recognition molecule PTX3 in a tissue repair mode. *J Exp Med* 212(6):905–925
- Ebana Y, Ozaki K, Inoue K, Sato H, Iida A, Lwin H et al (2007) A functional SNP in ITIH3 is associated with susceptibility to myocardial infarction. *J Hum Genet* 52(3):220–229
- Enghild JJ, Salvesen G, Hefta SA, Thøgersen IB, Rutherford S, Pizzo SV (1991) Chondroitin 4-sulfate covalently cross-links the chains of the human blood protein pre-alpha-inhibitor. *J Biol Chem* 266(2):747–751
- Enghild JJ, Salvesen G, Thøgersen IB, Valnickova Z, Pizzo SV, Hefta SA (1993) Presence of the protein-glycosaminoglycan-protein covalent cross-link in the inter-alpha-inhibitor-related proteinase inhibitor heavy chain 2/bikunin. *J Biol Chem* 268(12):8711–8716
- Enghild JJ, Thøgersen IB, Cheng F, Fransson LA, Roepstorff P, Rahbek-Nielsen H (1999) Organization of the inter-alpha-inhibitor heavy chains on the chondroitin sulfate originating from Ser(10) of bikunin: posttranslational modification of IalphaI-derived bikunin. *Biochemistry* 38(36):11804–11813
- Enghild JJ, Thøgersen IB, Pizzo SV, Salvesen G (1989) Analysis of inter-alpha-trypsin inhibitor and a novel trypsin inhibitor, pre-alpha-trypsin inhibitor, from human plasma. Polypeptide chain stoichiometry and assembly by glycan. *J Biol Chem* 264(27):15975–15981
- Eum S, Hill SK, Alliey-Rodriguez N, Stevenson JM, Rubin LH, Lee AM et al (2021) Genome-wide association study accounting for anticholinergic burden to examine cognitive dysfunction in psychotic disorders. *Neuropsychopharmacology* 46:1802
- Fries E, Blom AM (2000) Bikunin—not just a plasma proteinase inhibitor. *Int J Biochem Cell Biol* 32(2):125–137
- Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS et al (2003) Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 130(10):2253–2261
- Garantziotis S, Hollingsworth JW, Ghanayem RB, Timberlake S, Zhuo L, Kimata K et al (2007) Inter-alpha-trypsin inhibitor attenuates complement activation and complement-induced lung injury. *J Immunol* 179(6):4187–4192

- Goulding DR, Nikolova VD, Mishra L, Zhuo L, Kimata K, McBride SJ et al (2019) Inter-alpha-inhibitor deficiency in the mouse is associated with alterations in anxiety-like behavior, exploration and social approach. *Genes Brain Behav* 18(1):e12505
- Guo S, Zhu Q, Jiang T, Wang R, Shen Y, Zhu X et al (2016) Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis. *Modern rheumatology / The Japan Rheumatism Association* 1-7
- Hamm A, Veeck J, Bektas N, Wild PJ, Hartmann A, Heindrichs U et al (2008) Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. *BMC Cancer* 8:25
- Hamshere ML, Walters JT, Smith R, Richards AL, Green E, Grozeva D et al (2013) Genome-wide significant associations in schizophrenia to ITIH3/4, CACNA1C and SDCCAG8, and extensive replication of associations reported by the schizophrenia PGC. *Mol Psychiatry* 18(6):708–712
- Hatayama K, Chen RH, Hanson J, Teshigawara K, Qiu J, Santoso A et al (2021) High-mobility group box-1 and inter-alpha inhibitor proteins: in vitro binding and co-localization in cerebral cortex after hypoxic-ischemic injury. *FASEB J* 35(3):e21399
- He H, Li W, Tseng DY, Zhang S, Chen SY, Day AJ et al (2009) Biochemical characterization and function of complexes formed by hyaluronan and the heavy chains of inter-alpha-inhibitor (HC*HA) purified from extracts of human amniotic membrane. *J Biol Chem* 284(30):20136–20146
- Heron A, Bourguignon J, Calle A, Borghi H, Sesboue R, Diarra-Mehrpour M et al (1994) Post-translational processing of the inter-alpha-trypsin inhibitor in the human hepatoma HepG2 cell line. *Biochem J* 302(Pt 2):573–580
- Himmelfarb M, Klopocki E, Grube S, Staub E, Klamann I, Hinzmann B et al (2004) ITIH5, a novel member of the inter-alpha-trypsin inhibitor heavy chain family is downregulated in breast cancer. *Cancer Lett* 204(1):69–77
- Hirose J, Ozawa T, Miura T, Isaji M, Nagao Y, Yamashiro K et al (1998) Human neutrophil elastase degrades inter-alpha-trypsin inhibitor to liberate urinary trypsin inhibitor related proteins. *Biol Pharm Bull* 21(7):651–656
- Hochstrasser K, Schonberger OL, Rossmanith I, Wachter E (1981) Kunitz-type proteinase inhibitors derived by limited proteolysis of the inter-alpha-trypsin inhibitor, V. attachments of carbohydrates in the human urinary trypsin inhibitor isolated by affinity chromatography. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* 362(10):1357–1362
- Huerta V, Ramos Y, Yero A, Pupo D, Martin D, Toledo P et al (2016) Novel interactions of domain III from the envelope glycoprotein of dengue 2 virus with human plasma proteins. *J Proteome* 131:205–213
- Jaillon S, Mancuso G, Hamon Y, Beauvillain C, Cotici V, Midiri A et al (2013) Prototypic long pentraxin PTX3 is present in breast milk, spreads in tissues, and protects neonate mice from *Pseudomonas aeruginosa* lung infection. *J Immunol* 191(4):1873–1882
- Jean L, Smih F, Olivier E, Soury E, Simon-Chazottes D, Guenet JL et al (1997) Comparative assignments of the genes of the inter-alpha-inhibitor family in human and mouse: ITIH4 is close to ITIH1 and ITIH3, on HSA 3 and MMU 14. *Genomics* 41(1):139–140
- Jessen TE, Faarvang KL, Ploug M (1988) Carbohydrate as covalent crosslink in human inter-alpha-trypsin inhibitor: a novel plasma protein structure. *FEBS Lett* 230(1–2):195–200
- Jonsson-Berling BM, Ohlsson K (1991) Distribution and elimination of intravenously injected urinary trypsin inhibitor. *Scand J Clin Lab Invest* 51(6):549–557
- Kanayama N, el Maradny E, Halim A, Liping S, Maehara K, Kajiware Y et al (1995) Urinary trypsin inhibitor prevents uterine muscle contraction by inhibition of Ca^{++} influx. *Am J Obstet Gynecol* 173(1):192–199
- Kanayama N, Maehara K, She L, Belayet HM, Khatun S, Tokunaga N et al (1998) Urinary trypsin inhibitor suppresses vascular smooth muscle contraction by inhibition of Ca^{2+} influx. *Biochim Biophys Acta* 1381(2):139–146

- Kanayama N, Maehara K, Suzuki M, Fujise Y, Terao T (1997) The role of chondroitin sulfate chains of urinary trypsin inhibitor in inhibition of LPS-induced increase of cytosolic free Ca^{2+} in HL60 cells and HUVEC cells. *Biochem Biophys Res Commun* 238(2):560–564
- Kanayama S, Yamada Y, Onogi A, Shigetomi H, Ueda S, Tsuji Y et al (2007) Bikunin suppresses expression of pro-inflammatory cytokines induced by lipopolysaccharide in neutrophils. *J Endotoxin Res* 13(6):369–376
- Salier JP, Rouet P, Raguenez G, Daveau M (1996) The inter-alpha-inhibitor family: from structure to regulation. *Biochem J* 315(Pt 1):1–9
- Zhuo L, Hascall VC, Kimata K (2004) Inter-alpha-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex. *J Biol Chem* 279(37):38079–38082
- Zhuo L, Kimata K (2008) Structure and function of inter-alpha-trypsin inhibitor heavy chains. *Connect Tissue Res* 49(5):311–320
- Kobayashi H (2006) Endogenous anti-inflammatory substances, inter-alpha-inhibitor and bikunin. *Biol Chem* 387(12):1545–1549
- Pugia MJ, Valdes R Jr, Jortani SA (2007) Bikunin (urinary trypsin inhibitor): structure, biological relevance, and measurement. *Adv Clin Chem* 44:223–245
- Lord MS, Melrose J, Day AJ, Whitelock JM (2020) The Inter-alpha-trypsin inhibitor family: versatile molecules in biology and pathology. *J Histochem Cytochem* 68(12):907–927
- Toyoda H, Kobayashi S, Sakamoto S, Toida T, Imanari T (1993) Structural analysis of a low-sulfated chondroitin sulfate chain in human urinary trypsin inhibitor. *Biol Pharm Bull* 16(9):945–947
- Scavenius C, Nikolajsen CL, Stenvang M, Thogersen IB, Wyrozemski L, Wisniewski HG et al (2016) The compact and biologically relevant structure of Inter-alpha-inhibitor is maintained by the chondroitin sulfate chain and divalent cations. *J Biol Chem* 291(9):4658–4670
- Yamamoto T, Yamamoto K, Sinozawa H (1996) Inter-alpha-trypsin inhibitor and its related proteins in Syrian hamster urine and plasma. *J Biochem* 120(1):145–152
- Thuveson M, Fries E (1999) Intracellular proteolytic processing of the heavy chain of rat pre-alpha-inhibitor. The COOH-terminal propeptide is required for coupling to bikunin. *J Biol Chem* 274(10):6741–6746
- Kobayashi H, Sun GW, Terao T (1999) Immunolocalization of hyaluronic acid and inter-alpha-trypsin inhibitor in mice. *Cell Tissue Res* 296(3):587–597
- Lindqvist A, Bratt L, Altieri M, Kastern W, Akerstrom B (1992) Rat alpha 1-microglobulin: co-expression in liver with the light chain of inter-alpha-trypsin inhibitor. *Biochim Biophys Acta* 1130(1):63–67
- Thogersen IB, Enghild JJ (1995) Biosynthesis of bikunin proteins in the human carcinoma cell line HepG2 and in primary human hepatocytes. Polypeptide assembly by glycosaminoglycan. *J Biol Chem* 270(31):18700–18709
- Odum L (1990) Inter-alpha-trypsin inhibitor and pre-alpha-trypsin inhibitor in health and disease. Determination by immunoelectrophoresis and immunoblotting. *Biol Chem Hoppe Seyler* 371(12):1153–1158
- Rouet P, Raguenez G, Tronche F, Yaniv M, N'Guyen C, Salier JP (1992) A potent enhancer made of clustered liver-specific elements in the transcription control sequences of human alpha 1-microglobulin/bikunin gene. *J Biol Chem* 267(29):20765–20773
- Rouet P, Raguenez G, Tronche F, Mfou'ou V, Salier JP (1995) Hierarchy and positive/negative interplays of the hepatocyte nuclear factors HNF-1, -3 and -4 in the liver-specific enhancer for the human alpha-1-microglobulin/bikunin precursor. *Nucleic Acids Res* 23(3):395–404
- Ruminy P, Rouet P, Salier JP (2003) An interplay of Sp1, GSKF and CREB-2 controls human pre-alpha-inhibitor gene (ITIH3) transcription. *Gene* 315:133–144
- Kato M, Seki N, Sugano S, Hashimoto K, Masuho Y, Muramatsu M et al (2001) Identification of sonic hedgehog-responsive genes using cDNA microarray. *Biochem Biophys Res Commun* 289(2):472–478

- Sarafan N, Martin JP, Bourguignon J, Borghi H, Calle A, Sesboue R et al (1995) The human inter-alpha-trypsin inhibitor genes respond differently to interleukin-6 in HepG2 cells. *Eur J Biochem/ FEBS* 227(3):808–815
- Pratt CW, Pizzo SV (1986) In vivo metabolism of inter-alpha-trypsin inhibitor and its proteinase complexes: evidence for proteinase transfer to alpha 2-macroglobulin and alpha 1-proteinase inhibitor. *Arch Biochem Biophys* 248(2):587–596
- Lim YP, Bendelja K, Opal SM, Siryaporn E, Hixson DC, Palardy JE (2003) Correlation between mortality and the levels of inter-alpha inhibitors in the plasma of patients with severe sepsis. *J Infect Dis* 188(6):919–926
- Opal SM, Lim YP, Siryaporn E, Moldawer LL, Pribble JP, Palardy JE et al (2007) Longitudinal studies of inter-alpha inhibitor proteins in severely septic patients: a potential clinical marker and mediator of severe sepsis. *Crit Care Med* 35(2):387–392
- Zhuo L, Kimata K (2001) Cumulus oophorus extracellular matrix: its construction and regulation. *Cell Struct Funct* 26(4):189–196
- Sanggaard KW, Scavenius C, Rasmussen AJ, Wisniewski HG, Thøgersen IB, Enghild JJ (2010a) The TSG-6/HC2-mediated transfer is a dynamic process shuffling heavy chains between glycosaminoglycans. *J Biol Chem* 285(29):21988–21993
- Sanggaard KW, Sonne-Schmidt CS, Krogager TP, Lorentzen KA, Wisniewski HG, Thøgersen IB et al (2008) The transfer of heavy chains from bikunin proteins to hyaluronan requires both TSG-6 and HC2. *J Biol Chem* 283(27):18530–18537
- Pijuan-Galito S, Tamm C, Anneren C (2014) Serum Inter-alpha-inhibitor activates the yes tyrosine kinase and YAP/TEAD transcriptional complex in mouse embryonic stem cells. *J Biol Chem* 289(48):33492–33502
- Pijuan-Galito S, Tamm C, Schuster J, Sobol M, Forsberg L, Merry CL et al (2016) Human serum-derived protein removes the need for coating in defined human pluripotent stem cell culture. *Nat Commun* 7:12170
- Mizon C, Piva F, Queyrel V, Balduyck M, Hachulla E, Mizon J (2002) Urinary bikunin determination provides insight into proteinase/proteinase inhibitor imbalance in patients with inflammatory diseases. *Clin Chem Lab Med* 40(6):579–586
- Okroj M, Holmquist E, Sjölander J, Corrales L, Saxne T, Wisniewski HG et al (2012) Heavy chains of inter alpha inhibitor (I α h α) inhibit the human complement system at early stages of the cascade. *J Biol Chem* 287(24):20100–20110
- Scavenius C, Sanggaard KW, Nikolajsen CL, Bak S, Valnickova Z, Thøgersen IB et al (2011) Human inter-alpha-inhibitor is a substrate for factor XIIIa and tissue transglutaminase. *Biochim Biophys Acta* 1814(12):1624–1630
- Stober VP, Lim YP, Opal S, Zhuo L, Kimata K, Garantziotis S (2019) Inter- α -inhibitor ameliorates endothelial inflammation in sepsis. *Lung* 197:361–369
- Winkler CJ, Jorba N, Shitanishi KT, Herring SW (2013) Protein sieving characteristics of sub-20-nm pore size filters at varying ionic strength during nanofiltration of coagulation factor IX. *Biol J Int Assoc Biol Standard* 41(3):176–183
- McKay EJ, Laurell CB (1980) The interaction of heparin with plasma proteins. Demonstration of different binding sites for antithrombin III complexes and antithrombin III. *J Lab Clin Med* 95(1):69–80
- Pemberton AD, Brown JK, Inglis NF (2010) Proteomic identification of interactions between histones and plasma proteins: implications for cytoprotection. *Proteomics* 10(7):1484–1493
- Potempa J, Kwon K, Chawla R, Travis J, Inter-alpha-trypsin inhibitor. (1989) Inhibition spectrum of native and derived forms. *J Biol Chem* 264(25):15109–15114
- Kobayashi H, Gotoh J, Kanayama N, Hirashima Y, Terao T, Sugino D (1995) Inhibition of tumor cell invasion through matrigel by a peptide derived from the domain II region in urinary trypsin inhibition. *Cancer Res* 55(9):1847–1852
- Kobayashi H, Shinohara H, Takeuchi K, Itoh M, Fujie M, Saitoh M et al (1994) Inhibition of the soluble and the tumor cell receptor-bound plasmin by urinary trypsin inhibitor and subsequent effects on tumor cell invasion and metastasis. *Cancer Res* 54(3):844–849
- Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, Terao T (2003) A Kunitz-type protease inhibitor, bikunin, inhibits ovarian cancer cell invasion by blocking the calcium-dependent transforming growth factor-beta 1 signaling cascade. *J Biol Chem* 278(10):7790–7799

- Wilharm E, Parry MA, Friebe R, Tschesche H, Matschiner G, Sommerhoff CP et al (1999) Generation of catalytically active granzyme K from *Escherichia coli* inclusion bodies and identification of efficient granzyme K inhibitors in human plasma. *J Biol Chem* 274(38):27331–27337
- el Maradny E, Kanayama N, Halim A, Maehara K, Terao T (1994) Urinary trypsin inhibitor has a protective effect on the amnion. *Gynecol Obstet Invest* 38(3):169–172
- Sugiki M, Sumi H, Maruyama M, Yoshida E, Mihara H (1989) Clearance and distribution of acid-stable trypsin inhibitor (ASTI). *Enzyme* 42(1):31–38
- Sjoberg EM, Blom A, Larsson BS, Alston-Smith J, Sjoquist M, Fries E (1995) Plasma clearance of rat bikunin: evidence for receptor-mediated uptake. *Biochem J* 308(Pt 3):881–887
- Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y et al (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. *J Biol Chem* 276(11):7693–7696
- Sanggaard KW, Hansen L, Scavenius C, Wisniewski HG, Kristensen T, Thøgersen IB et al (2010b) Evolutionary conservation of heavy chain protein transfer between glycosaminoglycans. *Biochim Biophys Acta* 1804(4):1011–1019
- Milner CM, Tongsoongnoen W, Rugg MS, Day AJ (2007) The molecular basis of inter-alpha-inhibitor heavy chain transfer on to hyaluronan. *Biochem Soc Trans* 35(Pt 4):672–676
- Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B et al (2004) PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. *Development* 131(7):1577–1586
- Lord MS, Day AJ, Youssef P, Zhuo L, Watanabe H, Caterson B et al (2013) Sulfation of the bikunin chondroitin sulfate chain determines heavy chain. Hyaluronan complex formation. *J Biol Chem* 288(32):22930–22941
- Kluft C (1976) Occurrence of C1 inactivator and other proteinase inhibitors in euglobulin fractions and their influence on fibrinolytic activity. *Haemostasis* 5(3):136–146
- Steinbuch M, Pejaudier L, Kichenin V, Boffa MC (1984) Studies on prothrombin complex concentrates contact factors, complement components and proteinase inhibitors. *Thromb Haemost* 52(3):256–262
- Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P et al (2005) Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 202(2):271–281
- Tucker EI, Gailani D, Hurst S, Cheng Q, Hanson SR, Gruber A (2008) Survival advantage of coagulation factor XI-deficient mice during peritoneal sepsis. *J Infect Dis* 198(2):271–274
- Koraka P, Lim YP, Shin MD, Setiati TE, Mairuhu AT, van Gorp EC et al (2010) Plasma levels of inter-alpha inhibitor proteins in children with acute dengue virus infection. *PLoS One* 5(4):e9967
- Parker AL, Waddington SN, Nicol CG, Shayakhmetov DM, Buckley SM, Denby L et al (2006) Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood* 108(8):2554–2561
- Munch J, Rucker E, Standker L, Adermann K, Goffinet C, Schindler M et al (2007) Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 131(6):1059–1071
- Torihashi S, Ho M, Kawakubo Y, Komatsu K, Nagai M, Hirayama Y et al (2015) Acute and temporal expression of tumor necrosis factor (TNF)-alpha-stimulated gene 6 product, TSG6, in mesenchymal stem cells creates microenvironments required for their successful transplantation into muscle tissue. *J Biol Chem* 290(37):22771–22781
- Tseng SC (2016) HC-HA/PTX3 purified from amniotic membrane as novel regenerative matrix: insight into relationship between inflammation and regeneration. *Invest Ophthalmol Vis Sci* 57(5):ORSFh1-8
- Scott LJ, Muglia P, Kong XQ, Guan W, Flickinger M, Upmanyu R et al (2009) Genome-wide association and meta-analysis of bipolar disorder in individuals of European ancestry. *Proc Natl Acad Sci U S A* 106(18):7501–7506
- Psychiatric GCBWDG (2011) Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. *Nat Genet* 43(10):977–983
- Schizophrenia Psychiatric Genome-Wide Association Study C (2011) Genome-wide association study identifies five new schizophrenia loci. *Nat Genet* 43(10):969–976

- Witt SH, Juraeva D, Sticht C, Strohmaier J, Meier S, Treutlein J et al (2014) Investigation of manic and euthymic episodes identifies state- and trait-specific gene expression and STAB1 as a new candidate gene for bipolar disorder. *Transl Psychiatry* 4:e426
- Xie X, Meng H, Wu H, Hou F, Chen Y, Zhou Y et al (2020) Integrative analyses indicate an association between ITIH3 polymorphisms with autism spectrum disorder. *Sci Rep* 10(1):5223
- Katzeff JS, Bright F, Lo K, Kril JJ, Connolly A, Crossett B et al (2020) Altered serum protein levels in frontotemporal dementia and amyotrophic lateral sclerosis indicate calcium and immunity dysregulation. *Sci Rep* 10(1):13741
- Tang M, Alaniz ME, Felsky D, Vardarajan B, Reyes-Dumeyer D, Lantigua R et al (2020) Synonymous variants associated with Alzheimer disease in multiplex families. *Neurol Genet* 6(4):e450
- Schuffels S, Nakada S, Wu Y, Lim YP, Chen X, Stonestreet BS (2020) Effects of inter-alpha inhibitor proteins on brain injury after exposure of neonatal rats to severe hypoxia-ischemia. *Exp Neurol* 334:113442
- Threlkeld SW, Lim YP, La Rue M, Gaudet C, Stonestreet BS (2017) Immuno-modulator inter-alpha inhibitor proteins ameliorate complex auditory processing deficits in rats with neonatal hypoxic-ischemic brain injury. *Brain Behav Immun* 64:173–179
- Werbowski-Ogilvie TE, Agar NY, Waldkircher de Oliveira RM, Faury D, Antel JP, Jabado N et al (2006) Isolation of a natural inhibitor of human malignant glial cell invasion: inter alpha-trypsin inhibitor heavy chain 2. *Cancer Res* 66(3):1464–1472
- Obayashi Y, Yabushita H, Kanyama K, Noguchi M, Zhuo L, Kimata K et al (2008) Role of serum-derived hyaluronan-associated protein-hyaluronan complex in ovarian cancer. *Oncol Rep* 19(5):1245–1251
- Yabushita H, Iwasaki K, Kanyama K, Obayashi Y, Zhuo L, Itano N et al (2011) Clinicopathological role of serum-derived Hyaluronan-associated protein (SHAP)-Hyaluronan complex in endometrial cancer. *Obstet Gynecol Int* 2011:739150
- Wu X, Wang L, Ye Y, Aakre JA, Pu X, Chang GC et al (2013) Genome-wide association study of genetic predictors of overall survival for non-small cell lung cancer in never smokers. *Cancer Res* 73(13):4028–4038
- Uen YH, Lin KY, Sun DP, Liao CC, Hsieh MS, Huang YK et al (2013) Comparative proteomics, network analysis and post-translational modification identification reveal differential profiles of plasma con A-bound glycoprotein biomarkers in gastric cancer. *J Proteome* 83:197–213
- Völlmy F, Van Den Toorn H, Chiozzini RZ, Zuchetti O, Papi A, Volta CA, Marracino L, Dalla Sega FV, Fortini F, Demichev V, Tober-Lau P (2021) A serum proteome signature to predict mortality in severe COVID-19 patients. *Life Sci Alliance* 4(9):e202101099
- Lourido L, Ayoglu B, Fernandez-Tajes J, Oreiro N, Henjes F, Hellstrom C et al (2017) Discovery of circulating proteins associated to knee radiographic osteoarthritis. *Sci Rep* 7(1):137
- Liao CC, Chou PL, Cheng CW, Chang YS, Chi WM, Tsai KL et al (2016) Comparative analysis of novel autoantibody isotypes against citrullinated-inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3)(542-556) peptide in serum from Taiwanese females with rheumatoid arthritis, primary Sjogren's syndrome and secondary Sjogren's syndrome in rheumatoid arthritis. *J Proteome* 141:1–11
- Kim TH, Koo JH, Heo MJ, Han CY, Kim YI, Park SY et al (2019) Overproduction of inter-alpha-trypsin inhibitor heavy chain 1 after loss of Galpha13 in liver exacerbates systemic insulin resistance in mice. *Sci Transl Med* 11(513)
- Masi LN, Lotufo PA, Ferreira FM, Rodrigues AC, Serdan TDA, Souza-Siqueira T et al (2021) Profiling plasma-extracellular vesicle proteins and microRNAs in diabetes onset in middle-aged male participants in the ELSA-Brasil study. *Physiol Rep* 9(3):e14731
- Wakahara K, Kobayashi H, Yagyu T, Matsuzaki H, Kondo T, Kurita N et al (2005) Bikunin suppresses lipopolysaccharide-induced lethality through down-regulation of tumor necrosis factor- α and interleukin-1 β in macrophages. *J Infect Dis* 191(6):930–938
- Zhu L, Zhuo L, Kimata K, Yamaguchi E, Watanabe H, Aronica MA et al (2010) Deficiency in the serum-derived hyaluronan-associated protein-hyaluronan complex enhances airway hyperresponsiveness in a murine model of asthma. *Int Arch Allergy Immunol* 153(3):223–233

Chapter 7

CD44: Does CD44v6 Adversely Impact the Prognosis of Cancer Patients?



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Abstract Colorectal cancer (CRC) is the third leading cause of cancer-related death in the USA. This high level of CRC death is primarily due to chemoresistance and metastasis. The ability of cancers to maintain tumorigenesis for a long term depends on a subpopulation of cancer cells, the self-renewing cancer-initiating cells (CICs) with cancer stem cell-like properties responsible for tumor initiation, progression, and drug resistance. The CICs can be identified and isolated by CIC-specific cell-surface adhesion marker expression, and CD44v6 is the most frequently functional marker on CICs. Knockout and knockdown of CD44v6 in mice accompanied loss of tumor progression but did not fully regress primary tumor growth indicating that CD44/CD44v6 (Variant 6 of CD44) may have an indispensable role in maintaining CIC turnover. We have recently shown that CRC-CIC enrichment markers and CD44v6 support the maintenance of CICs through communication of the CRC-CIC/niche with the cancer-associated stromal microenvironment. These positive CD44v6 activities for CICs depend on CD44v6's ability to join tissue micro-environmental cues with growth factor/cytokine signals that transduce signals to membrane receptor proteins or to the nucleus to regulate a variety of transcription factors and their target gene expression levels necessary to maintain the stemness of CICs and to convert differentiated tumor cells into CICs. In this perspective we encapsulate the current findings regarding the potential role of CD44/CD44v6 in the regulation of CICs stemness and the current status of therapies that target

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CD44v6-positive/CIC mechanisms, which will hold great potential to optimize therapeutic targeting of CRC.

Keywords CRC Colorectal cancer · CICs Cancer-initiating cells · CD44v6 Variant 6 of CD44 · TM transmembrane domain · ICD intracellular domain · Tf transferrin · TfR transferrin receptor · S sensitive · FR FOLFOX resistant · CTOS: c-Myc, Twist1, Oct 4, and SOX2 · Twist1 Oct 4 and SOX2

Abbreviations

CD44v6	variant 6 of CD44
CICs	cancer-initiating cells
CRC	colorectal cancer
CTOS	c-Myc, Twist1, Oct 4, and SOX2
FR	FOLFOX resistant
ICD	intracellular domain
S	sensitive
Tf	transferrin
TfR	transferrin receptor
TM	transmembrane domain

7.1 Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death in Western countries with incidences increasing by 2% annually (Siegel et al. 2017; Siegel and Miller 2016) and 5-year survival rate is 10% (Schmitt et al. 2015). Early detection by colonoscopy and surgical removal of precancerous lesions have led to a substantial reduction in incidence of CRC (Cunningham et al. 2010; Lieberman et al. 2012; Winawer 2005; Winawer et al. 1993). FOLFOX (5-fluorouracil [5-FU] + oxaliplatin [OXA] + leucovorin) has been used as the first-line agent for the treatment of CRC. However, after treatment repeats 40% of patients with stage II or III CRC acquire FOLFOX resistance within five years, and most of these patients die due to drug resistance (Augestad et al. 2014; Renouf et al. 2013; Sargent et al. 2009). The mechanism of drug resistance has been attributed to the following: increased expression of CD44v6 (Van der Jeught et al. 2018); drug influx/efflux/metabolism and drug target alteration (Longley and Johnston 2005; De Mattia et al. 2015); drug transporters, such as ABCB1, ABCC1, and ABCG2 overexpression (Perez-Tomas 2006; Glavinas et al. 2004); detoxification by glutathione-S-transferase enzymes (De Mattia et al. 2015; Townsend and Tew 2003); and generation of reactive oxygen species that induce apoptosis resistance through tumor cytokines (Masuda et al. 1994; Kruidering M, Van de Water B, de Heer E, Mulder GJ, Nagelkerke JF 1997;

Weng et al. 2018; Chen et al. 2019) or activation of NF κ B (Robinson et al. 2011). Tumors with intrinsic resistance are less likely to show any therapeutic response following initial therapy, while tumors with acquired resistance developed after repeated exposures to a particular drug may also become cross-resistant to other drugs despite different mechanisms of action (Holohan et al. 2013). Accordingly, the survival benefits of adjuvant chemotherapy are currently only reaching a minor population of CRC patients (Longley and Johnston 2005). Thus, understanding the molecular mechanisms contributing to FOLFOX resistance and identifying an intervention approach to overcome subsequent drug resistance are not only clinically important for chemotherapeutic drug delivery as a specific anti-cancer therapeutic but also could develop novel therapeutic modalities for CRC malignancy that may overcome drug resistance and enhance drug efficacy. Since CD44v6 is a functional CIC marker in cancers including CRC (Yan et al. 2015; Zoller 2011; Todaro et al. 2014; Ghatak et al. 2021; Ghatak et al. 2022), this review: (1) will introduce the CD44/CD44v6 hyaluronan receptor family; (2) will establish the relation between CD44/CD44v6 and CIC activities with significance on the requirement of a CIC niche (Prasetyanti and Medema 2017; Prasetyanti et al. 2013) for multiple cell types (Vermeulen et al. 2010; Medema and Vermeulen 2011) and apoptosis resistance (Ramdass et al. 2013; Colak and Medema 2014; Vlashi and Pajonk 2015); and (3) will show evidence that CD44v6 is required for transcriptional modulation through its function as a transcription-related coactivator for transcription factors that maintain the stemness of CICs and revert the differentiated tumor cells into cancer stem cells (Ghatak et al. 2021). Finally, we will focus on the significance of CD44v6 knockdown-based therapeutic approaches in colon tumor cells.

7.2 Structure of CD44/CD44 Variants

CD44, a non-kinase single-chain type-1 transmembrane glycoprotein, is a receptor for hyaluronan, an anionic non-sulfated linear glycosaminoglycan that is an extra-cellular physiological ligand involved in cell–cell and cell–matrix adhesion (Aruffo et al. 1990; Stamenkovic et al. 1989). Based on the interactions of an anti-CD44 antibody on T lymphocytes, CD44 proteins exist in three states with respect to hyaluronan binding: non-binding; non-binding unless activated by physiological stimuli; or constitutively binding (Lesley et al. 2000a; Lesley et al. 2000b; Lesley and Hyman 1992). On the basis of studies by physical techniques, CD44 exists in two states—ordered and partially ordered (Ogino et al. 2010) or low affinity and high affinity forms (Jamison 2nd et al. 2011). Affinity binding of CD44 is necessary for physiological functions of leukocyte trafficking and metastasis. Nuclear magnetic resonance studies have shown that ordered to the partially disordered forms of conformation occur in the CD44-hyaluronan-binding domain. Experimental demonstration has revealed an equilibrium between the two conformation forms controls CD44 mediated rolling of cells in blood streams and migration into the adjacent inflammatory sites (Ogino et al. 2010). Crystal structures of the CD44 hyaluronan-

binding domain complexed with HA oligomers suggested low affinity to high affinity conformational switches (Jamison 2nd et al. 2011).

The CD44 gene is encoded by a single gene located on chromosome 11 in humans and on chromosome 2 in mice (Fig. 7.1a). The full-length mouse CD44 gene contains 20 exons and 19 introns (Orian-Rousseau and Sleeman 2014). The human CD44 gene is comprised of 19 exons in humans and 20 in mice since a homolog of exon 6 (variant 1) is not found in humans as it encodes a stop codon (Naor et al. 2002). The first five exons (1–5) and the last five exons (16–20), known as constant exons, encode the N-terminal and C-terminal regions of the CD44 molecule (85–95 kDa) called CD44 standard (CD44s) without any alternate splicing (Orian-Rousseau and Sleeman 2014) (Fig. 7.1b). Ten central exons, 6–15 (commonly called v1–v10) of CD44 gene undergo alternate splicing to form a wide variety of CD44 variant (CD44v) isoforms with multiple sizes of the CD44v and with different functions and structures in the stem region. These alternate spliced variants of CD44 have N- and O- sites accessible to glycosylation by addition of heparin sulfate or chondroitin sulfate (Screaton et al. 1992; Naor et al. 2008; Ghatak et al. 2017a; Stamenkovic et al. 1991; Bartolazzi et al. 1996; Bennett et al. 1995b; Bennett et al. 1995a; Greenfield et al. 1999). Variant exons of CD44 are assembled with constant exons to form different mRNAs for different CD44 variants (Idzerda et al. 1989; Goldstein and Butcher 1990; Screaton et al. 1992). At least twenty different isoforms have been identified. CD44v isoforms can be inserted as a single variant exon or combined with other variant exons that code for peptides located in the juxta membrane domain (Fig. 7.1a).

The CD44 protein consists of three functional domains: an extracellular domain (ectodomain), a transmembrane domain (TM), and an intracellular domain (ICD) (Fig. 7.1a, b) (Goldstein et al. 1989). The first five exons of CD44 constitute the globular structure of the N-terminal region, which is stabilized by conserved cysteines. The two cysteines in the flanking region are responsible for the folding of the linkage domain (Ishii et al. 1993). The ectodomain, which serves as a binding motif for hyaluronan, is a major component of extracellular matrices (Aruffo et al. 1990). The N-terminal globular region of CD44s is separated from the plasma membrane by a short stem structure, which contains putative proteolytic cleavage sites (Neame and Isacke 1993; Ruiz et al. 1995; Ghatak et al. 2005). It is here that variant exons are inserted, which add binding sites for several proteins shown in Fig. 7.1c. The single-pass TM domain provides a platform for the formation of CD44/CD44v oligomers and supports the recruitment of CD44 to glycolipid-enriched membrane domains. This is important for the interaction of CD44s/CD44v with extracellular ligands and for its association with other transmembrane and cytoplasmic molecules, such as cofactors, adaptor proteins, receptor tyrosine kinases, or nonreceptor protein-tyrosine kinases, to promote CD44-mediated signaling (Liu and Sy 1997; Foger et al. 2001; Ilangumaran et al. 1999). The rather short cytoplasmic tail of CD44 binds to ankyrin and ezrin-radixin-moesin (ERM) proteins providing a link to these cytoskeletal binding proteins (Bai et al. 2007; Lokeshwar et al. 1994; Oliferenko et al. 2000) (Fig. 7.1b).

Ubiquitously expressed membrane presenilin γ -secretase is known to cleave the intracellular domain (ICD) of at least 8 type 1 membrane proteins, including CD44 (Lammich et al. 2002; Medina and Dotti 2003). The MT1-MMP and ADAM-like proteases cleave the extracellular domain of CD44/CD44v (Kajita et al. 2001) (Fig. 7.2). The γ -secretase subsequently cleaves the intracellular domain of CD44/CD44v6 (CD44/CD44v6-ICD) (Lammich et al. 2002; Murakami et al. 2003; Okamoto et al. 2001). CD44/CD44v6 can regulate gene expression in part via intracytoplasmic CD44/CD44v6-ICD domain. The CD44/CD44v6-ICD acts as a coactivator as well as a co-repressor. CD44/CD44v6-ICD interacts with the transcriptional machinery, resulting in the upregulation of genes containing the 12-O-tetradecanoylphorbol 13-acetate response element, including CD44/CD44v6 itself (Okamoto et al. 2001). Since the size of CD44-ICD is only 72 residues, the specific sequence of the CD44-ICD response element (CIRE) to which it can bind is not usual. This type of protein–DNA interaction is usually associated with larger DNA-binding proteins such as transcription factors. The dual ability of CD44-ICD to interact with proteins in the transcriptional machinery and to interact with its own response element on promoter sequences could place CD44-ICD in a unique category of transcriptional regulators (Fig. 7.2). The nuclear localization signal present in CD44v6-ICD (³⁶⁰RRRCGQKKK³⁶⁸) has important biological functions including complex formation with transcription factors and activation of stemness factors in cells. The novel function of CD44-ICD in transcriptional modulation is due to nuclear translocation and formation of a complex with transcription factors CBP/p300 or STAT3, regulating target genes including CD44, Cyclin D1, MMP-9, HIF-2 α , c-Myc, and Twist1 (Su et al. 2011; Pietras et al. 2014; Okamoto et al. 2001; Bourguignon et al. 2012a). CD44-ICD activates stemness factors Nanog, Sox2, and Oct4, which promotes the tumorigenesis of breast cancer (Cho et al. 2015) and shows the function of CD44 in supporting CIC stemness (Gao et al. 2018) (Fig. 7.2).

The cytoplasmic tail of CD44/CD44v6 binds to the cytoskeletal proteins ankyrin and ERM. This links CD44 to the actin-cytoskeleton and spectrin, which regulate cell adhesion and motility, and cell migration and invasion in hyaluronan-dependent pathways (Tsukita et al. 1994; Lokeshwar et al. 1994). The N-terminus region of activated ERM proteins is recruited to the region between the CD44-TM region and the CD44-ankyrin binding site, and the C-terminus of ERM binds to F-actin (Mori et al. 2008) and induces CD44 downstream signaling pathways (Mori et al. 2008; Fehon et al. 2010; Bourguignon et al. 2014). In contrast, the tumor suppressor protein Merlin also binds to the ERM-binding domain of the CD44-cytoplasmic tail (Bai et al. 2007).

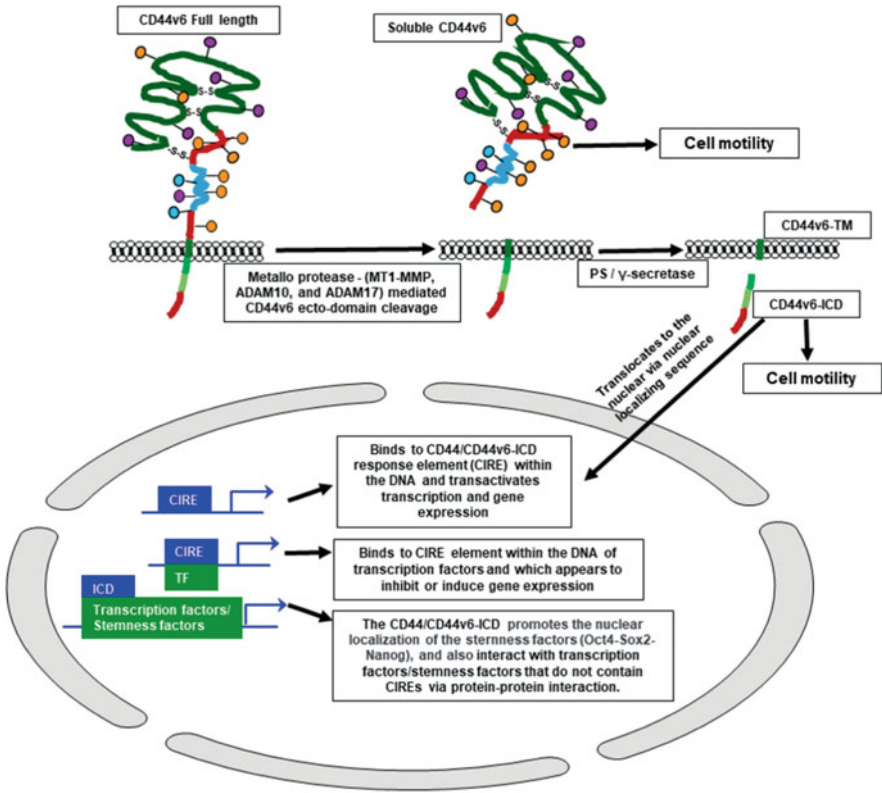


Fig. 7.2 Proposed model for CD44/CD44v6-ICD regulation. The extracellular stem region (ectodomain) of CD44/CD44v6 is proteolytically cleaved by MT1-MMP, ADAM-10, and ADAM-17, leaving a CD44 C-terminal fragment inserted in the plasma membrane. The cleaved extracellular region is soluble CD44/CD44v6 and is either secreted into the microenvironment fluid phase or sequestered to the extracellular matrix and is known to modulate cell migration. Extracellular domain is cleaved by intramembraneous proteolysis by presenilin-1/γ-secretase activity for further processing of the secretion of a CD44/CD44v6β-like peptide and the generation of a CD44/CD44v6 intracellular domain fragment (ICD). The CD44-ICD has been shown to be involved in nuclear signaling. The CD44/CD44v6-ICD is translocated to the nucleus, and the nuclear translocation of the CD44-ICD has been shown to be essential for the signaling proteins Notch and SREBPs (sterol regulatory element-binding proteins) that upon translocating to the nucleus can regulate transcription. The CD44/CD44v6-ICD dependent regulation of gene expression is likely to be mediated by protein–protein interactions with factors of the transcriptional machinery, which makes the CD44/CD44v6-ICD a transcriptional coactivator/regulator. CD44/CD44v6-ICD activates transcription through the 12-O-tetradecanoylphorbol 13-acetate-response element (TPA) resulting in the upregulation of genes containing the TPA-responsive element (TRE), including CD44/CD44v6 itself. However, the existence of the CD44/CD44v6-ICD response element (CIRE) and its reported binding to it makes the CD44/CD44v6-ICD a transcription factor. The ability of the CD44/CD44v6-ICD to interact with proteins of the transcriptional machinery as well as to interact with its own response element CIRE on promoter sequences might place the CD44-ICD in a novel category of transcriptional regulators

7.3 Ligands of CD44/CD44 Variants

Hyaluronan, a major component of extracellular matrices, is a high molecular weight (10^6 – 10^7 Dalton) linear polymer of D-Glucuronic acid and N-Acetylglucosamine repeating disaccharides (Laurent and Fraser 1992)], and it binds to a basic motif outside in the link domain (Aruffo et al. 1990) (Fig. 7.1b). Interaction of hyaluronan with CD44 depends on CD44 conformation (Schrijvers et al. 1993) and induces signaling when it binds to constitutively activated CD44/CD44 variants (CD44v) (He et al. 1992; Sleeman et al. 1996). For example, the sequence encoded by exon 5-7 of CD44v3 binds various forms of heparan sulfate-bound growth factors, including fibroblast growth factor (FGF) and heparin-bound epidermal growth factor (EGF) (Bennett et al. 1995a; Williams et al. 2013), while the sequence encoded by exon v6 contains binding sites for hepatocyte growth factor (HGF) (Orian-Rousseau et al. 2002), vascular endothelial growth factor (VEGF) (Tremmel et al. 2009), EGF (Cheng et al. 2006), and TGF β 1 (Ghatak et al. 2014; Ghatak et al. 2017b) (Fig. 7.1c). CD44v6 functions as a co-receptor for receptor tyrosine kinase (RTK) and serine-threonine-kinase activations. The link domain of CD44/CD44v6 has other ligands including collagen (Faassen et al. 1992), fibronectin (Jalkanen and Jalkanen 1992), and laminin (Jalkanen and Jalkanen 1992). Other important ligands of CD44/CD44v6 are mucosal addressin (Picker et al. 1989), MIP1beta (Tanaka et al. 1993), chondroitin sulfate (CS) (Naujokas et al. 1993), proteoglycan (Toyama-Sorimachi and Miyasaka 1994), serglycin (Toyama-Sorimachi et al. 1995), and osteopontin (Weber et al. 1996).

7.4 CD44/CD44v6-associated Signaling Pathways

CD44s is endogenously expressed in low levels on normal tissues (Cichy and Pure 2003; Mackay et al. 1994) and before binding to hyaluronan, its activation is necessary (Lesley et al. 2000b; Lesley and Hyman 1992; Lesley and Hyman 1998; Sneath and Mangham 1998). CD44 has been demonstrated to work as a co-receptor controlling the signals originating from a group of receptor tyrosine kinases, including c-Met and members of the ERBB family (Orian-Rousseau et al. 2002). CD44 associates with ABC transporters (Kozovska et al. 2014), antiapoptotic proteins (Bates et al. 2001; Bates et al. 1998; Bourguignon 2008), CXCR4 (G-protein-coupled receptors) (Katoh 2017), MT1-MMP proteases (Kajita et al. 2001), and hyaluronidase-2 (Heldin et al. 2014) and activates WNT signaling pathway via CD44-associated LRP6 (Xu et al. 2015). Many of the activities that have been ascribed to CD44, those relevant to cancer cell growth, invasion, and metastasis, could be attributed in part to these interactions involving CD44v6 in CRC. CD44v isoforms are involved in specific signaling pathways that are determined by the variant exon-encoded sequences (v2-v10), which are inserted by alternative splicing in different combinations at a single site in the stem region (Goldstein et al. 1989).

Glycosylation is required for introduction of new exons into this region to form spliced variants of CD44v. These variants have high affinity in hyaluronan binding as showed in certain cell types by inducing conformational changes or by allowing CD44v isoforms to have a new function as a co-receptor by generating new binding sites for many growth factors/cytokines, receptors, and nonreceptor protein-tyrosine kinases. For example, CD44v3 binds to fibroblast growth factors and heparin-binding epidermal growth factor (Bennett et al. 1995a; Williams et al. 2013), whereas CD44v6 contains a binding site for HGF (Orian-Rousseau et al. 2002), VEGF (Tremmel et al. 2009), EGF (Cheng et al. 2006), and TGF β 1 (Ghatak et al. 2014; Ghatak et al. 2017b). CD44v6-ECM binding also promotes PI3K/Akt pathway activation (Krause and Van Etten 2005; Misra et al. 2008a; Misra et al. 2009; Misra et al. 2008b; Weber 2008) and MET transcription (Adamia et al. 2005). CD44v6 crosslinking similarly regulates activation of insulin-like growth factor 1 receptor and platelet-derived growth factor receptor (Misra et al. 2006). The mitogenic or oncogenic signaling also regulates alternative splicing (Cheng et al. 2006; Prochazka et al. 2014; Weg-Remers et al. 2001) indicating that cancer cells often express a large variety of CD44 variants, especially in a metastatic stage. Systemic application of antibodies directed against the variant 6 epitope and the expression of antisense CD44v6 retard colon tumor growth and metastasis in vivo (Reeder et al. 1998; Seiter et al. 1993). The association of CD44v6 with metastasis was established in pancreatic cancer BSp73AS cells over expressing CD44v4-v7 both in vitro and in vivo (Gunthert et al. 1991). Similarly, overexpressing CD44v6 among CRC cells increased their resistance to anti-cancer therapy by activating PI3K/Akt, MAPK/ERK, EMT, and autophagy-related signaling pathways (Lv et al. 2016). In addition, CD44v6 stabilized the exchange of cysteine-glutamate on the cell membrane, thus protecting cancer cells against oxidative stress (Sugano et al. 2015). Overexpression of CD44variants, especially CD44v6 in various cancers (Gunthert et al. 1991; Naor et al. 1998; Ochiai et al. 1997; Kurozumi et al. 1998; Ishida 2000; Foekens et al. 1999; Misra et al. 2009) (for more details, see Heider et.al. (2004)) as well as downregulation of the standard CD44 isoform (CD44s) in CRC is postulated to result in increased tumorigenicity (Choi et al. 2000), emphasizing the potential importance of CD44 splice variants in cancer.

7.5 Defining CICs Association with Niche

In recent years, an increasing body of evidence has provided support for the existence of tumors as complex tissues composed of heterogeneous aberrant cell types containing a hierarchy of cells that differ in morphology, gene expression, proliferative capacity, and invasiveness (Singh et al. 2015). Solid tumors display the heterogeneity that originated from a small subset of cancer cells that display cellular hierarchies from which tumor clones originate with self-renewing, tumorigenic stemlike cells, or cancer-initiating cells (CICs) at the top. Cancer stem cells (CSCs) are also defined as CICs, which are the unique source of all tumor cells

and responsible for tumor propagation and relapse (Valent et al. 2012). Since the first identification of CSCs/CICs in breast cancer CD44/CD24 has been used as marker (Al-Hajj et al. 2003). CICs have been identified in a variety of solid tumors, such as colon carcinoma (Wei et al. 2010; Todaro et al. 2007; Todaro et al. 2014), glioblastoma (Singh et al. 2003), melanoma (Fang et al. 2005), osteosarcoma (Tirino et al. 2008), chondrosarcoma (Tirino et al. 2011), prostate cancer (Collins et al. 2005), ovarian cancer (Bapat et al. 2005), gastric cancer (Takaishi et al. 2009), and lung cancer (Eramo et al. 2008; Tirino et al. 2009). Tumor maintenance depends on a stromal cell compartment. Importantly, different cancer types CD44/CD44v6 have been identified as a typical CIC surface marker, individually or in combination with other marker(s) such as CD24, CD133, and CD34, (Yan et al. 2015). The tumor microenvironment surrounding CICs is called the CICs-niche, and this microenvironment nurtures the fate of CICs and the growth of cancer cells (Hanahan and Weinberg 2011; Sneddon and Werb 2007). Anchoring CICs to the niche through cell–cell contacts is critical because when the niche is sequestered from CICs, they remain close to niche factors that are crucial for self-renewal, replication, and differentiation in an effort to restore the tumor mass and its microenvironment (Rich 2016; Vermeulen et al. 2010; Vermeulen et al. 2008a). Evidence for CD44/CD44v6 assisting CICs in fulfilling these tasks originates through cytokines (hepatocyte growth factor, osteopontin, and stromal-derived factor- α) secreted by cancer-associated fibroblasts (CAFs) that increase CD44v6 expression in CRC CICs, which in turn promotes metastasis in distant tumors (Todaro et al. 2014).

Another study demonstrated that chemotherapy-treated human CAFs promoted CIC self-renewal, associated in vivo with tumor growth and secretion of IL-17a (Lotti et al. 2013). In a recent unpublished study, we showed that CRC-CICs share with CICs apoptosis resistance and altered expression of transcription factors that contribute to drug resistance through transcription of MDR1 and CD44v6 (Figs. 7.3, 7.4, 7.5, 7.6, and 7.7) (Ghatak et al. 2022). Further, we also showed that FOLFOX therapy-treated human CAFs promoted CRC-CICs autonomous resistance to chemotherapy, self-renewal, and in vivo tumor growth associated with increased secretion of specific cytokines and matricellular proteins including WNT3A, IL17A, and periostin. This justifies the importance of CD44/CD44v6 supporting CICs (Figs. 7.3, 7.4, 7.5, 7.6, and 7.7) (Ghatak et al. 2022) and indicates that chemotherapy induces remodeling of the tumor microenvironment to support the CIC maintenance through secreted factors.

7.6 CD44/CD44v6 Defines CICs

Recent studies indicate that, in mouse models (K19-Wnt1/C2Me [Gan] mice, in which both WNT and PGE2 signaling pathways are stimulated in the gastric mucosa) of gastric cancer, CD44+/+ Gan mice developed large gastric tumors and increased expression of CD44v8-10 compared to CD44/- Gan mice. Similarly, in a mouse model of glioma, CD44+/+ animals developed significantly more high-grade

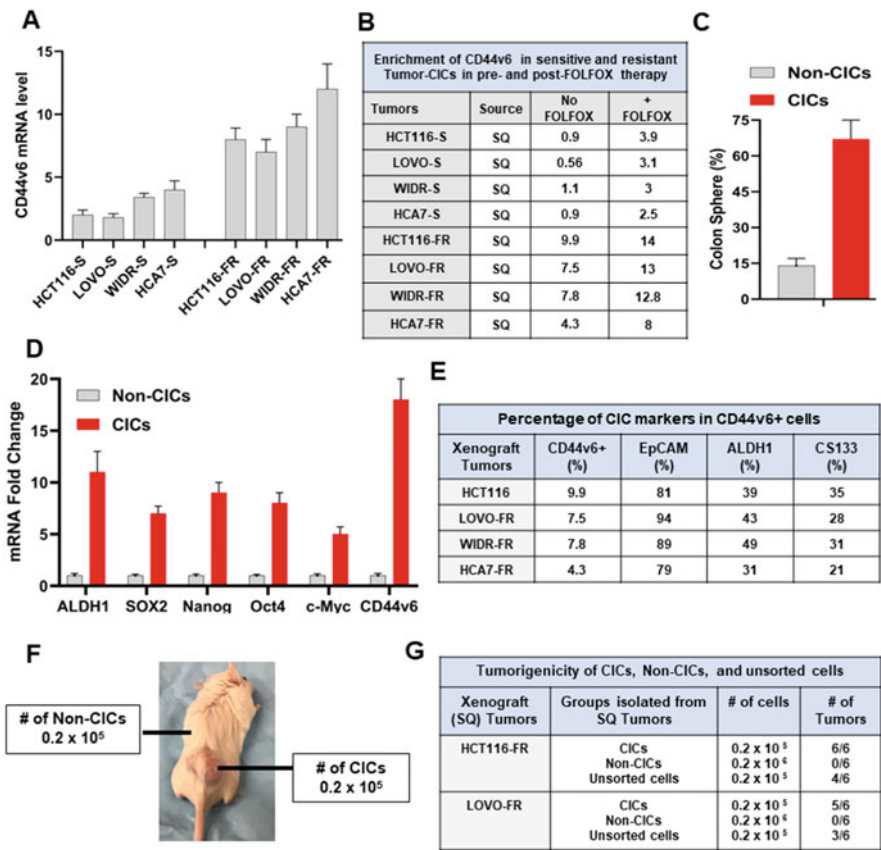


Fig. 7.3 CD44v6 identifies CRC-CICs. (a), QRT-PCR analysis of CD44v6 mRNA expression in the indicated 7 CRC cells. (b), Single-cell suspensions of CICs from dissociated fresh xenograft (SQ) tumors implanted with Folfox resistant and sensitive cells were analyzed by FACS. Enrichment of CD44v6^{high} CICs in bulk cells from indicated sensitive (S) and FOLFOX resistant (FR) SQ tumors was assessed by FACS analysis for CD44v6. (c), Quantification of tumor sphere-formation assay demonstrates that CICs have elevated tumor sphere formation efficiency. CICs and non-CICs isolated from HCT116-FR SQ tumors were plated in 96-well plates for a period of 14 days. Data are presented as a percentage of wells containing tumor spheres compared to the total number of wells. (d), Quantitative RT-PCR analysis of indicated CIC markers including CD44v6 was performed on CICs and non-CICs isolated from HCT116-FR SQ tumors. (e), Colorectal tumor from indicated four FOFOX-resistant cells were subjected to dissociation to single cells and FACS sorting for high expression of CD44v6 (CD44v6⁺). CD44⁺ cells were sequentially analyzed for the expression of the epithelial marker EpCAM, and for stem cells markers ALDH1 and CD133. The percentages of EpCAM, ALDH1, and CD133 in CD44⁺ cells obtained are listed in the Table. (f), Representative images of HCT116-FR SQ tumors initiated from CICs implanted into immunocompromised SCID mice. (g), CICs were tumorigenic, whereas 10 fold more non-CICs were unable to generate tumors in three independent experiments. Six mice were used per each experimental group

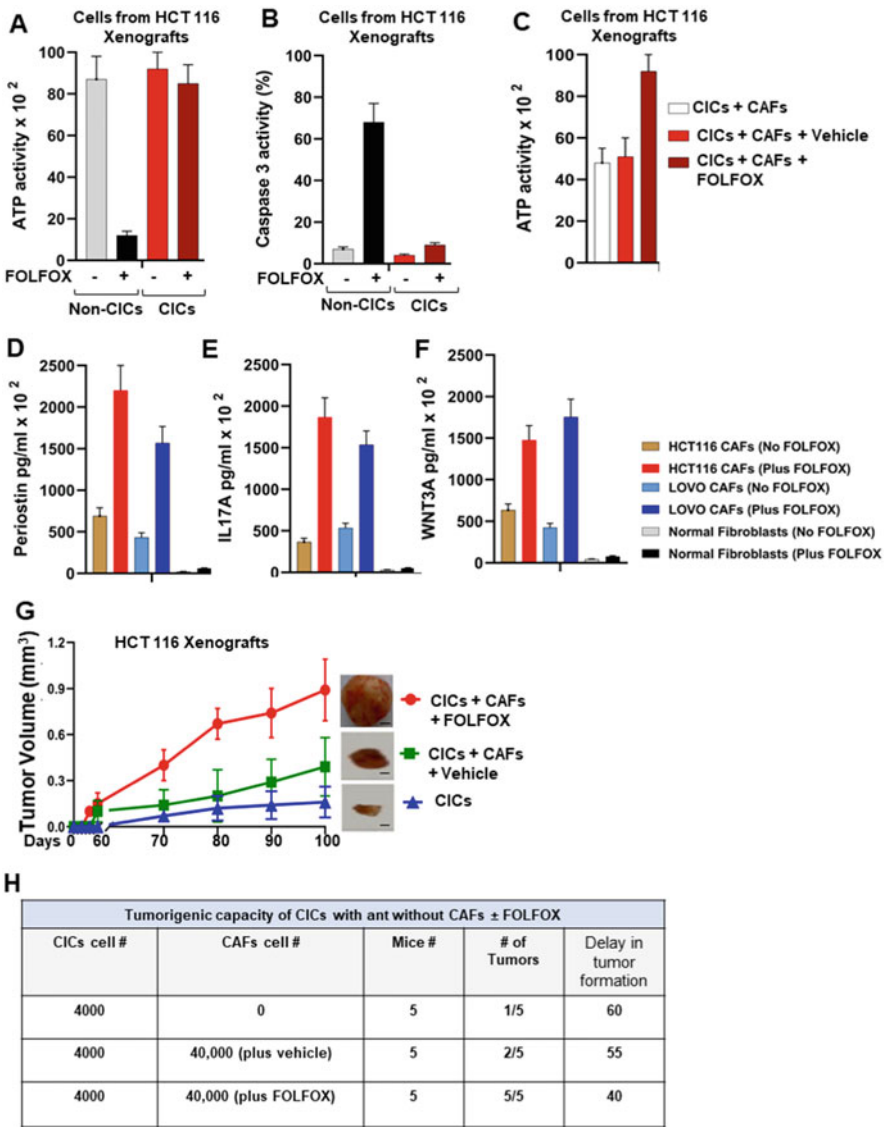


Fig. 7.4 FOLFOX treatment induces increased frequency of CICs and CAFs with autonomous chemoresistance in CICs. (a), Cell growth of CICs and non-CICs from HCT116-FR/SQ tumors was assessed by ATP-based assay (CellTiter-Glo) following treatment of FOLFOX (50 μ g/ml 5-fluorouracil + 10 μ M oxaliplatin + 1 μ M leucovorin). (b), Apoptosis of CICs and non-CICs from HCT116-FR/SQ tumors were assessed by caspase 3 activation (by ELISA assay) after treatment with FOLFOX. (c), 1×10^4 CICs were cultured with 2×10^4 cancer-associated fibroblasts (CAFs) or with normal human fibroblasts treated with vehicle (DMSO) or chemotherapy for 12 days. Cellular viability was measured by Cell Titer-Glo assay. (d–f), Secreted periostin, IL-17A, and WNT3A in the freshly isolated HCT116-FR SQ, and LOVO-FR SQ tumors were measured by ELISA assay. (g), The ability of the CAFs to affect the tumorigenic capacity of CICs from HCT11-FR SQ was tested in vivo. 40,000 CAFs treated with vehicle (DMSO) or with

gliomas than CD44⁺/⁻ and CD44⁻/⁻ mice and had a much shorter survival time (25). Even though CD44s, CD44v6, and CD44v4-10 are detected in the human gut epithelium (Zeilstra et al. 2014), CD44v4-v10, but not CD44s, drives gut adenoma formation in APC/Min⁺ mouse models (Joosten et al. 2017; Zeilstra et al. 2014; Zeilstra et al. 2008), and among CD44v4-v10, CD44v6 appears confined to intestinal CSC/CICs (Zeilstra et al. 2014) and has a major role in the development of the intestinal epithelium (Joosten et al. 2017; Zeilstra et al. 2014). In familial adenomatous polyposis patients, among CD44v4-v10, CD44v6 is highly expressed in adenomas compared to normal crypts, and CD44s expression is decreased (Zeilstra et al. 2014). This appears to be true in APC/Min⁺ mouse models (Misra et al. 2009). As demonstrated, freshly isolated CD44v6⁺ cells were able to generate orthotopic tumors and metastases, while their CD44v6⁻ counterparts grew locally as small tumors, without forming distant metastatic lesions in mouse xenografts. Furthermore, the tumorigenic capacity of the CD44v6⁺ population was completely lost in secondary recipients, suggesting that CD44v6⁻ cells include transient amplifying progenitors and more differentiated non-tumorigenic cells, whereas metastatic CRC-CSCs are limited to the small CD44v6⁺ cell population (Todaro et al. 2014).

To understand the mechanism of CRC resistance to the clinically relevant chemotherapy combination (FOLFOX: fluorouracil [5-FU], oxaliplatin, and leucovorin), we developed a cellular model of FOLFOX resistance and screened seven CRC cell lines for CD44v6 expression (Fig. 7.3a) and generated FOLFOX resistant cells by using increasing concentrations of FOLFOX, where $1 \times \text{FOLFOX} = \text{IC}_{50} \text{ of } 5\text{-FU} + \text{IC}_{50} \text{ OXA} + \text{leucovorin}$. In these FOLFOX-resistant (FR) cells, CD44v6 was consistently enriched (Fig. 7.3b) for self-renewing, tumorigenic CICs. FACS analysis showed that a small population of our generated FOLFOX-resistant cells (FR) express increased levels of CD44v6 (abbreviated as CD44v6^{high+}CICs) that overlapped with cells that expressed the epithelial marker, EpCAM as well as ALDH1 and CD133 antigen expression (Figs. 7.3b, e) (Ghatak et al. 2022). These CD44v6^{high+} cells co-expressed higher levels of known CIC marker genes (Fig. 7.3d). Colon sphere formation assay evaluated the ability of CD44v6^{high+} CICs to be tumorigenic. Data in Fig. 7.3c show that CD44v6^{high+} CICs formed colon-spheres within 14 days at high efficacy compared with CD44v6^{low} non-CICs. When implanted into immunocompromised SCID mice, only 2×10^4 CD44v6^{high+} CICs formed tumors compared to 2×10^4 CD44v6^{low} non-CICs (Fig. 7.3f). Moreover, 2×10^4 CD44v6^{high+} CICs generated tumors in 80–90% SCID mice injected from six different HCT116-xenograft derived cells, whereas 2×10^5 CD44v6^{low} non-CICs did not show any tumor formation (Fig. 7.3g). Overall, these data suggest that CRC-CICs can be prospectively identified and characterized in the CD44v6^{high+} cell population, whereas CRC-non-CICs are CD44v6^{low} cell populations. Briefly,



Fig. 7.4 (continued) chemotherapy for 72 h were subcutaneously co-implanted in immunocompromised mice with 4000 CICs. Tumors were monitored every other day to evaluate the latency, harvested every 10 days, and volumes measured to evaluate the kinetics of growth

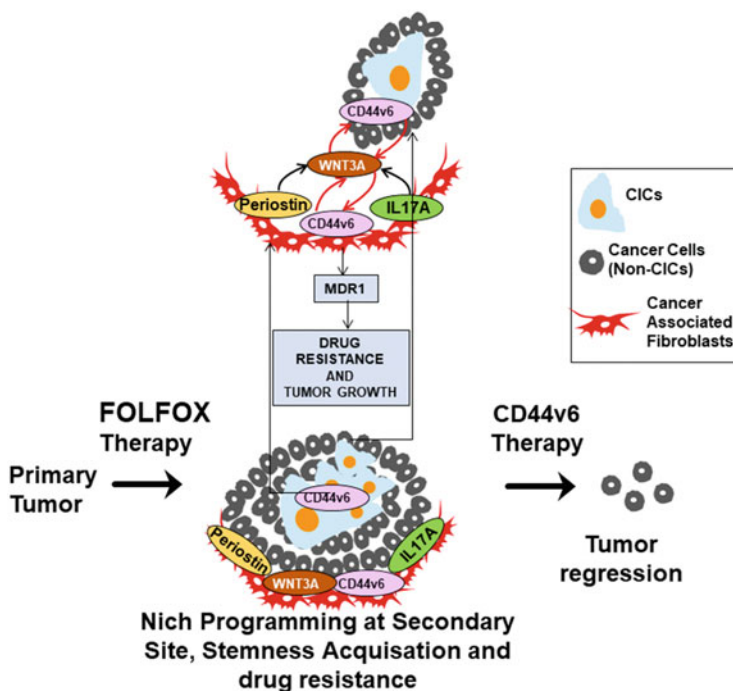


Fig. 7.5 CIC and cancer-associated fibroblast induced tumor progression and drug resistance. Colon cancer-associated fibroblast derived periostin and IL17A promote WNT3A induced CD44v6 expression in tumor cells and CICs. CD44v6 positive cancer-initiating cells (CICs) induce drug resistance and colorectal tumorigenesis by enhancing CD44v6/ β catenin nuclear localization and MDR1 gene expression in tumor cells and CICs. CD44v6 regulated IL17A, PN, and WNT3A derived from chemotherapy induced CAFs contribute to CICs maintenance through induction of CD44v6 receptor. This work identifies crosstalk between stromal cells and tumor CIC cells via IL17A, PN, and WNT3A during colorectal tumorigenesis

CD44v6 expression appears as part of a more complex system that confers colonization, self-renewal, and metastatic properties to CRC-CICs.

7.7 CD44/CD44v6 Is a Functional Marker for CICs

There are two models for tumor development. The Traditional Stochastic model envisions that any tumor cell type is able to repopulate and regenerate the tumor itself (Greaves and Maley 2012). The CIC model hypothesizes that only a subset of cells can generate a new tumor (Clevers 2011). CICs may originate from normal tissue stem cells by sharing many features, including self-renewal, differentiation, drug resistance, and migration capacity (Oliveira et al. 2010). Moreover, the long life of somatic stem cells makes them susceptible to alteration of genetic and epigenetic

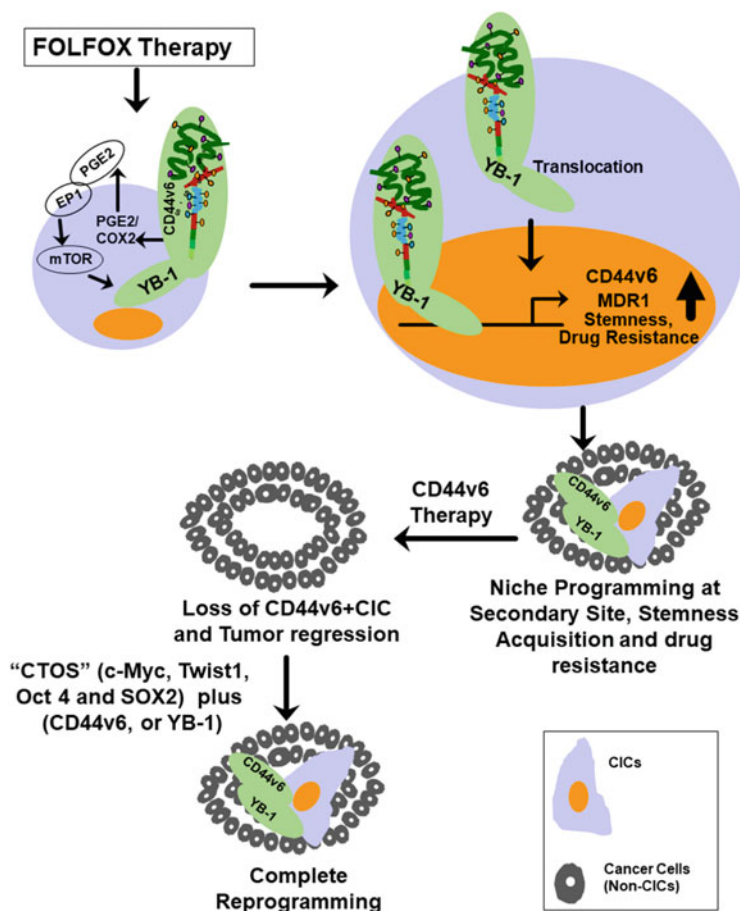


Fig. 7.6 Requirement of the CD44v6 mediated transcriptional modulation for maintaining the stemness of cancer-initiating cells and reverting differentiated cancer cells into cancer-initiating cells. FOLFOX therapy induces overexpression of CD44v6, and of YB-1, a key oncogenic transcription factor, in order to maintain stemness of CRC CICs. CD44v6-induced PGE2 is linked with mTOR signaling to induce YB-1 expression. CD44v6 is translocated into the nucleus and associated with stemness-associated transcription factors, including YB-1 in CICs, where YB-1 exerts its specific functions in CICs via CD44v6 and MDR1 DNA transcription. The resulting positive feedback loop couples YB-1 activation and CD44 alternative splicing to sustain CD44v6 and FOLFOX resistance through MDR1 expression. In CD44v6 Mu1 SW948-FR CICs, ectopic expression of the YB-1 and CD44v6-rescue plasmids enhanced expression of proliferation/antiapoptosis/invasion/stemness associated Cyclin D1, BCL2, FZD1, GINS-1, and MMP9 genes providing evidence that expression of these stemness related genes is mediated through CD44v6 and YB-1. Therefore, CD44v6-YB-1 signaling has an essential role in the activation and reversion of the pluripotency of differentiated cancer cells. Owing to their potential roles in chemoresistance, targeting CD44v6 significantly reduces tumor formation in vitro and in vivo. Thus, CD44v6 signaling could be a potential adjuvant target molecule combined with conventional chemotherapy to elevate the chemosensitivity of CRC

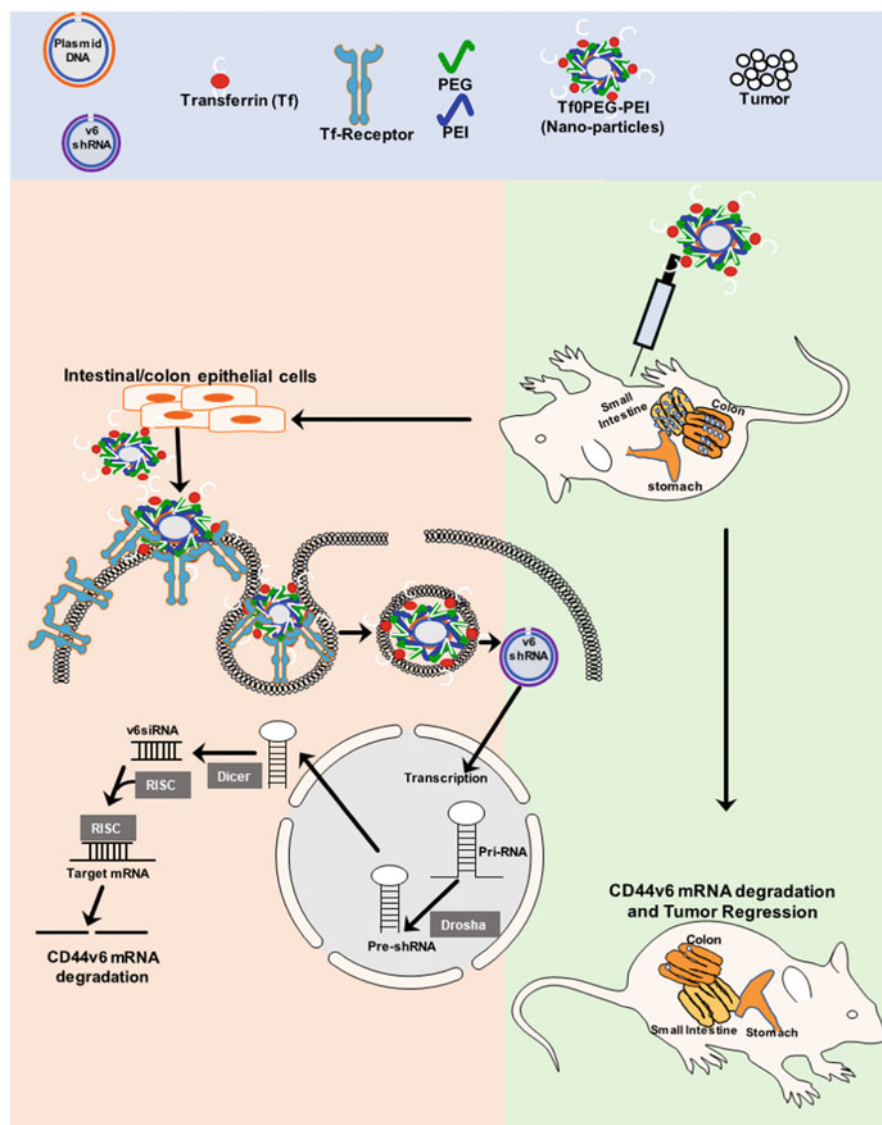


Fig. 7.7 Schematic illustration of cellular uptake of plasmid DNA/Tf-PEG-PEI (nanoparticles) polyplexes, their shielding from nonspecific interaction, and mechanism of action of shRNA. Internalization of PEG-shielded and Tf-R-targeted polyplexes into target cells occurs by receptor-mediated endocytosis after association of polyplex ligand Tf to Tf-R present on the target cell plasma membrane. Internalized particles are trafficked to endosomes followed by endosomal release of the particles and/or the shRNA vector into cytoplasm. The shRNA vector is transported into the nucleus and transcribed into pri-shRNA, which is further processed into pre-shRNA by the RNase III enzyme Drosha. With the help of exportin-5, pre-shRNA is transported to the cytoplasm and cleaved into a mature shRNA by Dicer. Released siRNA will be induced to RNA-induced silencing complex and will be guided for cleavage of complementary target mRNA in the cytoplasm. The siRNA (antisense) guide strand will direct the targeted RNAs to be cleaved by RNA

reparations in such a way that they become good candidates for the development of the neoplastic transformation (Tirino et al. 2013). Nevertheless, unlike somatic stem cells, CICs induce tumor formation and demonstrate tumorigenic activity when transplanted into animals (Natarajan et al. 2010; Prasetyanti and Medema 2017). Moreover, the variation in genetics and epigenetic damage in CRC patients is so diverse that markers to differentiate CICs from more differentiated progeny have not been fully informative for all patient tumors (Ogino et al. 2012; Sadanandam et al. 2013; Sanchez et al. 2009).

Regardless of the marker used, CICs are the parental source from which all other malignant cells within a given tumor arise and are responsible for CRC tumor growth/maintenance, metastatic spread, resistance to conventional chemotherapies, and recurrence after cancer therapy (Brabletz et al. 2005; Valent et al. 2012). Most CIC enhancement markers mediate interactions between a tumor cell and its stromal environment, indicating that tumorigenic features associated with that marker may be lost after depletion of CICs from their microenvironment. Similarly, human pancreatic CICs expressing the marker CD133 and the chemokine SDF-1 receptor CXCR4 lost their metastatic activity when the CXCR4+/CIC population was depleted, implying that CICs may take over marker-associated information (in this case, SDF-1/CXCR4-controlled signaling pathways) to initiate metastasis (Hermann et al. 2007). The cell-surface marker CD133 has been used to selectively identify CRC-CICs (O'Brien et al. 2007; Ricci-Vitiani et al. 2009). However, another group identified that CD133 expression was also common for differentiated, non-CICs and characterized a CD133^{negative} CD44⁺ population of CICs that behaved more aggressively than CD133+/CICs (Shmelkov et al. 2008). Additional studies have also reported that CRC cells expressing CD166 (Vermeulen et al. 2008b), CD44 (Ohata et al. 2012), CD44v6 (Todaro et al. 2014), CD66c (Vermeulen et al. 2008b), and aldehyde dehydrogenase (ALDH1) (Huang et al. 2009) also have CRC/CIC characteristics.

CD44v6-positive (+) CICs have been associated with increased metastatic potential in both pancreatic cancer (Gherardi et al. 2012; Hermann et al. 2007; Zoller



Fig. 7.7 (continued) endonuclease. Finally plasmid/Tf-PEG-PEI-nanoparticles delivery in the target cell shows reporter gene expression/activity and CD44v6 mRNA degradation

This approach of delivering CD44v6shRNA in mice bearing intestine/colon involves: (1) a key (a colon-specific Fabp1-promoter controlling Cre-recombinase expression), (2) a lock (conditionally silenced pSico-CD44v6shRNA that when acted upon by Cre-recombinase is unfloxed, thereby allowing expression of CD44v6shRNA); and 3) a target–transferrin (Tf)-tagged nanoparticle that places the lock and key plasmids in proximity and protects these plasmids from degradation in vivo. Tf helps targeting the nanoparticles to colon tumor cells because the Tf-receptor is 100-fold over expressed in tumor cells than in normal colon cells (Prost et al. 1998; Shinohara et al. 2000). When these nanoparticles are injected systemically into *Apc Min/+* mice, or a mouse bearing a xenograft tumor, they seek out Tf-receptor-positive cells (such as cells undergoing transformation) and express Cre-recombinase ONLY in cells of intestine/colon origin. This results in the sequential expression of CD44v6shRNA, knockdown of CD44v6 expression, and inhibition of CD44v6 signaling, which induces apoptosis of transformed cells and the prevention of tumor initiation

2009; Zoller 2011) and CRC (Todaro et al. 2014), suggesting that CICs may appropriate the apoptosis resistance and migration signaling pathways regulated by CD44v6 to initiate metastasis. Experiments in knock-in mice expressing CD44v4-10 or CD44s isoforms have shown that CD44v isoforms, but not the CD44s isoform, promote adenoma formation in Apc/Min/+ mice (Zeilstra et al. 2014). Thus, CD44v6 predicts poor prognosis and is a marker for constitutive and reprogrammed CICs that drive CRC metastasis (Todaro et al. 2014). CD44v8-10 is a specific marker for gastric CICs (Lau et al. 2014), and CD44v3 is a specific CIC marker of head and neck cancers (Bourguignon et al. 2012b). These studies provide supporting examples that various CD44v isoforms are especially expressed on cancer cells and are required for tumorigenesis and progression. Thus, the CD44v6 isoform is likely to be a better CIC marker than the CD44s isoform in CRCs. We have demonstrated that cell-surface marker CD44v6 identifies CRC-CICs that have cell-autonomous drug resistance and have a functional role in the signaling events between CICs and the microenvironment (Ghatak et al. 2022). Thus, the resistance to chemotherapeutic treatment in CRC is initiated by CICs through the expression of CD44v6, which is both a functional biomarker and therapeutic target (Ghatak et al. 2022) (Fig. 7.5).

7.8 Role of CD44/CD44v6 in Interaction of CICs-Niche with Tumor Microenvironment

CICs residing in niches of the tumor microenvironment regulates CIC fate by providing signals in the form of both cell–cell contacts and secreted factors (Sneddon and Werb 2007; Voog and Jones 2010). Niches have been identified for mammalian CICs in various epithelial tissues including intestine (Voog and Jones 2010). CICs exist in interactive niches that are specialized microenvironments comprised of various cell types including fibroblasts (Medema and Vermeulen 2011), immune cells (Holzel et al. 2013), endothelial cells (Lu et al. 2013), and perivascular cells (Korkaya et al. 2011) or their progenitors (Korkaya et al. 2011). Each cell type supports the CIC resistance, recurrence, and maintenance. Mechanisms driving CIC maintenance and resistance are controlled by cell–cell interactions mediated through various mechanisms that involve extracellular matrix components and networks of growth factors, cytokines, and chemokines (Korkaya et al. 2011; Li et al. 2012; Todaro et al. 2014; Vermeulen et al. 2010). Cytokines and chemokines function as both paracrine and autocrine factors, supporting tumor microenvironment secreted molecules as ideal mediators of interactions between the CICs and other tumor cellular components. As mentioned above, CD44v6 increases pathologically in the gut of the APC/Min+ mouse model (Zeilstra et al. 2014). Tumorigenic conditions and chemotherapy stimulate CD44 gene alternative splicing, resulting in the existence of several CD44 variants within tumors cells (Ghatak et al. 2022; Naor et al. 2002; Prochazka et al. 2014). Among these isoforms, CD44v6 exhibits higher affinity to HA compared to CD44s (Ghatak et al. 2022;

Misra et al. 2011). Functionally, hyaluronan interacting with CD44v6 regulates multiple receptor tyrosine kinase stimulated cell survival pathways (Misra et al. 2009; Misra et al. 2011; Misra et al. 2008b; Misra et al. 2006; Orian-Rousseau 2010; Orian-Rousseau and Sleeman 2014). In addition, CD44v6 stimulates HA synthase 3 gene expression in a feedback manner (Adamia et al. 2005). Furthermore, the CD44v6-ectodomain can bind to growth factors/cytokines, such as HGF (Orian-Rousseau et al. 2002), VEGF (Tremmel et al. 2009), EGF (Cheng et al. 2006), and TGF β 1 (Ghatak et al. 2014; Ghatak et al. 2017b) and potentiates RTK-associated signaling pathways where CD44v6 acts as a co-receptor. In these studies, knock-down of CD44v6 down regulated CD44v6-downstream PI3K/MAPK pathways resulting in antiapoptotic signaling. Thus, in a recent unpublished study (Ghatak et al. 2022), we focused on CD44v6 expression in CRC/CICs because it functions as a signaling platform that responds to microenvironmental paracrine cues through growth factor/cytokines/matricellular proteins and transduces these signals to membrane-associated proteins that induce a variety of genes related to apoptosis resistance (Bourguignon et al. 1997; Ghatak et al. 2017b; Misra et al. 2006; Orian-Rousseau 2010; Orian-Rousseau et al. 2002; Tremmel et al. 2009) including ABC transporters like MDR1 (Misra et al. 2005; Misra et al. 2003; Orian-Rousseau and Sleeman 2014; Safa 2016). CD44v6 also engages with WNT signaling (Schmitt et al. 2015), and in CRC, the hyaluronan-CD44v6 interaction induces β -catenin function (Misra et al. 2008a; Misra et al. 2008b).

We showed that CICs were significantly less sensitive to FOLFOX treatment, whereas non-CICs showed a decrease in relative viability (Fig. 7.4a). To further confirm that CICs are antiapoptotic phenotype, we quantified apoptosis and found that FOLFOX-induced apoptosis was significantly reduced in the CICs relative to non-CICs (Fig. 7.4b). The relative resistance of CICs to chemotherapy provides evidence that CICs may be enriched after chemotherapy (FOLFOX) treatment. Indeed, the CIC phenotype (CD44v6^{high+} cells) was increased 1.7–4-fold after FOLFOX treatment in four different sensitive and FOLFOX-resistant CRC cell types (Fig. 7.3b). These results (Fig. 7.4a–c, and Fig. 7.3b) provide evidence for a cell-autonomous chemoresistant phenotype of CRC-CICs, which are CD44v6^{high+} cell populations. The predominant function of CD44v6 in apoptosis resistance and in metastasis led us to test the potential role of paracrine signaling between cancer-associated fibroblasts (CAFs) and CD44v6+/CICs in modulating WNT/TCF-mediated MDR1 gene expression and whether WNT signaling regulates CD44v6 splicing to sustain MDR1 expression for FOLFOX resistance (Ghatak et al. 2022). In this study, we isolated viable CAFs from freshly resected xenograft (SQ) tumor tissues by FACS using platelet-derived growth factor receptor- α (PDGFR α) and an EpCAM antibody. These SQ tumor-derived CAF cultures expressed activated fibroblast markers, including PDGFR α , FAP, and FSP1 via QPCR analyses, but negligible epithelial cell marker EPCAM expression (Ghatak et al. 2022). Co-culture of FOLFOX-treated CAFs increased the viability of CICs compared to control CAFs (Fig. 7.4c). As interactions between CICs and CAFs can be mediated through paracrine factors that regulate the tumor hierarchy, we investigated whether the FOLFOX-induced CAFs secrete factors that are necessary to maintain CICs and

found that conditioned media from CAFs also increased the growth of CICs (Ghatak et al. 2022). More importantly, FOLFOX preferentially induces CAFs to produce and secrete periostin, WNT3A and IL17A significantly (Fig. 7.4c–e). To determine the effects of CAFs on CIC induced tumor growth in vivo, we implanted 4,000 CICs alone or in combination with CAFs pretreated with FOLFOX or vehicle control. In accordance with cell culture results in Fig. 7.4c, FOLFOX-treated CAFs enhanced the ability of CICs to initiate tumors in SCID mice (Fig. 7.4g). The FOLFOX-treated CAFs increased tumor incidence and reduced the latency of tumor formation by CICs with increased tumor weight/volume (Fig. 7.4g–h). Thus, while there are multiple pathways to explain why tumors become resistant to chemotherapeutic approaches such as FOLFOX, we propose that a common denominator is the cell-surface marker CD44v6 that identifies CRC-CICs and is highly expressed in CAFs (unpublished work (Ghatak et al. 2022)). In this work, we identified crosstalk between stromal cells and tumor cells via periostin, WNT3A, IL17A, and CD44v6 during colorectal tumorigenesis. We suggest that cellular niches that are normally involved in the regulation of stem cell functions are abnormally recruited to participate as cancer-initiating cells (CICs) at sites of primary tumor formation. Conventional chemotherapy (FOLFOX) that kills most cancer cells but not CICs which remain viable. Chemotherapy involves further activation and/or expansion of the niche in parallel to the increase in numbers of CD44v6 positive CICs. Upon extravasation, circulating CICs can enter secondary sites of tumor tissue and, together with CAFs “reprogram” these niches to become secondary niche, resulting in the cancer returning. Periostin and IL17A secreted by CAFs contribute to the tumorigenic reprogramming of secondary niches by upregulating the expression of CD44v6 through a reciprocal inductive interaction between CICs and CAFs through a WNT3A signaling mechanism. The elevated expression of CD44v6 is shown to directly regulate CIC maintenance. Not all potential secondary tissue sites can be expected to fully facilitate generation of tumor niche, which would be consistent with a non-random distribution of metastases in organs. The returning tumor can then be expected to demonstrate drug resistance due to over production of CD44v6 via its downstream WNT3A/ β -catenin/TCF4 mediated MDR1 and CD44v6 gene expressions. Therapeutic intervention can be done in two ways. Rather than attacking all of the tumor cells or the elements secreted CAFs at once (as is the case for traditional cancer therapy), we propose a strategy in which tissue-specific delivering therapeutic drugs that are targeted to CD44v6+CICs would slowly shrink the tumor, since the CICs would no longer be able to replenish (or reprogram) cells that naturally die within the cancer niche. Thus, in this strategy, interfering specifically with CD44v6 functions (by intraperitoneal delivery of shRNA/Nanoparticles) would inhibit CD44v6 of both CICs and CAFs and CD44v6-dependent secretion of WNT3A. However, as this strategy might take time to eradicate the tumor completely, a faster approach could be to employ a combination of targeted CD44v6 therapy along with blocking stromal periostin, IL17A, and WNT3A in CRC where these factors are functionally important (Figs. 7.5, 7.7, and 7.8) (Ghatak et al. 2022).

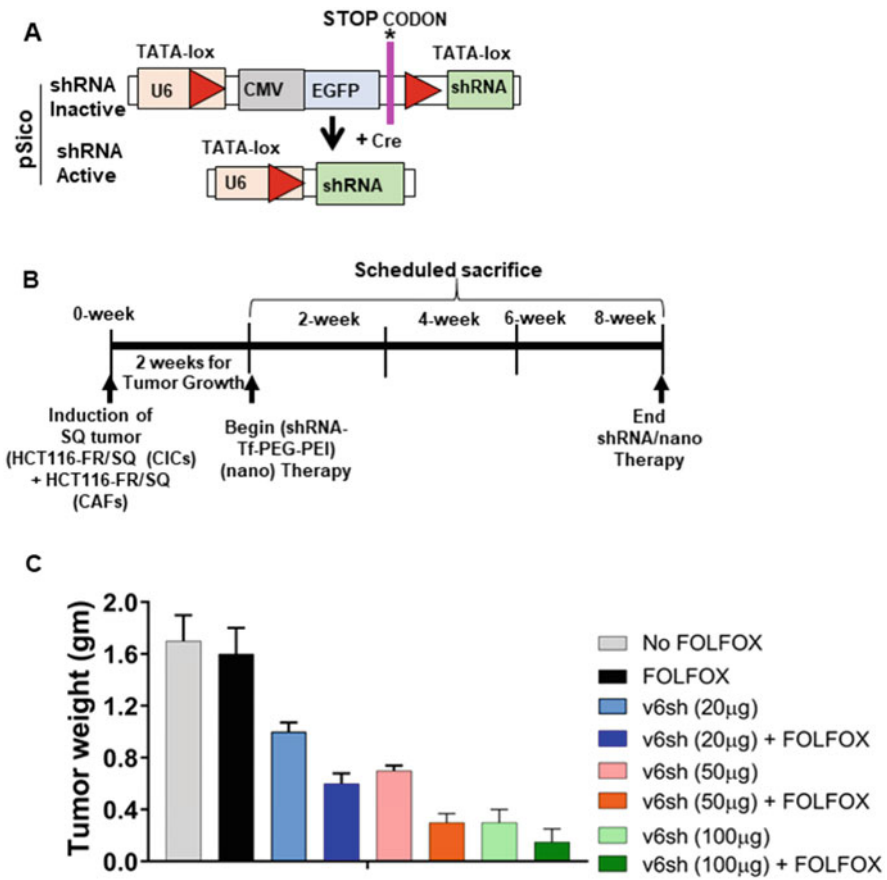


Fig. 7.8 Tissue-specific knockdown of CD44v6 by pFabpl-Cre inhibits tumor growth of implanted HCT116-FR/SQ/CICs plus HCT116-FR/SQ/CAFs in immune compromised mice. (a), Schematic representation of pSico after Cre-mediated recombination to synthesize active shRNA is shown. (b), Timeline is shown for pSico-v6-sh/Nano treatment with and without pFabpl-Cre/Nano in xenograft tumors derived from HCT116-FR/SQ tumor CICs plus HCT116-FR/SQ tumor CAFs. The dependence of CICs and CAFs on CD44v6 was evaluated in vivo. 2×10^4 CICs and 6×10^4 CAFs were injected into mice. When tumors were established to approximately 0.3 cm^3 in volume, treatment was initiated. Seven SCID mice per group were used. Mice were weighed every other day and the tumor weight was measured every week for 4 weeks. (c), Kinetics of relative tumor weights with time during in vivo SQ tumor growth at the indicated weeks generated by HCT116-FR SQ CICs plus HCT116-FR SQ CAFs treated with pSico-NT sh/Nano, pSico-v6 sh/Nano \pm pFabpl-Cre/Nano are shown

7.9 Role of CD44/CD44v6 in Transcriptional Modulation Through Regulation of Transcription Factors

Although CICs and somatic stem cells have similar transcription factors (TFs), including SOX2, OCT4, NANOG, KLF4, and c-Myc, several studies have shown that abnormal expression of some distinctive TFs (LaTulippe et al. 2002) plays a crucial role in the reprogramming of differentiated cancer cells (Padua et al. 2020). Distinctive TFs (LaTulippe et al. 2002) play a crucial role in the reprogramming of differentiated tumor cells (Padua et al. 2020). In this case, overexpression of TFs results in dysregulation of associated signaling pathways that are linked to the lineage and differentiation phenotype of CICs (Farabaugh et al. 2015). Transcriptional regulation occurs when certain TFs bind to DNA at promoter binding sites and influence the transcription of regulated genes through interactions with their gene promoters (Farabaugh et al. 2015; Padua et al. 2020). In this circumstance, TFs may play a crucial role in maintaining the stemness of CIC. Networks of transcription factors may be involved in gene regulatory networks, which consist of epigenetic, topological, and transcriptional regulation. Epigenetic regulation of chromatin involves irretrievable change of differentiated status of a cell under normal conditions (Perino and Veenstra 2016). Topological regulation of chromatin controls global gene expression models (Acemel et al. 2017), whereas TF network dictates cell phenotypes when expressed in various settings in an ectopic manner (Morris 2016; Niwa 2007; Niwa 2018). Several core transcription factors, including TWIST1, Snail, Zeb 1 (Beck and Blanpain 2013; Peinado et al. 2007) as well as OCT3/4 (Nichols et al. 1998; Niwa et al. 2000), Sox2 (Avilion et al. 2003), and Nanog (Chambers et al. 2003; Mitsui et al. 2003) have a critical function in maintenance of pluripotency in both early embryos, and in ES cells, adult stem cells, and CICs.

The CIC subpopulation can differentiate into non-CIC tumor cells promoting phenotypic and functional heterogeneity in the tumor. In 2006, Shinya Yamanaka's laboratory described that terminally differentiated fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSCs) using four transcription factors OCT4, Sox2, KLF4, and c-Myc, initiating several synergistic processes (Takahashi et al. 2006; Takahashi et al. 2007; Takahashi and Yamanaka 2006). Thus, any somatic cell can be reprogrammed into iPSCs by co-expression of fundamental stemness-related transcription factors specific to a cancer cell type. TFs can act in collaboration to regulate the expression of discrete genes specific for maintaining pluripotency and self-renewal of stem cells (Aoi et al. 2008; Avery et al. 2006; Masui et al. 2007; Yu et al. 2007). These tumor type-specific factors maintain the dynamic balance between CICs and differentiated cells. Dedifferentiation can alter this balance resulting in metastasis/aggression as CICs are resistant to chemotherapy and radiation. Our recent study showed that knockout of CD44v6 induced differentiation of CICs, which reduced their ability to form spheres and inhibited xenograft tumor growth by downregulation of CIC-related stemness genes. However, rescuing CD44v6 alone in CD44v6-knockout CICs did not reprogram differentiated cells

into CICs, in agreement with previous studies (Takahashi et al. 2007; Takahashi and Yamanaka 2006). Our results revealed that CD44v6-knockout CICs significantly promoted the expression of differentiation genes (CK20 and MUC2) (Ghatak et al. 2021). We also revealed that the simultaneous expression of c-Myc, Twist1, Oct 4 and SOX2 [CTOS] and a CD44v6-rescue plasmid restored CD44v6-knockout CICs. This shows that differentiated cells can be reprogrammed into CICs through the combined expression of CTOS-TFs and CD44v6 (Ghatak et al. 2021). This study also showed that YB-1 induced by CD44v6 is required for the reversion of differentiated tumor cells into CIC. Overall, we can conclude that after FOLFOX stimulation, internalized CD44v6 complexes with YB-1 and the combined signaling complex reaches the nucleus, where YB-1 regulated by CD44v6 stimulates the MDR1 and CD44v6 gene promoters. This supports resistance to FOLFOX and tumor formation through overexpression of CICs by CD44v6. Therefore, CD44v6-YB-1 signaling plays a key role in the activation and reversion of differentiated tumor cells into CICs (Ghatak et al. 2021) (Fig. 7.6).

7.10 CD44/CD44v6 as a Biomarker

As mentioned above, CD44v isoforms are overexpressed in various cancers, especially in CICs. Thus, CD44 studies have drawn much attention and indicate that CD44v6 could be clinically exploited both as a biomarker and a therapeutic target in CRC. A meta-analysis of 3,098 patients showed that CD44s is not a prognostic marker in colorectal cancer in terms of 5-year overall survival rates, histologic grade, and metastasis, whereas CD44v6 is associated with poor overall survival (Fan et al. 2012; Saito et al. 2013). Moreover, another multivariate study showed a significant negative correlation between CD44v6 expression and survival probability of CRC patients (Todaro et al. 2014). This study also showed that HGF, OPN, and SDF-1 induce the expression of CD44v6 in immature CRC cells. Therefore, the possible correlation of these cytokines with CD44v6 in tumor specimens from CRC patients has been addressed in our laboratory. The analysis of ten patients showed that hepatocyte growth factor, osteopontin, and stromal-derived factor 1 α are present at higher levels in tumors with increased number of CD44v6+CICs as compared with tumors with fewer or no CD44v6+CICs, indicating that the three cytokines contribute to increase the number of CD44v6+ CICs in tumors of CRC patients (Todaro et al. 2014). Our recent study demonstrated that cell–cell interactions such as those between CRC-CICs and colon cancer-associated fibroblasts can be mediated through paracrine factors (periostin, IL-17A, and WNT3A) that regulate and maintain the colon tumor hierarchy (CICs) (Ghatak et al. 2022). Thus, CRC-CICs overexpress CD44v6, which is a functional marker able to confer the ability to stimulate CRC-chemoresistance.

7.11 CD44/CD44v6 as Therapeutic Target

7.11.1 *Inhibition of Hyaluronan /CD44 Interactions*

Four different methods were used to address the mechanism of constitutive hyaluronan/CD44 interactions and the consequent outcomes in cancer cells. The first method used small hyaluronan oligosaccharides (O-hyaluronan; ~2.5 kDa) that compete with the endogenous hyaluronan polymer (Ghatak et al. 2002) and temporarily inhibited tumor growth in vivo (Zeng et al. 1998; Ghatak et al. 2002). The second method involves the expression of soluble hyaluronan-binding proteins (e.g., soluble CD44) that act as competitive decoys for CD44 and thus bind to endogenous hyaluronan inhibiting carcinoma cell growth and suppressing lung nodule formation (Ghatak et al. 2002; Peterson et al. 2000; Yu et al. 1997).

The third method blocks hyaluronan/CD44 interaction specifically by a blocking antibody against the hyaluronan-binding site of CD44 to inhibit tumor growth in preclinical animal models of human xenograft tumors (Ghatak et al. 2002). Over the past decades, various monoclonal antibodies (mAb) and antibodies against CD44 or CD44v isoforms have been shown to exhibit significant antitumor activity in vitro and in xenograft tumors. Administration of the mouse mAb-H90 directed against human CD44 reduced leukemia tumor growth in a xenograft model transplanted with human acute myelogenous leukemia cells. The absence of leukemia tumor in serially transplanted mice demonstrated that this mAb-H90 directly targets leukemia stem cells (Jin et al. 2006). Furthermore, administration of another mouse mAb-p245 directed against human CD44 substantially reduced tumor growth in xenograft model transplants with human triple-negative basal-like breast cancer cells (Marangoni et al. 2009). Since CD44 isoform expressions, especially CD44v6 expression, are significantly high in advanced stages of carcinomas (Naor et al. 2002; Misra et al. 2011; Orian-Rousseau 2010), two monoclonal antibodies (VFF18 [BIWA 1] and U36) for human CD44v6 were investigated. BIWA was derived from mice injected with the human CD44v6 (Heider et al. 1996) and U36 was obtained from a screen for specific epitopes expressed on human head and neck carcinoma cells and is also specific for CD44v6 (Van Hal et al. 1996; Schrijvers et al. 1993). On the basis of the promising preclinical antitumor studies, bivatuzumab (a humanized monoclonal antibody against CD44v6) was labeled with ¹⁸⁶Re or conjugated with the non-radioactive cytotoxic drug mertansine, an anti-microtubule agent that was first selected for clinical trials in patients with incurable squamous cell carcinoma of the head and neck or with esophagus cancer (Sauter et al. 2007). In the phase I trial, although bivatuzumab-mertansine showed antitumor effects, the clinical trial studies were ended due to serious skin toxicity development in non-tumor areas as well as death of a patient due to development of toxic epidermal necrolysis.

The fourth approach adoptive cell therapy was developed to target tumor antigens. In this approach a chimeric antigen receptor on CAR-T cells was genetically engineered to express receptors directed against the tumor antigen, such as CD44v6, and this adoptive therapy was used to target CD44v6 bearing tumor cells. This

method utilizes the binding moiety of a high-affinity CD44v6 monoclonal antibody cloned into a CAR-T cell receptor lentiviral vector (scFv-LNGFR-CD28-CD3z) where scFV is single-chain antibody, LNGFR is linker peptide, CD28 provides costimulatory signal for T cells, and CD3z is one of the components of T-cell receptor-CD3 complex. These engineered T cells can detect surface antigens like CD44v6 without the need for antigen presentation via a major histocompatibility complex. Binding of antigen by the CAR T-cell results in activation and release of cytotoxic molecules that kill the engaged tumor cell. This approach eliminated CD44v6 positive acute myeloid leukemia and multiple myeloma cells in murine xenografts (Casucci et al. 2013). The limitation of this approach is the off-tumor/on-target toxicity due to the expression of the tumor antigen on normal tissue.

7.11.2 CD44v6 Peptide-Approach

The identification of CD44v6 in the activation of RTKs such as Met and VEGFR-2 led to the identification of CD44v6 peptides that inhibit HGF/c-Met (Matzke et al. 2005) and VEGF/VEGFR2 (Tremmel et al. 2009). These peptides with the minimal length of five amino acids interfere with the co-receptor function of CD44v6 and inhibit vascularization of pancreatic tumors (Matzke et al. 2005; Tremmel et al. 2009). Further, we developed a CD44v6-mimetic peptide to suppress CD44v6 signaling in preclinical models of TGF β 1-mediated lung fibrosis (Ghatak et al. 2017b) and to inhibit colorectal tumor growth and drug resistance (Patent Pending). These peptides with the minimal length of 10-14 amino acids with the co-receptor function of CD44v6 with TGF β R1 inhibit fibrogenic fibroblast activation, apoptotic resistance and suppress 30% of lung fibrosis in a preclinical model of lung fibrosis. In addition, these peptides inhibit colon tumor growth by interfering co-receptor function of CD44v6 by inhibiting CD44v6-Sphingosine receptor 1 signaling (Patent Pending).

7.11.3 Tissue-Specific Inhibition of CD44 Expression

Instead of inhibiting functions of CD44v6 protein by antibody treatment, tissue-specific inhibition of CD44v6 expression is a powerful method to knock down CD44v6 by using specific shRNA against CD44v6 and delivering it tissue specifically into the tumor cells. The technique of using shRNA in an expression vector is an alternative strategy to stably suppress the expression of a selected gene, which suggests that the use of shRNA expression vectors is potentially promising for therapeutic approaches to silence disease-causing genes (Paul et al. 2002). There are two ways to deliver shRNA into cancer cells: using a viral vector or a non-viral vector. Viral vectors have been used to achieve this proof of principle in animal models and, in selected cases, in human clinical studies (Raper et al. 2003). Systemic

targeting of viral vectors to the desired tissue is difficult because host immune responses activate viral clearance. Systemic administration of a large amount of adenovirus (e.g., into the liver) can pose a serious health risk and has even caused the death of a patient (Raper et al. 2003). Nevertheless, there has been considerable interest in the development of non-viral vectors for gene therapy. In this regard, non-viral vectors, such as positively charged and screened polyethyleneimine (PEI) complexes with polyethylene glycol (PEG), can be safely used to avoid nonspecific interactions with non-target cells and blood components (Kursa et al. 2003). Non-viral vectors were once limited due to their low gene transfer efficiency. However, the incorporation of various ligands, such as peptides, growth factors and proteins, or antibodies to highly expressed targets on tumor cells, circumvented this obstacle (Bellocq et al. 2003). Furthermore, the increased permeability due to aberrant vascularization in solid tumors and the retention (known as the EPR effect) of ligand-coated vectors around tumor cell receptors may increase the chances of a high probability of interaction with cells (Misra et al. 2009). Therefore, non-viral vectors can acquire a high gene transfer efficiency (Naor et al. 2008). This concept was tested by preparing non-viral vector nanoparticles with plasmids packed within an outer layer of PEG-PEI coated with transferrin (Tf), an iron-transporting protein (Bellocq et al. 2003; Misra et al. 2009) that binds to Tf receptors (Tf-R) with high affinity. Tf-R is present at much higher levels in tumor cells (Misra et al. 2009) than in phenotypically normal epithelial cells. The association of transferrin with nanoparticles significantly increases the transfection efficiency of shRNA-generating plasmids, promoting nanoparticle internalization into dividing and non-dividing cells via receptor-mediated endocytosis (Bellocq et al. 2003). Finally, uptake of nanoparticles carrying multiple functional domains (Tf-PEG-PEI surface-screening particles, shRNA-generating plasmids, tissue-specific promoter-driven Cre-recombinase and conditionally silenced plasmid) can overcome intracellular barriers to successful shRNA gene delivery.

We have also developed *in vivo* approaches to deliver shRNA to specific cellular populations to suppress CD44v6 expression (Misra et al. 2009). This cell-specific shRNA delivery approach (Misra et al. 2009) confirmed that targeting the hyaluronan/CD44v6-induced signaling pathway inhibited distant colon tumor growth in *Apc Min/+* mice.

In this study, we engineered two unique vectors and encapsulated them into transferrin-coated nanoparticles. One vector (pSico) contained the shRNA for CD44v6 that was preceded by a stop codon flanked by two LoxP sites resulted in a construct that is inherently inactive. The second vector expressed Cre-recombinase under control of a tissue-specific promoter for FABPL, which is a gene uniquely expressed in intestinal epithelium. When nanoparticles (Transferrin-PEG-PEI) containing these two vectors are injected intraperitoneally, there is ubiquitous cellular uptake. However, only cells expressing FABPL (e.g., intestinal epithelium) will activate the Cre-recombinase and express the CD44v6 shRNA. We demonstrated the efficacy of this approach in suppressing CD44v6 expression and reducing CD44v6 + CICs tumor growth developed in a xenograft model bearing CICs plus cancer-associated fibroblasts isolated from a fresh patient derived colon tumor

(Ghatak et al. 2022; Ghatak et al. 2021) as well as in Apc/Min mice (Misra et al. 2009) (Figs. 7.7 and 7.8). After verifying this approach in Apc/Min mice (Misra et al. 2009), in an unpublished study, we tested the use of these nanoparticles *in vivo* and examined the therapeutic potential of targeting CD44v6 in tumors transplanted in mice using FOLFOX-resistant CICs plus CAFs. We first established SQ tumors by injecting HCT116-FR/CICs, or SW480-FR/CICs in combination with their CAFs subcutaneously in immunocompromised mice (experimental timeline is presented in Fig. 7.8. Cre-mediated conditional silencing of endogenous CD44v6 was used in combination with FOLFOX therapy. We have shown that periostin or IL17A interaction with WNT regulates CD44v6 and MDR1 expression (Ghatak et al. 2022) (Fig. 7.4a–c), and these stromal factors are required for CIC maintenance (Fig. 7.4d–h). Thus, we determined whether silencing the expression of CD44v6 and CD44v6-containing variants by using the Tf-nanoparticle delivery of CD44v6 shRNA can inhibit the tumor cell survival and growth by reducing WNT-CD44v6-MDR1 signaling. Administration of Cre-mediated CD44v6-shRNA nanoparticles prevented tumor growth and inhibited CD44v6 and MDR1 expression in a dose-dependent manner (Ghatak et al. 2022) (Fig. 7.8c). Moreover, 100 µg/ml plasmid concentration + FOLFOX had the predominant effect in reducing tumor growth (Fig. 7.8c) and inhibiting MDR1 expression (Ghatak et al. 2022). shRNA knock-down of CD44v6 in SQ tumors depended on the inclusion of a pFabpl-Cre plasmid, whereas substitution with a pARR2-Probasin-Cre plasmid, which is specific for prostate tissue, could not induce recombination of the pSico-CD44v6-shRNA plasmid, further demonstrating tissue specificity of this delivery system (Ghatak et al. 2022). Importantly, human pSico-v6-shRNA/Nano i.p. administration showed no evidence of toxicity within these experiments or in previous studies using CD44v6 shRNA nanoparticles (Ghatak et al. 2017b; Ghatak et al. 2017a). These results provide direct relevance for CD44v6 as a therapeutic target to maintain FOLFOX sensitivity and reduce CRC growth by reducing MDR1 expression.

This new approach opens up new perspectives in the fight against cancer and the understanding of tumorigenesis *in vivo* for the following reasons: (1) cell-specific shRNA release through the application of a Cre-lox mechanism driven by a tissue-specific promoter, (2) silencing of the expression of the selected CD44 variant in tumor cells of the target tissue, (3) no effect on normal cells of the target tissue, which do not express CD44v6, and rely on the CD44s (standard) form, which is not affected by the plasmids, (4) target CD44v6shRNA is not expressed in other cell types because the tissue-specific promoter unblocks Cre-recombinase only in the target tissue cells thus reducing potential side effects (Misra et al. 2009), (5) the plasmid-carrying nanoparticles are biodegradable and are eliminated from the system, (6) addresses the pathophysiological role of hyaluronan–CD44v interactions in cancer, (7) can establish diagnostic markers for targeted cancer, including CD44v6, soluble CD44, and hyaluronan, and (8) can establish CD44v/hyaluronan interactions as a novel therapeutic target against cancer progression. Therefore, conditional suppression of gene expression by a plasmid expressing CD44vshRNA is potentially promising for therapeutic approaches aimed at silencing CD44v signaling and thus

its downstream signaling that promotes disease-causing genes (Picker et al. 1989) (Figs. 7.7 and 7.8).

7.12 Significance

Hyaluronan receptor CD44 is a common component of the CIC niche and exists as a CD44s and a range of alternately spliced isoforms of CD44. CD44 alters signaling when it primarily interacts with hyaluronan, whereas CD44v modulates signal transduction through its co-receptor function regulating the signals originating from a group of receptor tyrosine kinases or serine-threonine kinases including c-MET, EGFR, VEGFR, and TGF β family. The function of CD44 in normal hematopoietic stem cell has been studied in detail, whereas the knowledge of CD44v6's novel function in intestinal stem cell niche, in transcriptional modulation where CD44/CD44v6 acting as transcription modulator remains limited. In this perspective, we reviewed the role of CD44, especially CD44v isoforms in : (1) defining CIC niche (Ghatak et al. 2022), (2) maintenance of stemness of CICs (Ghatak et al. 2022), (3) reversion of differentiated cancer cells into the pluripotent state (Ghatak et al. 2021), (4) induction of cell–matrix interactions, signal transduction during tumor progression, and (5) significance of using CD44/CD44v6 as biomarker and therapeutic targets were discussed. Our unpublished results suggest that chemotherapy can have both favorable and unfavorable consequences due to effects on enriching CD44v6^{high}+CICs that shows autonomous resistance, modulates the niche microenvironment through the effects of CAFs secreted factors on CICs. Furthermore we discussed the role of CD44v6 in disseminated CICs that can lead to chemoresistance and metastasis (Fig. 7.5) (Ghatak et al. 2022). Thus, if CICs can be identified and isolated using CD44v6 functional marker, then we should be able to conclude that CD44v6 is diagnostic biomarker and therapeutic target. A possible therapy using CD44v6 peptides especially using tissue-specific delivery of CD44v6 shRNA which will knock down the functions of its co-receptors and block the tumor growth, chemoresistance, and metastatic spreading might propose a new therapeutic route with fewer side effects.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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References

- Acemel RD, Maeso I, Gomez-Skarmeta JL (2017) Topologically associated domains: a successful scaffold for the evolution of gene regulation in animals. *Wiley Interdiscip Rev. Dev Biol* 6(3). <https://doi.org/10.1002/wdev.265>
- Adamia S, Maxwell CA, Pilarski LM (2005) Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer. *Curr Drug Targets Cardiovasc Haematol Disord* 5(1):3–14
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 100(7):3983–3988. <https://doi.org/10.1073/pnas.0530291100>
- Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 321(5889):699–702. <https://doi.org/10.1126/science.1154884>
- Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61(7):1303–1313
- Augustad KM, Rose J, Crawshaw B, Cooper G, Delaney C (2014) Do the benefits outweigh the side effects of colorectal cancer surveillance? A systematic review. *World J Gastrointest Oncol* 6(5):104–111. <https://doi.org/10.4251/wjgo.v6.i5.104>
- Avery S, Inniss K, Moore H (2006) The regulation of self-renewal in human embryonic stem cells. *Stem Cells Dev* 15(5):729–740. <https://doi.org/10.1089/scd.2006.15.729>
- Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* 17(1):126–140. <https://doi.org/10.1101/gad.224503>
- Bai Y, Liu YJ, Wang H, Xu Y, Stamenkovic I, Yu Q (2007) Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressor activity of merlin. *Oncogene* 26(6):836–850
- Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res* 65(8):3025–3029. <https://doi.org/10.1158/0008-5472.CAN-04-3931>
- Bartolazzi A, Nocks A, Aruffo A, Spring F, Stamenkovic I (1996) Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. *J Cell Biol* 132(6):1199–1208
- Bates RC, Edwards NS, Burns GF, Fisher DE (2001) A CD44 survival pathway triggers chemoresistance via lyn kinase and phosphoinositide 3-kinase/Akt in colon carcinoma cells. *Cancer Res* 61(13):5275–5283
- Bates RC, Elith CA, Thorne RF, Burns GF (1998) Engagement of variant CD44 confers resistance to anti-integrin antibody-mediated apoptosis in a colon carcinoma cell line. *Cell Adhes Commun* 6(1):21–38
- Beck B, Blanpain C (2013) Unravelling cancer stem cell potential. *Nat Rev Cancer* 13(10):727–738. <https://doi.org/10.1038/nrc3597>
- Belloq NC, Pun SH, Jensen GS, Davis ME (2003) Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjug Chem* 14(6):1122–1132
- Bennett KL, Jackson DG, Simon JC, Tanczos E, Peach R, Modrell B, Stamenkovic I, Plowman G, Aruffo A (1995a) CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. *J Cell Biol* 128(4):687–698
- Bennett KL, Modrell B, Greenfield B, Bartolazzi A, Stamenkovic I, Peach R, Jackson DG, Spring F, Aruffo A (1995b) Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. *J Cell Biol* 131(6 Pt 1):1623–1633
- Bourguignon LY (2008) Hyaluronan-mediated CD44 activation of RhoGTPase signaling and cytoskeleton function promotes tumor progression. *Semin Cancer Biol* 18(4):251–259
- Bourguignon LY, Earle C, Wong G, Spevak CC, Krueger K (2012a) Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. *Oncogene*

- Bourguignon LY, Shiina M, Li JJ (2014) Hyaluronan-CD44 interaction promotes oncogenic signaling, microRNA functions, chemoresistance, and radiation resistance in cancer stem cells leading to tumor progression. *Adv Cancer Res* 123:255–275. <https://doi.org/10.1016/B978-0-12-800092-2.00010-1>
- Bourguignon LY, Wong G, Earle C, Chen L (2012b) Hyaluronan-CD44v3 interaction with Oct4-Sox2-Nanog promotes miR-302 expression leading to self-renewal, clonal formation, and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma. *J Biol Chem* 287(39):32800–32824. <https://doi.org/10.1074/jbc.M111.308528>
- Bourguignon LY, Zhu H, Chu A, Iida N, Zhang L, Hung MC (1997) Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. *J Biol Chem* 272(44):27913–27918
- Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T (2005) Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* 5(9):744–749. <https://doi.org/10.1038/nrc1694>
- Casucci M, Nicolis di Robilant B, Falcone L, Camisa B, Norelli M, Genovese P, Gentner B, Gullotta F, Ponzoni M, Bernardi M, Marcatti M, Saudemont A, Bordignon C, Savoldo B, Ciceri F, Naldini L, Dotti G, Bonini C, Bondanza A (2013) CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. *Blood* 122(20):3461–3472. <https://doi.org/10.1182/blood-2013-04-493361>
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113(5):643–655. [https://doi.org/10.1016/S0092-8674\(03\)00392-1](https://doi.org/10.1016/S0092-8674(03)00392-1)
- Chen MK, Du Y, Sun L, Hsu JL, Wang YH, Gao Y, Huang J, Hung MC (2019) H2O2 induces nuclear transport of the receptor tyrosine kinase c-MET in breast cancer cells via a membrane-bound retrograde trafficking mechanism. *J Biol Chem* 294(21):8516–8528. <https://doi.org/10.1074/jbc.RA118.005953>
- Cheng C, Yaffe MB, Sharp PA (2006) A positive feedback loop couples Ras activation and CD44 alternative splicing. *Genes Dev* 20(13):1715–1720
- Cho Y, Lee HW, Kang HG, Kim HY, Kim SJ, Chun KH (2015) Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. *Oncotarget* 6(11):8709–8721. <https://doi.org/10.18632/oncotarget.3325>
- Choi SH, Takahashi K, Eto H, Yoon SS, Tanabe KK (2000) CD44s expression in human colon carcinomas influences growth of liver metastases. *Int J Cancer* 85(4):523–526
- Cichy J, Pure E (2003) The liberation of CD44. *J Cell Biol* 161(5):839–843
- Clevers H (2011) The cancer stem cell: premises, promises and challenges. *Nat Med* 17(3):313–319. <https://doi.org/10.1038/nm.2304>
- Colak S, Medema JP (2014) Cancer stem cells--important players in tumor therapy resistance. *FEBS J* 281 (21):4779–4791. doi:<https://doi.org/10.1111/febs.13023>
- Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 65(23):10946–10951
- Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N (2010) Colorectal cancer. *Lancet* 375(9719):1030–1047. [https://doi.org/10.1016/S0140-6736\(10\)60353-4](https://doi.org/10.1016/S0140-6736(10)60353-4)
- De Mattia E, Cecchin E, Toffoli G (2015) Pharmacogenomics of intrinsic and acquired pharmacoresistance in colorectal cancer: Toward targeted personalized therapy. *Drug Resist Updat* 20:39–70. <https://doi.org/10.1016/j.drug.2015.05.003>
- Eramo A, Lotti F, Sette G, Pilozi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R (2008) Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 15(3):504–514. <https://doi.org/10.1038/sj.cdd.4402283>
- Faassen AE, Schrager JA, Klein DJ, Oegema TR, Couchman JR, McCarthy JB (1992) A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. *J Cell Biol* 116(2):521–531. <https://doi.org/10.1083/jcb.116.2.521>

- Fan CW, Wen L, Qiang ZD, Chen T, Zhou ZG, Mo XM, Hu JK (2012) Prognostic significance of relevant markers of cancer stem cells in colorectal cancer - a meta analysis. *Hepatogastroenterology* 59(117):1421–1427. <https://doi.org/10.5754/hge10727>
- Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M (2005) A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 65(20):9328–9337. <https://doi.org/10.1158/0008-5472.CAN-05-1343>
- Farabaugh SM, Boone DN, Lee AV (2015) Role of IGF1R in Breast Cancer Subtypes, Stemness, and Lineage Differentiation. *Front Endocrinol (Lausanne)* 6:59. <https://doi.org/10.3389/fendo.2015.00059>
- Fehon RG, McClatchey AI, Bretscher A (2010) Organizing the cell cortex: the role of ERM proteins. *Nat Rev Mol Cell Biol* 11(4):276–287. <https://doi.org/10.1038/nrm2866>
- Foekens JA, Dall P, Klijn JG, Skroch-Angel P, Claassen CJ, Look MP, Ponta H, Van Putten WL, Herrlich P, Henzen-Logmans SC (1999) Prognostic value of CD44 variant expression in primary breast cancer. *Int J Cancer* 84(3):209–215
- Foger N, Marhaba R, Zoller M (2001) Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells. *J Cell Sci* 114(Pt 6):1169–1178
- Gao R, Li D, Xun J, Zhou W, Li J, Wang J, Liu C, Li X, Shen W, Qiao H, Stupack DG, Luo N (2018) CD44ICD promotes breast cancer stemness via PFKFB4-mediated glucose metabolism. *Theranostics* 8(22):6248–6262. <https://doi.org/10.7150/thno.28721>
- Ghatak S, Bogatkevich GS, Atnelishvili I, Akter T, Feghali-Bostwick C, Hoffman S, Fresco VM, Fuchs JC, Visconti RP, Markwald RR, Padhye SB, Silver RM, Hascall VC, Misra S (2014) Overexpression of c-Met and CD44v6 receptors contributes to autocrine TGF-beta1 signaling in interstitial lung disease. *J Biol Chem* 289(11):7856–7872. <https://doi.org/10.1074/jbc.M113.505065>
- Ghatak S, Hascall VC, Markwald RR, Feghali-Bostwick C, Artlett CM, Gooz M, Bogatkevich GS, Atanelishvili I, Silver RM, Wood J, Thannickal VJ, Misra S (2017b) TGF beta-1 induced CD44v6-NOX4 signaling in pathogenesis of idiopathic pulmonary fibrosis. *J Biol Chem*. <https://doi.org/10.1074/jbc.M116.752469>
- Ghatak S, Hascall VC, Markwald RR, Misra S (2021) FOLFOX Therapy Induces Feedback Upregulation of CD44v6 through YB-1 to Maintain Stemness in Colon Initiating Cells. *Int J Mol Sci*. 22(2):753
- Ghatak S, Hascall VC, Karamanos N, Markwald RR, Misra S (2022) Interplay between chemotherapy-activated cancer associated fibroblasts and cancer initiating cells expressing CD44v6 promotes colon cancer resistance. *Front Oncol* 2(12):906415. <https://doi.org/10.3389/fonc.2022.906415>
- Ghatak S, Markwald RR, Hascall VC, Dowling W, Lottes RG, Baatz JE, Beeson G, Beeson CC, Perrella MA, Thannickal VJ, Misra S (2017a) TGF beta-1 regulates CD44v6 expression and activity through ERK-induced EGR1 in pulmonary fibrogenic fibroblasts. *J Biol Chem*. <https://doi.org/10.1074/jbc.M116.752451>
- Ghatak S, Misra S, Toole BP (2002) Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. *J Biol Chem* 277(41):38013–38020
- Ghatak S, Misra S, Toole BP (2005) Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. *J Biol Chem* 280(10):8875–8883
- Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G (2012) Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 12(2):89–103. <https://doi.org/10.1038/nrc3205>
- Glavinas H, Krajcsi P, Cserepes J, Sarkadi B (2004) The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr Drug Deliv* 1(1):27–42. <https://doi.org/10.2174/1567201043480036>
- Goldstein LA, Butcher EC (1990) Identification of mRNA that encodes an alternative form of H-CAM(CD44) in lymphoid and nonlymphoid tissues. *Immunogenetics* 32(6):389–397

- Goldstein LA, Zhou DF, Picker LJ, Minty CN, Bargatze RF, Ding JF, Butcher EC (1989) A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell* 56(6):1063–1072. [https://doi.org/10.1016/0092-8674\(89\)90639-9](https://doi.org/10.1016/0092-8674(89)90639-9)
- Greaves M, Maley CC (2012) Clonal evolution in cancer. *Nature* 481(7381):306–313. <https://doi.org/10.1038/nature10762>
- Greenfield B, Wang WC, Marquardt H, Piepkorn M, Wolff EA, Aruffo A, Bennett KL (1999) Characterization of the heparan sulfate and chondroitin sulfate assembly sites in CD44. *J Biol Chem* 274(4):2511–2517
- Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P (1991) A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 65(1):13–24
- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646–674
- He Q, Lesley J, Hyman R, Ishihara K, Kincade PW (1992) Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. *J Cell Biol* 119(6):1711–1719
- Heider KH, Kuthan H, Stehle G, Munzert G (2004) CD44v6: a target for antibody-based cancer therapy. *Cancer Immunol Immunother* 53(7):567–579. <https://doi.org/10.1007/s00262-003-0494-4>
- Heider KH, Sproll M, Susani S, Patzelt E, Beaumier P, Ostermann E, Ahorn H, Adolf GR (1996) Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas. *Cancer Immunol Immunother* 43(4):245–253
- Heldin P, Basu K, Kozlova I, Porsch H (2014) HAS2 and CD44 in breast tumorigenesis. *Adv Cancer Res* 123:211–229. <https://doi.org/10.1016/B978-0-12-800092-2.00008-3>
- Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1(3):313–323. <https://doi.org/10.1016/j.stem.2007.06.002>
- Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG (2013) Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 13(10):714–726. <https://doi.org/10.1038/nrc3599>
- Holzel M, Bovier A, Tuting T (2013) Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? *Nat Rev Cancer* 13(5):365–376. <https://doi.org/10.1038/nrc3498>
- Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM (2009) Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res* 69(8):3382–3389
- Idzerda RL, Carter WG, Nottenburg C, Wayner EA, Gallatin WM, St John T (1989) Isolation and DNA sequence of a cDNA clone encoding a lymphocyte adhesion receptor for high endothelium. *Proc Natl Acad Sci U S A* 86(12):4659–4663. <https://doi.org/10.1073/pnas.86.12.4659>
- Ilangumaran S, Borisch B, Hoessli DC (1999) Signal transduction via CD44: role of plasma membrane microdomains. *Leuk Lymphoma* 35(5-6):455–469
- Ishida T (2000) Immunohistochemical expression of the CD44 variant 6 in colorectal adenocarcinoma. *Surg Today* 30(1):28–32
- Ishii S, Ford R, Thomas P, Nachman A, Steele G Jr, Jessup JM (1993) CD44 participates in the adhesion of human colorectal carcinoma cells to laminin and type IV collagen. *Surg Oncol* 2(4):255–264
- Jalkanen S, Jalkanen M (1992) Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol* 116(3):817–825. <https://doi.org/10.1083/jcb.116.3.817>
- Jamison FW 2nd, Foster TJ, Barker JA, Hills RD Jr, Guvench O (2011) Mechanism of binding site conformational switching in the CD44-hyaluronan protein-carbohydrate binding interaction. *J Mol Biol* 406(4):631–647. <https://doi.org/10.1016/j.jmb.2010.12.040>
- Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* 12(10):1167–1174. <https://doi.org/10.1038/nm1483>

- Joosten SPJ, Zeilstra J, van Andel H, Mijns RC, Zaunbrecher J, Duivenvoorden AAM, van de Wetering M, Clevers H, Spaargaren M, Pals ST (2017) MET Signaling Mediates Intestinal Crypt-Villus Development, Regeneration, and Adenoma Formation and Is Promoted by Stem Cell CD44 Isoforms. *Gastroenterology* 153(4):1040–1053. e1044. <https://doi.org/10.1053/j.gastro.2017.07.008>
- Kajita M, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M (2001) Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol* 153(5):893–904
- Katoh M (2017) Canonical and non-canonical WNT signaling in cancer stem cells and their niches: Cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity (Review). *Int J Oncol* 51(5):1357–1369. <https://doi.org/10.3892/ijo.2017.4129>
- Korkaya H, Liu S, Wicha MS (2011) Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J Clin Invest* 121(10):3804–3809. <https://doi.org/10.1172/JCI57099>
- Kozovska Z, Gabrisova V, Kucerova L (2014) Colon cancer: cancer stem cells markers, drug resistance and treatment. *Biomed Pharmacother* 68(8):911–916. <https://doi.org/10.1016/j.biopha.2014.10.019>
- Krause DS, Van Etten RA (2005) Tyrosine kinases as targets for cancer therapy. *N Engl J Med* 353(2):172–187
- Kruidering M, Van de Water B, de Heer E, Mulder GJ, Nagelkerke JF (1997) Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory chain. *J Pharmacol Exp Ther* 280(2):638–649
- Kurozumi K, Nishida T, Nakao K, Nakahara M, Tsujimoto M (1998) Expression of CD44 variant 6 and lymphatic invasion: importance to lymph node metastasis in gastric cancer. *World J Surg* 22 (8):853-857:discussion 857-858
- Kursa M, Walker GF, Roessler V, Ogris M, Roedl W, Kircheis R, Wagner E (2003) Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. *Bioconj Chem* 14(1):222–231
- Lammich S, Okochi M, Takeda M, Kaether C, Capell A, Zimmer AK, Edbauer D, Walter J, Steiner H, Haass C (2002) Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. *J Biol Chem* 277(47):44754–44759. <https://doi.org/10.1074/jbc.M206872200>
- LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V, Gerald WL (2002) Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. *Cancer Res* 62(15):4499–4506
- Lau WM, Teng E, Chong HS, Lopez KA, Tay AY, Salto-Tellez M, Shabbir A, So JB, Chan SL (2014) CD44v8-10 is a cancer-specific marker for gastric cancer stem cells. *Cancer Res* 74(9):2630–2641. <https://doi.org/10.1158/0008-5472.CAN-13-2309>
- Laurent TC, Fraser JR (1992) Hyaluronan. *The FASEB Journal* 6(7):2397–2404
- Lesley J, English N, Charles C, Hyman R (2000a) The role of the CD44 cytoplasmic and transmembrane domains in constitutive and inducible hyaluronan binding. *Eur J Immunol* 30(1):245–253
- Lesley J, Hascall VC, Tammi M, Hyman R (2000b) Hyaluronan binding by cell surface CD44. *J Biol Chem* 275(35):26967–26975
- Lesley J, Hyman R (1992) CD44 can be activated to function as an hyaluronic acid receptor in normal murine T cells. *Eur J Immunol* 22(10):2719–2723
- Lesley J, Hyman R (1998) CD44 structure and function. *Front Biosci* 3:D616–D630
- Li HJ, Reinhardt F, Herschman HR, Weinberg RA (2012) Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. *Cancer Discov* 2(9):840–855. <https://doi.org/10.1158/2159-8290.CD-12-0101>
- Lieberman DA, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR (2012) Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer. *Gastroenterology* 143(3):844–857. <https://doi.org/10.1053/j.gastro.2012.06.001>

- Liu D, Sy MS (1997) Phorbol myristate acetate stimulates the dimerization of CD44 involving a cysteine in the transmembrane domain. *J Immunol* 159(6):2702–2711
- Lokeshwar VB, Fregien N, Bourguignon LY (1994) Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *J Cell Biol* 126(4): 1099–1109. <https://doi.org/10.1083/jcb.126.4.1099>
- Longley DB, Johnston PG (2005) Molecular mechanisms of drug resistance. *J Pathol* 205(2): 275–292. <https://doi.org/10.1002/path.1706>
- Lotti F, Jarrar AM, Pai RK, Hitomi M, Lathia J, Mace A, Gantt GA Jr, Sukhdeo K, DeVecchio J, Vasanji A, Leahy P, Hjelmeland AB, Kalady MF, Rich JN (2013) Chemotherapy activates cancer-associated fibroblasts to maintain colorectal cancer-initiating cells by IL-17A. *J Exp Med* 210(13):2851–2872. <https://doi.org/10.1084/jem.20131195>
- Lu J, Ye X, Fan F, Xia L, Bhattacharya R, Bellister S, Tozzi F, Sceusi E, Zhou Y, Tachibana I, Maru DM, Hawke DH, Rak J, Mani SA, Zweidler-McKay P, Ellis LM (2013) Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. *Cancer Cell* 23(2):171–185. <https://doi.org/10.1016/j.ccr.2012.12.021>
- Lv L, Liu HG, Dong SY, Yang F, Wang QX, Guo GL, Pan YF, Zhang XH (2016) Upregulation of CD44v6 contributes to acquired chemoresistance via the modulation of autophagy in colon cancer SW480 cells. *Tumour Biol* 37(7):8811–8824. <https://doi.org/10.1007/s13277-015-4755-6>
- Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Gunthert U (1994) Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol* 124(1-2):71–82
- Marangoni E, Lecomte N, Durand L, de Pinieux G, Decaudin D, Chomienne C, Smadja-Joffe F, Poupon MF (2009) CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancers xenografts. *Br J Cancer* 100(6):918–922. <https://doi.org/10.1038/sj.bjc.6604953>
- Masuda H, Tanaka T, Takahama U (1994) Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. *Biochem Biophys Res Commun* 203(2):1175–1180. <https://doi.org/10.1006/bbrc.1994.2306>
- Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS, Niwa H (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 9(6):625–635. <https://doi.org/10.1038/ncb1589>
- Matzke A, Herrlich P, Ponta H, Orian-Rousseau V (2005) A five-amino-acid peptide blocks Met- and Ron-dependent cell migration. *Cancer Res* 65(14):6105–6110
- Medema JP, Vermeulen L (2011) Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. *Nature* 474(7351):318–326. <https://doi.org/10.1038/nature10212>
- Medina M, Dotti CG (2003) RIPPed out by presenilin-dependent gamma-secretase. *Cell Signal* 15(9):829–841. [https://doi.org/10.1016/s0898-6568\(03\)00041-x](https://doi.org/10.1016/s0898-6568(03)00041-x)
- Misra S, Ghatak S, Toole BP (2005) Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. *J Biol Chem* 280(21):20310–20315
- Misra S, Ghatak S, Zoltan-Jones A, Toole BP (2003) Regulation of multidrug resistance in cancer cells by hyaluronan. *J Biol Chem* 278(28):25285–25288
- Misra S, Hascall VC, Berger FG, Markwald RR, Ghatak S (2008a) Hyaluronan, CD44, and cyclooxygenase-2 in colon cancer. *Connect Tissue Res* 49(3):219–224
- Misra S, Hascall VC, De Giovanni C, Markwald RR, Ghatak S (2009) Delivery of CD44 shRNA/nanoparticles within cancer cells: perturbation of hyaluronan/CD44v6 interactions and reduction in adenoma growth in Apc Min/+ MICE. *J Biol Chem* 284(18):12432–12446. <https://doi.org/10.1074/jbc.M806772200>
- Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, Ghatak S (2011) Hyaluronan-CD44 interactions as potential targets for cancer therapy. *Febs J* 278(9):1429–1443

- Misra S, Obeid LM, Hannun YA, Minamisawa S, Berger FG, Markwald RR, Toole BP, Ghatak S (2008b) Hyaluronan constitutively regulates activation of COX-2-mediated cell survival activity in intestinal epithelial and colon carcinoma cells. *J Biol Chem* 283(21):14335–14344
- Misra S, Toole BP, Ghatak S (2006) Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells. *J Biol Chem* 281(46):34936–34941
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113(5):631–642. [https://doi.org/10.1016/s0092-8674\(03\)00393-3](https://doi.org/10.1016/s0092-8674(03)00393-3)
- Mori T, Kitano K, Terawaki S, Maesaki R, Fukami Y, Hakoshima T (2008) Structural basis for CD44 recognition by ERM proteins. *J Biol Chem* 283(43):29602–29612
- Morris SA (2016) Direct lineage reprogramming via pioneer factors; a detour through developmental gene regulatory networks. *Development* 143(15):2696–2705. <https://doi.org/10.1242/dev.138263>
- Murakami D, Okamoto I, Nagano O, Kawano Y, Tomita T, Iwatsubo T, De Strooper B, Yumoto E, Saya H (2003) Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44. *Oncogene* 22(10):1511–1516
- Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y (2002) CD44 in cancer. *Crit Rev Clin Lab Sci* 39(6):527–579
- Naor D, Sionov RV, Zahalka M, Rochman M, Holzman B, Ish-Shalom D (1998) Organ-specific requirements for cell adhesion molecules during lymphoma cell dissemination. *Curr Top Microbiol Immunol* 231:143–166
- Naor D, Wallach-Dayana SB, Zahalka MA, Sionov RV (2008) Involvement of CD44, a molecule with a thousand faces, in cancer dissemination. *Semin Cancer Biol* 18(4):260–267
- Natarajan TG, Ganesan N, Fitzgerald KT (2010) Cancer stem cells and markers: new model of tumorigenesis with therapeutic implications. *Cancer Biomark* 9(1-6):65–99. <https://doi.org/10.3233/CBM-2011-0173>
- Naujokas MF, Morin M, Anderson MS, Peterson M, Miller J (1993) The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* 74(2):257–268. [https://doi.org/10.1016/0092-8674\(93\)90417-o](https://doi.org/10.1016/0092-8674(93)90417-o)
- Neame SJ, Isacke CM (1993) The cytoplasmic tail of CD44 is required for basolateral localization in epithelial MDCK cells but does not mediate association with the detergent-insoluble cytoskeleton of fibroblasts. *J Cell Biol* 121(6):1299–1310. <https://doi.org/10.1083/jcb.121.6.1299>
- Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95(3):379–391. [https://doi.org/10.1016/s0092-8674\(00\)81769-9](https://doi.org/10.1016/s0092-8674(00)81769-9)
- Niwa H (2007) How is pluripotency determined and maintained? *Development* 134(4):635–646. <https://doi.org/10.1242/dev.02787>
- Niwa H (2018) The principles that govern transcription factor network functions in stem cells. *Development* 145(6). <https://doi.org/10.1242/dev.157420>
- Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24(4):372–376. <https://doi.org/10.1038/74199>
- O'Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445(7123):106–110
- Ochiai S, Nakanishi Y, Mizuno K, Hashimoto S, Inutsuka S, Kawasaki M, Yatsunami J, Hara N (1997) Expression of CD44 standard and CD44 variant 6 in human lung cancer. *Nihon Kyobu Shikkan Gakkai Zasshi* 35(11):1179–1185
- Ogino S, Fuchs CS, Giovannucci E (2012) How many molecular subtypes? Implications of the unique tumor principle in personalized medicine. *Expert Rev Mol Diagn* 12(6):621–628. <https://doi.org/10.1586/erm.12.46>
- Ogino S, Nishida N, Umemoto R, Suzuki M, Takeda M, Terasawa H, Kitayama J, Matsumoto M, Hayasaka H, Miyasaka M, Shimada I (2010) Two-state conformations in the hyaluronan-

- binding domain regulate CD44 adhesiveness under flow condition. *Structure* 18(5):649–656. <https://doi.org/10.1016/j.str.2010.02.010>
- Ohata H, Ishiguro T, Aihara Y, Sato A, Sakai H, Sekine S, Taniguchi H, Akasu T, Fujita S, Nakagama H, Okamoto K (2012) Induction of the stem-like cell regulator CD44 by Rho kinase inhibition contributes to the maintenance of colon cancer-initiating cells. *Cancer Res* 72(19): 5101–5110. <https://doi.org/10.1158/0008-5472.CAN-11-3812>
- Okamoto I, Kawano Y, Murakami D, Sasayama T, Araki N, Miki T, Wong AJ, Saya H (2001) Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J Cell Biol* 155(5):755–762
- Oliferenko S, Kaverina I, Small JV, Huber LA (2000) Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. *J Cell Biol* 148(6):1159–1164. <https://doi.org/10.1083/jcb.148.6.1159>
- Oliveira LR, Jeffrey SS, Ribeiro-Silva A (2010) Stem cells in human breast cancer. *Histol Histopathol* 25(3):371–385. <https://doi.org/10.14670/HH-25.371>
- Orian-Rousseau V (2010) CD44, a therapeutic target for metastasising tumours. *Eur J Cancer* 46(7): 1271–1277
- Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* 16(23):3074–3086
- Orian-Rousseau V, Sleeman J (2014) CD44 is a multidomain signaling platform that integrates extracellular matrix cues with growth factor and cytokine signals. *Adv Cancer Res* 123:231–254. <https://doi.org/10.1016/B978-0-12-800092-2.00009-5>
- Padua D, Figueira P, Ribeiro I, Almeida R, Mesquita P (2020) The Relevance of Transcription Factors in Gastric and Colorectal Cancer Stem Cells Identification and Eradication. *Front Cell Dev Biol* 8:442. <https://doi.org/10.3389/fcell.2020.00442>
- Paul CP, Good PD, Winer I, Engelke DR (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol* 20(5):505–508
- Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7(6):415–428. <https://doi.org/10.1038/nrc2131>
- Perez-Tomas R (2006) Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr Med Chem* 13(16):1859–1876. <https://doi.org/10.2174/09298670677585077>
- Perino M, Veenstra GJ (2016) Chromatin Control of Developmental Dynamics and Plasticity. *Dev Cell* 38(6):610–620. <https://doi.org/10.1016/j.devcel.2016.08.004>
- Peterson RM, Yu Q, Stamenkovic I, Toole BP (2000) Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites. *Am J Pathol* 156(6):2159–2167
- Picker LJ, Nakache M, Butcher EC (1989) Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J Cell Biol* 109(2): 927–937. <https://doi.org/10.1083/jcb.109.2.927>
- Pietras A, Katz AM, Ekstrom EJ, Wee B, Halliday JJ, Pitter KL, Werbeck JL, Amankulor NM, Huse JT, Holland EC (2014) Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell* 14(3):357–369. <https://doi.org/10.1016/j.stem.2014.01.005>
- Prasetyanti PR, Medema JP (2017) Intra-tumor heterogeneity from a cancer stem cell perspective. *Mol Cancer* 16(1):41. <https://doi.org/10.1186/s12943-017-0600-4>
- Prasetyanti PR, Zimmerlin CD, Bots M, Vermeulen L, Melo Fde S, Medema JP (2013) Regulation of stem cell self-renewal and differentiation by Wnt and Notch are conserved throughout the adenoma-carcinoma sequence in the colon. *Mol Cancer* 12(1):126. <https://doi.org/10.1186/1476-4598-12-126>
- Prochazka L, Tesarik R, Turanek J (2014) Regulation of alternative splicing of CD44 in cancer. *Cell Signal* 26(10):2234–2239. <https://doi.org/10.1016/j.cellsig.2014.07.011>
- Prost AC, Menegaux F, Langlois P, Vidal JM, Koulibaly M, Jost JL, Duron JJ, Chigot JP, Vayre P, Aurengo A, Legrand JC, Rosselin G, Gespach C (1998) Differential transferrin receptor density

- in human colorectal cancer: A potential probe for diagnosis and therapy. *Int J Oncol* 13(4): 871–875
- Ramdas B, Duggal R, Minev B, Chowdhary A, Koka P (2013) Functional role of solid tumor stem cells in disease etiology and susceptibility to therapeutic interventions. *J Stem Cells* 8(3-4): 189–231. doi:jsc.2014.8.3/4.189
- Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80(1-2):148–158
- Reeder JA, Gotley DC, Walsh MD, Fawcett J, Antalis TM (1998) Expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis and tumor growth in a wound environment. *Cancer Res* 58(16):3719–3726
- Renouf DJ, Woods R, Speers C, Hay J, Phang PT, Fitzgerald C, Kennecke H (2013) Improvements in 5-year outcomes of stage II/III rectal cancer relative to colon cancer. *Am J Clin Oncol* 36(6): 558–564. <https://doi.org/10.1097/COC.0b013e318256f5dc>
- Ricci-Vitiani L, Fabrizio E, Palio E, De Maria R (2009) Colon cancer stem cells. *J Mol Med (Berl)* 87(11):1097–1104. <https://doi.org/10.1007/s00109-009-0518-4>
- Rich JN (2016) Cancer stem cells: understanding tumor hierarchy and heterogeneity. *Medicine (Baltimore)* 95(1 Suppl 1):S2–S7. <https://doi.org/10.1097/MD.0000000000004764>
- Robinson S, Manas D, Mann D, White S, Mann J (2011) Tumour cytokine production after FOLFOX chemotherapy is mediated by the NF- κ B DNA damage response-an important contribution to chemo induced liver injury. *EJSO* 37(11):P995
- Ruiz P, Schwarzler C, Gunthert U (1995) CD44 isoforms during differentiation and development. *Bioessays* 17(1):17–24
- Sadanandam A, Lyssiotis CA, Homicsko K, Collisson EA, Gibb WJ, Wullschlegel S, Ostos LC, Lannon WA, Grotzinger C, Del Rio M, Lhermitte B, Olshen AB, Wiedenmann B, Cantley LC, Gray JW, Hanahan D (2013) A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 19(5):619–625. <https://doi.org/10.1038/nm.3175>
- Safa AR (2016) Resistance to Cell Death and Its Modulation in Cancer Stem Cells. *Crit Rev Oncog* 21(3-4):203–219. <https://doi.org/10.1615/CritRevOncog.2016016976>
- Saito S, Okabe H, Watanabe M, Ishimoto T, Iwatsuki M, Baba Y, Tanaka Y, Kurashige J, Miyamoto Y, Baba H (2013) CD44v6 expression is related to mesenchymal phenotype and poor prognosis in patients with colorectal cancer. *Oncol Rep* 29(4):1570–1578. <https://doi.org/10.3892/or.2013.2273>
- Sanchez JA, Krumroy L, Plummer S, Aung P, Merkulova A, Skacel M, DeJulius KL, Manilich E, Church JM, Casey G, Kalady MF (2009) Genetic and epigenetic classifications define clinical phenotypes and determine patient outcomes in colorectal cancer. *Br J Surg* 96(10):1196–1204. <https://doi.org/10.1002/bjs.6683>
- Sargent D, Sobrero A, Grothey A, O'Connell MJ, Buyse M, Andre T, Zheng Y, Green E, Labianca R, O'Callaghan C, Seitz JF, Francini G, Haller D, Yothers G, Goldberg R, de Gramont A (2009) Evidence for cure by adjuvant therapy in colon cancer: observations based on individual patient data from 20,898 patients on 18 randomized trials. *J Clin Oncol* 27(6): 872–877. <https://doi.org/10.1200/JCO.2008.19.5362>
- Sauter A, Kloft C, Gronau S, Bogeschdorfer F, Erhardt T, Golze W, Schroen C, Staab A, Riechelmann H, Hoermann K (2007) Pharmacokinetics, immunogenicity and safety of bivatuzumab mertansine, a novel CD44v6-targeting immunoconjugate, in patients with squamous cell carcinoma of the head and neck. *Int J Oncol* 30(4):927–935
- Schmitt M, Metzger M, Gradl D, Davidson G, Orian-Rousseau V (2015) CD44 functions in Wnt signaling by regulating LRP6 localization and activation. *Cell Death Differ* 22(4):677–689. <https://doi.org/10.1038/cdd.2014.156>
- Schrijvers AH, Quak JJ, Uytendaele AM, van Walsum M, Meijer CJ, Snow GB, van Dongen GA (1993) MAb U36, a novel monoclonal antibody successful in immunotargeting of squamous cell carcinoma of the head and neck. *Cancer Res* 53(18):4383–4390

- Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci U S A* 89(24):12160–12164
- Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzku S, Zoller M (1993) Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 177(2): 443–455
- Shinohara H, Fan D, Ozawa S, Yano S, Van Arsdell M, Viner JL, Beers R, Pastan I, Fidler IJ (2000) Site-specific expression of transferrin receptor by human colon cancer cells directly correlates with eradication by antitransferrin recombinant immunotoxin. *Int J Oncol* 17(4):643–651
- Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, St Clair R, Baljevic M, White I, Jin DK, Chadburn A, Murphy AJ, Valenzuela DM, Gale NW, Thurston G, Yancopoulos GD, D'Angelica M, Kemeny N, Lyden D, Rafii S (2008) CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. *J Clin Invest* 118(6):2111–2120. <https://doi.org/10.1172/JCI34401>
- Siegel RL, Miller KD (2016) Jemal A (2016) Cancer statistics. *CA Cancer J Clin* 66(1):7–30. <https://doi.org/10.3322/caac.21332>
- Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A (2017) Jemal A (2017) Colorectal cancer statistics. *CA Cancer J Clin* 67(3):177–193. <https://doi.org/10.3322/caac.21395>
- Singh AK, Arya RK, Maheshwari S, Singh A, Meena S, Pandey P, Dormond O, Datta D (2015) Tumor heterogeneity and cancer stem cell paradigm: updates in concept, controversies and clinical relevance. *Int J Cancer* 136(9):1991–2000. <https://doi.org/10.1002/ijc.28804>
- Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res* 63(18):5821–5828
- Sleeman J, Rudy W, Hofmann M, Moll J, Herrlich P, Ponta H (1996) Regulated clustering of variant CD44 proteins increases their hyaluronate binding capacity. *J Cell Biol* 135(4): 1139–1150
- Sneath RJ, Mangham DC (1998) The normal structure and function of CD44 and its role in neoplasia. *Mol Pathol* 51(4):191–200
- Sneddon JB, Werb Z (2007) Location, location, location: the cancer stem cell niche. *Cell Stem Cell* 1(6):607–611. <https://doi.org/10.1016/j.stem.2007.11.009>
- Stamenkovic I, Amiot M, Pesando JM, Seed B (1989) A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell* 56(6):1057–1062
- Stamenkovic I, Aruffo A, Amiot M, Seed B (1991) The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J* 10(2):343–348
- Su YJ, Lai HM, Chang YW, Chen GY, Lee JL (2011) Direct reprogramming of stem cell properties in colon cancer cells by CD44. *EMBO J* 30(15):3186–3199. <https://doi.org/10.1038/emboj.2011.211>
- Sugano K, Maeda K, Ohtani H, Nagahara H, Shibutani M, Hirakawa K (2015) Expression of xCT as a predictor of disease recurrence in patients with colorectal cancer. *Anticancer Res* 35(2): 677–682
- Takahashi A, Ohtani N, Yamakoshi K, Iida S, Tahara H, Nakayama K, Nakayama KI, Ide T, Saya H, Hara E (2006) Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat Cell Biol* 8(11):1291–1297. <https://doi.org/10.1038/ncb1491>
- Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2(12):3081–3089. <https://doi.org/10.1038/nprot.2007.418>
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126(4):663–676. <https://doi.org/10.1016/j.cell.2006.07.024>

- Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 27(5):1006–1020. <https://doi.org/10.1002/stem.30>
- Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S (1993) T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. *Nature* 361(6407):79–82
- Tirino V, Camerlingo R, Franco R, Malanga D, La Rocca A, Viglietto G, Rocco G, Pirozzi G (2009) The role of CD133 in the identification and characterisation of tumour-initiating cells in non-small-cell lung cancer. *Eur J Cardiothorac Surg* 36(3):446–453. <https://doi.org/10.1016/j.ejcts.2009.03.063>
- Tirino V, Desiderio V, d'Aquino R, De Francesco F, Pirozzi G, Graziano A, Galderisi U, Cavaliere C, De Rosa A, Papaccio G, Giordano A (2008) Detection and characterization of CD133+ cancer stem cells in human solid tumours. *PLoS One* 3(10):e3469. <https://doi.org/10.1371/journal.pone.0003469>
- Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, Fazioli F, Pirozzi G, Papaccio G (2011) Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity in vivo. *FASEB J* 25(6):2022–2030. <https://doi.org/10.1096/fj.10-179036>
- Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, Laino L, De Francesco F, Papaccio G (2013) Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J* 27(1):13–24. <https://doi.org/10.1096/fj.12-218222>
- Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G (2007) Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 1(4):389–402
- Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G (2014) CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. *Cell Stem Cell* 14(3):342–356
- Townsend DM, Tew KD (2003) The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* 22(47):7369–7375. <https://doi.org/10.1038/sj.onc.1206940>
- Toyama-Sorimachi N, Miyasaka M (1994) A novel ligand for CD44 is sulfated proteoglycan. *Int Immunol* 6(4):655–660
- Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K, Miyasaka M (1995) A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *J Biol Chem* 270(13):7437–7444. <https://doi.org/10.1074/jbc.270.13.7437>
- Tremmel M, Matzke A, Albrecht I, Laib AM, Olaku V, Ballmer-Hofer K, Christofori G, Heroult M, Augustin HG, Ponta H, Orian-Rousseau V (2009) A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. *Blood* 114(25):5236–5244
- Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 126(2):391–401. <https://doi.org/10.1083/jcb.126.2.391>
- Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, Chomienne C, Ishikawa F, Schuringa JJ, Stassi G, Huntly B, Herrmann H, Soulier J, Roesch A, Schuurhuis GJ, Wohrer S, Arock M, Zuber J, Cerny-Reiterer S, Johnsen HE, Andreeff M, Eaves C (2012) Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer* 12(11):767–775. <https://doi.org/10.1038/nrc3368>
- Van der Jeught K, Xu HC, Li YJ, Lu XB, Ji G (2018) Drug resistance and new therapies in colorectal cancer. *World J Gastroenterol* 24(34):3834–3848. <https://doi.org/10.3748/wjg.v24.i34.3834>
- Van Hal NL, Van Dongen GA, Rood-Knippels EM, Van Der Valk P, Snow GB, Brakenhoff RH (1996) Monoclonal antibody U36, a suitable candidate for clinical immunotherapy of squamous-cell carcinoma, recognizes a CD44 isoform. *Int J Cancer* 68(4):520–527. [https://doi.org/10.1002/\(SICI\)1097-0215\(19961115\)68:4<520::AID-IJC19>3.0.CO;2-8](https://doi.org/10.1002/(SICI)1097-0215(19961115)68:4<520::AID-IJC19>3.0.CO;2-8)

- Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 12(5):468–476
- Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP (2008a) Cancer stem cells--old concepts, new insights. *Cell Death Differ* 15(6):947–958
- Vermeulen L, Todaro M, de Sousa MF, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, Medema JP (2008b) Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci U S A* 105(36):13427–13432
- Vlashi E, Pajonk F (2015) Cancer stem cells, cancer cell plasticity and radiation therapy. *Semin Cancer Biol* 31:28–35. <https://doi.org/10.1016/j.semcancer.2014.07.001>
- Voog J, Jones DL (2010) Stem cells and the niche: a dynamic duo. *Cell Stem Cell* 6(2):103–115. <https://doi.org/10.1016/j.stem.2010.01.011>
- Weber GF (2008) Molecular mechanisms of metastasis. *Cancer Lett* 270(2):181–190. <https://doi.org/10.1016/j.canlet.2008.04.030>
- Weber GF, Ashkar S, Glimcher MJ, Cantor H (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271(5248):509–512. <https://doi.org/10.1126/science.271.5248.509>
- Weg-Remers S, Ponta H, Herrlich P, König H (2001) Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway. *EMBO J* 20(15):4194–4203. <https://doi.org/10.1093/emboj/20.15.4194>
- Wei B, Chen L, Li R, Tian J (2010) Stem cells in gastrointestinal cancers: a matter of choice in cell fate determination. *Expert Rev Anticancer Ther* 10(10):1621–1633. <https://doi.org/10.1586/era.10.52>
- Weng MS, Chang JH, Hung WY, Yang YC, Chien MH (2018) The interplay of reactive oxygen species and the epidermal growth factor receptor in tumor progression and drug resistance. *J Exp Clin Cancer Res* 37(1):61. <https://doi.org/10.1186/s13046-018-0728-0>
- Williams K, Motiani K, Giridhar PV, Kasper S (2013) CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches. *Exp Biol Med* (Maywood) 238(3):324–338. <https://doi.org/10.1177/1535370213480714>
- Winawer SJ (2005) Screening of colorectal cancer. *Surg Oncol Clin N Am* 14(4):699–722. <https://doi.org/10.1016/j.soc.2005.05.009>
- Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF et al (1993) Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 329(27):1977–1981. <https://doi.org/10.1056/NEJM199312303292701>
- Xu H, Tian Y, Yuan X, Wu H, Liu Q, Pestell RG, Wu K (2015) The role of CD44 in epithelial-mesenchymal transition and cancer development. *Onco Targets Ther* 8:3783–3792. <https://doi.org/10.2147/OTT.S95470>
- Yan Y, Zuo X, Wei D (2015) Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med* 4(9):1033–1043. <https://doi.org/10.5966/sctm.2015-0048>
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318(5858):1917–1920. <https://doi.org/10.1126/science.1151526>
- Yu Q, Toole BP, Stamenkovic I (1997) Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. *J Exp Med* 186(12):1985–1996
- Zeilstra J, Joosten SP, Dokter M, Verwiel E, Spaargaren M, Pals ST (2008) Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. *Cancer Res* 68(10):3655–3661

- Zeilstra J, Joosten SP, van Andel H, Tolg C, Berns A, Snoek M, van de Wetering M, Spaargaren M, Clevers H, Pals ST (2014) Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling. *Oncogene* 33(5):665–670. <https://doi.org/10.1038/onc.2012.611>
- Zeng C, Toole BP, Kinney SD, Kuo JW, Stamenkovic I (1998) Inhibition of tumor growth in vivo by hyaluronan oligomers. *Int J Cancer* 77(3):396–401
- Zoller M (2009) Tetraspanins: push and pull in suppressing and promoting metastasis. *Nat Rev Cancer* 9(1):40–55. <https://doi.org/10.1038/nrc2543>
- Zoller M (2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 11(4):254–267

Chapter 8

The Pharmacokinetics and Pharmacodynamics of 4-Methylumbelliferone and its Glucuronide Metabolite in Mice



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Abstract Hyaluronan (HA) is an extracellular matrix glycosaminoglycan, with important roles in chronic inflammation, cancer, and autoimmunity. 4-methylumbelliferone (4-MU), a small molecule inhibitor of HA synthases, is widely used to study HA's interactions with the surrounding tissues and the immune cells. There is substantial experimental and therapeutic interest in using oral 4-MU to inhibit HA synthesis, but pharmacokinetic and pharmacodynamic data on treatment routes have been lacking. Moreover, it recently became clear that the main metabolite of 4-MU, 4-methylumbelliferyl glucuronide (4-MUG), is bioactive. We, therefore, sought to define the pharmacokinetics and pharmacodynamics of 4-MU and its active metabolite 4-MUG in mice. Single-dose mouse studies showed that 4-MU administered intravenously (i.v.) resulted in 100-fold higher 4-MU exposure compared to oral (p.o.) administration. The 4-MU ratio AUC i.v./AUC p.o. was 96/1. 4-MUG exposures were much higher than 4-MU exposures after both 4-MU i.v. and p.o. administration, but only small differences in 4-MU exposure were seen after 4-MU i.v. versus p.o. administration. The 4-MUG metabolite was also administered as a single dose both i.v. and p.o. and showed a 25.9 % bioavailability. Compared to 4-MUG p.o. dosing, 1.14 higher 4-MUG exposures were seen after 4-MU p.o. dosing. 4-MU exposure after 4-MUG p.o. administration was minimal but

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similar to 4-MU exposure after 4-MU p.o. administration. In mice treated for several weeks with 4-MU in chow, the 4-MU concentration immediately drops after treatment was stopped, whereas the 4-MUG concentration showed a peak 1 h after treatment stops. In a build-up study, 4-MU and 4-MUG treatment in mice lead to a plateau of 4-MU concentration starting at 4 days post treatment start. These 4-MU and 4-MUG concentration findings *in vivo* will inform future clinical studies and experimental work with 4-MU.

Abbreviations

4-MU	4-methylumbelliferone
4-MUG	4-methylumbelliferyl glucuronide
HA	hyaluronan

8.1 Introduction

4-Methylumbelliferone (4-MU) is a small molecule inhibitor of hyaluronan (HA) synthesis (Kuipers et al. 2015), which has been shown to inhibit HA production in multiple cell lines and tissue types *in vitro* and *in vivo* (Yoshioka et al. 2013; Bollyky et al. 2010; Nagy et al. 2010). HA is an extracellular matrix glycosaminoglycan with many roles in tissue function and development (Fraser et al. 1997; Jiang et al. 2011; Termeer et al. 2003), which has been identified as a driving factor in inflammation (Yoshioka et al. 2013; Hull et al. 2012; Kuipers et al. 2016). Greatly elevated HA levels have been found in chronically inflamed tissues (Cheng et al. 2011; Kang et al. 2013; Mine et al. 2006), the tumor microenvironment, in fibrosis, and at sites of autoimmunity (Bollyky et al. 2012; Nagy et al. 2015). There is substantial experimental and therapeutic interest in inhibiting HA synthesis. The mechanism of action by which 4-MU inhibits HA synthesis has been described (Kakizaki et al. 2004). UDP-GlcUA and UDP-GlcNAc are the substrates of HA synthesis, their availability thereby limits HA synthesis. 4-MU mainly functions by depleting the HA precursor UDP-GlcUA by activation of UDP-glucuronyl transferases (UGTs) (Kakizaki et al. 2004), and 4-MU also downregulates the HAS expression (Kultti et al. 2009).

4-MU is a commercially available drug in Europe and Asia and is approved for use in humans to prevent biliary spasm (Tanayama and Kanai 1977; Takeda and Aburada 1980; Fontaine et al. 1968; Stacchino et al. 1983). Several clinical trials in humans have been published on 4-MU, all demonstrated excellent safety data (Abate et al. 2001; Camarri and Marchettini 1988; Krawzak et al. 1994; Quaranta et al. 1984; Trabucchi et al. 1986; Walter and Seidel 1978; Hoffmann et al. 2005). Right now, 4-MU is under investigation in human clinical trials as a safety study at multiple doses, and the treatment for HA-associated fibrotic liver and autoimmune

biliary diseases ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00225537) Identifiers: NCT00225537, NCT02780752). The biggest downside to 4-MU is its poor pharmacokinetics, which is thought to limit its use outside the biliary tract. The use of 4-MU in other diseases at other sites of the body is desirable due to HA's involvement in many different chronic and acute diseases. The systemic oral bioavailability of 4-MU is reported to be <3 %, mostly due to extensive first pass glucuronidation in the liver and small intestine (Takeda and Aburada 1980; Garrett et al. 1993). If first pass metabolism is bypassed by giving the dose intravenously (i.v.), the systemic exposure achieved can be more than 10–30 fold higher than after the same dose given orally (p.o.) (Garrett and Venitz 1994). However, due to the high clearance of 4-MU, systemic concentrations will decrease rapidly after an i.v. dose and peripheral exposures will likely be almost undetectable by 4–6 h after a 4-MU dose. The pharmacodynamic (PD) and pharmacokinetic (PK) properties of p.o. vs i.v. 4-MU administration are of great importance and will be investigated in this study.

Any 4-MU that reaches the systemic circulation is rapidly metabolized with a half-life of 28 mins in humans and < 1 % of a given dose is excreted unchanged in the urine (Takeda and Aburada 1980; Garrett et al. 1993). 4-MU's half-life in mice is with 3 mins even shorter. Consequently, the median plasma concentration of the 4-MU metabolite, 4-methylumbelliferyl glucuronide (4-MUG), is more than 3000-fold higher than that of 4-MU in mouse models (Kuipers et al. 2015). Analogous findings have been reported in healthy human volunteers (Garrett et al. 1993). Metabolism of the drug occurs via conjugation to either a glucuronic (4-MUG), or a sulfate (4-MUS). The glucuronide (4-MUG) is the predominant pathway and accounts for over 90% of its metabolism (Garrett and Venitz 1994; Mulder et al. 1985). 4-MUG is more hydrophilic and gets eliminated in the bile and urine (Mulder et al. 1985). Biliary eliminated 4-MUG likely undergoes further enterohepatic recirculation with reabsorption of the metabolite from the intestine and ultimate elimination in the urine via the kidney. This is supported by a healthy volunteer PK study in which 93 % of a single i.v. dose of 4-MU was eliminated as the 4-MUG metabolite in the urine (Garrett and Venitz 1994). The PK and PD properties of 4-MU and 4-MUG will be investigated in this study.

Despite the poor bioavailability and a short half-life, oral administration of 4-MU nonetheless inhibits HA synthesis *in vivo* and has demonstrated a reduction of severity and frequency in different chronic and autoimmune diseases (Kuipers et al. 2015; Kuipers et al. 2016; Nagy et al. 2015). Future animal studies and clinical studies would benefit from a more thorough understanding of the PK and PD of 4-MU and the 4-MUG metabolite to help support dosing and target exposure. Furthermore, to our knowledge PK/PD data for multiple dosing with 4-MU and 4-MUG have not been reported previously. Here, we have used mass spectrometry to interrogate the PK and PD of 4-MU and 4-MUG in mice with single and multiple dosing.

8.2 Materials and Methods

8.2.1 Mice

All animals were bred and maintained under specific pathogen-free conditions, with free access to food and water, in the animal facilities at Bioduro (Beijing, China) and Stanford University Medical School (Stanford, CA). Male CD-1 mice were bred in house at Bioduro, and male C57Bl/6 J mice were purchased from The Jackson Laboratories (Bar Harbor, Me).

8.2.2 4-MU and 4-MUG Treatment

4-MU (Alfa Aesar) was pressed into the mouse chow by TestDiet® and irradiated before shipment, as previously described (Nagy et al. 2010). We previously determined that this chow formulation delivers 250 mg/mouse/day, yielding a serum drug concentration of 640.3 ± 17.2 nmol/L in mice, as measured by HPLC-MS. 4-MUG (ChemImpex) was distributed in the drinking water at a concentration of 2 mg/ml, delivering 10 mg/mouse/day, yielding a serum drug concentration of 357.1 ± 72.6 ng/mL in mice, as measured by LC-MS/MS. Mice were initiated on 4-MU and 4-MUG at 8 weeks of age, unless otherwise noted, and were maintained on this diet until they were euthanized, unless otherwise noted.

8.2.3 Pharmacokinetic Study in CD-1 Mice for 4-MU

Male CD-1 mice, 7–9 weeks of age, were given 4-MU and 4-MUG at 25 mg/kg i.v. at a vehicle concentration of 5 mg/mL in an injection volume of 5 mL/kg. 10 % DMSO (Sigma-Aldrich and Tedia)/ 50 % PEG400 (Fluka)/ 40 % water was used as vehicle. Mice were fed at the time of injection. Blood samples were collected at 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h from the saphenous vein, temporally put on ice until plasma was processed. Plasma samples were stored at -80°C until analysis.

CD-1 mice were given 4-MU at 25 mg/kg p.o. via gavage at a vehicle concentration of 2.5 mg/mL in a volume of 10 mL/kg. 5 % methyl cellulose (Sigma) was used as vehicle. Mice were fed at the time of experiment. Blood samples were collected at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h, from the saphenous vein, temporally put on ice until plasma was processed. Plasma samples were stored at -80°C until analysis.

The bioanalysis was performed via LC/MS/MS (AB Science API 5500 and AB Science Triple Quad 6500+) using the Analyst Software 1.7.1 a Shimadzu pump (LC-20 AD) and Acquity Ultra Performance LC System (Waters) and a Kinetex

column (2.6u C18 100A 30 mm x 3 mm) and an Aquity UPLC BEH C18 2.1*50 mm column (Waters). 50 ng/mL Tolbutamide in methanol/acetonitrile (1:1, v/v) was used as an internal standard.

8.2.4 *Caco-2 Permeability Assessment*

Caco-2 cells were cultured to confluency, trypsinized, and seeded onto filter transwell inserts at a density of 32 K cells/well in DMEM cell culture medium (Gibco). Following an overnight attachment period, the cell medium was replaced every other day. The cell monolayer was ready for assessment 22 days post seeding. A 25 mM HBSS (Sigma)/HEPES (Gibco) solution pH 7.4 and a HBSS/HEPES 0.1 % BSA (VWR Life Science) solution were prepared. 10 uM 4-MU (Sigma) and 4-MUG (Sigma) working solutions were prepared as well as internal standards and quenching solutions (25/50 ng/mL Terfenadine (Sigma)/Tolbutamide. The TEER (Millicell ERS-2) value of each well was measured, the wells were only used when their TEER values were greater than 600 Ohms/cm². The cell plates were prepared as follows, the cell culture medium from the basolateral side (B) and apical side (A) were aspirated. For A to B direction, the donor plate A was rinsed with 400 ul HBSS/Hepes, the recipient plate B was rinsed with 400 ul HBSS/Hepes/0.1 % BSA. For the B to A direction, the donor plate B was rinsed with 1200 ul HBSS/Hepes solution and the recipient plate A was rinsed with 400 ul HBSS/Hepes/0.1 % BSA. The plates were dosed as follows, for the A to B direction, 500 ul of the compound or control working solution was added to the donor plate A. 1300 ul of HBSS/Hepes/0.1 BSA solution was added to the recipient plate B. This was vice versa for the B to A direction. Plates were pre-incubated for 10 min and 100 ul sample was collected as 0 min sample for future analysis. The cell plates were incubated for another 90 min and samples were taken after that. The samples were prepared for LC-MS/MS analysis. The MS detection was performed using Sciex API 4500 Q Trap and 5500 instruments. Each compound was analyzed by reverse phase HPLC.

8.2.5 *Take Away Study*

4-MU (Alfa Aesar) was pressed into the mouse chow by TestDiet® and irradiated before shipment, as previously described (Nagy et al. 2010). We previously determined that this chow formulation delivers 250 mg/mouse/day. 4-MUG (ChemImpex) was distributed in the drinking water at a concentration of 2 mg/ml, delivering 10 mg/mouse/day. Male C57Bl/6 J mice were treated with 5 % 4-MU (Alpha Aesar) and 2 mg/ml 4-MUG (Sigma) starting at 8 weeks of age and maintained on this diet until they were euthanized, unless otherwise noted. The mice were treated for 5 weeks. At the end of the 5 weeks of treatment, the mice were transferred to clean cages with regular chow and blood samples were taken at T0, T1,

T2, T6, T12, and T24 hours and processed to plasma. Plasma samples were stored at -80°C for further analysis.

8.2.6 Built-up Study

Male C57Bl/6 J mice were treated with 5 % 4-MU (Alpha Aesar) and 2 mg/ml 4-MUG (Sigma) starting at 8 weeks of age for 20 days. During the time of treatment blood samples were taken from the mice at T0, T1, T4, T7, T15, and T20 days after treatment start. Plasma samples were stored at -80°C for further analysis.

8.2.7 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis of 4-MU and 4-MUG Concentrations in Mouse Serum

4-MU and 4-MUG concentrations were measured via mass spectrometry. 4-methylumbelliferone- $^{13}\text{C}_4$ (Toronto Research Chemicals, Ontario, Canada) was used as the IS for 4-MU and 7-hydroxycoumarin β -D-glucuronide (Toronto Research Chemicals, Ontario, Canada) as the IS for 4-MUG. The neat stock solutions of 4-MU and 4-MUG were mixed and diluted in 50 % methanol to prepare the spiking solutions ranging from 1 ng/mL to 5000 ng/mL for each compound.

For calibration standards, 25 μL of blank serum was mixed with 25 μL of the spiking solutions. For samples to be tested, 25 μL of serum was mixed with 25 μL of 50 % methanol to make up the volume. 25 μL of a mixture of the two IS (1000 ng/mL each in 50 % methanol) was then added. After vortexing all standards and samples, 150 μL of methanol/acetonitrile 20:80 (v/v) was added to the mixture and the sample was further vortexed vigorously for 1 min followed by centrifugation at 3000 rpm for 10 min. 100 μL of the supernatant was taken and diluted with 200 μL of Milli Q water.

The LC-MS/MS system consists of an AB SCIEX QTRAP 4000 mass spectrometer linked to a Shimadzu UFLC system. Mobile phase A is HPLC grade water. Mobile phase B is HPLC grade acetonitrile. LC separation was carried out on a Phenomenex Luna PFP (Yoshioka et al. 2013) column (3 μm , 150×2 mm) with isocratic elution using 45 % mobile phase B and a flow rate of 0.4 mL/min at room temperature. The analysis time was 2.5 min. 10 μL of the extracted sample was injected. The mass spectrometer was operated in the negative mode with the following multiple-reaction monitoring (MRM) transitions: m/z 174.7 \rightarrow 132.9 for 4-MU, m/z 178.7 \rightarrow 134.9 for 4-MU- $^{13}\text{C}_4$ (IS), m/z 350.8 \rightarrow 174.9 for 4-MUG and m/z 336.9 \rightarrow 160.9 for 7-hydroxy coumarin β -D-glucuronide (IS). Data acquisition and analysis were performed using the Analyst 1.6.1 software (AB SCIEX).

8.2.8 Pharmacokinetic Analysis

The C_{\max} (maximum plasma concentration) and T_{\max} (time of maximum concentration) were obtained directly from the observed data. The terminal rate constant (λ_z) was determined by linear regression analysis of the terminal portion of the log plasma concentration–time curve. The terminal half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda_z$. $AUC_{0-\text{last}}$ was found using the linear/logarithmic trapezoidal method. Summation of AUC_{last} and the concentration at the last measured point divided by λ_z yielded $AUC_{0-\infty}$. Bioavailability (F) was calculated as $AUC_{0-\infty,\text{iv}} / AUC_{0-\infty,\text{po}}$. To allow direct comparisons of $AUC_{0-\infty}$ across 4MU and 4MUG dose formulations, $AUC_{0-\infty}$ after 4MUG dose was dose-adjusted for molar equivalent of 4MU using a factor of 2. The mean residence time (MRT) was calculated as the ratio of the area under the first moment curve ($AUMC_{0-\infty}$) divided by $AUC_{0-\infty}$ minus the estimated mean absorption time (MAT) (i.e., $MRT = (AUMC_{0-\infty} / AUC_{0-\infty}) - MAT$). MAT was the reciprocal of the first-order absorption rate (k_a) constant estimated when fitting concentration data to a two-compartmental model with first-order absorption.

8.2.9 Statistical Analysis

Data are expressed as means \pm SEM of n independent measurements. A p value of <0.05 was considered significant. Significance of the difference between the means of two or three groups of data was evaluated using a two-tailed t test or one-way ANOVA with Šidák's multiple comparisons post-test. GraphPad Prism 8.1.2 software was used to perform data analysis.

8.3 Results

8.3.1 4-MU Administration i.v. and p.o., 4-MU Measured

To establish the basic PK properties of 4-MU in mice, a single dose of 25 mg/kg 4-MU was administered i.v. and p.o. (Tables 8.1 and 8.2). The mean terminal half-life ($t_{1/2}$) of 4-MU in plasma was 0.182 h for i.v. and 0.346 h for p.o. administration. The initial plasma drug concentration at time zero following the bolus i.v. injection (C_0) was 21,852 ng/mL. The time to reach maximal plasma concentration (t_{\max}) following p.o. administration was 0.139 h and the maximal plasma 4-MU concentration (C_{\max}) was 100 ng/mL. The area under the plasma concentration–time curve extrapolated from time t to infinity as a percentage of total AUC (AUC_{last}) was 2875 h*ng/mL for i.v. and 25.9 h*ng/mL for p.o. administration. Similarly, the area under the plasma concentration–time curve from time zero to infinity (AUC_{Inf}) was 2876 h*ng/mL for i.v. and 27.8 h*ng/mL for p.o. administration. The mean infinite

Table 8.1 4-MU administration i.v. 4-MU measured

Animal	Treatment	Administration	measured	t1/2 (h)	C0 (ng/mL)	AUClast (h*ng/mL)	AUCInf (h*ng/mL)	AUCEXtr (%)	Vz (L/kg)	Vss (L/kg)	CL (mL/min/ kg)	MRTInf (h)
Mouse 1	4-MU (25 mg/ kg)	i.v.	4-MU	0.315	20,195	2818	2820	0.0607	4.04	0.942	148	0.106
Mouse 2	4-MU (25 mg/ kg)	i.v.	4-MU	0.118	26,334	3111	3113	0.0584	1.36	0.607	134	0.0756
Mouse 3	4-MU (25 mg/ kg)	i.v.	4-MU	0.114	19,027	2695	2696	0.0485	1.53	0.722	155	0.0779
Mean	4-MU (25 mg/ kg)	i.v.	4-MU	0.182	21,852	2875	2876	0.0559	2.31	0.757	145	0.0866
SD	4-MU (25 mg/ kg)	i.v.	4-MU	0.115	3925	214	214	0.0065	1.50	0.170	11	0.0171

Table 8.2 4-MU administration p.o. 4-MU measured

Animal	Treatment	Administration	measured	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC_{Ext} (%)	MR_{Tinf} (h)	AUC_{Inf}/D (h*kg*ng/mL/ mg)	F (%)
Mouse 4	4-MU (25 mg/ kg)	p.o.	4-MU	0.405	0.25	31.3	19.5	20.1	2.99	0.52	0.804	0.699
Mouse 5	4-MU (25 mg/ kg)	p.o.	4-MU	0.446	0.083	55.3	17.7	22.4	21.1	0.62	0.896	0.779
Mouse 6	4-MU (25 mg/ kg)	p.o.	4-MU	0.188	0.083	214	40.4	40.9	1.28	0.21	1.64	1.42
Mean	4-MU (25 mg/ kg)	p.o.	4-MU	0.346	0.139	100	25.9	27.8	8.47	0.451	1.11	0.967
SD	4-MU (25 mg/ kg)	p.o.	4-MU	0.139	0.096	99.3	12.6	11.4	11.0	0.216	0.46	0.397

residence time (MRT_{Inf}) for 4-MU after i.v. administration was 0.0866 h and 0.451 h for p.o. administration. The dose-normalized area under the plasma concentration–time curve from time 0 to infinity (AUC_{Inf}/D) for 4-MU administered p.o. was 1.11 h*kg*ng/mL/mg. The systemic bioavailability (F) for the p.o. administered 4-MU dose was 0.967%. For the 4-MU administration i.v. the apparent volume of distribution during the terminal phase (V_Z) was 2.31 L/kg, and the apparent volume of distribution at steady state (V_{SS}) was 0.757 L/kg. The apparent total body clearance of the drug from plasma (CL) was 145 mL/min/kg for i.v. 4-MU administration.

8.3.2 4-MU Administration i.v. and p.o., 4-MUG Measured

In the same experiment as described above, 4-MU's major metabolite 4-MUG was assessed after 25 mg/kg 4-MU was administered i.v. and p.o. (Tables 8.3 and 8.4). The mean half-life ($t_{1/2}$) of 4-MUG in the serum was 1.29 h for i.v. and 3.79 h for p.o. administration. The initial plasma 4-MUG concentration at time zero following the bolus i.v. injection (C_0) was 25,400 ng/mL. The time to reach maximal plasma concentration following p.o. administration (t_{max}) was 0.25 h and the maximal plasma 4-MU concentration (C_{max}) was 28,733 ng/mL. The area under the plasma concentration–time curve extrapolated from time t to infinity as a percentage of total AUC (AUC_{last}) was 45,389 h*ng/mL for i.v. and 20,009 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve from time zero to infinity (AUC_{Inf}) was 45,842 h*ng/mL for i.v. and 27,086 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve extrapolated from time to infinity as a percentage of total AUC (AUC_{Extr}) was 1.01% for i.v. and 17.6% for p.o. 4-MU administration. The mean infinite residence time (MRT_{Inf}) for 4-MU after i.v. administration was 0.638 h and 4.000 h for p.o. administration.

8.3.3 4-MU and 4-MUG Concentration in Mice over Time after 4-MU i.v. and p.o. Administration

4-MU was administered to mice at 25 mg/kg i.v. and p.o., and subsequently the serum concentration of 4-MU and 4-MUG was measured over time (Fig. 8.1). The mice dosed 4-MU i.v. started at 0.033 h with 17,800 ng/mL 4-MU whereas the mice dosed 4-MU p.o. starting at 0.083 h had a mean 4-MU concentration of 100 ng/mL (Fig. 8.1a–c). At 0.5 h the 4-MU concentration after i.v. administration went down to 72.7 ng/mL and 16.7 ng/mL was measured for p.o. administration (Fig. 8.1a–c). At the 2 h timepoint 4-MU was barely detectable in the serum independent of route of

Table 8.3 4-MU administration i.v. 4-MUG measured

Animal	Administration	Administration	measured	$t_{1/2}$ (h)	C0 (ng/mL)	AUClast (h*ng/ mL)	AUCInf (h*ng/ mL)	AUCEXtr (%)	MRTInf (h)
Mouse 1	4-MU (25 mg/kg)	i.v.	4-MUG	1.46	16,100	54,570	54,982	0.751	0.611
Mouse 2	4-MU (25 mg/kg)	i.v.	4-MUG	0.961	30,200	41,093	41,194	0.246	0.503
Mouse 3	4-MU (25 mg/kg)	i.v.	4-MUG	1.44	29,900	40,505	41,351	2.05	0.800
Mean	4-MU (25 mg/kg)	i.v.	4-MUG	1.29	25,400	45,389	45,842	1.01	0.638
SD	4-MU (25 mg/kg)	i.v.	4-MUG	0.28	8055	7956	7916	0.93	0.150

Table 8.4 4-MU administration p.o. 4-MUG measured

Animal	Administration	Administration	measured	$t_{1/2}$ (h)	t_{max} (h)	Cmax (ng/mL)	AUClast (h*ng/ mL)	AUCInf (h*ng/ mL)	AUCExtr (%)	MRTInf (hr)
Mouse 1	4-MU (25 mg/ kg)	p.o.	4-MUG	1.72	0.25	22,100	18,470	18,911	2.33	1.10
Mouse 2	4-MU (25 mg/ kg)	p.o.	4-MUG	8.94	0.25	20,900	20,490	41,259	50.3	10.4
Mouse 3	4-MU (25 mg/ kg)	p.o.	4-MUG	0.705	0.25	43,200	21,067	21,089	0.105	0.55
Mean	4-MU (25 mg/ kg)	p.o.	4-MUG	3.79	0.25	28,733	20,009	27,086	17.6	4.00
SD	4-MU (25 mg/ kg)	p.o.	4-MUG	4.49	0.00	12,543	1363	12,323	28.4	5.51

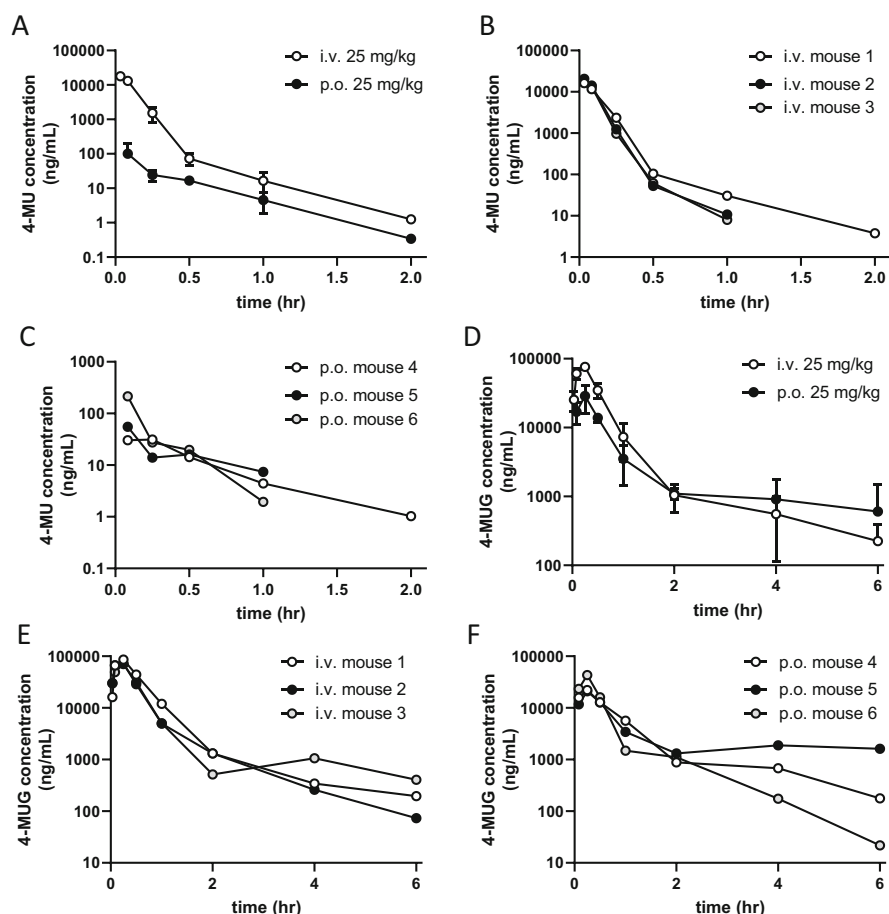


Fig. 8.1 4-MU i.v. and p.o. administration. CD-1 mice, 7–9 weeks of age, were given 4-MU i.v. and p.o. Blood samples were collected at 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. The 4-MU and 4-MUG concentration in the blood samples was determined via LC-MS/MS. (a) Mean 4-MU concentration after i.v. and p.o. administration at different time points. (b) 4-MU concentration after i.v. administration, individual animals. (c) 4-MU concentration after p.o. administration, individual animals. (d) Mean 4-MUG concentration after i.v. and p.o. administration at different time points. 1344-MUG concentration after i.v. administration, individual animals. (f) 4-MUG concentration after p.o. administration, individual animals. $N = 3$ mice per group

administration, 1.25 ng/mL was measured for i.v. and 0.34 ng/mL for p.o. (Fig. 8.1a–c).

After 4-MU administration, 4-MUG was detected at 0.033 h at 25400 ng/mL in the i.v. group, compared to 16,933 ng/mL at 0.083 h for the p.o. administration

group (Fig. 8.1d–f). One hour after 4-MU administration, the 4-MUG concentration in the mouse serum after i.v. and p.o. administration was almost identical with 1045 ng/mL for i.v. and 1110 ng/mL for p.o. (Fig. 8.1d–f). At the last measurement time point of 6 h the 4-MUG concentration after 4-MU i.v. injection was 225 ng/mL and 603 ng/mL for p.o. administration (Fig. 8.1d–f).

8.3.4 4-MUG Administration i.v. and p.o., 4-MUG Measured

To establish the basic PK properties of 4-MUG in mice, 25 mg/kg 4-MUG, the major metabolite of 4-MU, was administered i.v. and p.o. (Tables 8.5 and 8.6). The mean half-life ($t_{1/2}$) of 4-MUG in the serum was 3.17 h for i.v. and 1.14 h for p.o. administration. The initial plasma drug concentration at time zero (C_0) following the bolus i.v. injection was 102,477 ng/mL. The time to reach maximal plasma concentration (t_{max}) following p.o. administration was 3.33 h and the maximal plasma 4-MUG concentration (C_{max}) was 2800 ng/mL. The area under the plasma concentration–time curve extrapolated from time t to infinity as a percentage of total AUC (AUC_{last}) was 33,783 h*ng/mL for i.v. and 8506 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve from time zero to infinity (AUC_{Inf}) was 33,792 h*ng/mL for i.v. and 8766 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve extrapolated from time t to infinity as a percentage of total AUC (AUC_{Extr}) was 0.0262% for i.v. and 2.91% for p.o. 4-MU administration. The mean infinite residence time (MRT_{Inf}) for 4-MUG after i.v. administration was 0.524 h and 3.46 h for p.o. administration. The dose-normalized area under the plasma concentration–time curve from time 0 to infinity (AUC_{Inf}/D) for 4-MUG administered p.o. was 351 h*kg*ng/mL/mg. The systemic availability (F) for the p.o. administered 4-MUG dose was 25.9%. For the 4-MUG administration i.v. the apparent volume of distribution during the terminal phase (V_Z) was 0.681 L/kg, and the apparent volume of distribution at steady state (V_{ss}) was 0.0805 L/kg. The apparent total body clearance of the drug from plasma (CL) was 2.51 mL/min/kg for i.v. 4-MUG administration.

8.3.5 4-MUG Administration i.v. and p.o., 4-MU Measured

In the same experiment to establish the basic PK properties of 4-MUG, 4-MU was assessed after 25 mg/kg 4-MUG was administered i.v. and p.o. (Tables 8.7 and 8.8). The mean half-life ($t_{1/2}$) of 4-MU in the serum was 0.265 h for i.v. and 0.00 for p.o. administration. The initial plasma 4-MUG concentration at time zero (C_0) following the bolus i.v. injection was 504 ng/mL. The time to reach maximal plasma concentration (t_{max}) following p.o. administration was 0.27 h and the maximal plasma 4-MU concentration (C_{max}) was 10.2 ng/mL. The area under the plasma concentration–time curve extrapolated from time t to infinity as a percentage of total

Table 8.5 4-MUG administration i.v. 4-MUG measured

Animal	Treatment	Administration	measured	t1/2 (h)	C0 (ng/mL)	AUClast (h*ng/mL)	AUCInf (h*ng/mL)	AUCExtr (%)	Vz (L/kg)	Vss (L/kg)	CL (mL/min/ kg)	MRTInf (h)
Mouse 1	4-MUG (25 mg/kg)	i.v.	4-MUG	3.09	90,225	31,708	31,717	0.0267	0.704	0.0820	2.63	0.520
Mouse 2	4-MUG (25 mg/kg)	i.v.	4-MUG	3.46	123,807	39,900	39,907	0.0192	0.625	0.0501	2.09	0.400
Mouse 3	4-MUG (25 mg/kg)	i.v.	4-MUG	2.95	93,398	29,741	29,751	0.0325	0.716	0.109	2.80	0.650
Mean	4-MUG (25 mg/kg)	i.v.	4-MUG	3.17	102,477	33,783	33,792	0.0262	0.681	0.0805	2.51	0.524
SD	4-MUG (25 mg/kg)	i.v.	4-MUG	0.26	18,540	5388	5387	0.0066	0.050	0.0296	0.37	0.125

Table 8.6 4-MUG administration p.o. 4-MUG measured

Animal	Treatment	Administration	measured	$t_{1/2}$ (h)	t_{\max} (h)	C_{\max} (ng/mL)	AUClast (h*ng/mL)	AUCInf (h*ng/mL)	AUCExtr (%)	MRTInf (h)	AUCInf/D (h*kg*ng/m L/mg)	F (%)
Mouse 4	4-MUG (25 mg/kg)	p.o.	4-MUG	1.04	4	2960	10,023	10,330	2.97	4.14	413	30.6
Mouse 5	4-MUG (25 mg/kg)	p.o.	4-MUG	1.43	2	3970	8135	8494	4.24	2.94	340	25.1
Mouse 6	4-MUG (25 mg/kg)	p.o.	4-MUG	0.959	4	1470	7362	7475	1.51	3.30	299	22.1
Mean	4-MUG (25 mg/kg)	p.o.	4-MUG	1.14	3.33	2800	8506	8766	2.91	3.46	351	25.9
SD	4-MUG (25 mg/kg)	p.o.	4-MUG	0.25	1.15	1258	1369	1447	1.36	0.61	58	4.3

Table 8.7 4-MUG administration i.v. 4-MU measured

Animal	Treatment	Administration	measured	$t_{1/2}$ (hr)	C0 (ng/mL)	AUClast (hr*ng/ mL)	AUCInf (h*ng/ mL)	AUCEXtr (%)	MRTInf (h)
Mouse 1	4-MUG (25 mg/ kg)	i.v.	4-MU	0.232	340	254	255	0.309	0.381
Mouse 2	4-MUG (25 mg/ kg)	i.v.	4-MU	0.215	1012	272	273	0.128	0.249
Mouse 3	4-MUG (25 mg/ kg)	i.v.	4-MU	0.348	161	128	130	1.30	0.399
Mean	4-MUG (25 mg/ kg)	i.v.	4-MU	0.265	504	218	219	0.579	0.343
SD	4-MUG (25 mg/ kg)	i.v.	4-MU	0.072	449	78	78	0.631	0.082

Table 8.8 4-MUG administration p.o. 4-MU measured

Animal	Treatment	Administration	measured	$t_{1/2}$ (h)	t_{max} (h)	C _{max} (ng/mL)	AUC _{last} (h*ng/ mL)	AUC _{Inf} (h*ng/ mL)	AUC _{Extr} (%)	MRT _{Inf} (h)
Mouse 1	4-MUG (25 mg/ kg)	p.o.	4-MU	0.00	4	4.96	7.73	0.00	0.00	0.00
Mouse 2	4-MUG (25 mg/ kg)	p.o.	4-MU	0.00	2	19.2	19.2	0.00	0.00	0.00
Mouse 3	4-MUG (25 mg/ kg)	p.o.	4-MU	0.00	2	6.37	16.0	0.00	0.00	0.00
Mean	4-MUG (25 mg/ kg)	p.o.	4-MU	0.00	2.67	10.2	14.3	0.00	0.00	0.00
SD	4-MUG (25 mg/ kg)	p.o.	4-MU	NA	1.15	7.8	5.9	NA	NA	NA

Caco-2 permeability assessment 4-MU & 4-MUG

AUC (AUC_{last}) was 218 h*ng/mL for i.v. and 14.3 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve from time zero to infinity (AUC_{Inf}) was 219 h*ng/mL for i.v. and 0.00 h*ng/mL for p.o. administration. The area under the plasma concentration–time curve extrapolated from time to infinity as a percentage of total AUC (AUC_{Ext}) was 0.579% for i.v. and 0.00% for 4-MU measured after p.o. 4-MUG administration. The mean infinite residence time (MRT_{Inf}) for 4-MU after i.v. administration of 4-MUG was 0.082 h and 0.00 h for p.o. administration.

8.3.6 4-MUG and 4-MU Concentration in Mice over Time after 4-MUG I.V. and P.O. Administration

4-MUG was administered to mice at 25 mg/kg i.v. and p.o., the serum concentration of 4-MUG and 4-MU was measured (Fig. 8.2). The mice dosed i.v. started at 0.083 h with 74,133 ng/mL 4-MUG whereas the mice dosed p.o. starting at 0.25 h had a mean 4-MUG concentration of 209 ng/mL (Fig. 8.2a–c). At 1 h the 4-MUG concentration after i.v. administration went down to 3590 ng/mL and the 4-MUG concentration after 1 h p.o. administration went up to 359 ng/mL (Fig. 8.2a–c). At the 8 h timepoint 4-MUG was barely detectable in the serum after i.v. administration, with 28.1 ng/mL compared to 154 ng/mL still detectable after 8 h after p.o. administration of 4-MUG (Fig. 8.2a–c).

After 4-MUG administration, 4-MU was detected at 0.083 h at 399 ng/mL in the i.v. group, compared to 0.00 ng/mL for the p.o. administration group (Fig. 8.2d–f). One hour after 4-MUG administration, the 4-MU concentration in the mouse serum after i.v. was 22.2 ng/mL and p.o. barely detectable with 1.26 ng/mL (Fig. 8.2d–f). At the last measurement time point with usable data at 4 h the 4-MU concentration after 4-MUG i.v. injection was not detectable and 2.66 ng/mL for p.o. administration (Fig. 8.2d–f).

8.3.7 4-MU and 4-MUG Permeability Assessment

In a Caco-2 permeability experiment we assessed the permeability and Pgp substrate classification for 4-MU and 4-MUG (Table 8.9). For 4-MU the permeability was classified as high and the 4-MUG permeability was classified as low, both substances were not classified as Pgp substrates. The % recovery from A \rightarrow B and from B \rightarrow A was between 86.9 and 93.4 for 4-MU and 104 and 103 for 4-MUG. The parameter apparent permeability P_{app} from A \rightarrow B and from B \rightarrow A was 42.9 and 37.2 $\times 10^6$ cm/s for 4-MU and 0.156 and 0.129 $\times 10^6$ cm/s for 4-MUG. The efflux ratio was 0.865 for 4-MU and 0.823 for 4-MUG.

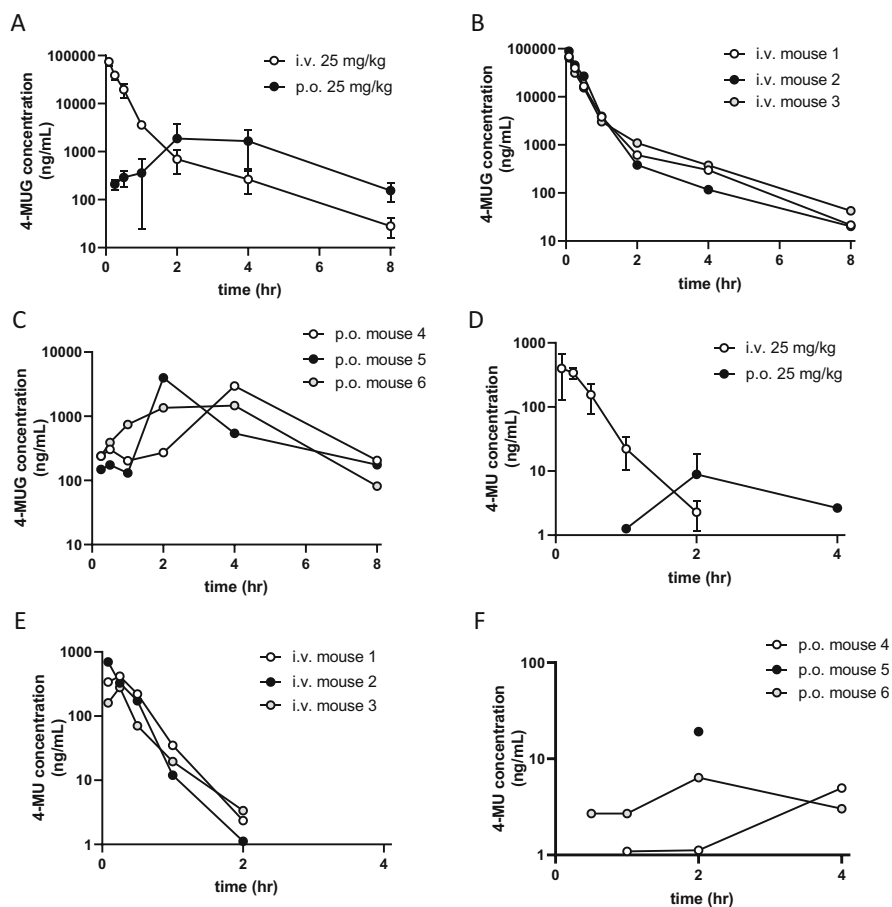


Fig. 8.2 4-MUG i.v. and p.o. administration. CD-1 mice, 7–9 weeks of age, were given 4-MUG i.v. and p.o. Blood samples were collected at 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. The 4-MU and 4-MUG concentration in the blood samples was determined via LC-MS/MS. (a) Mean 4-MUG concentration after i.v. and p.o. administration at different time points. (b) 4-MUG concentration after i.v. administration, individual animals. (c) 4-MUG concentration after p.o. administration, individual animals. (d) Mean 4-MU concentration after i.v. and p.o. administration at different time points. (e) 4-MU concentration after i.v. administration, individual animals. (f) 4-MU concentration after p.o. administration, individual animals. $N = 3$ mice per group

Table 8.9 Caco-2 permeability assessment 4-MU & 4-MUG

Compound ID	% Recovery (N = 2)		Papp ($\times 10^{-6}$ cm/s) (N = 2)		Efflux ratio	Permeability Classification	Pgp substrate
	A \rightarrow B	B \rightarrow A	A \rightarrow B	B \rightarrow A			
4-MU	86.9	93.3	42.9	37.2	0.865	High	NO
4-MUG	104	103	0.156	0.129	0.823	Low	NO
Atenolol	103	93.4	0.300	0.402	1.34	Low	NO

8.3.8 4-MU and 4-MUG Treatment Stop Study

To evaluate how fast after treatment stop the 4-MU and 4-MUG plasma concentration decreases, we treated mice for 5 weeks with 4-MU and 4-MUG and then stopped the treatment (Fig. 8.3). We took blood samples at different timepoints after treatment stop up to 24 hrs. We found that the 4-MU concentration after 4-MU treatment stop significantly decreased after 1 h from 4960 ng/mL to 1156 ng/mL (Fig. 8.3a). At 2, 6, and 12 h after treatment stop the 4-MU concentration was 460 ng/mL, 220 ng/mL, and 92 ng/mL (Fig. 8.3a). At 24 h after treatment stop the 4-MU concentration in the serum was close to background noise with 4.4 ng/mL (Fig. 8.3a). In the same 4-MU treated animals we measured the 4-MUG concentration in the serum after treatment stop (Fig. 8.3b). Here we saw a slightly different drug concentration profile. Immediately after treatment stop the 4-MUG concentration in the serum was 220,333 ng/mL and increased to 354,667 ng/mL 1 h after treatment stop (Fig. 8.3b). This increase was followed by a rapid decrease to 31,133 ng/mL at 2 h after treatment stop and declined further at 6 and 12 h were 4-MUG concentrations of 1461 ng/mL and 520 ng/mL were measured (Fig. 8.3b). Twenty-four h after 4-MU treatment stop the 4-MUG concentration was with 140 ng/mL at the level of background noise (Fig. 8.3b).

In the 4-MUG treatment group we found after treatment stop very low levels of 4-MU (Fig. 8.3c). At treatment stop the 4-MU concentration was 17 ng/mL, increased to 40 ng/mL 1 h after treatment stop and declined to background noise at 6, 12, and 24 h ranging between 3 and 1 ng/mL (Fig. 8.3c). We saw much higher concentrations of 4-MUG after 4-MG treatment stop (Fig. 8.3d). At the time of 4-MUG treatment stop, the 4-MUG concentration was 2796 ng/mL, which increased to 9223 ng/mL 1 h after treatment stop (Fig. 8.3d). 2 h after treatment stop the 4-MUG concentration declined to 3752 ng/mL and at 6 h after treatment stop the 4-MUG concentration declined further almost to background level to 199 ng/mL, followed by 44 ng/mL and 57 ng/mL at the 12 h and 24 h time points (Fig. 8.3d). The AUC calculations of this experiment revealed a bigger AUC when 4-MUG was measured, after the initial 4-MUG treatment, the highest AUC was calculated for 4-MUG measurement when the initial treatment was 4-MU (Fig. 8.3e).

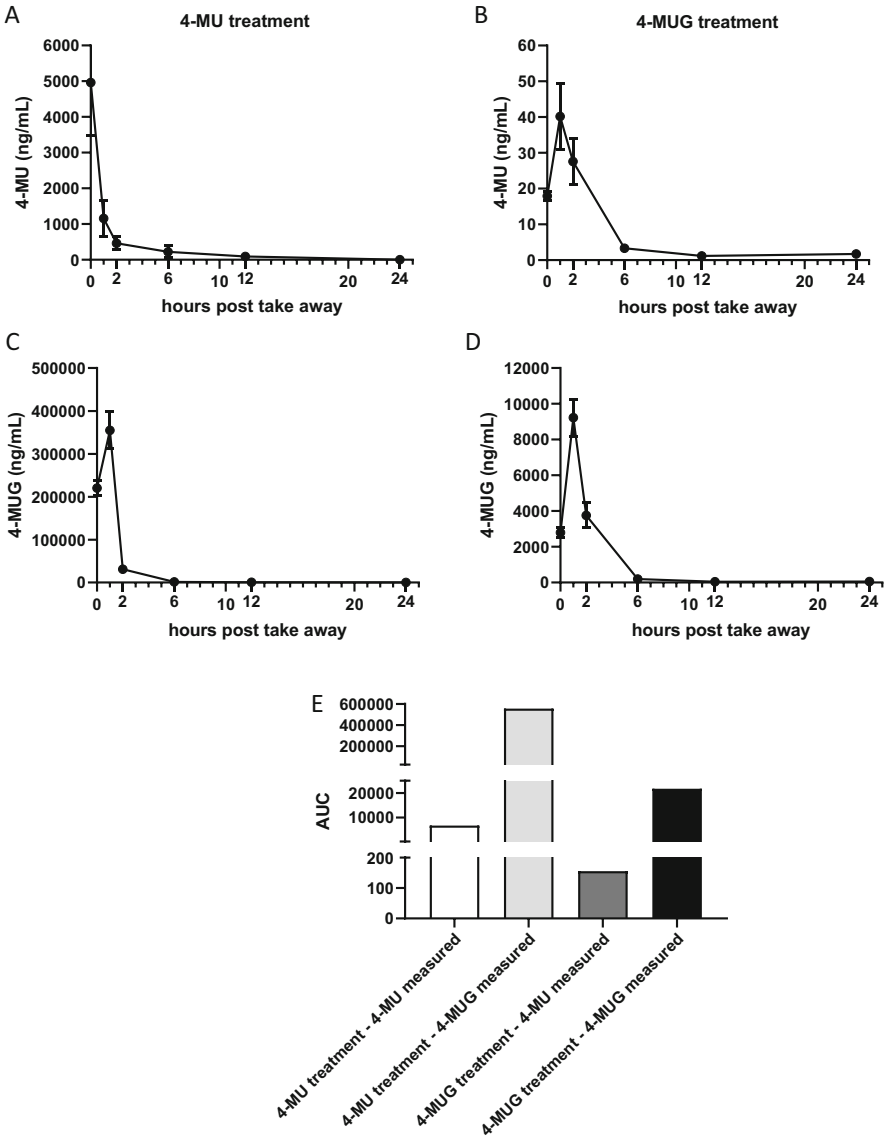


Fig. 8.3 4-MU and 4-MUG treatment stop study. C57Bl/6 J mice were treated with 4-MU and 4-MUG starting at 8 weeks of age. The mice were treated for 5 weeks, and at the end of 5 weeks the treatment was stopped and blood samples were taken at T0, T1, T2, T6, T12, and T24 hours. The 4-MU and 4-MUG concentration in the blood samples was determined via LC-MS/MS. (a) 4-MU treated mice, 4-MU measured. (b) 4-MUG treated mice, 4-MU measured. (c) 4-MU treated mice, 4-MUG measured. (d) 4-MUG treated mice, 4-MUG measured. (e) AUC for all treatment groups. $N = 5$ mice per group

8.3.9 4-MU and 4-MUG Treatment Built-up Study

To evaluate how fast and to what concentrations 4-MU and 4-MUG would built up in the plasma, we treated mice for 20 days with 4-MU and 4-MUG (Fig. 8.4). We took blood samples at different timepoints from treatment start up to 20 days later. We found that the 4-MU concentration after 4-MU treatment reached a plateau after 4 days of treatment (Fig. 8.4a). One day after treatment start a 4-MU concentration of 541 ng/mL was detected, which increased to 1371 ng/mL at 4 days after treatment start (Fig. 8.4a). The 4-MU measurement at 7 days and 15 days post treatment start showed with 1228 ng/mL and 1516 ng/mL similar concentration level (Fig. 8.4a). We noticed a decline in 4-MU concentration to 931 ng/mL at day 20 post treatment start (Fig. 8.4a). In the same 4-MU treated animals we measured the 4-MUG concentration from treatment start and saw the same plateau starting at day 4, with a slight increase at day 15 (Fig. 8.4b). The 4-MUG concentration after 1 day of 4-MU treatment was 27,228 ng/mL, which increased to 262,400 ng/mL 4 days after treatment start (Fig. 8.4b). The 4-MUG concentrations at day 7 and day 20 after treatment start were with 297,800 ng/mL and 268,540 ng/mL very similar (Fig. 8.4b). An increase in 4-MUG concentration could be detected at 15 days post treatment start, here the concentration reached 386,460 ng/mL (Fig. 8.4b).

In the 4-MUG treatment group the 4-MU concentration was in general very low, here a plateau was reached after 1 day of treatment (Fig. 8.4c). One day post treatment start 4-MU reached concentrations of 21 ng/mL, which increased to 26 ng/mL at day 4 post treatment (Fig. 8.4c). The 4-MU concentrations after 4-MUG treatment at days 7, 15, and 20 post treatment start were with 17 ng/mL, 20 ng/mL, and 19 ng/mL quite similar (Fig. 8.4c). When we looked at the 4-MUG concentration after 4-MUG treatment, we found a massive drop at day 7 post treatment start followed by another increase at day 20 (Fig. 8.4d). One day after treatment start the 4-MUG concentration was at 1907 ng/mL and increased further to 4546 ng/mL 4 days after treatment start (Fig. 8.4d). This was followed by a huge decrease in 4-MUG concentration at day 7 post treatment start to 685 ng/mL (Fig. 8.4d). The 4-MUG concentration after 4-MUG treatment increased again at day 7 and day 20 post treatment start to 1740 ng/mL and 4332 ng/mL (Fig. 8.4d). The AUC calculations of this experiment revealed a bigger AUC when 4-MUG was measured, after the initial 4-MUG treatment, the highest AUC was calculated for 4-MUG measurement when the initial treatment was 4-MU (Fig. 8.4e).

8.4 Discussion

In these studies, we have examined the PK for both oral and i.v. 4-MU and 4-MUG in mice when given as single as well as multiple dosing protocols.

We find that most of the circulating drug present in vivo exists as 4-MUG. This is consistent with previous PK studies in animals that demonstrated the extraction of

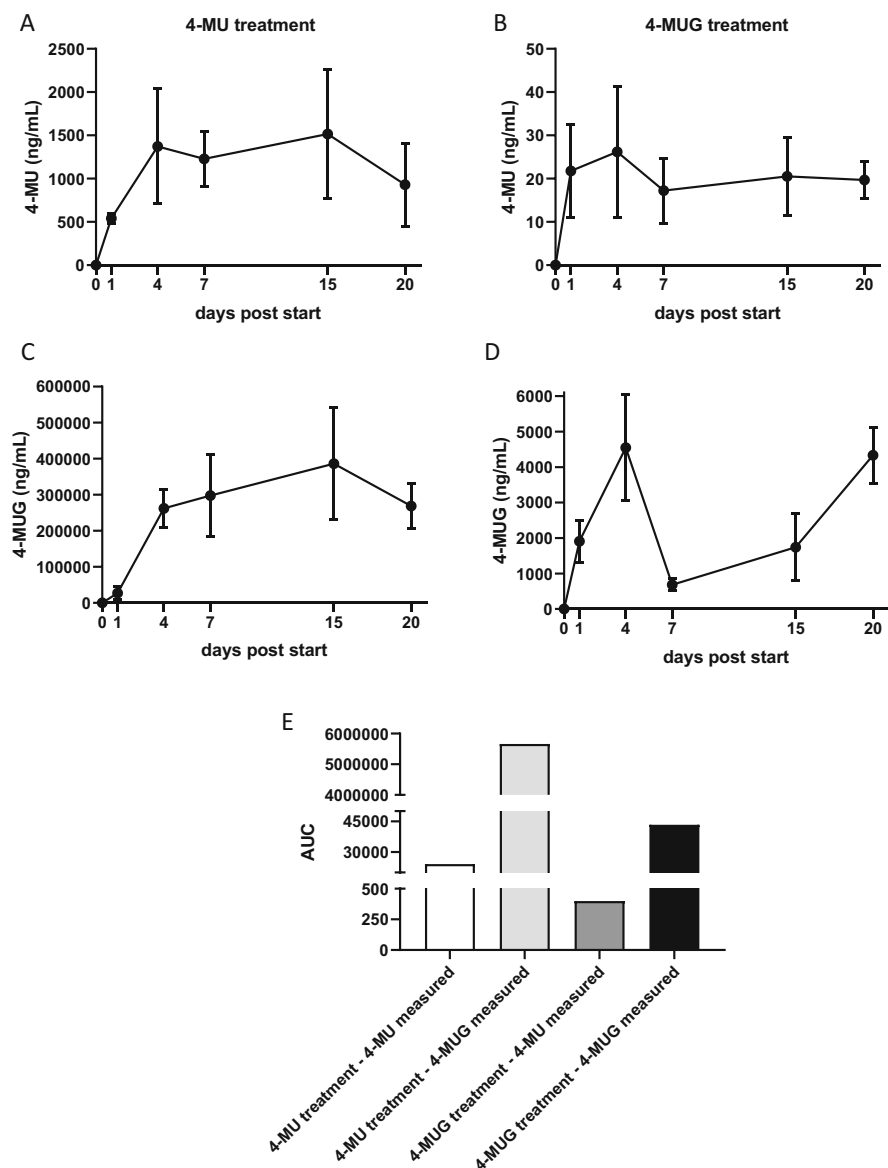


Fig. 8.4 4-MU and 4-MUG treatment built up study. C57Bl/6 J mice were treated with 4-MU and 4-MUG starting at 8 weeks of age for 20 days. During the time of treatment blood samples were taken from the mice at T0, T1, T4, T7, T15, and T20 days after treatment start. The 4-MU and 4-MUG concentration in the blood samples was determined via LC-MS/MS. (a) 4-MU treated mice, 4-MU measured. (b) 4-MUG treated mice, 4-MU measured. (c) 4-MU treated mice, 4-MUG measured. (d) 4-MUG treated mice, 4-MUG measured. (e) AUC for all treatment groups. $N = 5$ mice per group

4-MU by the gastrointestinal system (pre-hepatic) to be ~40% and extraction by the liver as high as 97% (Stacchino et al. 1983) due to the major hepatic and intestinal UGTs involved in drug metabolism (Uchaipichat et al. 2004). As a result of this high extraction, the fraction of an administered oral dose of hymecromone (4-MU) that reaches the systemic circulation (post-hepatic) as unchanged drug (i.e. the bioavailability) is very low. These data indicate that It is important to understand the 4-MUG PK during oral 4-MU therapy, given the expected much higher exposures of the metabolite relative to the parent in peripheral tissues.

In our studies comparing p.o. and i.v. delivery of 4-MU, we report that the half-life of 4-MU given as a single dose via i.v. administration (0.182 h) was doubled when given p.o. (0.346 h). The half-life of the resulting 4-MUG metabolite given as a single dose i.v. (1.29 h) was almost tripled when given p.o. (3.79 h) was one-third. Comparing p.o. and i.v. delivery of 4-MUG, we report the half-life was almost three times higher for i.v. (3.17 h) administration compared to p.o. (1.14 h). When we dosed 4-MUG and measured 4-MU, only a low half-life for i.v. administration could be observed, the oral administration did not yield in measurable 4-MU concentrations. Consistent with this, in a Caco-2 experiment we found that the permeability of 4-MU is high and the permeability for 4-MUG is low. These data suggest that oral delivery of 4-MU is an effective way to achieve longer half-lives of the drug. Conversely, oral 4-MUG is not absorbed effectively.

In a study where mice were treated with 4-MU and 4-MUG for several weeks, we observed that mice that received 4-MU reached far higher drug levels than mice treated with 4-MUG. In this study, we see a rapid increase in serum 4-MU as well as 4-MUG after 4 days of treatment. We also observed a plateau from that day on until the end of our measurements more than 2 weeks later. When we treated with 4-MUG and looked at the serum concentrations of 4-MU we saw a fast increase after 1 day which stayed at that relatively low level for the time of our experiment.

In a study where mice were treated multiple weeks with 4-MU and 4-MUG and the treatment was stopped, after cessation of therapy, we observed a rapid drop of 4-MU in the serum in the 4-MU treated mice. In those mice, the 4-MUG concentration reached a peak at 1 h after treatment stop and was non-detectable at 6 h. When mice were treated with 4-MUG and this was stopped, both 4-MU and 4-MUG were again detectable for less than 6 h. These data indicate that drug persists in circulation for several hours after continuous dosing of 4-MU or 4-MUG.

While these studies illuminate the PK of 4-MU and 4-MUG in mice, the PK of 4-MUG in humans are not well studied. In healthy volunteers, the systemic exposure of 4-MUG after an i.v. dose was shown to be higher than that of 4-MU (Garrett et al. 1993). Future clinical studies with 4-MU would benefit from a more thorough understanding of the PK of 4-MU itself and its metabolite 4-MUG.

We believe that our performed PK studies will aid the understanding and development of dosing strategies including appropriate dose strength and frequency in animal models using 4-MU and 4-MUG. And hopefully these studies will ultimately better inform us about the use of 4-MU and maybe even 4-MUG in human clinical trials targeting HA synthesis in chronic and acute diseases (Medina et al. 2018).

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Conflict of Interest Statement

N.N., G.K., and P.L.B. are listed as inventors of the patents-pending (PCT/US2014/050770, S17-131US/BLSU-1-65,422, PCT/US2019/067911) filed by the Board of Trustees of the Leland Stanford Junior University.

Contribution Statement N.N., M.A.U., S.V.M., and P.L.B. conceptualization; N.N., G.K., P.L.B. supervision; N.N., and P.L.B. funding acquisition; N.N., G.K., N.L.H., A.H., M.A.U., J. R., and P.L.B. investigation; N.N., G.K., N.L.H., and P.L.B. visualization; N.N., M.A.U., and G.K., methodology; N.N. and P.L.B. writing-original draft; N.N., G.K., and P.L.B. project administration.

Data Availability The data are available from the corresponding author.

References

- Abate A, Dimartino V, Spina P, Costa PL, Lombardo C, Santini A, Del Piano M, Alimonti P (2001) Hymecromone in the treatment of motor disorders of the bile ducts: a multicenter, double-blind, placebo-controlled clinical study. *Drugs Exp Clin Res* 27:223–231
- Bollyky PL, Bogdani M, Bollyky JB, Hull RL, Wight TN (2012) The role of hyaluronan and the extracellular matrix in islet inflammation and immune regulation. *Curr Diab Rep* 12:471–480
- Bollyky PL, Evanko SP, Wu RP, Potter-Perigo S, Long SA, Kinsella B, Reijonen H, Guebtner K, Teng B, Chan CK, Braun KR, Gebe JA, Nepom GT, Wight TN (2010) Th1 cytokines promote T-cell binding to antigen-presenting cells via enhanced hyaluronan production and accumulation at the immune synapse. *Cell Mol Immunol* 7:211–220
- Camarri E, Marchettini G (1988) Hymecromone in the treatment of symptoms following surgery of the bile ducts. *Recenti Prog Med* 79:198–202
- Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA (2011) Hyaluronan deposition and correlation with inflammation in a murine ovalbumin model of asthma. *Matrix Biol* 30, Cross Ref Medline:126–134
- Fontaine L, Grand M, Molho D, Chabert MJ, Boschetti E (1968) Choleretic, spasmolytic and general pharmacologic activities of 4-methylumbelliferone. *Therapie* 23:51–62
- Fraser JR, Laurent TC, Laurent UB (1997) Hyaluronan: its nature, distribution, functions and turnover. *J Inter Des. Med.* 242,27–33CrossRef Medline 242:27
- Garrett ER, Venitz J (1994) Comparisons of detections, stabilities, and kinetics of degradation of hymecromone and its glucuronide and sulfate metabolites. *J Pharm Sci* 83:115–116
- Garrett ER, Venitz J, Eberst K, Cerda JJ (1993) Pharmacokinetics and bioavailabilities of hymecromone in human volunteers. *Biopharm Drug Dispos* 14:13–39
- Hoffmann RM, Schwarz G, Pohl C, Ziegenhagen DJ, Kruis W (2005) Bile acid-independent effect of hymecromone on bile secretion and common bile duct motility. *Dtsch Med Wochenschr* 130: 1938–1943. <https://doi.org/10.1055/s-2005-872606>
- Hull RL, Johnson PY, Braun KR, Day AJ, Wight TN (2012) Hyaluronan and hyaluronan binding proteins are normal components of mouse pancreatic islets and are differentially expressed by islet endocrine cell types. *J Histochem Cytochem* 60,749–760CrossRef Medline 60:749

- Jiang D, Liang J, Noble PW (2011) Hyaluronan as an immune regulator in human diseases. *Physiol Rev* 91:221–264 [CrossRef](#) [Medline](#)
- Kakizaki I, Kojima K, Takagaki K, Endo M, Kannagi R, Ito M, Maruo Y, Sato H, Yasuda T, Mita S, Kimata K, Itano N (2004) A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. *J. Biol Chem* 279:33281–33289
- Kang L, Lantier L, Kennedy A, Bonner JS, Mayes WH, Bracy DP, Bookbinder LH, Hasty AH, Thompson CB, Wasserman DH (2013) Hyaluronan accumulates with high-fat feeding and contributes to insulin resistance. *Diabetes* 62:1888–1896
- Krawzak HW, Heistermann HP, Andrejewski K, Hohlbach G (1994) Postprandial bile-duct kinetics under the influence of 4-methylumbelliferone (hymecromone). *Int J Clin Pharmacol Ther* 33: 569–572
- Nagy N, Kuipers HF, Frymoyer AR, Ishak HD, Bollyky JB, Wight TN, Bollyky PL (2015) 4-methylumbelliferone treatment and hyaluronan inhibition as a therapeutic strategy in inflammation, autoimmunity, and cancer. *Front Immunol* 6:123
- Kuipers HF, Rieck M, Gurevich I, Nagy N, Butte MJ, Negrin RS, Wight TN, Steinman L, Bollyky PL (2016) Hyaluronan synthesis is necessary for autoreactive T-cell trafficking, activation, and Th1 polarization. *Proc Natl Acad Sci U.S.A.* 113:1339–1344
- Kultti A, Pasonen-Seppänen S, Jauhainen M, Rilla KJ, Kärnä R, Pyöriä E, Tammi RH, Tammi MI (2009) 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3. *Exp Cell Res* 315: 1914–1923
- Medina CO, Nagy N, Bollyky PL (2018) Extracellular matrix and the maintenance and loss of peripheral immune tolerance in autoimmune insulinitis. *Curr Opin Immunol* 55:22–30
- Mine S, Okada Y, Kawahara C, Tabata T, Tanaka Y (2006) Serum hyaluronan concentration as a marker of angiopathy in patients with diabetes mellitus. *Endocr. J* 53:761–766
- Mulder GJ, Brouwer S, Weiting JG, Scholtens E, Pang KS (1985) Glucuronidation and sulfation in the rat in vivo. The role of the liver and the intestine in the in vivo clearance of 4-methylumbelliferone. *Biochem Pharmacol* 34:1325–1329
- Nagy N, Freudenberger T, Melchior-Becker A, Röck K, Ter Braak M, Jastrow H, Kinzig M, Lucke S, Suvorava T, Kojda G, Weber AA, Sörgel F, Levkau B, Ergün S, Fischer JW (2010) Inhibition of hyaluronan synthesis accelerates murine atherosclerosis: Novel insights into the role of hyaluronan synthesis. *Circulation* 122:2313–2322
- Nagy N, Kaber G, Johnson PY, Gebe JA, Preisinger A, Falk BA, Sunkari VG, Gooden MD, Vernon RB, Bogdani M, Kuipers HF, Day AJ, Campbell DJ, Wight TN, Bollyky PL (2015) Inhibition of hyaluronan synthesis restores immune tolerance during autoimmune insulinitis. *J Clin Invest* 125:3928–3940
- Quaranta S, Rossetti S, Camarri E (1984) Double-blind clinical study on hymecromone and placebo in motor disorders of the bile ducts after cholecystectomy. *Clin Ter* 108:513–517
- Stacchino C, Spanò R, Pettiti A (1983) Spasmolytic activity of some 4-methylumbelliferone derivatives. *Boll Chim Farm* 122:158–160
- Takeda S, Aburada M (1980) The choleretic mechanism of coumarin compounds and phenolic compounds. *J Pharmacobiodyn* 4:724–734
- Tanayama S, Kanai Y (1977) Studies on increased bile formation produced by polyoxybenzenes in rats. *Jpn J Pharmacol* 27:71–78
- Termeer C, Sleeman JP, Simon JC (2003) Hyaluronan—magic glue for the regulation of the immune response? *Trends Immunol* 24:112–114

- Trabucchi E, Baratti C, Centemero A, Zuin M, Rizzitelli E, Colombo R (1986) Controlled study of the effects of tiotropium on biliary dyskinesia. *Pharmatherapeutica* 4:541–550
- Uchaipichat V, Mackenzie PI, Guo X-H, Gardner-Stephen D, Galetin A, Houston JB, Miners JO (2004) Human udp-glucuronosyltransferases: isoform selectivity and kinetics of 4-methylumbelliferone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by diclofenac and probenecid. *Drug Metab Dispos* 32:413–423
- Walter P, Seidel W (1978) Studies on the effect of 4-methyl-umbelliferon (Hymecromone) in patients following surgical revision of the biliary pathways. *Chirurg* 50:436–440
- Yoshioka Y, Kozawa E, Urakawa H, Arai E, Futamura N, Zhuo L, Kimata K, Ishiguro N, Nishida Y (2013) Suppression of hyaluronan synthesis alleviates inflammatory responses in murine arthritis and in hu-man rheumatoid synovial fibroblasts. *Arthritis Rheum* 65:1160–1170

Chapter 9

The Role of Hyaluronan in Skin Wound Healing



Yan Wang and Edward V. Maytin

Abstract Skin wound healing is a complex process involving many types of cells at different specific times. HA is a linear un-sulfated glycosaminoglycan synthesized by three HA synthases (HAS) and degraded by hyaluronidases. By binding to its receptors on cell surfaces, HA regulates the function of various types of cells at different phases of wound healing. During the first phase of wounding (inflammation phase), levels of HA increase rapidly at the wound site. Platelets cleave HMW-HA into fragments and stimulate the extrinsic clotting cascade, achieving hemostasis. The local accumulation of high molecular weight HA (HMW-HA) promotes diffusion of water molecules and facilitates the migration and infiltration of inflammatory cells into the wound area. Low molecular weight HA (LMW-HA) stimulates the secretion of proinflammatory cytokines, which induces vasodilation and increases vascular permeability, leading to more inflammatory cell migration into the wound and facilitating proinflammatory cascades. During the second (proliferative) and third (remodeling) phases of wound healing, HA has crucial roles in regulating the repair of damaged tissue elements. By binding to its receptors such as CD44, ICAM-1 and RHAMM, HA influences the activities of keratinocytes (re-epithelialization) and fibroblasts (migration, proliferation, fibroblast turnover, myofibroblast differentiation) through modulation of signaling cascades such as the TGF-beta receptor pathway. In diabetes, under hyperglycemic condition, HA and other GAGs are degraded into small fragments and become more proinflammatory, leading to damage and loss of the ECM glycocalyx in small vessels, which has a detrimental effect on endothelial cells. Due to its special physical and chemical characteristics such as good biocompatibility, biodegradability, and water-absorbing properties, HA has been widely used in various types of topical dressings and demonstrates a promising therapeutic function in promoting wound healing.

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9.1 Introduction

9.1.1 Skin Wound Healing

Wound healing of the skin is a complex process initiated by the disruption of the integrity of skin structure, and it involves multiple types of cells working collaboratively at different phases or stages (Aya and Stern 2014; Singer and Clark 1999; Schafer and Werner 2008); see Fig. 9.1. Briefly, in the first stage (called the “inflammatory” phase), hemostasis starts immediately after wounding as platelets aggregate at the wound site and trigger the formation of a fibrin clot, which functions to stop the bleeding and also to act as a temporary and provisional matrix for migrating cells. Within hours after wounding, leukocytes travel to the site of injury from the blood stream to clear invading pathogens. Monocytes and macrophages are also recruited to the wound site to phagocytose dead neutrophils and cellular debris. In the second stage (called the “proliferative” or “tissue repair” phase), keratinocytes in epidermis migrate from the wound edges toward the center to help to seal the defect, prevent fluid loss, and exclude infection. Dermal fibroblasts start to proliferate at the wound edges and migrate into the provisional matrix, where a subset of fibroblasts transform into myofibroblasts that express the neo-expression of alpha smooth muscle actin (a-SMA), in a process known as myofibroblast differentiation. Neoangiogenesis is also important during this phase, especially for granulation tissue formation. In the final stage of wound healing (the “remodeling” phase), a completely closed wound takes several more months to progress into fully-mature scar tissue, during which

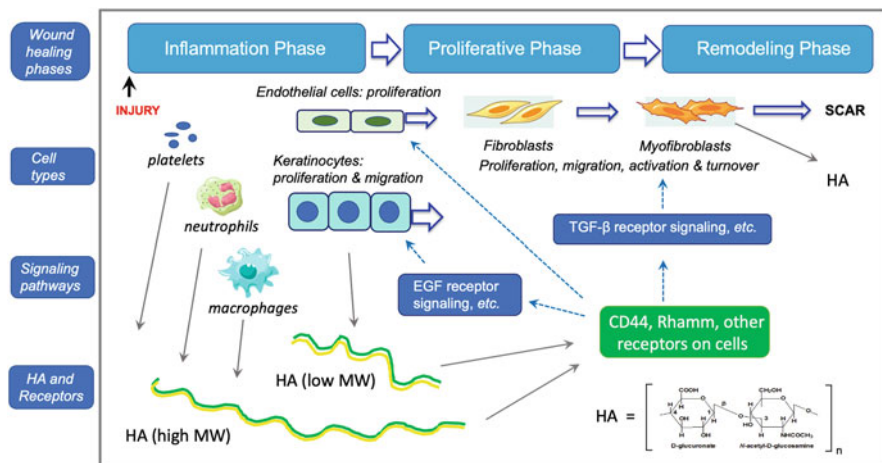


Fig. 9.1 Wound healing of the skin involves different timed phases and requires the participation of many cell types. Each of these cells produce hyaluronan (HA, of both low and high molecular weight) that interacts with receptors, mainly (CD44 and RHAMM) on cell surfaces. Such interactions trigger downstream signaling pathways that regulate the various activities of the cells that are necessary to bring about successful wound repair

extensive changes in extracellular matrix (ECM), including the assembly and maturation of fully developed collagen fibrils, and the formation of complex structures that contain various proteoglycans and glycosaminoglycans (GAGs).

9.1.2 HA Structure, Synthesis, and Degradation

Hyaluronan (or Hyaluronic Acid, HA) was first isolated by Karl Meyer and John Palmer in 1934 (Hascall 2000). The chemical structure of HA was later determined to be a linear unsulfated glycosaminoglycan (GAG) composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine joined by β -linkages. HA is the only GAG that is not sulfated and not linked to a core protein or synthesized by the Golgi pathway. Instead, HA is synthesized on the cytoplasmic surface of the plasma membrane by three HA synthases (HAS) (Itano and Kimata 2002). All three of these enzymes are membrane embedded, and each of their genes resides at a different chromosomal location, namely: HAS1 on human chromosome 19q13.4/mouse chromosome 17; HAS2 on human chromosome 8q24.12/mouse chromosome 15; and HAS3 on human chromosome 16q22.1/mouse chromosome 8 (Spicer et al. 1997). HAS2 is the predominant HAS in most tissues including the skin, and the only one for which genetic depletion results in embryonic lethality (Camenisch et al. 2000). HAS1 and HAS3 synthesize HA with an estimated molecular mass of 2×10^5 to $\sim 2 \times 10^6$ Da. On the other hand, very large molecules (over 2×10^6 Da) are synthesized by HAS2 (Itano et al. 1999). The polymer is transported out of the cell after being synthesized. HA is the most abundant GAG in the human body (~ 15 grams in a 70 kg man) and its turnover rate is very rapid—nearly one-third of HA in the body is degraded and removed each day (Fraser et al. 1997). The estimated half-life of HA in the epidermis is one day (Tammi et al. 1991). As for HA catabolism, there are six hyaluronidase-like genes identified in the human genome. HYAL1, HYAL2, and HYAL3 are clustered at the chromosomal locus 3P21.3, whereas HYAL4, 5, and 6 are localized on chromosome 7q31.3 (Csoka et al. 1999). Among the six HYALs, HYAL1 and HYAL2 are expressed ubiquitously and therefore are the primary hyaluronidases that degrade HA in somatic tissues. As for the other HYALs, the expression levels of HYALs3, 4, and 5 are very low, and HYAL6 is a pseudogene; therefore, none of these are likely to have any major role in HA degradation (Csoka et al. 1999). In recent years, two more proteins, CEMIP (cell migration inducing protein, or KIAA1199) and TMEM2 (a transmembrane protein) were also found to have HA-degradation capability (Kaul et al. 2021). Reactive oxygen species can also break HA into fragments (Soltes et al. 2006); therefore, although synthesized as very large polymers, HA can be degraded into fragments with various molecular weights. The sizes of these HA species vary widely and include HA oligosaccharides (O-HA, $<1 \times 10^4$ Da), low-molecular-weight HA (LMW-HA, $1\text{--}25 \times 10^4$ Da), medium-molecular-weight HA (MMW-HA, $25\text{--}100 \times 10^4$ Da), high-molecular-weight HA (HMW-HA, $>1 \times 10^6$ Da), and very high-molecular-weight HA (vHMW-HA, $>6 \times 10^6$ Da). Each of these HA

size categories is associated with distinct functions (Stern et al. 2006; Tavianatou et al. 2019). HMW-HA displays immunosuppressive (Delmage et al. 1986; McBride and Bard 1979), anti-inflammatory (Nakamura et al. 2004), and anti-angiogenic effects (Feinberg and Beebe 1983), maintains a hydrated environment, and regulates growth factor activity. On the other hand, LMW-HA exerts strong proinflammatory effects by binding and activating different receptors, including CD44, TLR-4, and RHAMM. Such differences could be explained by differences in binding affinity when the variously sized HA interact with a variety of HA receptors (Aruffo et al. 1990; Hardwick et al. 1992; Prevo et al. 2001; Jiang et al. 2005).

There are multiple types of HA receptors on cell surfaces. Among all HA receptors, CD44 is the predominant one for HA and probably the most well-studied. CD44 is a type I transmembrane proteoglycan with multiple isoforms that arise from alternative splicing of 10 variable exons; the most widely expressed isoform is the standard form (CD44s) (Ponta et al. 2003). CD44 mediates cell–cell and cell–matrix interactions and binds growth factors and interacts with growth factor receptors within the cell membrane, thereby exerting a complex role in the regulation of cell growth, survival, and differentiation (Ponta et al. 2003; Jordan et al. 2015; Orian-Rousseau 2015). Although CD44 is the principal receptor for HA, HA is actually not the only ligand binding to CD44. Another relatively well-studied receptor for HA is RHAMM (receptor for HA-mediated motility), for which its role in connecting intracellularly with cytoskeletal proteins, activating protein kinases (e.g., protein kinase C and *c-src*), and regulating cell motility have been established (Hall et al. 2001; Hall et al. 1996).

9.2 HA in the Skin

9.2.1 *Location/Distribution in Normal Skin*

Over 50% of total body HA is located in the skin (Fraser et al. 1997; Reed et al. 1988) and is present in both of the major cutaneous compartments (dermis and epidermis). In the dermis, HA is very abundant and diffusely distributed throughout the ECM. In the epidermis, HA levels are much lower overall than in the dermis. However, while the intracellular HA within epidermal keratinocytes is low, the extracellular HA concentration between keratinocytes is even higher than between fibroblasts in the dermis, as keratinocytes are densely packed compared to the loose distribution of fibroblasts within the dermal ECM (Tammi et al. 1988). In the epidermis, HA is mainly detected in proliferating basal regions and extracellular cell–cell junctions. Within the dermis, HA has the highest concentration in the papillary region. Dermal fibroblasts can produce HA at a rate of 12–60 pg/cell/24 h, whereas the HA production rate of keratinocytes is much lower (Averbeck et al. 2007). HAS2 is the predominant HAS in skin fibroblasts (Wang et al. 2014).

9.3 HA in Skin Wound Healing

9.3.1 *Role of HA in Hemostasis and the Inflammatory Phase of Wound Healing*

After wounding, levels of hyaluronan increase rapidly at the wounding site, reach peak levels at Day 3, and then decrease (Oksala et al. 1995). The main sources of HA are platelets and megakaryocytes that contain large amounts of HA in cytoplasm (Shirali and Goldstein 2008). Another source of HA is keratinocytes. Laboratory studies from the Tammi group and the Maytin group showed that upon wounding, keratinocytes produce increased amounts of HA, corresponding to a 3 ~ 6-fold increase in epidermal HA on Day 3 following wounding, associated with a strong induction of gene expression of Has2 and Has3 (Tammi et al. 2005; Monslow et al. 2009). This increase in HA production is partially mediated by the activation of epidermal growth factor (EGF) receptors due to binding of a specific ligand, heparin-binding EGF (HB-EGF) (Monslow et al. 2009).

The accumulation of hydrophilic HMW-HA at wound sites promotes the passive diffusion of water molecules and leads to local edema, which further facilitates the migration and infiltration of inflammatory cells into the wound area, including neutrophils and monocytes (Tavianatou et al. 2019; Mikecz et al. 1995). Platelets also contain Hyaluronidase 2 (de la Motte et al. 2009), which cleaves HMW-HA into fragments and stimulates the extrinsic clotting cascade by binding to tissue factors and fibrinogen (Weigel et al. 1986; Frost and Weigel 1990). LMW-HA stimulates the secretion of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-8. Local accumulation of these cytokines induces vasodilation and increased vascular permeability, leading to more inflammatory cell migration into the wound and facilitating proinflammatory cascades (Weigel et al. 1988; Lennon and Singleton 2011).

Depending upon the tissue-specific context, the regulatory effects of HMW-HA can be anti-inflammatory and inhibitory to vascular leakiness (Singleton et al. 2010). Mack et al. showed that in a HAS1 and HAS3 double-knockout mouse model (Has1/3 null mice in which functional Has2 is still expressed), the wound closure rate was significantly faster than in wild-type mice after excisional wounding (Mack et al. 2012). Coincidentally, a marked decrease in epidermal and dermal HA and a marked increase in neutrophil efflux from cutaneous blood vessels were observed in Has1/3-null skin versus wild-type skin, suggesting that loss of Has1 and Has3 leads to enhanced recruitment of neutrophils and other proinflammatory changes in the dermis (Mack et al. 2012).

Another mechanism for HMW-HA's anti-inflammatory function is mediated via binding to hyaladherins such as TNF-stimulated-gene-6 (TSG-6). TSG-6 is produced by cells such as keratinocytes and endothelial cells in response to the increased expression of TNF- α and IL-1 (Milner and Day 2003). It binds to inter-alpha-inhibitor (I α I) to form a TSG-6/I α I complex that is stable in nature (Lauer et al. 2013). HMW-HA binds to this complex, which facilitates and prolongs the anti-

inflammatory properties of TSG-6 during wound healing (Shakya et al. 2020). Shakya et al. showed that TSG-6 is constitutively expressed in normal skin and its expression increases upon wounding (Shakya et al. 2020). Further, as compared with WT mice, the TSG-6 null mice demonstrated an interesting wound-healing phenotype including: (1) a significantly delayed wound closure rate; (2) neutrophil recruitment that was delayed in early wounds (12 h and day 1) but elevated in late wounds (day 7); (3) elevated expression of the proinflammatory cytokine TNF- α (up > 3-fold in unwounded TSG-6 null skin, and increasing further after wounding at 12 h to 7 days) before returning to baseline by day 10. All the findings indicate that the HA-binding protein TSG-6 has a crucial role in regulating wound closure and inflammation during skin wound healing (Shakya et al. 2020).

9.3.2 Role of HA During the Proliferative Phase of Wound Healing

The main events in the proliferative phase include fibroblast migration, granulation tissue formation, wound contraction, and reepithelialization (Aya and Stern 2014; Singer and Clark 1999; Schafer and Werner 2008). HA has important but diverse functions throughout this phase. HA regulates fibroblast migration by binding to different receptors including CD44, ICAM-1, and RHAMM. Acharya et al. (2008) compared the difference in cytoskeletal structures and migration behavior between wild-type (WT) and CD44 knockout (CD44KO) fibroblasts and found that CD44KO fibroblasts formed fewer stress fibers and focal adhesion complexes, migrating with increased velocity but poor directionality, as compared with WT fibroblasts. They further showed that CD44KO cells secreted less active TGF- β ligands than did the WT cells, and exposure of CD44KO cells to exogenous active TGF- β rescued the defect in stress fibers and migrational velocity (but was not able to restore directionality of migration), suggesting that CD44 may be critical for the recruitment of fibroblasts to sites of injury and the normal function of fibroblasts in tissue remodeling and fibrosis. Tolg et al. (2006) showed that Rhamm $-/-$ fibroblasts failed to resurface scratch wounds or to invade hyaluronan-supplemented collagen gels in culture. They further identified a requirement for Rhamm in the localization of CD44 to the cell surface in the formation of CD44-ERK1/2 (extracellular regulated kinase 1,2) complexes. ERK1/2 activation and fibroblast migration/differentiation were also defective during repair of Rhamm $-/-$ excisional skin wounds, which resulted in aberrant granulation tissue in vivo. These results identify Rhamm as an essential regulator of CD44-ERK1,2 fibroblast motogenic signaling that is required for normal wound repair.

Whether different-sized HA molecules regulate migration and other functions of fibroblasts in a size-specific manner remains debatable. David-Raoudi et al. reported that HA fragments with different molecular weights had quite different impacts upon cellular adhesion and differentiation of human dermal fibroblasts. In addition, these

HA fragments with different lengths also stimulated the fibroblasts to synthesize different types of collagens, resulting in different outcomes during scar formation (David-Raoudi et al. 2008). In contrast, Ferguson et al. reported that it was molar concentration of HA rather than molecular size that was crucial for regulating the migration and proliferation of fibroblasts (Ferguson et al. 2011).

Certain subpopulations of fibroblasts undergo myofibroblast differentiation during wound healing. Myofibroblasts are characterized by the neo-expression of alpha smooth muscle actin, a marker of full functional maturation (Gabbiani 2003). As the primary receptor for HA on the cell surface, CD44 appears to play a crucial role in regulating myofibroblast differentiation. A series of studies from researchers at Cardiff University established the importance of HA and CD44 in facilitating the proliferation of fibroblasts and myofibroblast differentiation driven by TGF- β -mediated profibrotic signaling in human lung fibroblasts, which is mediated by the canonical Smad2/3-dependent pathway (Meran et al. 2008; Webber et al. 2009; Meran et al. 2011). In contrast, Wang et al. showed that inhibition of CD44 expression by RNA interference promotes myofibroblast differentiation in a serum response factor (SRF)/myocardin-related transcription factor (MRTF)-dependent manner in primary skin fibroblasts cultured *in vitro*. In addition, the subcellular localization and function of MRTF is controlled by the p38MAPK-mediated non-canonical TGF- β receptor signaling (Wang et al. 2019). These findings suggest that the regulatory effect of HA and CD44 on myofibroblast differentiation could be tissue or cell context dependent. Govindaraju et al. (2019) showed in a global CD44 knockout mouse model that there were increased inflammation and reduced fibrogenic responses during early phases of healing in excisional biopsy punch wounds. Furthermore, the CD44-null mice had reduced collagen degradation leading to increased accumulation of fibrillar collagen at wound closure resulting in reduced tensile strength and more severe scarring compared to WT mice (Govindaraju et al. 2019).

In addition to their effects on cell migration, proliferation, and differentiation, HA and HAS also regulate fibroblast turnover. Campo et al. showed that TGF- β 1-stimulated hyaluronan production had an antioxidant effect and protected fibroblasts against apoptosis induced by free radicals (Campo et al. 2008). Studies using a Has1 and Has3 double-knockout mouse (Has1/3 null model) showed that Has2 gene expression is upregulated as a compensation to the genetic depletion of both Has1 and Has3 (Mack et al. 2012) and that HA production is increased in primary mouse skin fibroblasts as compared with WT cells (Wang et al. 2014). Furthermore, the Has1/3 null fibroblasts are more resistant to apoptosis induced by environment stresses such as growth factor deprivation and UVB irradiation, in a Has2-dependent manner, suggesting that HA and HAS have crucial roles in regulating fibroblast turnover (Wang et al. 2014).

As the most dynamic tissue in later stages of wound healing, granulation tissue (so-called because multiple small blood vessels give it a “granular” appearance) is a complex of inflammatory cells, endothelial cells, fibroblasts, and myofibroblasts, along with collagen and other extracellular matrix components. Both HA and CD44 are abundant in granulation tissue. HA actually fills in the gaps between the fibrotic

scaffolds and binds to collagen, fibrin, and other matrix components (Oksala et al. 1995; Weigel et al. 1986; Weigel et al. 1988; Chen and Abatangelo 1999). Angiogenesis is important for granulation tissue formation. Interestingly HA fragments with different molecular weights have diverse regulatory effect on angiogenesis. Small HA fragments with lengths of only 6–20 saccharides can bind CD44 and not only promote endothelial cell proliferation, but also cleave and activate MMPs at the cell surface, which in turn degrade the basal lamina of existing vessels and components of the extracellular matrix to allow new vessels to grow (Pardue et al. 2008). In contrast, HMW-HA may suppress angiogenesis by various mechanisms including inhibition of early response gene such as c-fos in endothelial cells (EC), inhibition of EC proliferation, disruption of the newly-formed monolayer of EC (West and Kumar 1989). HMW-HA may also sheath cells to prevent their interaction with other cells and thereby block subsequent cell–cell communications (Pardue et al. 2008). Shakya et al. showed that resolution of the granulation phase was delayed, with persistent blood vessels and reduced dermal collagen at 10 days in TSG-6 null mice, as compared with WT mice (Shakya et al. 2020).

Reepithelialization is the resurfacing and closure of a skin wound with new epithelium, which requires keratinocytes to progress from the surrounding edges toward the center of the wound. HA and its receptors have been found to have a crucial role in regulating keratinocyte proliferation, differentiation, and migration. Using a 3-dimensional in vitro organotypic culture of rat epidermal keratinocytes (REK), Maytin et al. observed that HA production was induced in keratinocytes upon disruption of the stratum corneum barrier (Maytin et al. 2004). In addition, the presence of HA was found to suppress late markers of epidermal differentiation in keratinocytes (Passi et al. 2004). Bourguignon et al. showed that the binding of HA to CD44 triggers differentiation in cultured human keratinocytes (Bourguignon et al. 2006). Rilla et al. showed that treatment with an inhibitor to hyaluronan synthesis, 4-Methylumbelliferone, prevents keratinocyte activation and epidermal hyperproliferation induced by EGF (Rilla et al. 2004). Shatirishvili et al. demonstrated that in an epidermal-specific CD44 depletion mouse model, wound healing was delayed, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal thickening and stiffness was reduced; and at the cellular level, CD44 Δ ker keratinocytes demonstrated an impairment of adhesion and migration. All these findings suggest that CD44 is important in regulating keratinocyte adhesion, migration, and reepithelialization post-wounding (Shatirishvili et al. 2016).

9.3.3 Role of HA in the Remodeling Phase of Wound Healing

Beginning at about three weeks post-injury, the wound-healing program moves into a remodeling phase as tissue repair continues, wounds contract, and collagen is continuously synthesized and deposited. At this stage, scar formation occurs as cellular components (endothelial cells, fibroblasts, etc.) are replaced by avascular, collagenous tissues (Deodhar and Rana 1997). It is well recognized that fetal skin

wounds heal without a scar, as opposed to fibrosis and scarring that occurs in adult skin (Longaker and Adzick 1991; Mack et al. 2003). HA is an abundant component in amniotic fluid and in embryonic tissues that contain fibroblasts. Fetal fibroblasts display slower metabolism, lower apoptosis rates, and enhanced migration rates relative to adult fibroblasts. Interestingly, treatment with HA and IL-10 can transform adult fibroblasts into fibroblasts with a fetal phenotype, in a dose-dependent manner (Balaji et al. 2015), suggesting that HA indeed has a crucial role in regulating fibroblast function and scar formation.

9.3.4 HA in Diabetic Wounds

Diabetes affects ~24 million people or 8% of the population of the USA. Hyperglycemia results from either loss of pancreatic insulin-producing cells (as seen in type I diabetes) or from the loss of normal insulin-responsiveness of target cells in type II diabetes. Chronic hyperglycemia leads to multiple complications including renal failure, retinopathy, atherosclerosis, peripheral vascular disease, loss of peripheral sensory nerve function, and impaired wound healing. About 10% diabetic patients have ongoing unhealed wounds in their lower extremities and 25% of diabetic patients will develop foot or lower leg ulcers during their lifetimes. Among the patients with diabetic foot ulcers, 25% will eventually end up with a lower limb amputation due to chronic infection and gangrene (Jeffcoate and Harding 2003; Boulton 2008).

Hyperglycemia leads to the abnormal and prolonged presence of a hyper-inflammatory state that disrupts the normal progression of the three stages of wound healing. The inflammatory phase is prolonged as inflammation fails to resolve. This leads to enhanced myofibroblast differentiation and dysregulated fibrosis, disrupting normal ECM synthesis and reducing the quality of new collagen architecture. A hyperglycemic microenvironment generates reactive oxygen species (ROS), which in turn degrades HA and other GAGs and leads to damage and loss of the endothelial cell glycocalyx in small vessels of the skin (Nieuwdorp et al. 2006). The EC glycocalyx is a reservoir for various antithrombotic factors, and any reduction of this reservoir will disrupt the balance between pro- and anti-coagulatory factors. In addition, loss of the EC glycocalyx will promote the interaction between platelets and EC-surface receptors, thereby increasing platelet adhesion and aggregation. Hyaluronidase 2 released by platelets (de la Motte et al. 2009) will degrade HA into small fragments that in turn promote proinflammatory and angiogenic signaling. In addition, hyperglycemia promotes apoptosis of EC (Risso et al. 2001) and impairs the normal vascular endothelium, leading to loss of barrier function in the walls of small blood vessels (Zuurbier et al. 1985). In addition, hyperglycemia may also promote fibrosis by decreasing apoptosis in smooth muscle cells and fibroblasts and promoting their profibrotic capability (Shakya et al. 2015).

9.4 HA in Topical Wound Dressings

The beneficial role of HA has been established in both *in vitro* and *in vivo* models of wound healing, a testament to the continuous efforts of researchers in basic biomedical sciences around the world. Because of the special physical and chemical characteristics of HA, such as good biocompatibility, biodegradability, water-absorbing (hygroscopic) properties, and availability of multiple sites on the molecule for chemical modification, many types of HA-based dressings have been developed and shown promise for promoting wound healing. HA has been used as a component within decellularized matrix (e.g., ECM scaffolds made from pig peritoneum (Xu et al. 2009)) or by itself as a scaffold that can be crosslinked with other biomaterials or growth factors. At normal physiological pH, the anionic charges at carboxylic groups within the HA structure form hydrogen bonds with water molecules via the carboxyl and acetamino groups, providing a stable secondary structure for the macropolymer (Fallacara et al. 2018). The polymeric HA backbone can be crosslinked to many different functional groups, via the hydroxyl and carboxyl groups in HA, thereby improving the mechanical and rheological properties, and reducing the rate of degradation. The modified HA polymers can be fabricated into a variety of useful shapes such as sponges, films, and electrospun membranes.

HA-containing sponges have a higher porosity than other sponge materials, thereby enhancing oxygen and nutritional supply, cell migration, and proliferation at the wound site (Bruzauskaite et al. 2016; Orellana et al. 2016). Other biomaterials such as carboxymethylcellulose sodium (CMC-Na) were mixed with HA and crosslinked with adipic dihydrazide (ADH) to enhance the mechanical stability and decrease the vulnerability to degradation (Graca et al. 2020); Silver nanoparticles (AgNPs) were added to the chitosan/HA sponges to increase the bactericidal function and improve their performance in treating diabetic foot ulcers (Anisha et al. 2013). Similarly, bioactive materials were added to HA-based films to increase their stability and enhance their wound-healing function. Fahmy et al. (2018) demonstrated that HA/sodium alginate films crosslinked with AgNPs and sulfadiazine decreased the production of inflammatory mediators, lowered the level of oxidative stress, and reduced the open wound area relative to control groups. The porous, 3-dimensional structure of HA-based hydrogels has good water-absorbing capacity of absorbing water, allows the trafficking of nutrients and waste products, and thereby provides a good environment for cell migration, growth, and function.

Various biomaterials have been crosslinked with HA in an attempt to improve its mechanical stability and to slow degradation. Electrospinning of HA was performed by combining HA with other polymers, to generate scaffolds that have good mechanical strength and better biochemical performance (Kenar et al. 2019; Shin et al. 2016; Chanda et al. 2018). The addition of growth factors such as VEGF or EGF to various types of HA scaffold demonstrated promise for significantly facilitating wound closure and improving dermal thickness (Fiorica et al. 2017; Su et al. 2014; Wang et al. 2016; Zhou et al. 2016). At the current time, various types of

HA-based dressings such as HylaSponge® system, Hyalomatrix®, Hyalosafe®, Hyalofill®, etc. are being used in wound care hospitals (Graca et al. 2020).

In spite of the promising therapeutic outcomes mentioned above, there are still some remaining limitations and drawbacks with HA-based dressings. These include high production costs, possible contamination during the extraction process; low mechanical stability, rapid in vivo degradability, limited cell adhesion; and the presence of some potentially toxic agents (used during crosslinking with other biomaterials) that decrease biocompatibility and increase cytotoxicity (Graca et al. 2020). All of these concerns will need to be further addressed in the future.

9.5 Conclusion

As the most abundant GAG in the skin, HA has important roles in all the stages of wound healing. These roles are complex and vary with HA size. Long HA polymers function as structural scaffolds and porous networks that provide a favorable milieu for inflammatory, phagocytic, and regenerative cells. HA also fills the space in granulation tissues. Short HA fragments tend to display more stimulatory, proinflammatory, and proangiogenic functions. Based on the continuous efforts of scientists from multiple disciplines, the overall beneficial role of HA in promoting wound healing has been largely established. HA-based dressings or other therapeutic strategies that utilize HA to treat skin wounds appear to be a very active and promising area of applied medicine. However, many questions remain to be answered. A better understanding is needed about how HA structure affects its bioactivity, and especially how different-sized fragments of HA recognize and bind to various types of receptors. The molecular mechanisms that regulate the expression of HA receptors, e.g. the transcriptional regulation of different variant splice forms of CD44, require further investigation. Last but not least, improved knowledge about how HA associates and interacts with other GAGs and with other extracellular matrix components in the skin will require extensive and in-depth studies in the future.

References

- Acharya PS, Majumdar S, Jacob M, Hayden J, Mrass P, Weninger W, Assoian RK, Pure E (2008) Fibroblast migration is mediated by CD44-dependent TGF beta activation. *J Cell Sci* 121:1393–1402
- Anisha BS, Biswas R, Chennazhi KP, Jayakumar R (2013) Chitosan-hyaluronic acid/nano silver composite sponges for drug resistant bacteria infected diabetic wounds. *Int J Biol Macromol* 62: 310–320
- Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61:1303–1313

- Averbeck M, Gebhardt CA, Voigt S, Beilharz S, Anderegg U, Termeer CC, Sleeman JP, Simon JC (2007) Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation. *J Invest Dermatol* 127:687–697
- Aya KL, Stern R (2014) Hyaluronan in wound healing: rediscovering a major player. *Wound Repair Regen* 22:579–593
- Balaji S, King A, Marsh E, LeSaint M, Bhattacharya SS, Han N, Dhamija Y, Ranjan R, Le LD, Bollyky PL, Crombleholme TM, Keswani SG (2015) The role of interleukin-10 and hyaluronan in murine fetal fibroblast function in vitro: implications for recapitulating fetal regenerative wound healing. *PLoS One* 10:e0124302
- Boulton AJ (2008) The diabetic foot: grand overview, epidemiology and pathogenesis. *Diabetes Metab Res Rev* 24(Suppl 1):S3–S6
- Bourguignon LY, Ramez M, Gilad E, Singleton PA, Man MQ, Crumrine DA, Elias PM, Feingold KR (2006) Hyaluronan-CD44 interaction stimulates keratinocyte differentiation, lamellar body formation/secretion, and permeability barrier homeostasis. *J Invest Dermatol* 126:1356–1365
- Bruzauskaite I, Bironaite D, Bagdonas E, Bernotiene E (2016) Scaffolds and cells for tissue regeneration: different scaffold pore sizes-different cell effects. *Cytotechnology* 68:355–369
- Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 106:349–360
- Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Sama D, Calatroni A (2008) The antioxidant effect exerted by TGF-1 β -stimulated hyaluronan production reduced NF- κ B activation and apoptosis in human fibroblasts exposed to FeSo₄ plus ascorbate. *Mol Cell Biochem* 311:167–177
- Chanda A, Adhikari J, Ghosh A, Chowdhury SR, Thomas S, Datta P, Saha P (2018) Electrospun chitosan/polycaprolactone-hyaluronic acid bilayered scaffold for potential wound healing applications. *Int J Biol Macromol* 116:774–785
- Chen WY, Abatangelo G (1999) Functions of hyaluronan in wound repair. *Wound Repair Regen* 7:79–89
- Csoka AB, Scherer SW, Stern R (1999) Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics* 60:356–361
- David-Raoudi M, Tranchepain F, Deschrevel B, Vincent JC, Bogdanowicz P, Boumediene K, Pujol JP (2008) Differential effects of hyaluronan and its fragments on fibroblasts: relation to wound healing. *Wound Repair Regen* 16:274–287
- Delmage JM, Powars DR, Jaynes PK, Allerton SE (1986) The selective suppression of immunogenicity by hyaluronic acid. *Ann Clin Lab Sci* 16:303–310
- Deodhar AK, Rana RE (1997) Surgical physiology of wound healing: a review. *J Postgrad Med* 43:52–56
- Fahmy HM, Aly AA, Abou-Okeil A (2018) A non-woven fabric wound dressing containing layer - by - layer deposited hyaluronic acid and chitosan. *Int J Biol Macromol* 114:929–934
- Fallacara A, Marchetti F, Pozzoli M, Citernes UR, Manfredini S, Vertuani AS (2018) Formulation and characterization of native and crosslinked hyaluronic acid microspheres for dermal delivery of sodium Ascorbyl phosphate: a comparative study. *Pharmaceutics* 10:254
- Feinberg RN, Beebe DC (1983) Hyaluronate in vasculogenesis. *Science* 220:1177–1179
- Ferguson EL, Roberts JL, Moseley R, Griffiths PC, Thomas DW (2011) Evaluation of the physical and biological properties of hyaluronan and hyaluronan fragments. *Int J Pharm* 420:84–92
- Fiorica C, Palumbo FS, Pitarresi G, Bongiovi F, Giammona G (2017) Hyaluronic acid and beta cyclodextrins films for the release of corneal epithelial cells and dexamethasone. *Carbohydr Polym* 166:281–290
- Fraser JR, Laurent TC, Laurent UB (1997) Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* 242:27–33
- Frost SJ, Weigel PH (1990) Binding of hyaluronic acid to mammalian fibrinogens. *Biochim Biophys Acta* 1034:39–45

- Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200: 500–503
- Govindaraju P, Todd L, Shetye S, Monslow J, Pure E (2019) CD44-dependent inflammation, fibrogenesis, and collagenolysis regulates extracellular matrix remodeling and tensile strength during cutaneous wound healing. *Matrix Biol* 75-76:314–330
- Graca MFP, Miguel SP, Cabral CSD, Correia IJ (2020) Hyaluronic acid-based wound dressings: a review. *Carbohydr Polym* 241:116364
- Hall CL, Collis LA, Bo AJ, Lange L, McNicol A, Gerrard JM, Turley EA (2001) Fibroblasts require protein kinase C activation to respond to hyaluronan with increased locomotion. *Matrix Biol* 20: 183–192
- Hall CL, Lange LA, Prober DA, Zhang S, Turley EA (1996) pp60(c-src) is required for cell locomotion regulated by the hyaluronan receptor RHAMM. *Oncogene* 13:2213–2224
- Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM, Turley EA (1992) Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. *J Cell Biol* 117:1343–1350
- Hascall VC (2000) Hyaluronan, a common thread. *Glycoconj J* 17:607–616
- Itano N, Kimata K (2002) Mammalian hyaluronan synthases. *IUBMB Life* 54:195–199
- Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, Yoshida Y, Ohnuki Y, Miyauchi S, Spicer AP, McDonald JA, Kimata K (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274: 25085–25092
- Jeffcoate WJ, Harding KG (2003) Diabetic foot ulcers. *Lancet* 361:1545–1551
- Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA, Homer RJ, Goldstein DR, Bucala R, Lee PJ, Medzhitov R, Noble PW (2005) Regulation of lung injury and repair by toll-like receptors and hyaluronan. *Nat Med* 11:1173–1179
- Jordan AR, Racine RR, Hennig MJ, Lokeshwar VB (2015) The role of CD44 in disease pathophysiology and targeted treatment. *Front Immunol* 6:182
- Kaul A, Short WD, Wang X, Keswani SG (2021) Hyaluronidases in human diseases. *Int J Mol Sci* 22(6):3204
- Kenar H, Ozdogan CY, Dumlu C, Doger E, Kose GT, Hasirci V (2019) Microfibrous scaffolds from poly(l-lactide-co-epsilon-caprolactone) blended with xeno-free collagen/hyaluronic acid for improvement of vascularization in tissue engineering applications. *Mater Sci Eng C Mater Biol Appl* 97:31–44
- Lauer ME, Glant TT, Mikecz K, DeAngelis PL, Haller FM, Husni ME, Hascall VC, Calabro A (2013) Irreversible heavy chain transfer to hyaluronan oligosaccharides by tumor necrosis factor-stimulated gene-6. *J Biol Chem* 288:205–214
- Lennon FE, Singleton PA (2011) Hyaluronan regulation of vascular integrity. *Am J Cardiovasc Dis* 1:200–213
- Longaker MT, Adzick NS (1991) The biology of fetal wound healing: a review. *Plast Reconstr Surg* 87:788–798
- Mack JA, Abramson SR, Ben Y, Coffin JC, Rothrock JK, Maytin EV, Hascall VC, Largman C, Stelnicki EJ (2003) Hoxb13 knockout adult skin exhibits high levels of hyaluronan and enhanced wound healing. *FASEB J* 17:1352–1354
- Mack JA, Feldman RJ, Itano N, Kimata K, Lauer M, Hascall VC, Maytin EV (2012) Enhanced inflammation and accelerated wound closure following tetraphorbol ester application or full-thickness wounding in mice lacking hyaluronan synthases Has1 and Has3. *J Invest Dermatol* 132:198–207
- Maytin EV, Chung HH, Seetharaman VM (2004) Hyaluronan participates in the epidermal response to disruption of the permeability barrier in vivo. *Am J Pathol* 165:1331–1341
- McBride WH, Bard JB (1979) Hyaluronidase-sensitive halos around adherent cells. Their role in blocking lymphocyte-mediated cytotoxicity. *J Exp Med* 149:507–515

- Meran S, Luo DD, Simpson R, Martin J, Wells A, Steadman R, Phillips AO (2011) Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. *J Biol Chem* 286:17618–17630
- Meran S, Thomas DW, Stephens P, Enoch S, Martin J, Steadman R, Phillips AO (2008) Hyaluronan facilitates transforming growth factor-beta1-mediated fibroblast proliferation. *J Biol Chem* 283: 6530–6545
- Mikecz K, Brennan FR, Kim JH, Glant TT (1995) Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. *Nat Med* 1:558–563
- Milner CM, Day AJ (2003) TSG-6: a multifunctional protein associated with inflammation. *J Cell Sci* 116:1863–1873
- Monslow J, Sato N, Mack JA, Maytin EV (2009) Wounding-induced synthesis of hyaluronic acid in organotypic epidermal cultures requires the release of heparin-binding egf and activation of the EGFR. *J Invest Dermatol* 129:2046–2058
- de la Motte C, Nigro J, Vasanji A, Rho H, Kessler S, Bandyopadhyay S, Danese S, Fiocchi C, Stern R (2009) Platelet-derived hyaluronidase 2 cleaves hyaluronan into fragments that trigger monocyte-mediated production of proinflammatory cytokines. *Am J Pathol* 174:2254–2264
- Nakamura K, Yokohama S, Yoneda M, Okamoto S, Tamaki Y, Ito T, Okada M, Aso K, Makino I (2004) High, but not low, molecular weight hyaluronan prevents T-cell-mediated liver injury by reducing proinflammatory cytokines in mice. *J Gastroenterol* 39:346–354
- Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, Meijers JC, Holleman F, Hoekstra JB, Vink H, Kastelein JJ, Stroes ES (2006) Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. *Diabetes* 55:480–486
- Oksala O, Salo T, Tammi R, Hakkinen L, Jalkanen M, Inki P, Larjava H (1995) Expression of proteoglycans and hyaluronan during wound healing. *J Histochem Cytochem* 43:125–135
- Orellana SL, Giacaman A, Pavicic F, Vidal A, Moreno-Villoslada I, Concha M (2016) Relevance of charge balance and hyaluronic acid on alginate-chitosan sponge microstructure and its influence on fibroblast growth. *J Biomed Mater Res A* 104:2537–2543
- Orian-Rousseau V (2015) CD44 acts as a signaling platform controlling tumor progression and metastasis. *Front Immunol* 6:154
- Pardue EL, Ibrahim S, Ramamurthi A (2008) Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering. *Organogenesis* 4:203–214
- Passi A, Sadeghi P, Kawamura H, Anand S, Sato N, White LE, Hascall VC, Maytin EV (2004) Hyaluronan suppresses epidermal differentiation in organotypic cultures of rat keratinocytes. *Exp Cell Res* 296:123–134
- Ponta H, Sherman L, Herrlich PA (2003) CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 4:33–45
- Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG (2001) Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J Biol Chem* 276:19420–19430
- Reed RK, Lilja K, Laurent TC (1988) Hyaluronan in the rat with special reference to the skin. *Acta Physiol Scand* 134:405–411
- Rilla K, Pasonen-Seppanen S, Rieppo J, Tammi M, Tammi R (2004) The hyaluronan synthesis inhibitor 4-methylumbelliferone prevents keratinocyte activation and epidermal hyperproliferation induced by epidermal growth factor. *J Invest Dermatol* 123:708–714
- Risso A, Mercuri F, Quagliaro L, Damante G, Ceriello A (2001) Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture. *Am J Physiol Endocrinol Metab* 281:E924–E930
- Schafer M, Werner S (2008) Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol* 9:628–638
- Shakya S, Mack JA, Alipour M, Maytin EV (2020) Cutaneous wounds in mice lacking TSG-6 exhibit delayed closure and an abnormal inflammatory response. *J Invest Dermatol* 140:2505–2514

- Shakya S, Wang Y, Mack JA, Maytin EV (2015) Hyperglycemia-induced changes in Hyaluronan contribute to impaired skin wound healing in diabetes: review and perspective. *Int J Cell Biol* 2015:701738
- Shatirishvili M, Burk AS, Franz CM, Pace G, Kastilan T, Breuhahn K, Hinterseer E, Dierich A, Bakiri L, Wagner EF, Ponta H, Hartmann TN, Tanaka M, Orian-Rousseau V (2016) Epidermal-specific deletion of CD44 reveals a function in keratinocytes in response to mechanical stress. *Cell Death Dis* 7:e2461
- Shin YC, Shin DM, Lee EJ, Lee JH, Kim JE, Song SH, Hwang DY, Lee JJ, Kim B, Lim D, Hyon SH, Lim YJ, Han DW (2016) Hyaluronic acid/PLGA Core/Shell fiber matrices loaded with EGCG beneficial to diabetic wound healing. *Adv Healthc Mater* 5:3035–3045
- Shirali AC, Goldstein DR (2008) Activation of the innate immune system by the endogenous ligand hyaluronan. *Curr Opin Organ Transplant* 13:20–25
- Singer AJ, Clark RA (1999) Cutaneous wound healing. *N Engl J Med* 341:738–746
- Singleton PA, Mirzapoiazova T, Guo Y, Sammani S, Mambetsariev N, Lennon FE, Moreno-Vinasco L, Garcia JG (2010) High-molecular-weight hyaluronan is a novel inhibitor of pulmonary vascular leakiness. *Am J Physiol Lung Cell Mol Physiol* 299:L639–L651
- Soltes L, Mendichi R, Kogan G, Schiller J, Stankovska M, Arnhold J (2006) Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules* 7:659–668
- Spicer AP, Seldin MF, Olsen AS, Brown N, Wells DE, Doggett NA, Itano N, Kimata K, Inazawa J, McDonald JA (1997) Chromosomal localization of the human and mouse hyaluronan synthase genes. *Genomics* 41:493–497
- Stern R, Asari AA, Sugahara KN (2006) Hyaluronan fragments: an information-rich system. *Eur J Cell Biol* 85:699–715
- Su Z, Ma H, Wu Z, Zeng H, Li Z, Wang Y, Liu G, Xu B, Lin Y, Zhang P, Wei X (2014) Enhancement of skin wound healing with decellularized scaffolds loaded with hyaluronic acid and epidermal growth factor. *Mater Sci Eng C Mater Biol Appl* 44:440–448
- Tammi R, Pasonen-Seppanen S, Kolehmainen E, Tammi M (2005) Hyaluronan synthase induction and hyaluronan accumulation in mouse epidermis following skin injury. *J Invest Dermatol* 124: 898–905
- Tammi R, Ripellino JA, Margolis RU, Tammi M (1988) Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe. *J Invest Dermatol* 90:412–414
- Tammi R, Saamanen AM, Maibach HI, Tammi M (1991) Degradation of newly synthesized high molecular mass hyaluronan in the epidermal and dermal compartments of human skin in organ culture. *J Invest Dermatol* 97:126–130
- Tavianatou AG, Caon I, Franchi M, Piperigkou Z, Galesso D, Karamanos NK (2019) Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. *FEBS J* 286:2883–2908
- Tolg C, Hamilton SR, Nakrieko KA, Kooshesh F, Walton P, McCarthy JB, Bissell MJ, Turley EA (2006) Rhamm—/— fibroblasts are defective in CD44-mediated ERK1,2 mitogenic signaling, leading to defective skin wound repair. *J Cell Biol* 175:1017–1028
- Wang Y, Lauer ME, Anand S, Mack JA, Maytin EV (2014) Hyaluronan synthase 2 protects skin fibroblasts against apoptosis induced by environmental stress. *J Biol Chem* 289:32253–32265
- Wang Y, Mack JA, Maytin EV (2019) CD44 inhibits alpha-SMA gene expression via a novel G-actin/MRTF-mediated pathway that intersects with TGFbetaR/p38MAPK signaling in murine skin fibroblasts. *J Biol Chem* 294:12779–12794
- Wang Z, Qian Y, Li L, Pan L, Njunge LW, Dong L, Yang L (2016) Evaluation of emulsion electrospun polycaprolactone/hyaluronan/epidermal growth factor nanofibrous scaffolds for wound healing. *J Biomater Appl* 30:686–698
- Webber J, Jenkins RH, Meran S, Phillips A, Steadman R (2009) Modulation of TGFbeta1-dependent myofibroblast differentiation by hyaluronan. *Am J Pathol* 175:148–160
- Weigel PH, Frost SJ, McGary CT, LeBoeuf RD (1988) The role of hyaluronic acid in inflammation and wound healing. *Int J Tissue React* 10:355–365

- Weigel PH, Fuller GM, LeBoeuf RD (1986) A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. *J Theor Biol* 119:219–234
- West DC, Kumar S (1989) Hyaluronan and angiogenesis. *Ciba Found Symp* 143:187–201. discussion 201–187, 281–185
- Xu H, Wan H, Zuo W, Sun W, Owens RT, Harper JR, Ayares DL, McQuillan DJ (2009) A porcine-derived acellular dermal scaffold that supports soft tissue regeneration: removal of terminal galactose- α -(1,3)-galactose and retention of matrix structure. *Tissue Eng Part A* 15:1807–1819
- Zhou J, Zhang B, Liu X, Shi L, Zhu J, Wei D, Zhong J, Sun G, He D (2016) Facile method to prepare silk fibroin/hyaluronic acid films for vascular endothelial growth factor release. *Carbohydr Polym* 143:301–309
- Zuurbier CJ, Demirci C, Koeman A, Vink H, Ince C (2005) Short-term hyperglycemia increases endothelial glycocalyx permeability and acutely decreases lineal density of capillaries with flowing red blood cells. *J Appl Physiol* (1985) 99:1471–1476

Chapter 10

Sulfated Hyaluronan: A Novel Player in Cancer Therapeutic and Bioengineering Approaches



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Abstract Hyaluronan (HA) is a non-sulfated glycosaminoglycan ubiquitously distributed in cells and tissues. It plays a pivotal role in several cellular functions, such as proliferation and migration, with its binding to its receptors CD44 and RHAMM triggering various signaling cascades. HA modifications regulate cellular responses in a different manner compared to unmodified HA fragments. The modified fragments are synthesized through a variety of strategies and have several applications, including therapeutic strategies against cancer, enhancement of wound healing and osteogenesis, as well as preparation of innovative polymer matrices. Notably, sulfated HA (sHA) shows promising results in cancer treatment, exhibiting anti-cancer effects through different modes of action, while also interfering with the binding of matrix effectors controlling extracellular matrix remodeling. Additionally, sHA can be used in biomedical polymer matrices and different tissue-engineering applications to enhance bone formation when used for biomaterial coating. In this chapter we present the processes that sHA could contribute in for the development of future interventions and innovative bioengineering approaches.

Abbreviations

aECM	artificial extracellular matrix
CEMIP	Cell migration-inducing and hyaluronan binding protein
DOX	doxorubicin
DS	degree of sulfation
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptors

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GA	glycyrrhetinic acid
GAG	glycosaminoglycan
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
HA	hyaluronan
HBD	heparin-binding domain
HMW-HA	high molecular weight hyaluronan
HPSE	heparanase
HS	heparan sulfate
HYAL	hyaluronidase
LMW-HA	low molecular weight hyaluronan
MMPs	matrix metalloproteinases
sHA	sulfated hyaluronan
TGF- β 1	transforming growth factor- β 1
TME	tumor microenvironment
VEGF	vascular endothelial growth factor

10.1 Introduction

Hyaluronan (HA) is an extracellular matrix (ECM) glycosaminoglycan (GAG) composed of disaccharide repeats of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA). It plays an important role in a variety of biological processes, such as tissue hydration, maintenance of the elastoviscosity of connective tissues, tumor development, wound healing, and inflammation (Karamanos et al. 2021). Modifications of HA fragments such as sulfation, alkylation, esterification, and amidation lead to HA derivatives which differentially modulate cellular responses and can be used in biomedical polymer matrices, as the modifications attenuate HA degradation and differentially alter its properties.

Taking into account the size-depended regulatory role of HA in different biological processes like angiogenesis, inflammation, wound healing, apoptosis, and tumorigenesis, it is essential to tightly control the degradation of high molecular weight HA (HMW-HA) prior to chemical modifications, in order to produce HA molecules with the desired molecular weight (Tavianatou et al. 2019). The HA fragments can be generated via chemoenzymatic approaches, oxidative processes, or thermal stress. Subsequently, HA modifications can be performed via conjugation and/or crosslinking. In conjugation, an extra single bond onto a HA chain is formed. In this way, the introduction of functional groups or the incorporation of bioactive compounds as well as drugs can be achieved. In crosslinking, different HA chains are linked together by multiple bonds. Both methods can also be combined for the chemical modification of HA molecules (Hintze et al. 2022).

10.2 Structural Modification of HA by Incorporation of Sulfate Groups

Over the years, several techniques have been tested for the introduction of sulfate groups in HA structure. Sulfating agents like chlorosulfuric acid or sulfuric acid were used for the sulfation of HA, but complete sulfation of free OH-groups could not be achieved and the polymer chain was partly degraded due to reaction conditions (Hintze et al. 2022). Nowadays, the most frequently employed technique for the introduction of sulfate groups in HA is the use of sulfur trioxide (SO₃)-organic amine complexes (pyridine, trimethylamine, triethylamine) or SO₃-highly polar organic amide complexes since they are relatively stable reagents and do not provoke the degradation of HA, they are easy to handle, and they are compatible with highly polar aprotic solvents (DMF, DMSO), thus aiding in the solubility of HA (Caputo et al. 2019; Schnabelrauch et al. 2021).

The degree of sulfation (DS) at available -OH groups among the different sHA derivatives depends on the sulfating agent and the amount used, as well as the reaction time (Schnabelrauch et al. 2021). Depending on the reagents used as sulfating agents, a selectivity regarding the sulfation of certain -OH at C-4, C-6 of GlcNAc or C-2,3 of GlcA can be obtained. Recently, new methods to enable the introduction of sulfate groups into a specific position of HA hydroxyl groups have been developed, leading to sHA derivatives with controlled sulfation patterns (Caputo et al. 2019; Bedini et al. 2017).

10.3 The Role of Sulfated HA in Cancer

The unique capability of HA to trigger different cellular responses following its binding to the receptors CD44 and RHAMM has been extensively researched, with the effects of HA depending on its characteristics, such as its molecular size and modifications (Karamanos et al. 2021; Tavianatou et al. 2019). Adding to these research approaches, several scientific studies have pointed toward the use of sHA as a potential therapeutic in diseases, with the list continuously growing.

Cancer constitutes a complicated, heterogeneous disease with one of the most commonly reported death causes worldwide (Sung et al. 2021). Tumors are cellular masses formed through interactions between cancer cells and stromal cells. These interactions lead to the establishment of a peritumoral microenvironment that is capable of altering cellular behavior (Iozzo and Gubbiotti 2018). HA is a crucial modulator of this microenvironment. Through its interactions with the receptor CD44, HA triggers intracellular signaling pathways which promote cellular proliferation, migration, and invasion. Furthermore, the interplay between HA and cancer cells has been correlated with epithelial-to-mesenchymal transition (EMT) and ECM degradation, which lead to a more aggressive cancer cell phenotype, facilitating migration and metastasis. Elevated HA levels have been associated with poor

Table 10.1 The potential effects of sulfated HA in malignancies

Disease	Effect	Mode of action	Reference
Cancer	Decreased cancer cell invasion	Heparanase inhibition	(Shi et al. 2022)
Cancer	HYAL1 inhibition	Competitive & uncompetitive mechanism	(Isoyama et al. 2006)
Cancer	CEMP inhibition	Degree of sulfation proportional to the strength of inhibition	(Schmaus et al. 2022)
Cancer	Angiogenesis inhibition	Selectively binds to angiogenic VEGF _{165a} isoform	(Lim et al. 2016)
Prostate cancer	Antitumor effects	HYAL1 inhibition, downregulation of CD44 & RHAMM, PI3K/Akt inhibition	(Benitez et al. 2011)
Bladder cancer	Antitumor effects	HYAL1 inhibition, downregulation of CD44 & RHAMM, PI3K/Akt inhibition	(Jordan et al. 2014)
Breast cancer	Anti-cancer effects	Downregulation of MMPs, HAS2, modulation of EMT markers	(Koutsakis et al. 2021)
Breast cancer	Antitumor effects	Downregulation of angiogenic factors in macrophages/monocytes	(Spinelli et al. 2022)
Lung cancer			
Liver cancer	Drug delivery system	sHA-DOX/HA-GA micelles	(Li et al. 2019)
Radiation therapy	Radioprotective effects	Heparin substitute without anticoagulant action	(Miura et al. 2022)

outcome and resistance to chemotherapy in various malignancies such as breast, lung, ovarian, and prostate cancer (Karousou et al. [2017](#); Heldin et al. [2019](#)). Exogenously added HA fragments, depending on their molecular size, can attenuate these properties and inhibit disease progression (Tavianatou et al. [2019](#)). Taking the sHA properties into account, an emerging field is focusing on its effects on different types of cancer cells, with results looking promising. The different effects of sHA in cancer are summarized in Table [10.1](#).

A recent study has shown that sHA binds to the enzyme heparanase (HPSE) blocking its enzymatic and cellular effects in cancer cells in vitro (Shi et al. [2022](#)). HPSE is an endoglucuronidase which cleaves heparan sulfate (HS) chains. During cancer progression and development, its role is essential since it degrades HS proteoglycans of the ECM (Piperigkou et al. [2021](#)). The cleaved HS fragments bind to specific molecules, triggering signaling cascades such as the PKC and MAPK pathways, thus facilitating cancer cell migration and invasion. Moreover, HPSE induces the production of inflammatory cytokines and chemokines, while also interfering with intracellular signaling, initiating signaling pathways such as Akt, ERK, NF- κ B, and TLR-2,4 and leading to different cellular responses depending on the cell type context (Sanderson et al. [2017](#)). In colon-26 and 4 T1 cancer cell lines, sHA of 40–50 kDa modified with approximately 2.5 sulfate groups per disaccharide unit directly inhibits the enzymatic activity of HPSE, resulting in a significant suppression of cell invasiveness. Notably, this inhibition is stronger than heparin, a conventional HPSE inhibitor (Shi et al. [2022](#)).

Apart from HPSE, sHA fragments are also able to modulate the effects of other enzymes involved in cancer progression. Hyaluronidases (HYALs) play a crucial role during cancer development since they degrade pericellular HA in smaller fragments that induce angiogenesis, aiding in disease progression and metastasis. HYAL1 is the major tumor-derived HYAL in many cancer types, such as prostate and bladder cancer (Lokeshwar et al. 1999; Lokeshwar et al. 2001). The enzyme levels are elevated in these cases and serve as a biomarker for detecting intermediate- and high-grade bladder cancer and predicting prostate cancer progression. sHA has been shown to inhibit HYAL1 in a dose-dependent manner, with the inhibition consisting of competitive and uncompetitive mechanisms (Isoyama et al. 2006).

Recently another HA-degrading protein, CEMIP (cell migration-inducing and hyaluronan binding protein), has been characterized. CEMIP cleaves HA through a different mechanism than the other HYALs and its levels have been correlated with poor prognosis during breast, gastric, and pancreatic cancer, promoting cancer cell proliferation, migration, and invasion (Li et al. 2017; Zhang et al. 2017). Moreover, tumor cells secrete exosomes containing CEMIP to promote brain metastasis (Rodrigues et al. 2019). sHA of 20–110 kDa can inhibit the activity of CEMIP, with the degree of sulfation proportional to the strength of inhibition. The observed sHA effect is stronger compared to the one against HYAL1. Additionally, fibroblasts treated with sHA produce increased amounts of HMW-HA, while decreasing levels of low molecular weight HA (LMW-HA) fragments (Schmaus et al. 2022).

During cancer development, angiogenesis is essential to support tumor cell survival. VEGF₁₆₅ is an ECM protein involved in this process and exists in two different isoforms: the angiogenic VEGF_{165a} and the anti-angiogenic VEGF_{165b}. These two isoforms bear different heparin-binding domain (HBD) structures, allowing for selective binding of sulfated GAGs (Ferrara 2010). Therefore, it is of importance to develop therapeutic inhibitors against only the angiogenic VEGF_{165a}. Depending on its degree of sulfation, sHA of 150 kDa can selectively bind to VEGF_{165a} but not VEGF_{165b}, effectively preventing pathological angiogenesis. However, increasing the sulfation to more than 1 sulfate group per HA disaccharide, sHA binds to both VEGF_{165a} and VEGF_{165b}, highlighting the importance of all synthetic sHA characteristics (Lim et al. 2016).

In prostate cancer the levels of HYAL1 are increased, degrading pericellular HA into angiogenic fragments that further cause tumor progression. As sHA constitutes an HYAL1 inhibitor, the effects of the molecule were tested against prostate cancer cell lines with promising results. sHA of 320–490 kDa attenuates prostate cancer cells proliferation, migration, invasion, while also initiating caspase-8-dependent apoptosis. Notably, sHA interferes in signaling pathways involved in disease progression by affecting Akt signaling, NF- κ B activation, and VEGF expression. Additionally, sHA also inhibits PI3K activation and causes transcriptional downregulation of HA receptors CD44 and RHAMM. The antitumor effects of sHA in prostate cancer were also shown in animal models, with sHA inhibiting tumor growth without causing side effects and decreasing angiogenesis (Benitez et al. 2011).

Like prostate cancer, bladder cancer is another malignancy during which the HYAL1 levels are increased, having been correlated to high-grade bladder cancer and metastasis. By blocking the HYAL1 activity and thus stopping the production of angiogenic HA fragments, sHA inhibits bladder cancer cell proliferation, migration, and invasion, induces cancer cell apoptosis, and downregulates the expression of HA receptors CD44 and RHAMM. Additionally, sHA inhibits the formation of CD44/PI3K complex, further affecting intracellular signaling cascades. The antitumor activity of sHA is highlighted in xenograft models as well, since sHA reduces tumor growth and attenuates angiogenesis, suggesting a role as an intravesical agent to control bladder cancer progression (Jordan et al. 2014).

Breast cancer is one of the most common cancer types for women worldwide. Matrix metalloproteinases (MMPs) constitute another enzyme category implicated in ECM degradation and remodeling, facilitating cancer cell migration and metastasis (Piperigkou et al. 2021). The HA synthase, HAS2, is also increased during breast cancer, with its levels used as biomarkers and correlated to bad prognosis and metastasis (Passi et al. 2019). sHA fragments of 50 kDa were tested in vitro on breast cancer cells with different estrogen receptor (ER) status. Notably, sHA attenuates breast cancer cell proliferation, migration, and invasion, while increasing their adhesiveness. Moreover, sHA modulates the expression of EMT markers to a less aggressive phenotype, while also downregulating the levels of MT1-MMP, MMP-2, and MMP-9. Finally, sHA inhibits the expression of HAS2 (Koutsakis et al. 2021).

The effects of sHA in breast cancer have also been studied in 3D cultures. sHA fragments of 30–60 kDa reduce the size of tumor spheres and alter the total HA levels. During breast cancer development, various cell types are involved in the formation of the tumor and the surrounding microenvironment which supports tumor cell survival. Treating macrophages and monocytes, which are part of the tumor microenvironment (TME), with sHA downregulates the expression of angiogenic factors, attenuates endothelial cell migration, and modulates the HA-binding protein TSG-6. In vivo experiments also demonstrate that sHA decreases angiogenesis in the tumor niche and downregulates the levels of HA and TSG-6, as well (Spinelli et al. 2022). In the same study, the effects of sHA on lung cancer cells were also investigated with similar results, proposing another role for sHA, in the tumor-associated cells of the TME this time.

Sulfated HA has also been used in developing a drug delivery system for hepatoma. HA polymers can function as ideal non-immunotoxic drug carriers which can self-assemble into nano-sized micelles; however, they can be easily degraded by HYALs. In order to block this degradation, sHA of 210 kDa was conjugated with the drug doxorubicin (DOX) to create a system of micelles which also contained glycyrrhetinic acid (GA) for selective targeting of liver cancer cells. This sHA-DOX/HA-GA mixed micelles exhibited a promising antitumor effect in in vivo murine experiments (Li et al. 2019).

Radiation therapy is a treatment regime often used during cancer. Despite its effectiveness, it can cause tissue damage leading to severe side effects for the

patients. To prevent or treat this damage, fibroblast growth factors are often administered alongside heparin to stabilize their structure. However, while heparin exerts radioprotective effects, it also has an undesirable anticoagulant action. To this end, sHA with a high degree of sulfation has been shown to have similar protective effects against radiation-induced intestinal damage and promote tissue regeneration, without having an effect on blood coagulation (Miura et al. 2022).

10.4 Sulfated HA in Matrix-Based Biomaterials

Aside from its role in cancer, sHA is also involved in other processes, interacting with, and regulating molecules of high biological interest in health and disease. For example, sHA upregulates the expression of the cell adhesion molecules N-cadherin and Connexin43 in rat osteoblasts, resulting in the formation of aggregates and enhancing the activity of alkaline phosphatase (Nagahata et al. 2004). Additionally, sHA of low degree of sulfation can interact with fibronectin to influence the matrix assembly of human bone marrow stromal cells, exerting a pro-osteogenic effect (Vogel et al. 2016). In disease treatment, derivatives of sulfated HA have been used to synthesize a biocompatible micelle-forming polymer as a drug delivery system for inflammatory osteoarthritis (Guarise et al. 2022). Furthermore, high-sulfated HA has been implicated in the inhibition of SARS-CoV-2 virus, emerging as a promising therapeutic candidate for COVID-19 (Möller et al. 2022).

The multifaceted actions exerted by sHA naturally lead to its use in hydrogels and artificial extracellular matrices (aECM). Native HA already bears many desirable properties, as it has excellent biocompatibility and regulates a wide array of processes. However, HA is degraded fast *in vivo*, posing a serious limitation for biomedical applications. On the other hand, sHA biomaterials are less degradable, more durable, and offer a higher affinity for protein binding (Feng et al. 2017). aECM scaffolds coated with high-sulfated HA have been used for bone healing in animal models, leading to improved bone regeneration and formation of new bone volume (Förster et al. 2020). Further highlighting their pro-osteogenic properties, sHA biomaterials can scavenge and inactivate Dickkopf-1, a protein implicated in bone fracture defects (Gronbach et al. 2020). Moreover, sHA-containing aECMs can adsorb and control the release of TGF- β 1 to promote collagen synthesis in human mesenchymal stromal cells, in a DS-dependent manner (Hempel et al. 2012). Finally, hydrogels containing sHA are able to bind and slowly release heparin-binding EGF-like growth factor (HB-EGF), provoking keratinocyte migration in dermal fibroblasts and inducing epithelial tip growth in wound healing models (Thönes et al. 2019).

10.5 Concluding Remarks and Perspectives

Hyaluronan is a natural ECM macromolecule with significant effects in cell functional properties in health and disease. During the last decade various modifications of the HA structure have been developed as to either alter its effects in cells and tissues or produce innovative biomaterials. The main purposes for such modifications include the utilization of modified polymer as a pharmacologic agent to target cancer, inflammatory-based diseases and in regenerative medicine. The modified hyaluronan polymers are synthesized through a variety of strategies. Introducing sulfate groups into HA results in a potent biopolymer with promising capabilities. Sulfated HA, one of the most used modified HA, exhibits anti-cancer effects through different modes of action and can be used in biomedical polymer matrices and different tissue-engineering applications in regenerative medicine when used as biomaterials. A schematic representation of the various functions of sulfated hyaluronan is given in Fig. 10.1. Since its effects are closely tied to its properties, such as molecular weight, degree of sulfation, and sulfation pattern, sHA is characterized by versatility. To this day, sulfated HA has been used to enhance osteogenesis and wound healing, as disease treatment, in drug delivery systems, as an anti-cancer agent, and as a potent biomaterial. With the literature investigating the mechanisms behind its actions continuously expanding, sHA could be seen as an emerging player both in treatment strategies and bioengineering applications.

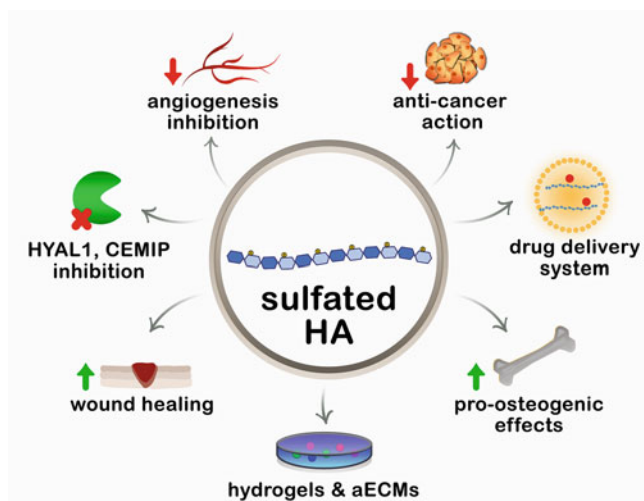


Fig. 10.1 A schematic presentation of the main actions of sulfated HA. It inhibits HA-degrading enzymes such as HYAL1 and CEMIP, inhibits angiogenesis either by selective binding to VEGF_{165a} or by downregulating angiogenic factors, exhibits *in vitro* anti-cancer action, can be used in targeted drug delivery systems, enhances osteogenesis and wound healing, and overall is a potent biomaterial in hydrogels and aECMs

References

- Bedini E, Laezza A, Parrilli M, Iadonisi A (2017) A review of chemical methods for the selective Sulfation and Desulfation of polysaccharides. *Carbohydr Polym* 174:1224–1239. <https://doi.org/10.1016/j.carbpol.2017.07.017>
- Benitez A, Yates TJ, Lopez LE, Cerwinka WH, Bakkar A, Lokeshwar VB (2011) Targeting hyaluronidase for cancer therapy: antitumor activity of sulfated hyaluronic acid in prostate cancer cells. *Cancer Res* 71:4085. <https://doi.org/10.1158/0008-5472.CAN-10-4610>
- Caputo HE, Straub JE, Grinstaff MW (2019) Design, synthesis, and biomedical applications of synthetic Sulphated polysaccharides. *Chem Soc Rev* 48:2338–2365. <https://doi.org/10.1039/C7CS00593H>
- Feng Q, Lin S, Zhang K, Dong C, Wu T, Huang H, Yan X, Zhang L, Li G, Bian L (2017) Sulfated hyaluronic acid hydrogels with retarded degradation and enhanced growth factor retention promote HMSC Chondrogenesis and articular cartilage integrity with reduced hypertrophy. *Acta Biomater* 53:329–342. <https://doi.org/10.1016/j.actbio.2017.02.015>
- Ferrara N (2010) Vascular endothelial growth factor and age-related macular degeneration: from basic science to therapy. *Nat Med* 16:1107–1111. <https://doi.org/10.1038/nm1010-1107>
- Förster Y, Schulze S, Penk A, Neuber C, Möller S, Hintze V, Scharnweber D, Schnabelrauch M, Pietzsch J, Huster D et al (2020) The influence of different artificial extracellular matrix implant coatings on the regeneration of a critical size femur defect in rats. *Mater Sci Eng C* 116:111157. <https://doi.org/10.1016/j.msec.2020.111157>
- Gronbach M, Mitrach F, Lidzba V, Müller B, Möller S, Rother S, Salbach-Hirsch J, Hofbauer LC, Schnabelrauch M, Hintze V et al (2020) Scavenging of Dickkopf-1 by Macromer-based bio-materials covalently decorated with sulfated Hyaluronan displays pro-osteogenic effects. *Acta Biomater* 114:76–89. <https://doi.org/10.1016/j.actbio.2020.07.017>
- Guarise C, Tessari M, Pavan M, Pluda S, Di Lucia A, Barbera C, Galesso D (2022) Hydrophobic derivatives of sulfated hyaluronic acid as drug delivery Systems for Multi-Target Intra-Articular Treatment of post-traumatic osteoarthritis. *J Pharm Sci* S0022354922001551:2505. <https://doi.org/10.1016/j.xphs.2022.04.003>
- Heldin P, Lin CY, Kolliopoulos C, Chen YH, Skandalis SS (2019) Regulation of Hyaluronan biosynthesis and clinical impact of excessive Hyaluronan production. *Matrix Biol* 78–79:100–117. <https://doi.org/10.1016/j.matbio.2018.01.017>
- Hempel U, Hintze V, Möller S, Schnabelrauch M, Scharnweber D, Dieter P (2012) Artificial extracellular matrices composed of collagen I and sulfated Hyaluronan with adsorbed transforming growth factor B1 promote collagen synthesis of human mesenchymal stromal cells. *Acta Biomater* 8:659–666. <https://doi.org/10.1016/j.actbio.2011.10.026>
- Hintze V, Schnabelrauch M, Rother S (2022) Chemical modification of Hyaluronan and their biomedical applications. *Front Chem* 10. <https://doi.org/10.3389/fchem.2022.830671>
- Iozzo RV, Gubbiotti MA (2018) Extracellular matrix: the driving force of mammalian diseases. *Matrix Biol* 71-72:1
- Isoyama T, Thwaites D, Selzer MG, Carey RI, Barbucci R, Lokeshwar VB (2006) Differential selectivity of hyaluronidase inhibitors toward acidic and basic hyaluronidases. *Glycobiology* 16: 11–21. <https://doi.org/10.1093/glycob/cwj036>
- Jordan AR, Lokeshwar SD, Lopez LE, Hennig M, Chipollini J, Yates T, Hupe MC, Merseburger AS, Shiedlin A, Cerwinka WH et al (2014) Antitumor activity of sulfated hyaluronic acid fragments in pre-clinical models of bladder cancer. *Oncotarget* 8:24262. <https://doi.org/10.18632/oncotarget.10529>
- Karamanos NK, Theocharis AD, Piperigkou Z, Manou D, Passi A, Skandalis SS, Vynios DH, Orian-Rousseau V, Ricard-Blum S, Schmelzer CEH et al (2021) A guide to the composition and functions of the extracellular matrix. *FEBS J* febs.15776:6850. <https://doi.org/10.1111/febs.15776>

- Karousou E, Misra S, Ghatak S, Dobra K, Götte M, Vigetti D, Passi A, Karamanos NK, Skandalis SS (2017) Roles and targeting of the HAS/Hyaluronan/CD44 molecular system in cancer. *Matrix Biol* 59:3–22. <https://doi.org/10.1016/j.matbio.2016.10.001>
- Koutsakis C, Tavianatou AG, Kokoretsis D, Baroutas G, Karamanos NK (2021) Sulfated Hyaluronan modulates the functional properties and matrix effectors expression of breast cancer cells with different estrogen receptor status. *Biomol Ther* 11. <https://doi.org/10.3390/biom11121916>
- Li L, Yan LH, Manoj S, Li Y, Lu L (2017) Central role of CEMIP in tumorigenesis and its potential as therapeutic target. *J Cancer* 8(12):2238
- Li Z, Tian G, Jiang H, Pan R, Lian B, Wang M, Gao Z, Zhang B, Wu J (2019) Liver-targeting and PH-sensitive sulfated hyaluronic acid mixed micelles for hepatoma therapy. *IJN* 14:9437–9452. <https://doi.org/10.2147/IJN.S214528>
- Lim D-K, Wylie RG, Langer R, Kohane DS (2016) Selective binding of C-6 OH sulfated hyaluronic acid to the Angiogenic isoform of VEGF165. *Biomaterials* 77:130–138. <https://doi.org/10.1016/j.biomaterials.2015.10.074>
- Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, Nadji M, Lokeshwar BL (2001) Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. *J Biol Chem* 276:11922. <https://doi.org/10.1074/jbc.M008432200>
- Lokeshwar VB, Young MJ, Goudarzi G, Iida N, Yudin AI, Cherr GN, Selzer MG (1999) Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1. *Cancer Res* 59:4464
- Miura T, Kawano M, Takahashi K, Yuasa N, Habu M, Kimura F, Imamura T, Nakayama F (2022) High-sulfated hyaluronic acid ameliorates radiation-induced intestinal damage without blood anticoagulation. *Adv Radiat Oncol* 7:100900. <https://doi.org/10.1016/j.adro.2022.100900>
- Möller S, Theiß J, Deinert TIL, Golat K, Heinze J, Niemeyer D, Wyrwa R, Schnabelrauch M, Bogner E (2022) High-sulfated glycosaminoglycans prevent coronavirus replication. *Viruses* 14:413. <https://doi.org/10.3390/v14020413>
- Nagahata M, Tsuchiya T, Ishiguro T, Matsuda N, Nakatsuchi Y, Teramoto A, Hachimori A, Abe K (2004) A novel function of N-cadherin and Connexin43: marked enhancement of alkaline phosphatase activity in rat Calvarial osteoblast exposed to sulfated Hyaluronan. *Biochem Biophys Res Commun* 9:603
- Passi A, Vigetti D, Buraschi S, Iozzo RV (2019) Dissecting the role of Hyaluronan synthases in the tumor microenvironment. *FEBS J* 286:2937–2949. <https://doi.org/10.1111/febs.14847>
- Piperigkou Z, Kyriakopoulou K, Koutsakis C, Mastronikolis S, Karamanos NK (2021) Key matrix remodeling enzymes: functions and targeting in cancer. *Cancers* 13:1441. <https://doi.org/10.3390/cancers13061441>
- Rodrigues G, Hoshino A, Kenific CM, Matei IR, Steiner L, Freitas D, Kim HS, Oxley PR, Scandariato I, Casanova-Salas I et al (2019) Tumour Exosomal CEMIP protein promotes cancer cell colonization in brain metastasis. *Nat Cell Biol* 21:1403. <https://doi.org/10.1038/s41556-019-0404-4>
- Sanderson RD, Elkin M, Rapraeger AC, Ilan N, Vlodavsky I (2017) Heparanase regulation of cancer, autophagy and inflammation: new mechanisms and targets for therapy. *FEBS J* 284(1): 42–55
- Schmaus A, Rothley M, Schreiber C, Möller S, Roßwag S, Franz S, Garvalov BK, Thiele W, Spataro S, Herskind C et al (2022) Sulfated hyaluronic acid inhibits the hyaluronidase CEMIP and regulates the HA metabolism, proliferation and differentiation of fibroblasts. *Matrix Biol* 109:173–191. <https://doi.org/10.1016/j.matbio.2022.04.001>
- Schnabelrauch M, Schiller J, Möller S, Scharnweber D, Hintze V (2021) Chemically modified glycosaminoglycan derivatives as building blocks for biomaterial coatings and hydrogels. *Biol Chem* 402:1385–1395. <https://doi.org/10.1515/hsz-2021-0171>

- Shi J, Kanoya R, Tani Y, Ishikawa S, Maeda R, Suzuki S, Kawanami F, Miyagawa N, Takahashi K, Oku T et al (2022) Sulfated Hyaluronan binds to Heparanase and blocks its enzymatic and cellular actions in carcinoma cells. *Int J Mol Sci* 23:1–19. <https://doi.org/10.3390/ijms23095055>
- Spinelli FM, Rosales P, Pluda S, Vitale DL, Icardi A, Guarise C, Reszegi A, Kovalszky I, García M, Sevic I et al (2022) The effects of sulfated Hyaluronan in breast, lung and colorectal carcinoma and monocytes/macrophages cells: its role in angiogenesis and tumor progression. *IUBMB Life* 74(10):927. <https://doi.org/10.1002/iub.2604>
- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71:209–249. <https://doi.org/10.3322/caac.21660>
- Tavianatou AG, Caon I, Franchi M, Piperigkou Z, Galesso D, Karamanos NK (2019) Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. *FEBS J* 286:2883–2908. <https://doi.org/10.1111/febs.14777>
- Thönes S, Rother S, Wippold T, Blaszkiewicz J, Balamurugan K, Moeller S, Ruiz-Gómez G, Schnabelrauch M, Scharnweber D, Saalbach A et al (2019) Hyaluronan/collagen hydrogels containing sulfated Hyaluronan improve wound healing by sustained release of heparin-binding EGF-like growth factor. *Acta Biomater* 86:135–147. <https://doi.org/10.1016/j.actbio.2019.01.029>
- Vogel S, Arnoldini S, Möller S, Schnabelrauch M, Hempel U (2016) Sulfated Hyaluronan alters fibronectin matrix assembly and promotes osteogenic differentiation of human bone marrow stromal cells. *Sci Rep* 6:36418. <https://doi.org/10.1038/srep36418>
- Zhang D, Zhao L, Shen Q, Lv Q, Jin M, Ma H, Nie X, Zheng X, Huang S, Zhou P et al (2017) Down-regulation of KIAA1199/CEMIP by MiR-216a suppresses tumor invasion and metastasis in colorectal cancer. *Int J Cancer* 140:2298. <https://doi.org/10.1002/ijc.30656>