Chapter 7
Heparanase: Cloning, Function and Regulation

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7.1 Introduction

7.1.1 Identification of Heparanase

Heparanase (HPSE) is an endo-β-glucuronidase that cleaves heparan sulfate (HS) polysaccharide chains at the cell surface and in the extracellular matrix. It was first described in 1952 as heparinase due to its heparin cleavage functionality [1]. However, it was not until 1975 that its HS cleavage activity was observed whilst investigating the metabolism of cell surface glycosaminoglycans [2]. This discovery was followed by studies describing HS degradative activity by platelets in 1976 [3]. The enzyme was renamed heparanase (HPSE, a.k.a. HPA, HPA1, HSE1) in 1983 when it was found to be expressed in the metastatic B16 melanoma cell line, suggesting a possible role in cancer [4]. To this day, HPSE remains the only known endoglycosidase enzyme able to directly cleave HS.

7.1.2 Normal Expression of Heparanase

HPSE is expressed ubiquitously across different animal species, including invertebrates, with high amino acid sequence conservation ranging from 53 to 98% [5]. Despite the prevalence of *HPSE* genes in the database for various animal species, studies have primarily focussed on humans and the common laboratory mouse,

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Mus musculus, given its use as a model for understanding human disease (mouse and human *HPSE* share 77% amino acid sequence identity).

Human tissue analyses reveal that expression of *HPSE* is low to undetectable in most non-immune tissues, including the kidney, pancreas, heart, brain, endometrium, lung, testis, trachea, colon, adrenal glands, salivary glands, and skeletal muscle, whereas the placenta exhibits high levels of transcript expression [6–10]. Investigations into HPSE expression in immune cells reveal high expression levels (constitutive or inducible) in nearly all haematopoietic cells, including platelets, monocytes, macrophages, neutrophils, mast cells, dendritic cells, activated lymphocytes, and eosinophils, with lower expression levels observed in the lymph node, bone marrow, spleen, liver and thymus [6, 11, 12]. This indicates a key role for HPSE in the function of the immune system. HPSE expression is also upregulated in a number of pathological settings, such as cancer and inflammation-related disease, which will be explored in Sect. 7.3.4.

7.2 Gene Cloning

7.2.1 Race of Four: The Cloning of Human Heparanase

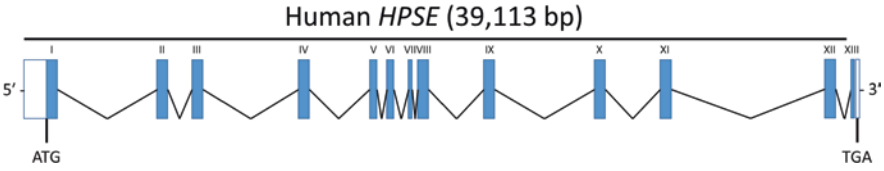
The year 2019 marks the 20th anniversary of the cloning of human HPSE. The *HPSE* gene was initially cloned in 1999 from a number of different cell lines and tissues, including placenta and the SV-40 transformed embryonic lung fibroblast cell line WI38/VA13 [6, 13–16]. Such was the high significance and strong competition of the 'race' to clone human HPSE, Hulett et al. (1999), Vlodavsky et al. (1999), Kussie et al. (1999), Toyoshima and Nakajima (1999) and Fairbanks et al. (1999) all published papers within a few months of each other [6, 14–17].

Using platelet-purified HPSE, Hulett et al. (1999) sequenced the purified 50 kDa protein via *in situ* tryptic digest and N-terminal sequencing [6, 18]. The tryptic digest generated 10 peptides, which were used in a BLASTN database search, resulting in the identification of a placenta-derived cDNA clone. This clone encoded for 5 of the observed HPSE peptides and matched to a 1.1 kb sequence that encodes for the 300 C-terminal HPSE amino acids. PCR amplification of a human placental cDNA library using primers based on the BLASTN-identified clone was successful in amplifying an 800 bp sequence containing the 5′ end of the gene and the corresponding 3′ overlapping sequence of the HPSE cDNA. This revealed an open reading frame of 1629 bp, encoding for a 543 amino acid protein. The identified sequence allowed for the prediction of six *N*-linked glycosylation sites, a second potential initiation site, and hydrophobic regions at the N- and C-terminus; characteristic of transmembrane and signal peptide sequences. The identical 1629 bp cDNA sequence was also identified in the human T-cell lymphoma Jurkat cell line. Comparison of this sequence to the 50 kDa N-terminal sequencing results initially suggested that the N-terminal domain may represent the active enzyme. However, this hypothesis

was dismissed when functional assays demonstrated that the 50 kDa domain was inactive, thus indicating the importance of amino acids 1–157 in producing a functional HPSE enzyme.

HPSE purified by Vlodavsky et al. (1999) from human hepatoma cell line SK-HEP-1 and placenta underwent tryptic digest, followed by HPLC and sequencing, resulting in the identification of a corresponding cDNA clone from a placental cDNA library [6, 14, 19]. This clone contained an insert of 1020 bp; a 973 bp open reading frame, a 27 bp 3′ UTR, and a 20 bp poly-A tail. The 5′ sequence was obtained using PCR amplification of DNA from placenta Marathon RACE cDNA composite, resulting in a 930 bp amplicon, containing the start AUG codon and overlap with the original clone. This yielded a 1758 bp cDNA with an open reading frame encoding a 543 amino acid protein of 61 kDa calculated molecular weight, which was consistent with the hypothesis from Hulett et al. (1999) that the open reading frame encodes for a proenzyme form of HPSE [6]. Vlodavsky et al. then screened a human genomic library in Lambda phage in an attempt to clone the *HPSE* gene. The coding region, except for a 2 kb sequence, was identified from three isolated plaques, and the missing 2 kb gap was completed using PCR amplification from human genomic DNA. Analysis of the *HPSE* gene and a 2.3 kb region upstream of the start site revealed a region of 39,113 bp, which was segregated into 13 exons and 12 introns (Fig. 7.1). The HS-cleavage functionality of the cloned *HPSE* gene was confirmed using radiolabelled HSPGs purified from the extracellular matrix (ECM) that were analyzed by gel filtration and gel shift assays.

Later in the same year, Kussie et al. (1999) published the cloning of active human HPSE from a placental cDNA library [15]. Using a similar experimental pipeline to Hulett et al. and Vlodavsky et al., HPSE was purified from the SK-HEP-1 cell line by sequential chromatography, and the resulting protein subjected to tryptic digest. A single EST clone was identified as a match to one of the peptides, with sequence



Exon	Exon Size (bp)	Intron	Intron Size (bp)	Exon	Exon Size (bp)	Intron	Intron Size (bp)
I	106	**1**	234	**VIII**	94	**8**	450
II	258	**2**	12191	**IX**	107	**9**	2527
III	146	**3**	2749	**X**	115	**10**	3934
IV	126	**4**	6056	**XI**	119	**11**	1043
V	174	**5**	2223	**XII**	147	**12**	5456
VI	169	**6**	643	**XIII**	3043		
VII	48	**7**	535				

Fig. 7.1 Gene structure of human *HPSE*: The human *HPSE* gene is 39,113 bp long on chromosome 4, and is composed of 13 exons (boxes, roman numerals) and 12 introns (connecting lines) [20]. The gene encodes for a predominant 1.7 kb transcript comprising a 224 bp 5′ UTR (empty box), a 1629 bp open reading frame (start ATG codon, filled boxes, stop TGA codon), and a 27 bp 3′ UTR (empty box). The sizes (bp) of the exons and introns are indicated in the table

analysis revealing it contained a 975 bp open reading frame, a stop codon, a poly-A tail, and when translated, matched with the remaining 5 peptide sequences. Absent from this clone was a start ATG codon and a Kozak sequence. To clone the complete *HPSE* gene, a pooled array placental cDNA library was screened using PCR primers designed against the identified EST clone. From this study, a 1.7 kb fragment was obtained and sequenced. Analysis of the sequence revealed a 1629 bp open reading frame encoding for a 543 amino acid enzyme, with the predicted molecular weight of HSPE. Expression of this 1.7 kb clone in COS-7 cells confirmed it encoded for a protein with the ability to cleave HS chains using ^{35}S radiolabelled HS.

In the same year, cloning of HPSE was also achieved by Toyoshima and Nakajima utilizing enzyme purified from SV-40 transformed WI38/VA13 embryonic fibroblast cells [16]. The 50 kDa fragment of the purified HPSE was subjected to N-terminal sequencing or *in situ* Lys-C endoproteinase digest. Sequences of the resulting peptides were used as search queries utilizing a TBLASTN database akin to the workflow undertaken by Hulett et al. (1999), Vlodavsky et al. (1999) and Kussie et al. (1999), yielding two overlapping ESTs (yw97a02.r1 and yw70a03.s1). PCR analysis identified a 731 bp amplicon in the WI38/VA13 cell line, corresponding to the overlapping ESTs. The amplicon was used as a probe to screen for the full-length cDNA sequence of HPSE from a WI38/VA13 cDNA library, resulting in the identification of a 3762 bp insert containing a 1629 bp open reading frame encoding for a 543 amino acid protein. HS-degradation assays confirmed the protein as HPSE.

7.2.2 Identification of an 8 kDa Peptide in the Active HPSE Enzyme

The cloning studies outlined in the previous section were successful in defining the gene arrangement and the open reading frame encoding the HPSE enzyme. However, the composition of the active enzyme, including the precise role of the 157 N-terminal residues, remained to be elucidated.

The apparent absence of the N-terminal domain 157 amino acids, predicted from the full-length open reading frame in the purified 'active' 50 kDa enzyme, led to the hypothesis that HPSE was synthesised as a proenzyme; containing a signal peptide and 'pro-domain' that regulate post-translational processing and intracellular trafficking [6, 14, 15]. Given that the 65 kDa pro-form of the protein was enzymatically inactive, it was proposed that upon proteolytic cleavage, the 50 kDa domain was released as the active form of HPSE [14]. As discussed in Sect. 7.2.1, the 50 kDa domain of HPSE alone has no HS-degrading activity, indicating that the 157 amino acid N-terminal domain is vital for enzymatic function [6].

The critical observation that the active form of HPSE contains an 8 kDa fragment derived from the N-terminal 157 amino acid domain, in addition to the previously described C-terminal 50 kDa peptide, was first suggested by Fairbanks et al. (1999) employing a novel sequential chromatography workflow for the purification of

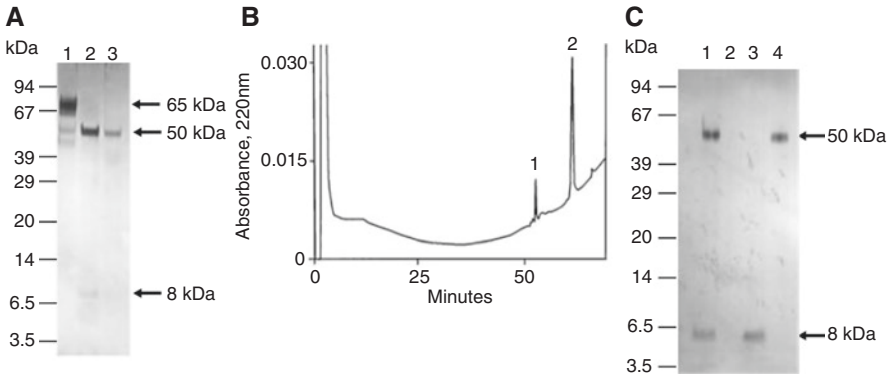


Fig. 7.2 Identification of an 8 kDa peptide in active HPSE: (**A**) SDS-PAGE of 3 distinct fractions from the purification of platelet-derived HPSE, obtained under non-reducing conditions, that exhibit HPSE activity. (**B**) Proteins were resolved on a Jupiter C4 reverse-phase column over 70 min with a linear gradient of increasing acetonitrile concentration from 0 to 70% in 0.15% TFA. (**C**) SDS-PAGE of the RP-HPLC peaks shows that peak 1 correlates with the 8-kDa (lane 3) and peak 2 with the 50-kDa chain (lane 4) identified in the load [1]. Adapted from [17]

HPSE from platelets (Fig. 7.2) [17]. RP-HPLC analysis revealed a 1:1 molar ratio of 50 kDa to 8 kDa peptide in the active form of HPSE. Alignment of the digested peptide sequences corroborated previous findings that the 50 kDa peptide resides at the C-terminal portion of the HPSE open reading frame, while the 8 kDa domain was found at the N-terminal end adjacent to the predicted signal peptide domain [6]. Analysis of the N- and C-terminal processing of the newly identified 8 kDa peptide led to the proposal that this domain consists of amino acids Gln^{36}s– Glu^{109}, with the interdigitating 6 kDa peptide unable to be observed in SDS-PAGE, predicted to be removed by proteolytic processing. Fairbanks et al. (1999) proposed that the 8 kDa and 50 kDa peptides non-covalently interact, forming a heterodimer, yet lacked the evidence to prove their hypothesis.

Following the hypothesis postulated by Fairbanks et al. (1999), Levy-Adam et al. (2003) were able to confirm the formation of a heterodimer using immunoprecipitation of tagged 8 kDa and 50 kDa domain peptides (Fig. 7.3) [21]. Further experiments identified that the amino acids responsible for the interaction among the 8 kDa and 50 kDa reside between residues 288 and 417. Expression of the 8 kDa and 50 kDa domains in cells was also found to be sufficient to increase HS degradation compared to expression of either of the domains alone, with the effect similar to that of transfection with full-length HPSE. Overall, these studies led to the proposal that the HPSE enzyme is synthesized as a pre-proenzyme, consisting of a signal peptide domain, followed by an 8 kDa domain (Ala^{35}-Glu^{109}), a 6 kDa linker region (Ser^{110}s–Gln^{157}) and a 50 kDa domain (Lys^{158}-Ile^{543}) (Fig. 7.4). The pre-proenzyme is initially processed to remove the signal peptide domain, then further processed to excise the 6 kDa linker domain, resulting in the formation of a heterodimer, which will be discussed in Sect. 7.4.4.

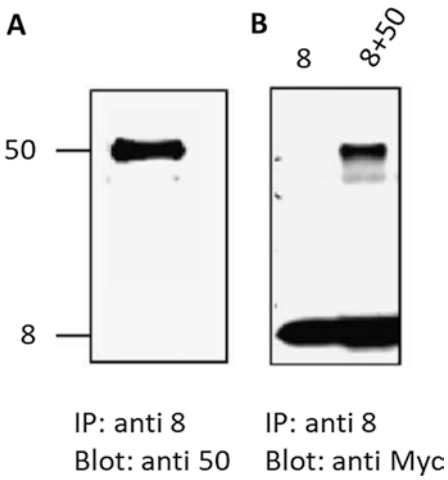


Fig. 7.3 The 8 and 50 kDa heparanase domains associate: **(A)** Total cell lysates of HEK293 cells stably transfected with the full-length heparanase were subjected to IP with anti-8 kDa heparanase antibodies, followed by immunoblotting with anti-50 kDa heparanase antibodies. (**B**) HEK293 cells were transfected with the c-Myc-tagged 8 kDa plasmid [8] or co-transfected with the c-Myc-tagged 8 and 50 kDa (8s+s50) plasmids. Total cell lysates were subjected to IP with anti-8 kDa antibodies, followed by anti-c-Myc immunoblotting. Adapted from [21]

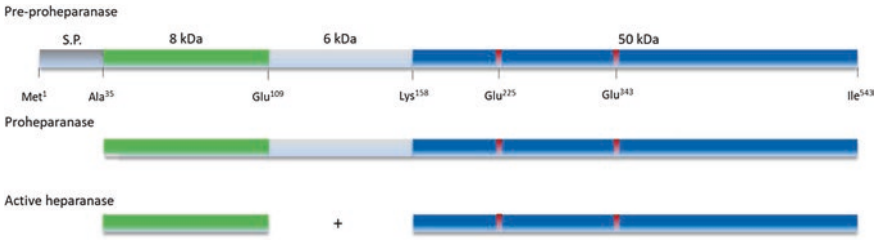


Fig. 7.4 Processing of the 65 kDa pre-proheparanase into the active heparanase enzyme: HPSE is translated as a pre-proenzyme including a C-terminal signal peptide sequence (S.P., dark grey), the 8 kDa domain (green), the 6 kDa linker domain (light grey), and the 50 kDa domain (blue) where the active site residues Glu^{225} and Glu^{343} sare located (red). Proteolytic processing removes the signal peptide sequence and the linker domain to produce the active enzyme

7.2.3 Cloning of Heparanase From Other Organisms

Using the human HPSE cDNA sequence identified in placenta and Jurkat cells, Hulett et al. (1999) attempted to clone mouse HPSE [6]. The cDNA sequence was screened against the dbEST database using BLASTN, which identified a homologous mouse HPSE. The database search returned five mouse ESTs corresponding to the 280 amino acids in the C-terminus of HPSE. Using first-strand cDNA derived from the RNA of activated mouse spleen cells, PCR amplification produced a 1368 bp cDNA sequence corresponding to the human HPSE sequence from amino acid

Table 7.1 HPSE is conserved across species: HPSE identified from various animal species, highlighting the size of the translated protein and the identity to human HPSE

Species	Protein size (amino acids)	AA identity to human HPSE
Gorilla gorilla	543	98%
Gallus gallus	523	62%
Bos taurus	545	78%
Sus scrofa	542	80%
Spalax	534	85%

158 to the poly-A tail. This sequence extended the combined EST sequence a further 532 bp in the 5′ direction, resulting in the identification of amino acids 158–543 in mouse HPSE. The use of primers based on the human HPSE sequence did not allow for the isolation of the remaining N-terminal sequence of mouse HPSE. The cloned sequence contained only the coding region of the 50 kDa domain that was shown not to be active when expressed. The complete mouse HPSE coding sequence was eventually cloned in 2002 [22]. A cDNA library from day 12.5 mouse embryo was prepared using oligo dT primers, resulting in the identification of a single clone containing an 1800 bp insert. Sequencing of this clone revealed a 1605 bp open reading frame encoding for a 535 amino acid protein, displaying a 77% amino acid homology to human HPSE.

While identifying and cloning the human and mouse HPSE proteins, Hulett et al. (1999) also attempted to clone HPSE from rat [6]. Using first-strand DNA from the highly metastatic rat mammary adenocarcinoma cell line MAT 13762, PCR amplification identified a cDNA fragment encoding for the same 386 amino acid C-terminal sequence of the protein identified in mouse HPSE. As expected, the rat HPSE sequence did not exhibit activity upon transfection into COS-7 cells (Table 7.1). To date, genes from 97 different species have been annotated as *HPSE* orthologues [23].

7.2.4 Cloning of Heparanase for In Vitro Analysis

The identification of a suitable expression system to produce functional recombinant HPSE has long been a challenge due to necessary post-translational modifications required for generating the active enzyme, including the proteolytic processing of the immature peptide as well as glycosylation required to facilitate the efficient secretion into the extracellular space [14, 16, 24–26]. Two approaches have been implemented to generate active recombinant heparanase. The first approach focused on expressing the 8 kDa and 50 kDa domains in bacteria [27, 28]. However, the putative Asn residues were unable to be glycosylated, which could explain why the 50 kDa domain was commonly found in inclusion bodies [27, 29]. The second approach utilized an insect cell expression system, allowing for glycosylation and increased solubility [29, 30].

Bacterial Expression Systems

Winkler et al. (2014) attempted to express the heterodimer peptides in separate vectors in *E. coli* BL21 (DE3)plys*S* cells [28]. The lysates were then mixed, revealing an increase in degraded HS chains, indicating the formation of active HPSE (Fig. 7.5). To further investigate the ability of this method to produce functional recombinant HPSE, both domains were purified using the hexa-histidine incorporated in the cloning and metal affinity chromatography. Mass spectrometry analysis revealed protein masses consistent with the expected mass of the domains. This mixture was unable to degrade HS, indicating that the cellular context and content, even upon cell lysis, was important for the 8 kDa and 50 kDa domains to adopt the active HPSE structure. Nevertheless, yields of approximately 20% and 15% for the purification of the 8 kDa and 50 kDa domains, respectively, were achieved, indicating the utility of this system for efficient purification of these domains.

Similarly, Pennacchio et al. (2017) expressed the 8 kDa and 50 kDa domains from separate vectors while incorporating a hexa-histidine onto the 50 kDa domain. Both plasmids were expressed separately in BL21 (DE3) *E. coli* cells and upon native lysis, approximately 85% of the 8 kDa domain was found in the soluble fraction, while most of the 50 kDa domain was insoluble. Despite this, the soluble fractions of both lysates were combined, and protein purified utilizing a heparin-sepharose column, followed by size exclusion chromatography. Mass spectrometry of the purified protein revealed molecular masses consistent with that of the 8 kDa and 50 kDa domains and activity was confirmed by assessing its ability to degrade HS [27].

Insect Cell Expression Systems

Expression of human HPSE in insect cells facilitates the post-translational processing and modification of recombinant HPSE. Accordingly, McKenzie et al. (2003) cloned the human 8 kDa and 50 kDa HPSE domains into a single expression vector, incorporating a GP67 secretory sequence to facilitate efficient purification, and created baculovirus containing these sequences in Sf9 insect cells [29]. The virus particles were then used to infect Tni cells to stably express the domains and the enzyme purified using a heparin-sepharose column. Yields of approximately 1 mg/L at >90% purity was achieved, and the enzyme was confirmed to be active.

The use of insect cells to produce recombinant HPSE has been utilized to significantly advance the understanding of HPSE, with this approach used to generate protein crystals to determine the structure of the human enzyme to 1.75 Å in 2015 [30] (Fig. 7.6). In this study, cDNA encoding the 8 kDa and the 50 kDa domains were cloned into a single bacmid under the control of separate viral promoters. Mellatin signal peptide was incorporated into the N-terminus of each domain, and baculovirus produced in Sf21 insect cells. Tni cells were infected and the secreted HPSE purified utilizing a 3-step protocol. While this protein was used to solve the structure of human HPSE for the first time (for more information, see chapter by Wu & Davies, Chap. 5; in this volume), no indication of yield, purity or activity was provided.

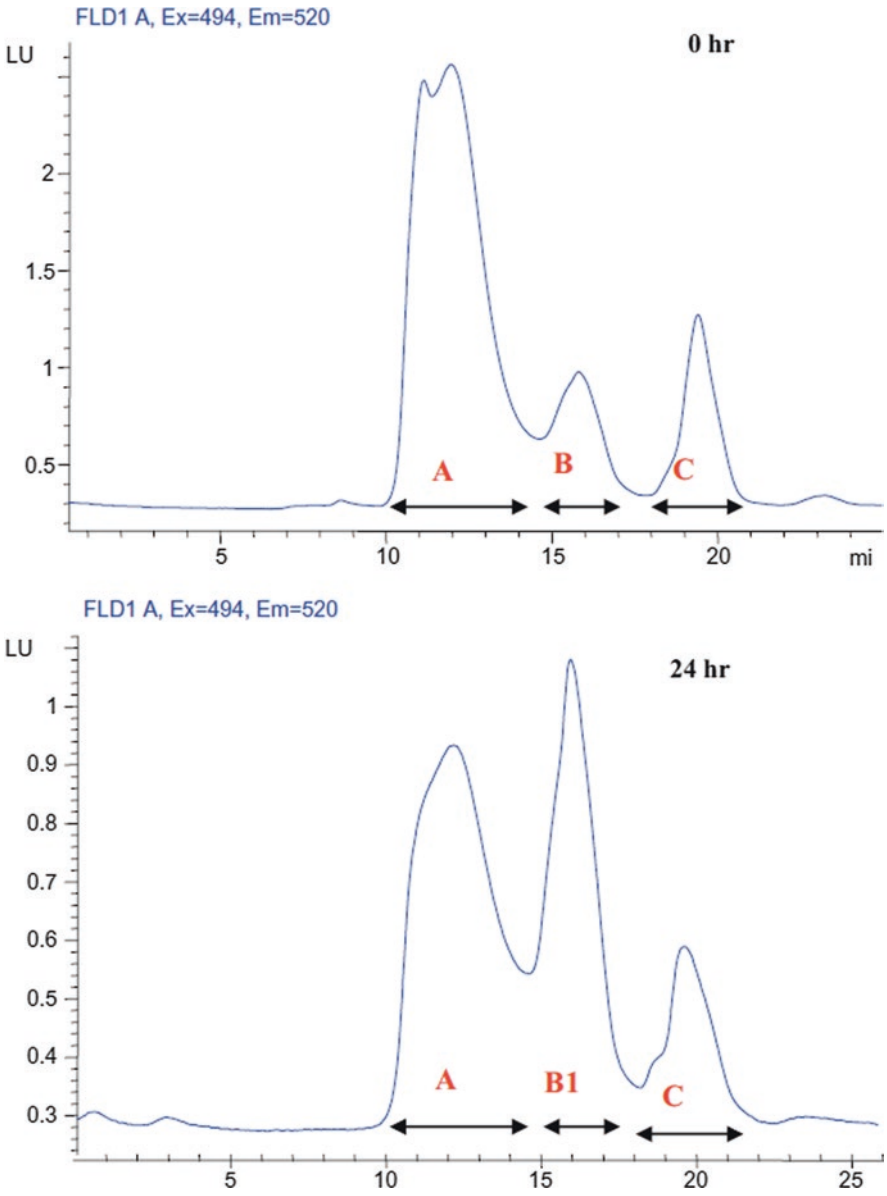


Fig. 7.5 Activity assay of the two recombinant HPSE subunits from mixed bacterial lysates: (0sh) Peak A represents the fluorescence of intact FITC-labelled heparan sulfate. Peaks B and C represent the fluorescent materials from the bacterial lysates; the control profile is not shown here. (24sh) Peak B1 contains the degraded amount of FITC heparan sulfate. Comparing the 0sh and 24sh of digestion, the peak of intact FITC-HS (**A**) decreases and the peak containing degraded heparan sulfate (**B1**) increases. Adapted from [28]

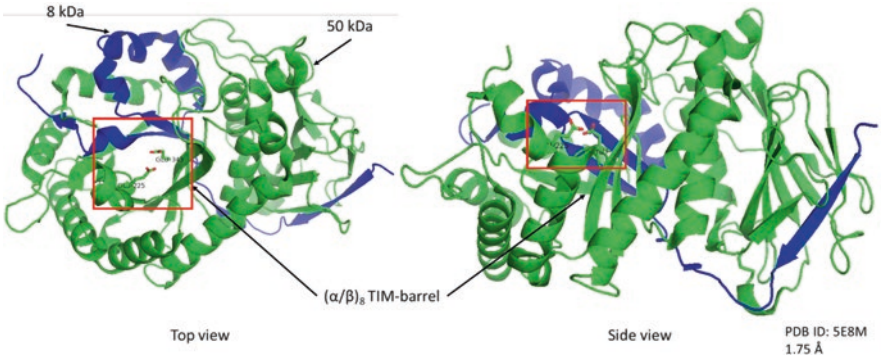


Fig. 7.6 HPSE adopts a (α/β)8 TIM-barrel conformation: The active HPSE enzyme consists of the 8 kDa and 50 kDa domain that form a $(\alpha/\beta)_8$ TIM-barrel structure, where the active site glutamate residues reside at the top of the barrel. The 8 kDa domain contributes two β-sheets, while the 50 kDa domain contributes six β-sheets. PDB ID: 5E8M [30]

7.3 Function

The role of HPSE is multi-faceted, performing a number of different functions both intra- and extracellularly. Various functions of HPSE require enzymatic activity, while others can be performed independent of this activity.

7.3.1 Heparan Sulfate Proteoglycans as HPSE Targets

The enzymatic activity of HPSE is localized predominantly to the extracellular space, where HS chains are ubiquitously found [31]. The ECM is a collection of secreted macromolecules, which form a three-dimensional lattice-like network implicated in biomechanical (e.g., providing structural scaffolding and a compression buffer to the tissue) and biochemical (e.g. regulation of cellular processes such as cell growth, migration, differentiation, and homeostasis) functions [32]. Approximately 300 proteins contribute to the composition of the ECM, with the majority of these being collagens, elastin, fibronectin, laminins, and glycosaminoglycans. Thirty-six of these constituents are proteoglycans, including heparan sulfate proteoglycans (HSPG) [33]. HS chains are linked to a protein core in the Golgi apparatus through a galactose-xylose linker, covalently bound to a serine residue in the protein core [34]. Generally, HSPGs contain between one and four chains of HS, consisting of a repeating glucuronic/iduronic acid and glucosamine disaccharide unit, which range from 40–300 residues in length (Fig. 7.7).

The glucuronic/iduronic acid and glucosamine disaccharide units share a 1 → 4 bond and allow for *N*- and *O*-sulfation and acetylation of the residues, resulting in

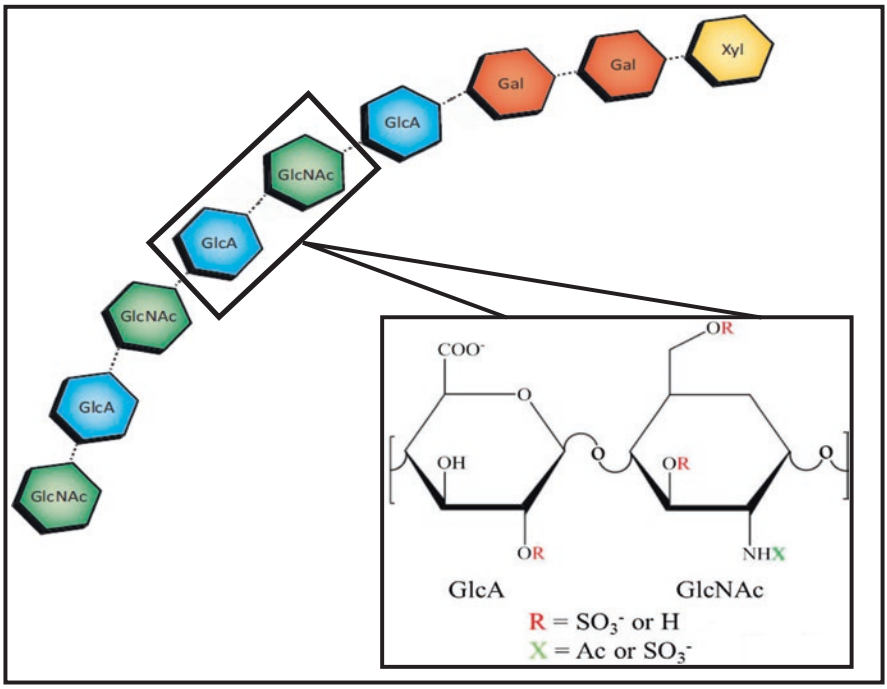


Fig. 7.7 Schematic representation of heparan sulfate chain structure: The repeating glucuronic/iduronic acid and glucosamine disaccharide units are bound to the protein core via a xylose-galactose linker and a serine residue in the protein core. Each of the saccharide units can undergo sulfation and/or acetylation. GlcAs=sglucuronic acid, GlcNs=sglucosamine, Acs=sacetylated. Adapted from [35, 36]

HS chains being amongst the most highly negatively charged biopolymers. Diversity in the structure and anionic character is provided by sulfation and acetylation of the HS chains [37, 38]. This diversity allows HS chains to act as an ECM reservoir by binding to approximately 300 different proteins, including growth factors, cytokines, and enzymes [39].

Extensive analysis of the HS chain structure revealed that HPSE recognizes inherent chain modifications for cleavage [18, 40, 41]. HS polysaccharides require a minimum -GlcN-GlcA-GlcN- trisaccharide in a highly sulfated region for HPSE recognition, with cleavage occurring between the GlcA and the GlcN carrying a 6-*O*-sulfo group [41, 42]. Conjecture surrounds the importance of 3-*O*-sulfation of the GlcN for HPSE activity [41, 42]. The sequence -GlcA-GlcNS- is also able to be cleaved by HPSE, reliant on the proximal presence of -GlcA2S-GlcN- [38]. Carboxyl-reduced HS is not a HPSE substrate, while the presence of 2-*O*-sulfated uronic acids is preferred for cleavage [18, 42, 43]. The recognition requirements for HPSE cleavage of HS result in the formation of ~5–12 kDa products (see also the chapter presented by Wu and Davies, Chap. 5; in this volume).

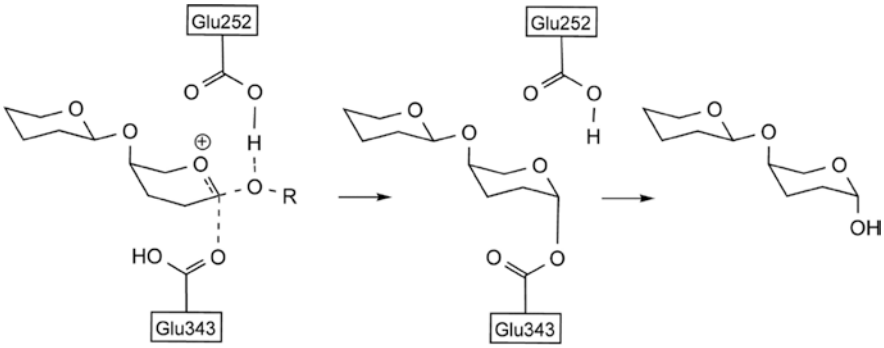


Fig. 7.8 HPSE cleaves HS using a hydrolytic mechanism: Glu^{225} donates a proton to the exocyclic oxygen within the glycosyl link, activating it as a leaving group. Glu^{343} then acts as a nucleophile by donating an electron pair, completing the cleavage of HS. Adapted from [46]

The HPSE active site contains two key residues; Glu^{225} which acts as a proton donor, and Glu^{343} which acts as a nucleophile [44]. Structural analysis reveals that these residues are located at the top of the TIM-barrel domain, allowing them to readily participate in HS cleavage [30, 45]. The cleavage of HS chains occurs via hydrolysis in a cleft that allows for flexibility in relation to configurational variations (Fig. 7.8).

The degradation of HS by HPSE not only begins to remove the physical barrier preventing cell invasion but also releases various proteins that bind to HS, promoting activation of cellular signaling pathways and responses. Interestingly, the protection of HSPGs from degradation by the intracellular scaffolding protein syntenin-1 is also able to promote these signaling events [47]. Seemingly counter-intuitive given that HS degradation by HPSE increases cell signaling, syntenin-1 is proposed to facilitate the recycling of HSPGs through trafficking to the cell surface or facilitating their inclusion in exosomes, resulting in increased receptor signaling activation [47].

Although not the focus of this chapter, it should be acknowledged that HPSE2 has been identified as a homolog of HPSE. HPSE2 is 592 amino acids in length, and the two proteins share 47% amino acid identity [9]. HPSE2 can bind to heparin and heparan sulfate but lacks the heparan sulfate cleavage ability [48] (Mckenzie; Roberts and Woolf, Chaps. 34 and 55; in this volume).

7.3.2 Regulation of Syndecan Function by HPSE

Exosomes are extracellular vesicles between 40 and 100 nm in diameter released from the endosome of cells [49]. Exosomes can carry payloads of cytoplasmic and membrane components, including DNA, proteins, enzymes, mRNA, miRNA, lipids and activated receptors [50–54]. Recipient cells endocytose the exosome, releasing their contents and therefore playing a role in cell-cell communication. A role for HPSE in exosome activities has been identified through its regulation of HS cleav-

age. Syndecans are proteoglycans with sulfated GAGs of either HS or chondroitin sulfate and have been strongly implicated in the formation of exosomes [55–57]. Binding of proteins to the HS chains on multiple syndecan HSPGs causes clustering of syndecans, resulting in the recruitment of adaptor proteins to facilitate intraluminal budding and formation of exosomes [55]. The enzymatic activity of HPSE results in cleavage of syndecan-1 HS chains, allowing clustering of syndecan-1, causing an increase in the production of exosomes [55, 57]. Increased HPSE expression also results in changes in the composition of exosomes from cancer cells [57] (David and Zimmermann, Chap. 10 in this volume).

The interplay between HPSE and syndecan-1 extends beyond the formation of exosomes. Increased HPSE expression results in an ERK signaling-dependent increase in MMP-9 expression; a protease able to cleave syndecan-1 from the cell surface [58–60]. Released syndecan-1 can be transported to the nucleus where the bound HS chains in conjunction with HPSE trafficked into the nucleus can influence numerous events, including the promotion of mitotic spindle formation and subsequent chromosome stability, inhibition of DNA topoisomerase I activity, and regulation of cell proliferation [58, 61–67]. In addition to these roles, HS has been shown to inhibit histone acetyltransferase (HAT) in a chain length and sulfation pattern dependent manner, resulting in decreased gene expression from the associated chromatin [68, 69]. Despite the optimal pH for HPSE activity being 5.5–6, HPSE can cleave the HS sidechains of syndecan-1 in the nucleus. High levels of HPSE causes low levels of nuclear syndecan-1 and HS, resulting in increased HAT activity [70, 71]. This increase in HAT activity causes an increase in tumor-proliferative genes including *VEGF*, *HIF1*, *HGF*, *VIM*, and *TGF*-β, while repressing expression of the major cellular iron transporter, *hepcidin*, indicative that HPSE can be a potential master regulator of pro-tumourigenic genes [72–75]. HPSE may also regulate gene expression by binding directly to gene promoters, with evidence of promiscuous binding to DNA independent of its enzymatic activity [58].

7.3.3 HPSE in the Immune System

As previously mentioned, several different immune cell types express HPSE. The role of HPSE in these cells is multi-faceted, with effects on immune cell gene regulation, differentiation, and migration/invasion of the cells. HPSE enters the nucleus of activated T lymphocytes, regulates histone methylation, and co-localises with RNA polymerase II at promoters of actively transcribed immune genes, including *CD69*, *IFNγ*, *IL-2* and *TNFα* [76, 77]. The nuclear localization of HPSE was found to be dependent on HSP90 activity and resulted in differentiation of leukemic cells *in vitro* [78]. HPSE is also vital for macrophage activation and function [79–81]. Incubation of peripheral mononuclear blood cells and splenocytes with purified HPSE resulted in increased release of proinflammatory cytokines, including IL-8, IL-10, and TNF, with a significant role for TLR4 identified in this effect [81]. This effect relied on HPSE enzymatic activity, as treatment with HPSE inhibitors prevented increased cytokine release, while addition of HS fragments to the system

drove cytokine release. These results were in contrast to previous findings by Blich et al., who discovered that addition of HPSE to macrophages was sufficient for their activation and increased cytokine release, yet their findings showed that the response by macrophages was independent of the enzymatic activity [80, 81]. Regarding function, macrophages from WT and HPSE-KO mice have been assessed for their ability to express cytokines and their effect on tumor cells. The WT macrophages exhibited higher cytokine expression than the HPSE-KO macrophages, while the knockout macrophages remain in the periphery of tumors in HPSE-KO mice due to their ability to cleave HS and invade the tumor microenvironment [79]. This results in tumor growth being unaffected by exposure to HPSE-KO macrophages, in contrast to the inhibitory effect on tumor growth by WT macrophages [79]. Dendritic cells (DCs), a key cell type in initiating innate and adaptive immune responses, possess migratory abilities to move between the various tissues to lymphatic vessels. Expression of HPSE by these cells is vital for their ability to migrate, with an absence of *HPSE* resulting in decreased migration of DCs from the skin to lymph nodes compared cells expressing *HPSE* [82]. Furthermore, HPSE is strongly implicated in the invasion/migration function of natural killer (NK) cells [83], Expression of HPSE is low, but is increased upon NK cell activation, facilitating NK cell invasion into tumors and tumor surveillance. Abolishment of HPSE in NK cells does not cause a difference in proliferation, survival, cytokine release, or degranulation of the cells, but does result in impaired migration/invasion, and inability to control metastases [83]. As seen here, the loss of HPSE activity results in the inability of various immune cells to maintain proper function. Further to the examples presented here, HPSE is vital for macrophage activation and polarisation, neutrophil granulocyte function, activity of T lymphocytes, and leukocyte recruitment to sites of inflammation, to name a few [79, 84–88]. Loss of HPSE function for these cells results in decreased ability for immune surveillance, inability to elicit an inflammatory response, and the overall depletion of immune system defenses.

7.3.4 HPSE Function in Pathogenesis

The enzymatic activity of HPSE contributes to various pathologies, including cancer and inflammatory diseases, for which supporting evidence is provided below. Other pathologies to which HPSE contributes have been extensively reviewed elsewhere [85, 89–103].

HPSE research has predominantly focussed on its role in cancer. This is not surprising, given that it is implicated in at least 5 of the 10 hallmarks of cancer, and is up-regulated in 90% of all cancers [10, 104–114]. Pre-clinical and clinical data demonstrate that high HPSE expression correlates with increased tumor size, tumor progression, tumor cell metastasis, lack of cell heterogeneity and poor patient prognosis [112, 114–118]. In the extracellular environment, HPSE cleaves HS chains from HSPGs, resulting in ECM remodeling and release of HS-bound cytokines and

growth factors [56]. The effect of this HS degradation is paired; the destruction of the ECM provides passage for tumor cells to begin invasion/metastasis, while the release of the cytokines and growth factors facilitates up-regulated receptor signaling, driving angiogenesis and tumor growth. The ability for HPSE to induce metastasis is demonstrated by increasing HPSE activity, through either transfection or treatment with exogenous enzyme, to induce increased metastasis, while knockout of HPSE or inhibition of HPSE activity decreases the metastatic nature dramatically [113, 119–125]. Expression of HPSE drives upregulation of VEGF, HGF, and MMP-9, promoting aggressive tumour behaviour [59, 126–130].

High expression of HPSE in tumor cells is also responsible for an increased shedding of syndecan-1 [123, 129, 131]. These shed HPSGs allow the formation of activated signaling complexes at the cell surface, driving tumor development [132]. Increased HPSE expression also allows for enhanced cleavage of syndecan-1 HS side-chains, promoting the formation of exosomes [57]. High HPSE expressing cells have been shown to produce increased amounts of exosomes than low HPSE expressing cells, facilitating enhanced inter-cellular signalling [57]. HPSE present in these exosomes may provide a mechanism for the establishment of a niche where metastatic tumor cells colonize. Strong evidence supports a role for HPSE in chemoresistance, with HPSE expression increased in resistant cancers and the application of a HPSE inhibitor sufficient to attenuate resistance [133]. Finally, increased HPSE expression has also been implicated in promoting chemoresistance through exosome binding to the recipient cell surface and increased autophagy [133–136].

As described in Sect. 7.3.3, HPSE plays a vital role in the immune system function and inflammation. It is, therefore, no surprise that HPSE functions in many inflammation-related pathologies, including atherosclerosis and diabetes. Using a mouse model of atherosclerosis, inhibition of HPSE employing a selective inhibitor resulted in lowered blood pressure (both systolic and diastolic), a modest increase in kidney function, decreased HDL-cholesterol levels, a decrease in oxidative stress, and induced weight loss in mice [137]. This provides evidence for a multi-faceted role for HPSE in atherosclerosis and provides reasoning for further investigation into the role of HPSE in this context.

The role of HPSE and HS in diabetes is quite broad and context-dependent. Islet beta cells in the pancreas are protected from free-radicals by a HS barrier. Increased HPSE activity in the pancreas leads to the degradation of this protective barrier, resulting in increased islet beta cell death. This causes a decrease in insulin production, leading to the development of type I diabetes [77] (Simeonovic et al., Chap. 23 in this volume). In contrast, it has also been suggested that high expression of HPSE can inhibit the development of diabetes, while also inducing glucagon resistance [138] (Shang et al., Chap. 30 in this volume).

Given HPSE has multiple roles in numerous pathologies, it is an attractive target for inhibitor development. Accordingly, a series of HPSE inhibitors have been developed, with some of these being discussed in Sect. 7.4.7.

7.4 Regulation of Heparanase

Regulation of HPSE expression and activity is achieved at the gene, transcript and protein level through a variety of mechanisms. In this section, we will explore these mechanisms.

7.4.1 A Lack of Methylation at the HPSE Promoter Increases HPSE Expression

DNA methylation is the epigenetic process of enzymatically adding a methyl group to the cytosine rings of DNA. These modifications are dynamic and can be added or removed to drive or suppress gene expression based on intracellular and extracellular signaling. Methylation of the *HPSE* promoter was identified as a potential regulator of *HPSE* expression in 2003, with a CpG island immediately upstream of the translation initiation site found to be methylated in a glioma cell line lacking HPSE activity [139]. The promoter of *HPSE* was found to be methylated in cell lines with low expression levels, with inhibition and removal of DNA methylation in these cell lines resulting in an increase in *HPSE* expression and activity (Fig. 7.9) [139, 140]. Demethylation of the *HPSE* promoter results in a translational effect *in vivo*, with glioma cells treated with the demethylation agent colonizing the lung nearly six-fold better as a result of increased HPSE expression and activity in mice [140].

The role of methylation of the *HPSE* promoter has been well investigated in cancer models, with demethylation in prostate, breast, and bladder cancers resulting in increased *HPSE* expression, activity, and pro-invasive and metastatic characteristics [109, 141–143]. Interestingly, no difference in *HPSE* promoter methylation is observed between normal colon cells and colon carcinoma cells, indicating other factors play a role in regulating *HPSE* expression [144].

7.4.2 Regulation of HPSE Expression by Transcription Factors

Various transcription factors have been shown to regulate the expression of *HPSE* and will be discussed in this section.

The binding of specificity protein 1 (Sp1) transcription factor to promoter recognition sequences has been shown to both maintain basal expression and induce or inhibit expression of a large number of target genes [145–147]. HPSE has been demonstrated to be one of these target genes, with three recognition sequences identified in the *HPSE* promoter, and binding and activity of Sp1 at the *HPSE* promoter required for *HPSE* expression (Fig. 7.10) [148].

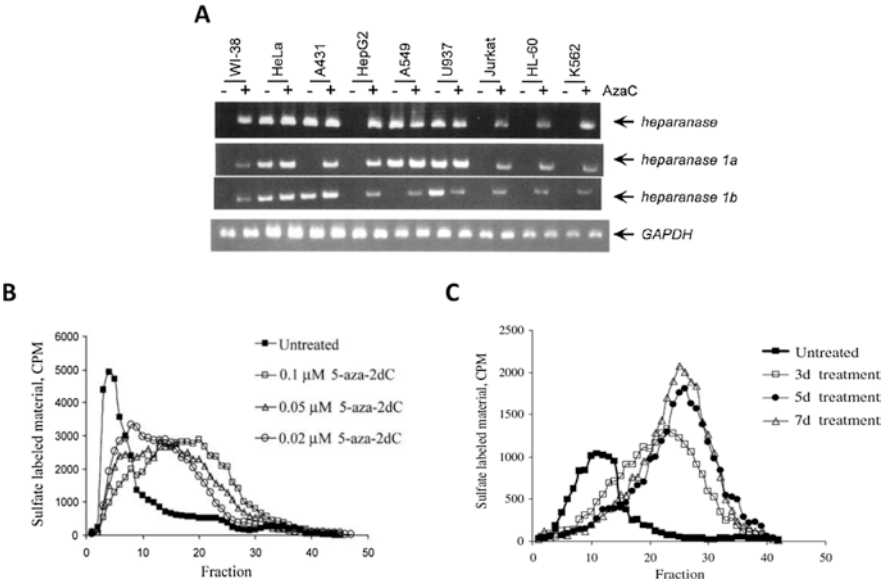


Fig. 7.9 Loss of *HPSE* methylation induces heparanase expression and activity: (**A**) Human tumor cell lines were treated with 10smM AzaC for 3sdays. The mRNAs were prepared from cells, and the relative levels of heparanase, heparanase 1a, heparanase 1b, and GAPDH mRNAs were analysed by semi-quantitative PCR. (**B**) Untreated human JAR cells (■) and cells treated with 0.02 (□), 0.05 (△), or 0.1 (○) mM 5-aza-2dC, were lysed and analysed for HPSE activity. (**C**) Untreated C-6 rat glioma cells (■); and C-6 glioma cells exposed to 0.2smM 5-aza-2dC for 3 (□), 5 (●), or 7 (△) days, were lysed and analysed for HPSE activity. Adapted from [139, 140]

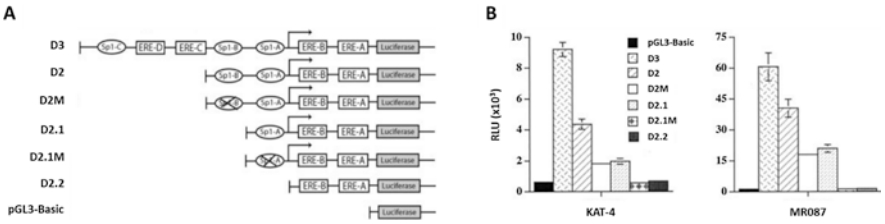


Fig. 7.10 Identification of putative Sp1 sites in the HPSE promoter required for the basal promoter activity of the *HPSE* gene: (**A**) schematic presentation of the luciferase reporter gene driven by the *HPSE* promoter containing mutated or truncated Sp1 sites. (**B**) Luciferase activity analysis of *HPSE* promoter activity in thyroid tumor cell lines KAT-4 and MR087. Adapted from [148]

As Sp1 sites are removed from the *HPSE* promoter sequence, promoter activation is decreased. Sp1 activity at the *HPSE* promoter has been shown to facilitate regulation of *HPSE* expression by various means, such as the binding of chemokine CCL19 to the CC chemokine receptor 7 (CCR7) [149]. The binding of CCL19 to CCR7 has been implicated in the invasive capacity of a variety of immune and tumor cells, including non-small cell lung cancer and oesophageal squamous cell

carcinoma, where overexpression of CCR7 correlates with increased metastasis, [150–153]. Incubating A549 squamous lung cancer cells with CCL19 resulted in an increase in HPSE mRNA and protein levels, while blocking CCR7 decreased HPSE expression [149]. Sp1 expression was also shown to be increased with CCL19 incubation and was found to be directly responsible for the increase in HPSE mRNA and protein expression upon exposure to CCL19. Another example is the regulation of *EGFR* expression, with binding of Sp1 to *EGFR* promoter recognition sites required for *EGFR* transcription [154]. EGF activation of the EGFR has been shown to create an environment conducive to tumor metastasis [155]. To facilitate this, it is not surprising that EGFR activation results in increased HPSE expression, promoting tumor metastasis [62].

Interestingly, Sp1 recognition sites are found within the promoter sequence for a variety of proteins which have been implicated in regulating *HPSE* expression, including EGFR, vitamin D, p53, and estrogen [156]. This may allow for rapid induction of *HPSE* via an initial increase in expression of a single transcription; Sp1. T-cell activation is required for differentiation and proliferation of naïve T-cells into cytotoxic CD8 T-cells and helper CD4 T-cells, facilitating normal immune system function. The ETS transcription factors are vital in differentiation of these types of T-cells and are responsible for the rapid induction of *Egr1* observed upon differentiation [157, 158]. T-cell activation has also been shown to induce *HPSE* expression, albeit in a more gradual and robust manner [95, 158]. Egr1 binds directly to a recognition sequence within the *HPSE* promoter, resulting in activation of *HPSE* expression in T-cells and tumor cell lines [109, 158, 159]. This was the first example of inducible *HPSE* expression. Interestingly, Egr1 was found to inhibit transcription of *HPSE* in melanoma cells, providing evidence that the role of Egr1 in regulation of *HPSE* expression is cell type dependent and more complex than initially thought [160]. In addition to the induction of *HPSE* expression by Egr1, ETS transcription factors ETS1 and ETS2 have also been demonstrated to bind to two functioning ETS binding sites in the *HPSE* promoter and strongly drive *HPSE* expression [161].

The vitamin D receptor (VDR) transcription factor binds 1,25-D3 (active vitamin D), interacts with retinoid X receptor (RXR) and subsequently binds to vitamin D response elements in DNA. Recently, two studies have identified an interaction between 1,25-D3 and HPSE expression in the renal system [162, 163]. Vitamin D has been shown to exert anti-proteinuric effects in experimental and human glomerular diseases. The development of proteinuria has been strongly attributed to the loss of HS in the glomerular filtration barrier and given that HPSE is the only human enzyme able to cleave HS, implies that HPSE is important in this pathology [164, 165]. Increased expression of HPSE has been observed in experimental and human glomerular diseases, with inhibition of HPSE resulting in decreased formation of proteinuria. This provides direct evidence for a role of HPSE in the development of proteinuria. Using rodent models, 1,25-D_3 has been shown to bind directly to the *HPSE* promoter, resulting in decreased HPSE transcript and protein levels (Fig. 7.11) [162]. This decrease in HPSE expression led to decreased transendothelial albumin passage, demonstrating a reduction in proteinuria formation. (Van der Vlag, et al., Chap. 26 in this volume).

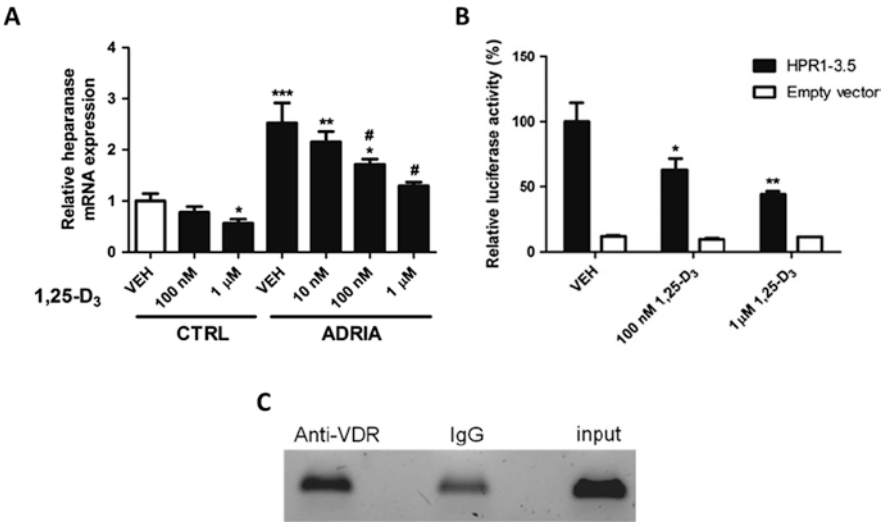


Fig. 7.11 Vitamin D receptor decreases *HPSE* expression by binding directly to the *HPSE* promoter: (**A**) *HPSE* mRNA levels in cultured mouse podocytes (CTRL) or stimulated with adriamycin (ADRIA, to induce a cellular FSGS model) in the absence or presence of 1,25-D_3 for 24sh. Statistical analysis conducted treatment VEH versus ADRIA. (**B**) Heparanase promoter activity determined by a luciferase reporter assay. Statistical analysis conducted treatment versus VEH (vehicle). *=<0.05, **=<0.01, and ***s=<0.001 versus VEH CTRL; #=<0.05 (**C**) ChIP using anti-VDR antibody compared with isotype control (IgG lane). Two percent of the chromatin used for the immunoprecipitation is shown (input lane). Adapted from [162]

Recently, the transcription factor SMAD4, a key protein in the TGF-β signaling pathway, was found to repress expression of *HPSE* by binding to the *HPSE* promoter region [166, 167]. In analyzing the *HPSE* promoter of neuroblastoma tissue, a potential SMAD4 binding site was identified approximately 2300 bp upstream of the *HPSE* transcription start site (Fig. 7.12A) [166]. Analysis of SMAD4 expression in normal dorsal ganglion (DG) and various neuroblastoma cell lines revealed an inverse correlation between SMAD4 and HPSE expression (Fig. 7.12B). Utilizing the two lowest SMAD4-expressing neuroblastoma cell lines, the effect of over-expression of SMAD4 on HPSE expression was explored. Over-expression of SMAD4 in these cell lines caused a decrease in HPSE expression when compared to the mock-transfected cells (Fig. 12C). shRNA knockdown of SMAD 4 confirmed a role for SMAD4 in regulation of HPSE expression, and the direct nature of this regulation was demonstrated using luciferase assays. An inverse relationship between SMAD4 and HPSE expression levels was also observed in gastric cancer cell lines compared to primary stomach epithelial cells [167]. ChIP analysis revealed enrichment around the SMAD4 binding site, confirming that SMAD4 is a direct negative regulator of *HPSE* expression.

The p53 transcription factor plays a vital role in the control of the cell cycle at the G1/S interface. It also plays an important role in apoptosis in response to severe

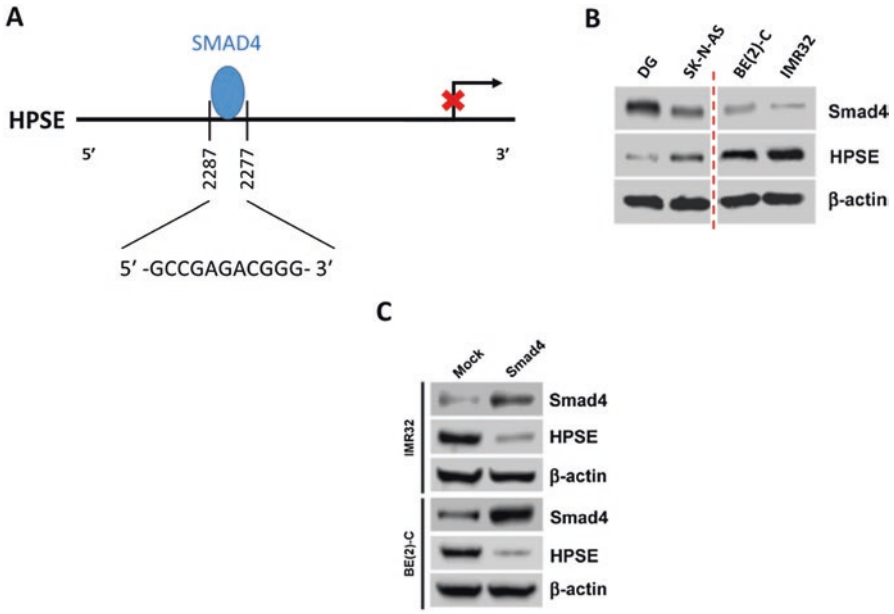


Fig. 7.12 SMAD4 potentially regulates expression of HPSE: (**A**) Scheme of the potential binding sites of Smad4 within HPSE promoter, locating at bases −2287/−2277 upstream the transcription start-site (arrow). (**B**) Western blot showing the expression levels of Smad4 and HPSE in normal dorsal ganglia (DG) and NB cell lines. (**C**) Western blot indicating the protein levels of Smad4 and HPSE in IMR32 and BE(2)-C cells stably transfected with empty vector (mock) or Smad4. Adapted from [166]

cellular DNA damage and is often mutated to provide cells with pro-oncogenic function. In 2006, HPSE transcript and activity were first observed to be increased in $p53^{-/}$ MEF cells, with this observation replicated in WT cells transfected with p53-targeting shRNA [168]. A non-functional conformational p53 mutant was created, rendering p53 protein functional at 32 °C and inactive at 37 °C. This mutant was shown to increase expression of *HPSE* in cultures grown at 37 °C, while p53 was shown to bind to the *HPSE* promoter between 2400 and 2700 bp upstream of the transcriptional start site (Fig. 7.13, HPSE-4). The observed inhibition of *HPSE* expression by p53 binding to the promoter also resulted in a decrease in HPSE activity, demonstrating that p53 is a potent regulator of *HPSE* expression and that loss of this protein alone is sufficient to increase *HPSE* transcript levels.

Estrogen signaling has been described as one of the key factors in the development of breast cancer, while HPSE has been shown to increase growth and invasion rates of breast cancer when over-expressed [14, 169, 170]. Estradiol, the predominant form of estrogen produced during reproductive years, has been demonstrated to be responsible for increasing *HPSE* expression in healthy endometrial and breast cancer cells [171–173]. Binding of estradiol to the estrogen receptor (ER) results in ER activation and relocation to the nucleus where it functions as a transcription factor [174]. The ER has been shown to bind to an ER recognition sequences

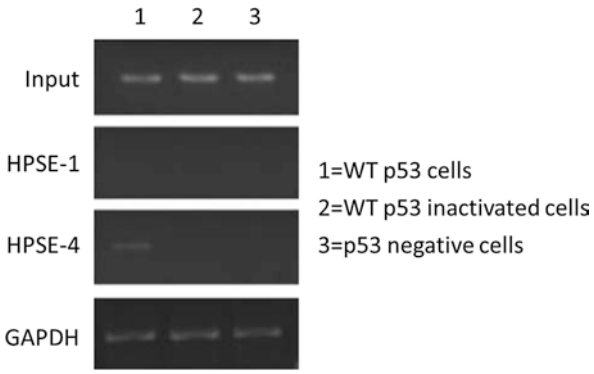


Fig. 7.13 p53 binds to the HPSE promoter: ChIP analysis was performed for WI-38/hTERT (WT p53), WI-38/hTERT/GSE56 (WT p53 inactivated), or H1299 (p53 negative) cells to demonstrate p53 binding to each of the heparanase promoter sites. Following crosslinking of proteins to DNA, DNA was fragmented, and the p53 protein was immunoprecipitated. PCR analysis was performed on the immunoprecipitated DNA samples using primers specific for regions of the heparanase promoter. Adapted from [168]

identified in the *HPSE* promoter and induce expression of *HPSE* at physiological levels of estradiol both in vitro and in vivo. This increase in *HPSE* expression results in increased cell surface HS degradation and tumor angiogenesis; typical of high HPSE expression. Tamoxifen (an antagonist of the ER) is the preferred treatment option for ER-positive breast cancer, yet treatment of breast tumor cells with tamoxifen still elicited an increase in HPSE expression, representing the first evidence of tamoxifen acting as an ER agonist [173]. Given the role of HPSE in tumor progression and metastasis, there appears to be a compromise in treating tumors with tamoxifen, with more attention to potential metastatic events required.

Hypoxia response pathways are activated under low oxygen conditions and have been shown to increase *HPSE* expression in the retina via NF-κβ signaling. This signaling has also been shown to induce tumor cell invasion in a HPSE-dependent manner. However, no definitive evidence has been provided for NF-κβ binding directly to the *HPSE* promoter and driving *HPSE* expression [60, 72, 175]. Contrary to this, NF-κβ localization to the nucleus has been shown to result in decreased *HPSE* expression in melanoma [58].

7.4.3 Regulation of Heparanase by MicroRNAs

MicroRNA (miRNA or miR) are short single-stranded non-coding RNA sequences 21–25 bp in length that regulate mRNA translation and mRNA stability predominantly via binding to the 3′ UTR [176]. Despite the short nature of the *HPSE* 3′ UTR (27 nucleotides), three miRs have been identified as regulators of HPSE expression [177].

The over-expression of miR-1258 in brain metastatic breast cancer (BMBC) cells results in a three-fold decrease in HPSE activity levels, while activity more than

doubles in cells treated with miR-1258 inhibitor [178]. Consequently, the over-expression of miR-1258 can decrease the number of brain metastases in HPSE-dependant manner, with invasive ductal carcinoma breast cancer patient samples revealing an inverse correlation of expression between miR-1258 and HPSE in both the primary tumor sections and metastatic lymph node sections. Patients with undetectable miR-1258 expression also experienced shorter postoperative survival than those with detectable expression, typical of patients with high HPSE expression [179, 180]. An inverse correlation was also observed in non-small cell lung cancer patient samples, and increased expression of miR-1258 in epithelial cell lines resulted in decreased HPSE expression and cell invasion [181]. It has also been suggested that HPSE expression is regulated by miR-1258 in gastric cancer cells [182].

Recently, miR-558 has been identified as a direct regulator of HPSE expression, functioning differently to other miRs [167, 183]. As mentioned earlier in this chapter, SMAD4 is a negative regulator of *HPSE* transcription, repressing LEF1-facilitated *HPSE* transcription [166]. Increased levels of HPSE mRNA, protein and degradative activity as a result of increased miR-558 expression have also been observed in neuroblastoma patient tissue and cell lines, and gastric cancer cell [167, 183] (Fig. 7.14A & B). Studies in gastric cancer cell lines have identified a miR-558 binding site adjacent to the SMAD4 binding site in the *HPSE* promoter, with over-expression of miR-558 resulting in decreased SMAD4 binding (Fig. 7.14C). Mechanistically, it is proposed that binding of miR-558 adjacent to the SMAD4 binding site in the *HPSE* promoter physically prevents SMAD4 binding, resulting in the increase of *HPSE* expression [167] (Fig. 7.14D). The effect of miR-558 on *HPSE* expression can be quenched by circHIPK3 expression, which contains two miR-558 binding sites [184].

Identified as a tumor suppressor gene in breast cancer, and correlating with decreased cisplatin sensitivity of endometrial endometrioid carcinoma, miR-429 is predicted to bind to the 3′ UTR of HPSE and decrease HPSE expression [185–187]. An inverse correlation between miR-429 and HPSE in gastric cancer tumor samples has been demonstrated, with expression of miR-429 observed to be low in tumors, compared to increased expression of *HPSE* [187]. The over-expression of miR-429 caused a decrease in HPSE mRNA and protein expression, while it also decreased the invasion capacity of gastric cancer cells, mirroring the effect of silencing HPSE mRNA [187].

A summary of the key transcription and miR mechanisms that regulate *HPSE* expression is presented in Fig. 7.15.

7.4.4 Regulation of Heparanase Activity by the Presence of the Linker Domain

Fairbanks et al. (1999) were the first to identify the 8 kDa domain in the active HPSE enzyme [17], leading to the proposed domain structure of the protein outlined in Fig. 7.4.

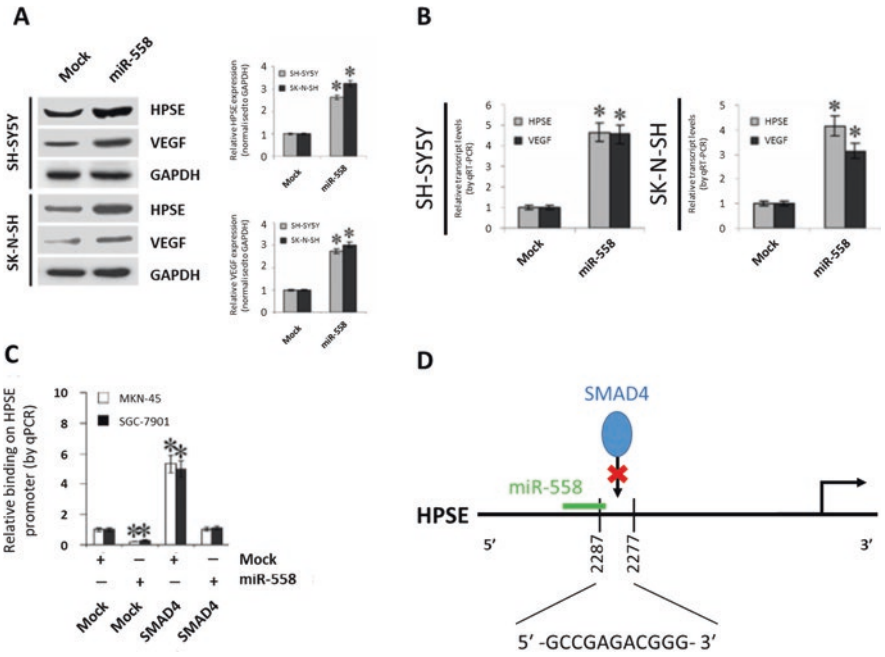


Fig. 7.14 Expression of miR-558 promotes HPSE expression by preventing SMAD4 binding to the *HPSE* promoter: (**A**) and (**B**) Western blot and real-time quantitative RT–PCR indicated that the stable transfection of the miR-558 precursor increased the HPSE and VEGF levels in SH-SY5Y and SK-N-SH neuroblastoma cells than those in mock cells (*P<0.01 versus mock). Adapted from [183]. (**C**) ChIP and qPCR assay showing the binding of SMAD4 to the HPSE promoter in MKN-45 and SGC-7901 gastric cancer cells, and those stably transfected with empty vector (mock), SMAD4 or miR-558 precursor. *p<0.01 versus mock. Adapted from [167]. (**D**) Schematic representation of the proposed mechanism for miR-558-driven expression of HPSE by inhibiting SMAD4 binding to the HPSE promoter

Proteolytic processing of the HPSE pre-pro form is required to form the active heterodimer, with the N-terminal signal peptide and 6 kDa linker domain not contributing to the active enzyme. HPSE is secreted from the cell initially as the inactive proenzyme (lacking the signal peptide domain), but upon binding to syndecan-1, LRP1 or the mannose-6-phosphate receptor, undergoes endocytosis into the cell for activation [188–190]. Cathepsin L was identified as responsible for the excision of the 6 kDa domain, with a bulky hydrophobic amino acid at position 156 found to be vital for the cleavage event at Gln^{157}-Lys^{158} [191, 192]. Structural determination revealed the 6 kDa domain resides in the substrate cleft of HPSE, blocking access of HS chains (Fig. 7.16). [207–210]. Cleavage of the 6 kDa domain was found to be confined to the lysosome and confirmed using multiple approaches, resulting in activation of HPSE [193]. Release of active HPSE from the cell is a tightly regulated process based on extracellular cues from molecules such as ATP and TNFα [194, 195].

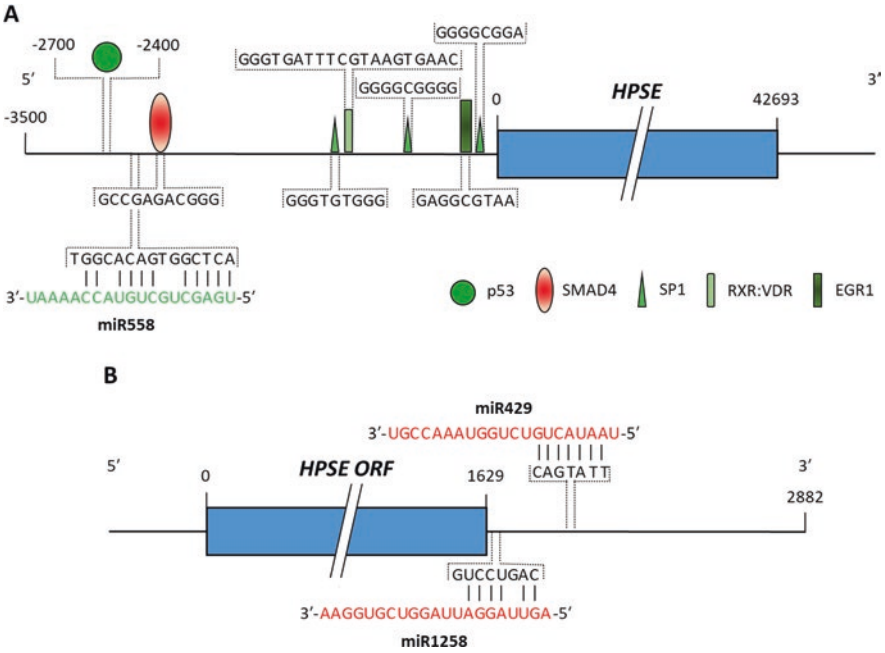


Fig. 7.15 Nucleotide level regulation of HPSE expression**: (A**) p53, SMAD4, SP1, RXR:VDR, and EGR1 are transcription factors that influence *HPSE* expression through the *HPSE* promoter region (up to 3.5 kb 5′ of the *HPSE* transcription start site), with miR558 also influencing *HPSE* expression through the promoter. (**B**) miR1258 and miR429 alter *HPSE* expression via the 3′ UTR. Green symbols and text indicate factors increasing *HPSE* expression, red symbols and text indicate factors inhibiting *HPSE* expression. Schematic is not to scale

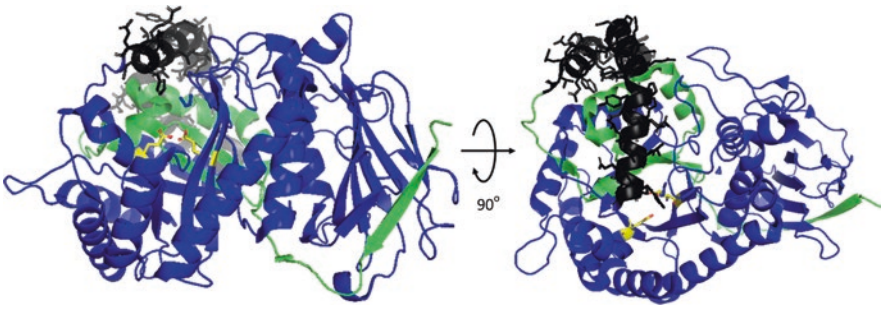


Fig. 7.16 Structural analysis of proHPSE reveals that the 6 kDa linker domain prevents binding to HS: The 6 kDa linker domain (black ribbon) resides in the substrate binding groove of proHPSE (green and blue ribbons, with cataytic Glu^{225} and Glu^{343} residues in yellow). This is proposed to inhibit binding of the HS substrate, inhibiting HS cleavage by the proenzyme. Adapted from [196]. PDB ID: 5LA4

7.4.5 Regulating HPSE Activity by HS Masking

Histidine-rich glycoprotein (HRG) is a multidomain protein and is one of the most abundant plasma proteins; found at concentrations of approximately 1.5μM. HRG is important in a large number of processes, including the formation of immune complexes, facilitating the removal of dying and dead cells, angiogenesis regulation, coagulation and cancer progression [197]. HRG binds to a variety of different molecules, including heparin, HS and HPSE. HRG can regulate the activity of HPSE by binding to HS and masking the HS cleavage sites, preventing the release of small HS fragments bound with growth factors and cytokines [198]. An interaction between HRG and HPSE has also been identified, with this interaction resulting in increased HPSE activity in neutral and acidic conditions [199]. Due to the abundance of HRG in the plasma and the ability for HRG to both inhibit and promote HPSE activity, HRG is thought to be an important regulator of HPSE activity [199].

7.4.6 The Effect of Small Biological Molecules on HPSE Expression

A number of non-protein biological molecules have been implicated in regulating HPSE expression, including reactive oxygen species (ROS), glucose, and fatty acids.

ROS were first identified to regulate expression of HPSE in 2006 during studies investigating the effect of proteinuria on renal damage [200]. As previously mentioned, the development of proteinuria is associated with the loss of HS attributed to the hydroxyl radical [201]. Kramer et al. (2006) investigated whether this loss of HS was a result of HPSE expression by treating rats with a hydroxyl radical scavenger. After 3sweeks of treatment, HPSE expression was decreased and HS loss was recovered [200]. Although a mechanism is not defined in this study, the evidence suggests that ROS can increase the expression of HPSE. The regulation of HPSE expression by ROS in the renal system was further proposed by Hoven et al. (2009) [202]. While ROS lack the functionality to exhibit a direct effect on the *HPSE* promoter, they have been shown to mediate an increase in HPSE expression during hyperglycaemic conditions [203].

For over a decade, the role of HPSE in diabetes has been of interest, with increasing glucose levels leading to an increase in HPSE expression and activity [75, 204]. Studies have revealed that glucose regulation of HPSE expression causes altered cell signaling and intestinal epithelial barrier damage [205–211]. HPSE secretion has also been shown to be regulated by high glucose levels, and that insulin cooperates with glucose to increase HPSE expressions and secretion [209, 211].

Fatty acids have been implicated in increased HPSE expression, subcellular localization, and secretion from endothelial cells [195, 209, 212]. Analysis of the media from oxidized low-density lipid-treated endothelial cells revealed a two-fold

increase in HPSE expression compared to untreated cells [195]. Cell exposure to oleic acid also resulted in an increase in HPSE expression, with elevated transcript and protein expression observed in a dose- and time-dependent manner [195]. This regulation is likely to occur through the activity of the Sp1 transcription factor, at least in part, as mutation of the Sp1 binding site within the *HPSE* promoter results in decreased promoter activation [195].

7.4.7 Active Site Inhibitors of Heparanase

Given the importance of HPSE in cancer progression, tumor metastasis, and immune cell migration/invasion, there has been a focussed effort on developing inhibitors of HPSE. Heparin, produced in humans exclusively by mast cells, was the first identified inhibitor of HS degradation, resulting in complete inhibition of HPSE activity at 5 μg/mL [213–215]. The sulfation of the heparin is critical for HPSE inhibition, with no inhibition of HPSE activity observed in desulfated heparin [214]. Given that heparin polysaccharides are HPSE cleavage targets and the inability of other glycosaminoglycans to inhibit HPSE activity, heparin is hypothesized to bind to the active site and compete for substrate binding [216]. Although the potent anti-coagulant activity of native heparin prevents its use as HPSE inhibitor *in vivo*, it provides the framework for the development of HPSE inhibitors lacking the anti-coagulant activity of heparin. While numerous inhibitors of HPSE have been developed in recent times, this section will focus on the most well-characterized molecules.

Therapeutic regulation of HPSE activity is an area of ongoing, competitive, and constantly evolving research [217]. As inhibition of HPSE is not cytotoxic, HPSE inhibitors would be utilized in the clinic in combination therapies to slow tumor progression and metastasis. Several compounds have been developed as HPSE inhibitors, including symmetrical benzazolyl derivatives [218, 219], triazolothiadiazoles [220], arylamidonaphtalene sulfonate analogs [221], sulf-2-endosulfatase inhibitor OKN-007 [222], various polymers of HS [223, 224], and suramin [225–228]. A recent report also suggests the regulation of HPSE activity by aspirin [229].

The HPSE inhibitors that have entered clinical trials have all been heparin derivatives, namely PI-88, PG545, and SST0001. PI-88 is a complex mixture of highly sulfonated oligosaccharides, SST001 is a mixture *N*-acetyl-reduced oxyheparins with 100% N-acetylation and 25% glycol split, while PG545 is single molecular identity composed of a fully sulfated tetrasaccharide with a cholestanyl aglycon [230–232]. In addition to inhibiting HPSE enzymatic activity, these compounds also compete with HS for binding of VEGF and FGF, resulting in a decrease in angiogenesis, while presenting with only minor anti-coagulation effects and resulting in a decrease in metastatic events. Complementing these features, PG545 has been shown to induce apoptosis in HPSE-independent manner [217]. SST0001 has also been shown to function by disrupting the HPSE/syndecan-1 axis [123]. PI-88 is the most successful HPSE inhibitor clinically tested to date, with the first phase I

studies published in 2002 [233]. The inhibitor mixture has twice entered phase III clinical trials in hepatocellular carcinoma patients (2008 [NCT00568308] and 2015 [NCT01402908]), but these trials had to be terminated early due to significant side-effects, most notably immune-related thrombocytopenia [233]. PG545 has been studied in two phase I clinical trials since 2011 ([NCT01252095], 2016 [NCT02042781]), and is currently recruiting for a third phase I study. Concerns over the emergence of unexpected site reactions caused the early termination of the trial in 2011 while undertaking multiple exposure studies (observed at both 25smg/week and 50smg/week for 7sweeks). To date, SST0001 has completed one phase I study in multiple myeloma patients in 2016, yet no results of this study have been published (NCT01764880) (Chhabra and Ferro; Hammond and Dredge; Giannini et al., and Noseda and Barbieri; Chap. 19, 21, 22, and 23 in this volume).

Although significant progress has been made in the past two decades into understanding heparanase biology, there is still much to be learned. More information into the structure, function, and regulation of heparanase will allow the development of new strategies for inhibition, and ultimately, the design of better therapeutic opportunities.

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