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Simple Primary Structure, Complex Turnover Regulation and Multiple Roles of Hyaluronan

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Hyaluronan is a major macromolecular polysaccharide component of the extracellular matrix that confers structural frameworks for cells. Despite its relatively simple chemical composition, hyaluronan mediates many other important functional aspects including signalling activity during embryonic morphogenesis, cellular regeneration and wound healing. Abnormalities in hyaluronan metabolism have been implicated in many diseases, such as inflammatory disorders, cardiovascular diseases and cancer. To date, it has become increasingly clear that hyaluronan production in vertebrates is tightly regulated by three hyaluronan synthases and that hyaluronan catabolism is regulated by an enzymatic degradation reaction involving several hyaluronidases. Together, these discoveries have provided key insights into the physiological roles of hyaluronan and a deeper understanding of the mechanisms underlying altered hyaluronan turnover in diseases. The central aim of this review article is therefore to highlight the multiple roles of hyaluronan in physiological and pathological states *via* its complex turnover regulation.

Key words: biosynthesis, cancer progression, cardiovascular disease, extracellular matrix, hyaluronan.

Abbreviations: MAP, mitogen-activated protein; cAMP, cyclic adenosine monophosphate; VEGF, vascular endothelial growth factor.

Hyaluronan (HA) was discovered by Karl Meyer and John Palmer in 1934 as a novel glycosaminoglycan in bovine vitreous (*1*), and it was found that HA is an extremely simple polysaccharide composed of repeating disaccharide units in which *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) are linked together by alternating β-1,3 and β-1,4 linkages (Fig. 1). Since its original description 70 years ago, there has been no doubt that the simple repeating structure of HA is involved in both a number of important cell behaviours and maintenance of tissue architecture (*2*, *3*); HA provides structural frameworks for cells as an abundant component of extracellular matrices (ECMs) in most supporting tissues of vertebrates (*4*). A wide variety of HA binding molecules contribute to the assembly of pericellular HA ECM and tightly regulate HA functions (*5*). It is now well known that HA functions as an extracellular molecule transmitting signals and regulates a variety of cell behaviours, such as cell adhesion, motility, growth and differentiation (*6*). Exposure of HA to the cells activates various intracellular signalling cascades such as c-Src, Ras and mitogen-activated protein (MAP) kinases through interaction with cell-surface receptors (Fig. 2). These in turn transcribe several genes relating to the cell growth and survival, and induce cytoskeletal rearrangement and membrane ruffling, leading to active cell migration (Fig. 2). The multiple properties of HA depend largely on its molecular size and tissue concentration both of which are firmly regulated by cooperated actions of HA biosynthetic and degradation processes. In vertebrates, HA is synthesized by three synthases and degraded by an enzymatic reaction involving several hyaluronidases (*7*).

Accumulating evidence has demonstrated that the production of HA is excessive in malignant cancers; increased HA serum levels and deposition in tumour tissue are often associated with malignant progression in breast and colorectal cancer (*8–10*). Excess HA accumulation has also been implicated in the promotion of cardiovascular diseases (*11*, *12*). Recently, animal models with genetically manipulated HA synthase and hyaluronidase genes have provided powerful tools for understanding the *in vivo* function of HA in connection with physiological and pathological events.

COMPLEX HA BIOSYNTHESIS

HA biosynthesis is regulated in a complex fashion based on substrate concentration, expression of HA synthase, transfer reaction of two substrates and elongation and secretion of the product (*13*). HA synthase (HAS), a key enzyme in HA biosynthesis, was first characterized in the bacterium *Streptococcus pyogenes* (designated SpHAS) (*14*). Subsequently, great progress in understanding the mechanism of HA biosynthesis in vertebrates has been made with the discovery of the three members of the HA synthase gene family, termed

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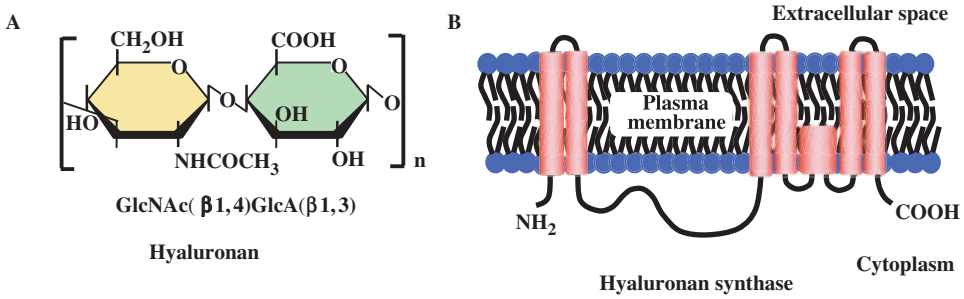

C

Enzymatic properties	HAS1	HAS2	HAS3
HA elongation rate	High	High	Low
Km for UDP-sugars	High	Low	Low
Enzymatic activity	Unstable	Stable	Stable
HA length	2×10^5 ~ 2×10^6 Da	2×10^6 Da <	2×10^5 ~ 2×10^6 Da
HA matrix formation	Low	High	High

Fig. 1. **HA structure (A), a predicted structure of mammalian HA synthases (B) and characteristics of three HA synthases (C).**

HAS1, HAS2 and HAS3, which share 55–71% amino acid sequence homology (*13*, *14*). Structurally, all HAS enzymes are integral membrane proteins composed of multiple membrane-spanning regions with hydrophobic amino acid clusters and large cytoplasmic loops (Fig. 1). Unlike typical glyscosyltransferases, this enzyme is localized in the plasma membrane. Since all HAS enzymes have conserved regions surrounding a DxD motif (x stands for any amino acid), a critical part of the nucleotide-sugar binding site for most glycosyltransferases, in their cytoplasmic loop, the HAS catalytic sites appear to locate to the intracellular face of the plasma membrane (*15*).

Recent biochemical studies using recombinant HAS enzymes have highlighted the unique process of HA biosynthesis and mode of chain elongation (*16*). In these studies, purified bacterial HAS protein retained significant activity and could link UDP-GlcNAc and UDP-GlcA substrates together in alternating β-1,3 and β-1,4 linkages. The mechanism for chain elongation is still controversial, but subunits are believed to excrete HA through a pore made by the enzyme itself along with lipid compounds on the plasma membrane. The phospholipid-dependent process of HA synthesis has been proposed by studies using a recombinant SpHAS protein (*17*). In this cell-free reaction, administration of caldiolipin increased activity of solubilized recombinant SpHAS in a dose-dependent manner. Therefore, the three-dimensional structure required for manifestation of full enzymatic activity appears to be ensured by interaction with phospholipids, and due to similarities in primary structure and configuration, the activities of mammalian HAS enzymes may also be affected by the lipid microenvironment. Current biochemical studies in our lab using recombinant human HAS2 have also revealed such a lipid-dependency (our unpublished data). Regarding the subcellular localization of HAS enzymes, a recent study has proposed that latent enzymes reside around the endoplasmic reticulum (ER) and nuclear membrane and synthesize cable-like HA structures upon particular cellular stresses, such as ER stress (*18*, *19*). This provides evidence for multiple regulatory mechanisms of HA biosynthesis, where structurally different HA molecules are synthesized in different subcellular compartments depending on cellular state.

The expression profiles of HAS genes are temporally and spatially regulated during embryogenesis and pathogenesis (*13*). Divergence in the transcriptional regulation of HAS genes can be explained to some extent by upstream signalling pathways that are triggered by various growth factors, cytokines, cellular stress and so on. Stimulation of a variety of cells with prostaglandins was shown to strongly induce HA biosynthesis *via* a cyclic adenosine monophosphate (cAMP)-dependent signalling pathway (*20*), and recent analysis of the proximal promoter sequences of HAS2 revealed several cAMP-responsive binding elements within a 500 bp region upstream of the transcriptional initiation site, implying transcriptional up-regulation in response to cAMP (*21*). In addition, several NFκB-binding sites are also present, allowing for cytokine- and growth factor-induced transcription of HAS2 (*22*).

Characterization of the three HAS isoforms has revealed several differences in enzymatic properties, particularly in the ability to form HA matrices and determine product

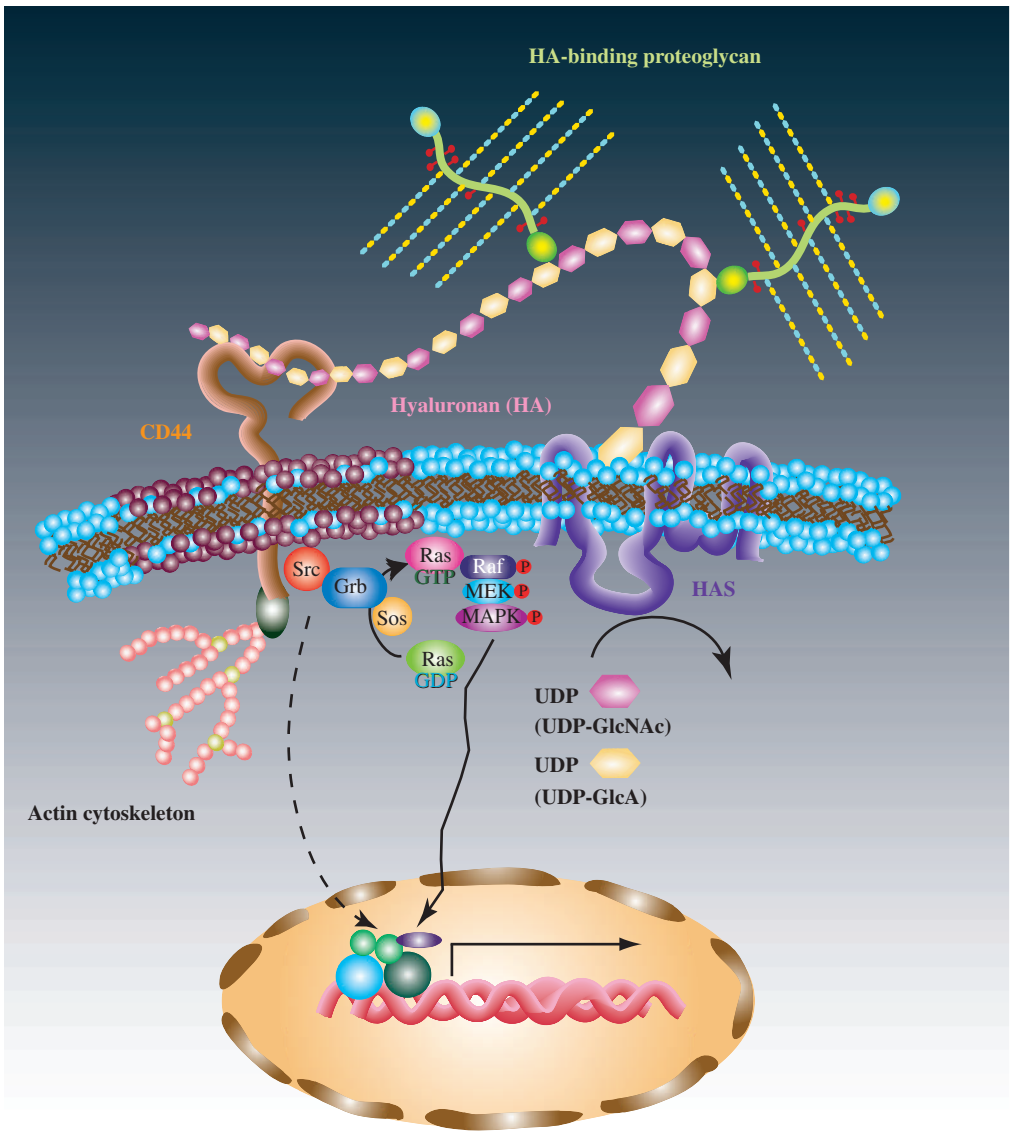

Fig. 2. **HA signalling cascades.** HA assembles HA-rich ECM by binding to its binding molecules. HA activates intracellular signalling cascades via its receptors such as CD44 and up-regulates several genes relating to the cell proliferation and survival. The interaction of HA with CD44 also induces rearrangement of actin cytoskeleton leading active cell migration.

molecular size (Fig. 1). One can speculate on the possibility that each HAS isoform synthesizes functionally distinct forms of HA. Although we do not know the detailed functional aspects of HA molecules synthesized by each HAS isoform, it is important to understand what mechanism causes these differences. To characterize the properties of each enzyme, we prepared membranous fractions from cells transfected with each HAS gene and investigated the *in vitro* stability, kinetics and rate of sugar chain elongation for each enzyme (*23*). Notably, kinetic analyses showed that HAS isoforms differ in their apparent K_m values for two substrates, UDP-GlcA and UDP-GlcNAc, and half-life of enzymatic activity (Fig. 1). As such, a broad spectrum of HA functions can be regulated by the concerted action of three enzymes with different enzymatic properties.

HA CATABOLISM

The dynamic turnover of HA molecules is tightly regulated during embryonic development and homoeostatic processes and is balanced by synthesis and catabolism, thereby maintaining a constant concentration in the tissue (*24*). In vertebrates, the half-life of HA molecules is short in certain tissues; for instance, rates are less than a day in skin and serum, and normally 2–3 weeks in cartilage. The turnover and removal of HA from the ECM occurs *via* local catabolism and/or drainage into the lymphatic system for catabolism in regional lymph nodes. HA catabolism is predominantly regulated by several hyaluronidases, which are classified as endo-β-*N*-acetylglucosaminidases according to their mammalian hydrolytic mechanisms (*25*). In mammals, at least six

hyaluronidase-like genes are clustered as two tightly linked triplets on two chromosomes: *HYAL1*, *HYAL2* and *HYAL3* on human chromosome 3p21 (*Hyal1*, *Hyal2* and *Hyal3* on mouse chromosome 9F1-F2) and *HYAL4*, *PH-20/SPAM1* and *HYALP1* on human chromosome 7q31 (*Hyal4*, *Ph-20* and *Hyalp1* on mouse chromosome 6A2) (*26*). There is now evidence that PH-20 is essential for sperm penetration of both the cumulus ECM and the zona pellucida. HYAL1 and HYAL2 are the major hyaluronidases of somatic tissues. HYAL1 is a lysosomal enzyme with an acidic pH optimum and ability to cleave HA into small oligosaccharides. The second hyaluronidase gene, *HYAL2*, was identified based on its homology to the previously described PH-20. HYAL2 is a glycosylphosphatidylinositol-(GPI-) anchored protein located on the surface of plasma membranes, and is able to degrade high-molecular-mass HA into intermediately sized products of 20 kDa. Since there is little evidence to support extracellular HA depolymerization, local turnover of HA is believed to occur intracellularly within lysosomes *via* a low pH-responsive hyaluronidase. Numerous studies support that receptor-mediated internalization of HA is the primary step for the turnover and catabolism in many tissues, and it is clear that at least a portion of HA is internalized *via* CD44 and delivered to lysosomes for degradation (*27*).

Several inherited disorders of hyaluronidase have been previously reported. In one case, a patient with a deficiency in plasma hyaluronidase was designated as having mucopolysaccharidosis IX, a lysosomal storage disorder (*28*). Serum hyaluronidase activity was noted to be deficient, and the concentration of HA in the serum was extremely elevated.

ROLES OF HA IN EMBRYONIC DEVELOPMENT

HA plays diverse roles both in the structural and physiological characteristics of embryonic morphogenesis. Highly hydrated HA pericellular matrices often surround migrating and proliferating cells, such as proliferating mesenchymal cells during embryonic limb development, neural crest cells migrating from the neural tube and endocardial cushion cells participating in the formation of heart valves (*29*). Targeted disruption of the murine Has2 gene revealed that the HA synthesized by this enzyme is essential for normal embryonic development (*30*, *31*). Has2 null embryos exhibit severe heart defects, and died at E9.5 due to a complete lack of endocardial cushions, unfolding of the ventricular wall and the absence of trabeculation (Fig. 3). Heart valve morphogenesis is achieved by the formation and remodelling of endocardial cushions. Endocardial cushion formation commences

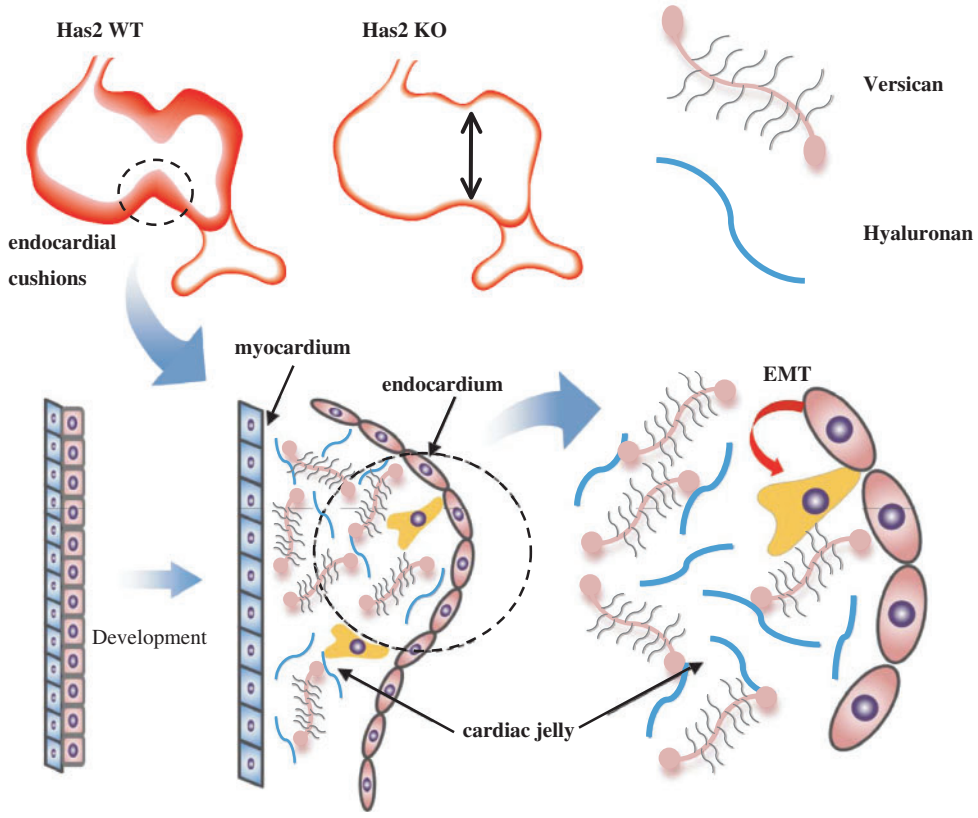

Fig. 3. **The role of HA in atrioventricular canal morphogenesis.** Has2 null embryo exhibits severe heart defect, due to the complete lack of endocardial cushions, the unfolding of the ventricular wall, and the absence of the trabeculation. HA matrices are synthesized by both myocardium and endocardium, and accumulated between the cell layers. The two layers are physically separated by the formation of cardiac jelly that is rich in HA and versican. Endocardial cushion cells undergo epithelial-to-mesenchymal transformation (EMT) and migrate into the cardiac jelly. In this process, HA plays an important role in the EMT event.

with the deposition of ECM, known as cardiac jelly, which provides a favourable environment for endothelial cells in the endocardium to transform into mesenchymal cells and migrate within the cardiac jelly. Has2 null embryos have defects in this process known as endothelial-to-mesenchymal transformation (EMT), resulting in heart valve formation abnormalities (Fig. 3). Modulating the expression of HAS genes in epithelial-like cells *in vitro* more directly assessed the role of HA in EMT (*32*, *33*). Cell culture studies showed that following endocardial cell EMT, high molecular weight (HMW) HA is processed to oligosaccharide, which stimulates vascular endothelial growth factor (VEGF) activity to attenuate cardiac developmental EMT (*34*). The observations thus clearly indicate the importance of cooperated actions of HAS and hyaluronidase in heart development. Additionally, a similar heart defect was observed in hdf gene trap mice deficient in an HA-binding chondroitin sulphate proteoglycan versican, suggesting a close association between HA and versican in cardiac morphogenesis (*35*).

DISEASES: CANCER PROGRESSION

Clinicopathological studies have indicated that increased serum levels and deposition of HA in tumour tissue are often associated with malignant progression in many cancers, including breast and colorectal cancer (*9*, *10*), and multiple transcriptional regulation of HAS genes may allow cancer cells to optimize the extracellular environment for tumour growth and invasion. During tumour initiation and progression, a transcriptional switch in HAS isoforms has been demonstrated in cells undergoing malignant transformation (*36*). In genetically manipulated tumour cells, forced expression of HAS genes enhanced tumuorigenic and sometimes metastatic abilities (*37–39*). In order to simulate the overproduction of HA found in human breast cancer, we generated a transgenic (T_g) mouse model allowing over-expression of murine Has2 in mammary glands (*40*). In this model, the expression of exogenous Has2 resulted in the aggressive growth of oncogene-initiated mammary tumours. Particularly, overproduction of HA accelerated tumour angiogenesis and lynphangiogenesis through stromal cell recruitment (*40*, *41*).

The above *in vivo* and *in vitro* data clearly demonstrate the important role of microenvironmental HA in tumuorigenesis and cancer progression. However, the tumour promoting ability of excess HA is somewhat controversial, since HAS2 over-expression has been found to rather suppress the tumuorigenesis of glioma cells (*42*). Because HA accumulation is the result of a balance between the activities of HAS and hyaluronidases, the presence of hyaluronidase may overcome the tumour suppression by excess amounts of HA. Indeed, expression of hyaluronidase HYAL1 restores the growth of human prostate cancer cells that are dramatically suppressed by HAS2 over-expression (*43*). Additional evidence for HYAL1-mediated tumour promotion has come from a xenograft experiment that showed how blocking HYAL1 expression decreased tumour growth of prostate cancer cells (*44*). Similarly to HAS2, high HYAL1 decreased growth of tumour xenografts in mice, implying that HYAL1 can be involved in both tumour promotion and suppression. Such biphasic effects of HA on tumuorigenesis can be explained by considering its dose-dependent properties through regulation by HAS and HYAL. This dose-dependency may also help contextualize statements regarding the physiological significance of changes in HA concentration with tumour grade or stage.

The tumour-promoting ability of HA may also depend on its molecular size that is balanced by the reactions of HAS and HYAL (*45*). It is well established that HA degradation products of specific sizes are potent stimulators of endothelial cell proliferation and migration, and thereby contribute to angiogenesis (*46*). In contrast, HMW native HA is anti-angiogenic and inhibits endothelial cell proliferation and migration and capillary formation in a three-dimensional matrix. Since angiogenesis is the result of complex interactions between positive and negative regulators, the balance of anti-angiogenic HMW HA and angiogenic HA oligosaccharides may be important for modulating the angiogenic response during tumour development.

CARDIOVASCULAR DISEASES

Atherosclerosis underlies much of the cardiovascular disease encountered in the developed world. The atherosclerotic process is initiated when low-density, cholesterol-containing lipoproteins accumulate in the intima and activate the endothelium. During atherosclerosis, lesions developed in the intima of these arteries are characterized by thickening of this layer due to accumulation of ECM and proliferation of smooth muscle cells (SMCs). In line with this, deposition of extracellular HA has been found to increase in regions of developing lesions where there is active SMC migration and proliferation (*47*). Evidence supporting the involvement of HA in the progression of atherosclerosis has come from experiments using genetically manipulated animal models (*48*). *In vivo* investigations using Tg mice, where HAS2 expression was driven by an αSMC-actin promoter, demonstrated that HA overproduction promoted atherosclerosis development in the aorta of an ApoE-deficient mouse strain. This is consistent with *in vitro* data revealing that HA influences the proliferation and migration of vascular SMCs (*49*). HA accumulation may also accelerate macrophage recruitment and activation, which are pivotal for the promotion of atherosclerosis (*50*). Together with previous findings, HA accumulation appears to be a key step in the initiation and promotion of atherosclerosis, implying that HA biosynthesis should be a main target for the prevention of atherosclerosis.

CONCLUSION AND PERSPECTIVES

In this article, we discuss the diverse physiological and pathological roles of HA by concerning a complex turnover regulation. Through the discovery of key enzymes regulating HA biosynthesis and degradation, many questions have been resolved; biochemical studies using recombinant enzymes highlight the unique process of HA biosynthesis and degradation, and genetic approaches

have enabled us to better understand the cellular roles of HA. Furthermore, animal models have allowed us to explore the *in vivo* function of HA in connection with physiological events. In spite of this, the underlying molecular mechanisms of altered HA turnover are still puzzling. Our future challenge is therefore to understand the entire compliment of diverse and complex HA functions, and further elucidation of HA biosynthetic and degradation processes will promise the generation of new drugs against cancer progression and cardiovascular diseases.

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