Review

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GPI-anchored serine proteases: essential roles in development, homeostasis, and disease

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Abstract: The glycosylphosphatidylinositol (GPI)-anchored serine proteases, prostasin and testisin, have essential roles in diverse physiological functions including development, reproduction, homeostasis and barrier function of epithelia, angiogenesis, coagulation, and fibrinolysis. Important functions in pathological conditions such as cancer, kidney disease and cardiovascular disease have also been reported. In this review, we summarize current knowledge of the cellular and *in vivo* roles of prostasin and testisin in physiology and pathophysiology and explore the underlying molecular mechanisms. We discuss how new insights of their role in cancer and cardiovascular disease may facilitate translation into clinical settings in the future.

Keywords: prostasin; testisin; GPI-anchored serine proteases

1 Introduction: GPI-anchored serine proteases – discovery, expression, and biochemical characteristics

1.1 Prostasin

Prostasin (also known as channel-activating protease 1, CAP1 and PRSS8) was first isolated in 1994 from human seminal fluid and described as a serine protease originating from the prostate gland with trypsin-like substrate specificity and inhibited by aprotinin, antipain, leupeptin, and benzamidine (Yu et al. 1994; 1996). Prostasin protein was also detected in urine, kidney, colon, lung, pancreas, salivary gland, liver, and bronchi, but not in the brain, muscle, testis, ventricle, atrium, and aorta (Yu et al. 1994). Later studies have described prostasin expression in additional tissues, predominantly in epithelial cells, including the epidermis of the skin, hair follicles, oral cavity, esophagus, trachea, ureter, bladder, placenta, and endometrium with similar expression patterns between humans and mice (Chang et al. 2020; Chen et al. 2006; Lai et al. 2016a; Lee et al. 2018; List et al. 2007; Ma et al. 2009; Vallet et al. 1997; Yu et al. 1995). Prostasin was cloned from the human prostate and *Xenopus laevis* A6 cells and also acquired its alternative name: channel-activating protease 1 (CAP1), because it was the first of several membrane serine peptidases found to activate the epithelial sodium channel ENaC (Vallet et al. 1997; Yu et al. 1995). It was later demonstrated that prostasin is glycosylphosphatidylinositol (GPI)-anchored to the cell surface and released through cleavage by phospholipase C (Chen et al. 2001b; Vallet et al. 1997). The crystal structure of the protease domain of prostasin is similar to other serine proteases, consisting of two β barrel-like subdomains with four conserved disulfide bonds (Rickert et al. 2008). Endogenous mouse prostasin (encoded by *Prss8*) is constitutively secreted from the apical surface of kidney cortical collecting duct cells and it was reported that secretion depends on GPI anchor cleavage by endogenous GPI-specific phospholipase D1 (Gpld1) (Verghese et al. 2006). Prostasin is produced as a zymogen with an N-terminal signal peptide, an amino acid pro-domain followed by a catalytic domain, and a C-terminal GPI-attachment signal (Rickert et al. 2008; Shipway et al. 2004; Verghese et al. 2006; Yu et al. 1996). Activation of the human zymogen occurs by endoproteolytic cleavage within the amino acid sequence QPR^{44}-ITG to generate a light chain and a heavy chain, that are covalently linked with a disulfide bond (Chen et al. 2010a; Friis et al. 2013; Rickert et al. 2008; Shipway et al. 2004; Yu et al. 1995). It was reported that the zymogen possesses low proteolytic activity that is capable of activating zymogen matriptase, but unable to activate its own zymogen form

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(Holt-Danborg et al. 2020). Proteases that can cleave and activate the prostasin zymogen include the type II transmembrane serine proteases hepsin, matriptase, and TMPRSS13 (Chen et al. 2010a; Murray et al. 2020; Netzel-Arnett et al. 2006). Prostasin is found in seminal fluid in a covalently linked complex with protease nexin-1 (PN-1), a serine protease inhibitor belonging to the serpin superfamily (Chen et al. 2004). PN-1 inhibits proteolytic activity of prostasin, is expressed by prostate epithelial cells and prostate cancer cells and is capable of binding to membrane-anchored prostasin (Chen et al. 2001b; Chen et al. 2004). The Kunitz-type inhibitors hepatocyte growth activator inhibitors (HAI)-1 and 2 were also found to form stable non-covalent inhibitor complexes with prostasin and to be important for its regulation *in vivo* (Chang et al. 2020; Chen et al. 2010b; Fan et al. 2005; Huang et al. 2024; Lai et al. 2016b; Shiao et al. 2017; Szabo et al. 2012).

1.2 Testisin

Testisin (serine protease 21, PRSS21, ESP-1, TESP5) was first cloned and characterized as a GPI-anchored trypsin-like serine protease in the human cervical adenocarcinoma cell line HeLa S3 and in eosinophils (eosinophil serine protease 1, ESP-1) (Hooper et al. 1999; Inoue et al. 1998; Nakamura et al. 2003; Scarman et al. 2001). Testisin was found to be highly expressed in human, mouse, and rat testis (Nakamura et al. 2003; Scarman et al. 2001). Testisin protein was also described in testicular germ cells and in lipid rafts of cauda epididymal sperm and was named testicular serine protease 5 (TESP5) (Honda et al. 2002). The most used name, however, is testisin which is encoded by the *PRSS21* gene. Testisin protein expression in normal tissues is more restrictive compared to prostasin in both mice and human, being highly expressed in testis and moderately expressed in the prostate with lower transcript expression in lung, spleen, pancreas, and peripheral blood leukocytes (Hooper et al. 1999; Inoue et al. 1998; Nakamura et al. 2003; Scarman et al. 2001). Testisin mRNA is also expressed in microvascular endothelial cells (Aimes et al. 2003; Antalis et al. 2016; Peroutka et al. 2020). Importantly, testisin is not detected in normal ovarian tissue or fallopian tubes but is expressed in ovarian cancer, which makes testisin a promising candidate for targeted therapy in this cancer type (see Section 6). Testisin is synthesized as a 43-kDa zymogen composed of an N-terminal signal peptide and a single protease domain that is linked to a GPI anchor. The human zymogen is converted into its active form upon cleavage within ITSR^{41}-IVGG (Honda et al. 2002; Hooper et al. 2000; Inoue et al. 1998). The activation occurs in the epididymis during sperm transport (Honda et al. 2002). Testisin was not reported to auto-activate and the physiological protease(s) that induces testisin zymogen activation has not been identified. Furthermore, testisin was not found to be naturally shed from the plasma membrane (Honda et al. 2002). Protease activated receptor (PAR)-2 was identified in cell culture models as a proteolytic substrate of testisin. Co-expression of testisin and PAR-2 in HeLa cells showed GPI-anchored testisin-mediated PAR-2 cleavage, exposing a new N-terminal sequence that acts as a tethered agonist for intramolecular PAR-2 activation followed by PAR-2 internalization (Driesbaugh et al. 2015). Conversely, shRNA-mediated silencing of endogenous testisin in NCI/ADR-Res ovarian carcinoma cells reduced PAR-2 proteolytic cleavage (Driesbaugh et al. 2015). Sequence analysis showed that two isoforms of testisin are generated by alternative precursor mRNA splicing (Inoue et al. 1999a; Manton et al. 2005).

2 Role in reproduction and embryonic development

2.1 Testisin in spermatozoa function

In male fertility, seminal proteases are important for spermatozoa penetration of cervical mucus, facilitation of migration, and the penetration of the zona pellucida (ZP). In a 2009 study by Netzel-Arnett and colleagues, spermatozoa of testisin-deficient mice displayed decreased motility, angulated and curled tails, fragile necks, and increased susceptibility to decapitation (Netzel-Arnett et al. 2009) (Table 1 provides an overview of expression, physiological, and pathological characteristics of prostasin and testisin). Cauda epididymal spermatozoa also failed to mount a swelling response when exposed to hypotonic conditions, suggesting an impaired ability to respond to osmotic challenges in the female reproductive tract. Together, these abnormalities led to reduced fertilization capabilities *in vitro*. When bred by continuous mating, the testisin-deficient mice displayed apparently normal fertility; however, in short-term mating studies, the number of pregnancies per mating attempt was significantly reduced (Netzel-Arnett et al. 2009). In a study by Yamashita and colleagues, normal fertility of male mice with testisin-deficiency was reported, but epididymal sperm showed decreased ability to penetrate the ZP *in vitro* (Yamashita et al. 2008). This phenotype was rescued by exposure of the sperm to the uterine microenvironment by injecting capacitated sperm into the uterus of female mice and by *in vitro* treatment of the sperm with uterine fluids (Yamashita et al. 2008). Interestingly, mice with double deficiency in testisin and acrosin, a serine proteinase

present in the acrosome of mature spermatozoa, displayed a complete loss of fertilization ability *in vitro* with double mutant epididymal sperm being incapable of undergoing surface acrosomal exocytosis and traversing the ZP (Kawano et al. 2010). In this model, the double mutant epididymal sperm were also capable of fertilizing the oocytes in the presence of uterine fluids *in vitro*. Males were sub-fertile *in vivo*, but still displayed the ability to impregnate females (Kawano et al. 2010). Based on these observations, it is plausible that the female reproductive tract partially rescued the loss of function, by providing one or more additional, yet unidentified, protease(s). Both testisin and PAR-2 are expressed in the head, midpiece, and tail regions of human spermatozoa (Netzel-Arnett et al. 2009; Swegen et al. 2019; Weidinger et al. 2003). Whether testisin functions as a physiological activator of PAR-2 in sperm is not known, but PAR-2 activation has previously been associated with the regulation of sperm motility following activation by tryptase (Weidinger et al. 2003). In equine spermatozoa, testisin formed several multiprotein complexes with a multitude of ZP-binding proteins identified by mass spectrometry, including zona pellucida binding protein, zonadhesin, acrosin, several heat-shock proteins, and components of the chaperonin-containing TCP1 complex (Swegen et al. 2019). In a 2024 study, the spermatid maturation (SPEM) family member 2, a testis-enriched gene, was found to be essential for spermiogenesis and male fertility. Male *Spem2* null mice developed infertility associated with abnormal acrosome development, cytoplasm removal, and sperm individualization (Li et al. 2024). Functionally, SPEM2 and testisin were linked because decreased testisin protein levels were observed in *Spem2* null sperm and co-immunoprecipitation analysis in cultured cells indicated an associated between SPEM2 and testisin proteins. The mechanisms by which SPEM2 regulates spermiogenesis and how it may regulate testisin expression and/or functions are not yet known.

2.2 Prostasin in embryonic development

Prostasin, matriptase, and PAR-2 are co-expressed in the surface ectoderm during neural tube closure in early embryonic development at embryonic day (E) 8.5–9.5 (Camerer et al. 2010; Szabo et al. 2012). It was proposed that prostasin might be involved in developmental activation of PAR-2, an important contributor to neural tube closure (Camerer et al. 2010). While prostasin did not directly activate PAR-2 *in vitro*, matriptase efficiently activated PAR-2 in a prostasin-dependent manner (Camerer et al. 2010). In a comprehensive genetic epistasis analysis study in mice, a dependency on this prostasin-matriptase pathway for neural tube closure could not be demonstrated and it is possible that one or more alternative serine proteases are sufficient for PAR-2 activation to complete this developmental process (Szabo et al. 2012, 2016). It was also shown that complementary, yet independent roles of the prostasin-matriptase pathway versus the PAR-2-dependent proteolytic signaling pathway, are essential for placental epithelial barrier function and for embryonic survival (Szabo et al. 2014). Important functional roles for prostasin in mid-gestational embryonic development using loss-of-function mouse models were described independently by two groups. In a 2013 study, it was reported that loss of prostasin in the inbred C57BL/6J strain caused embryonic lethality with only 50 % of the *Prss8*$^{-/-}$ embryos alive at day E12.5 or E13.5, and no living embryos from day E14.5 onwards (Hummler et al. 2013). No defects in the *Prss8*$^{-/-}$ embryo proper were evident, but the placentas exhibited significantly reduced vascular development and incomplete maturation of the syncytium (Hummler et al. 2013). Prostasin mRNA expression in the placenta was detected at E11.5 reaching the highest levels at E13.5 and then declining through E16.5 (Hummler et al. 2013). To further examine the role of prostasin during development, mice carrying *loxP*-flanked (floxed) *Prss8* were used to generate epiblast-targeted conditional knockout mice. Floxed mice were crossed with transgenic mice carrying the Cre recombinase (Cre) under the control of the Sox2 promoter (Sox2-Cre transgenic mice) to ablate prostasin in the embryo proper, but not in the placenta. Conditional knockout embryos were morphologically indistinguishable from the controls and born at the expected Mendelian ratio, which substantiates the hypothesis that placental insufficiency is the primary cause of embryonic lethality in wholesale prostasin-deficient mice (Hummler et al. 2013). Szabo and colleagues conducted a littermate-controlled study using mice in a genetically mixed background and reported partial pre-natal lethality with 46 % of *Prss8*$^{-/-}$ pups developing to term (Szabo et al. 2016). Detailed analysis of prenatal survival revealed that all *Prss8*$^{-/-}$ embryos extracted before E12.5 were alive and appeared normal with a steep decline in viability at E13.5, which suggested that prostasin is specifically required for mid-gestational development (Szabo et al. 2016). No developmental abnormalities were identified in *Prss8*$^{-/-}$ embryos at E13.5 and E14.5 in accordance with (Hummler et al. 2013); however, a major difference noted was the lack of detectable defects in the overall histological structure or differentiation of the placentas. Prostasin protein expression was detected only in the labyrinth layer of the placenta by immunohistochemistry (IHC), and it is plausible that functional placental defects, that were not visible histologically, were causing the lethality. The manifestation of prostasin deficiency during embryogenesis

Table 1: Overview of prostasin and testisin function(s) in physiological and cancerous conditions.

		Cell culture/Ex vivo models	Animal models	Human/patient studies
Prostasin	Bladder	Expressed in epithelial cultured cells[1]	Expressed in epithelia[2, 3]; transgenic OE = iNOS↓[2]	Expressed in epithelia[1]
		Expression ↓; ♀♀♀[1]; RE = E-cad. ↑[1]	N/A	Pt. low = E-cad↓[1]
	Breast	Expressed in epithelial cultured cells[4, 5]	Expressed in epithelia[3]	Expressed in epithelia[5]
		Expression ↓; ♀♀♀[4]; RE = invasiveness ↓[4] Loss assoc. EMT ↑[4, 5]; OE = survival ↓[6]	N/A	Expressed in epithelia and corr. With matriptase expression[5]
	Bronchus and lung	Cond. KO primary cells = ENaC activity ↓[7] CF primary cells = expression ↑, sputum[8, 9]	Expressed in epithelia[3] Regulation of ENaC mediated lung fluid homeostasis[7]	Expressed in tissue[10, 11]; IPF = prostasin serum level ↑[12]; IPF lung = expression ↓[13]
		Expression↓[14]; OE=invasion, EMT ↓[14] JAK/STAT3 regulation[14]	OE graft = tumor volume ↓[14]	N/A
	Esophagus	N/A	Expressed in epithelia[3]	Expressed in epithelia[15]
		Expression ↓; ♀♀♀[15]; RE = migration, prolif. ↓[15] RE =Snail/Twist ↓, E-cad↑[15]; KD=migration, prolif. ↑[15]	N/A	Pt. low corr. grade↑ and survival [15]
	Gastric	N/A	Expressed in epithelia[3]	Expressed in epithelia[16]
		Expression↓; ♀♀♀[16]	N/A	Expression ↓[16]; Pt. low corr. Survival ↓[16]
	Intestine and colon	Barrier function regulation[17-19]; KD = Claudin-2 ↑[17, 19] *SPINT2* mutations = prostasin inhibition↓[20, 21]	Expressed in epithelia[3]; *Prss8* mutant = ENaC activity↓[22]; Cond. KO = inflammation severity↑[23]	Expressed in epithelia[11,12,24 25, 26]; Ulcerative colitis, Crohn's disease=expression↓[23, 27]
		RE = invasion, migration, prolif. ↓[24, 26] KD = stemness markers ↑[26]; Sphk1 signaling[24]	RE graft = tumor vol. ↓[24, 26]; KD graft = tumor size ↑[26] Cond. KO = hyperplasia, adenoma ↑[26]; Sphk1 signaling[24]	Expression ↓[24-26]; HAI-1 ↓/PN-1 ↑[25] Pt. low corr. Survival ↓[24]
	Kidney	Expressed in epithelial cultured cells [11, 28, 29]; non-proteolytic function[30, 31]; ENaC activation[28, 29, 32]; TGF-β1 mediated prostasin inhibition[33]; PN-1 mediated ENaC inhibition[34]	Expressed in epithelia[3]; aldosterone treatment = urinary prostasin, hypertension ↑[35]; BP and electrolyte balance[36]; renal homeostasis[31]; cond. KO = unchanged ENaC activation[37, 38]	Expressed in tissue[10, 11] Adrenalectomy = urinary prostasin, urinary Na/K ratios ↓[35]; ENaC activation marker[39] Urinary prostasin corr. plasma aldosterone[40]
	Liver	Expressed in epithelial cultured cells[41;] inflammatory response attenuation (TLR4)[41]	WT HFD = expression ↓[41] Cond. KO = TLR4 ↑, inflam., glucose resistance, insulin resistance ↑[41]; RE = insulin resistance ↓[41] Transgenic OE = glucose tolerance, insulin sensitivity ↑[42]	Expressed in hepatocytes[10, 11, 43, 44]
		OE = invasiveness ↓, apop.↑[43] KD=PTEN, BAX, E-cad↓[43], BCL-2, MMP9, N-cad↑[43]	OE graft = tumor growth ↓[43] KD graft = tumor growth ↑[43]	Expression ↓[43, 44] Pt. low corr. Survival ↓[43]
	Oral cavity	N/A	Expressed in epithelia[3]	Expressed in epithelia[45]
		OE = migration/prolif. ↓ [45]; KD = prolif.↑ [45]; HAI-2 mediated prostasin inhibition[46]	N/A	Expression ↓[45]; Pt. low corr. grade↑ and survival↓[45]
	Ovary	N/A	Not detected in follicles, epithelium, or oviducts[3]	Expressed in tissue[, 47-49]
		Expression↑[50-52]; KD = cell viability, colony formation, cell migration↓[52]; Cell survival regulation[50, 51]; SREBF2-mediated regulation[52]	SREBF-2 KD graft in nude mice = prostasin expression ↓, tumor growth ↓[52]	Expression ↑[47-49] Serum levels ↑[47, 49]
	Pancreas	Glucose mediated protein degradation[53] OE = insulin secretion ↑[53]; KD = insulin secretion ↓[53]	Expressed in islets, exocrine cells, acini[3, 53]; pancreatic *β*-cell KO = sustained ↑ glucose levels, insulin secretion↓ [53]	Expressed in tissue[10, 11]

Table 1: (continued)

		Cell culture/Ex vivo models	Animal models	Human/patient studies
	Placenta	N/A	Expressed in embryonic development[54-56]; KO = embryonic death[56, 57]; Non-proteolytic function[57]; HAI regulation[55, 58-60]	Expressed in trophoblasts[61]
	Prostate	Expressed in epithelial cultured cells [11, 62]	Expressed in epithelia[3]	Expressed in epithelia[10, 11, 62-65]
		Expression ↓[64]; ♀♀♀ [66]; RE = slug, E-cad↑[67], COX-2, EGFR, iNOS, EMT ↓[67]	N/A	Expression↓[62, 64, 65]; Pt. low corr. grade↑[64, 65]
	Skin	Expressed in epithelial cultured cells [68]	Expressed in epithelia[3]; barrier function[69]; perinatal development[56,70]; hair formation/ growth[22, 71, 72]; Transgenic OE=hyperplasia, ichthyosis, inflam. ↑[73]; Proteolytic[74] and non-proteolytic functions[75-78]	Expressed in epithelia[68, 79, 80] *PRSS8* mutations in Pt. with autosomal recessive icthyosis[81]
	Vascular	N/A	Protease inhibitor treatment = BP ↓, urinary Na/K ratio ↑[82]	Gene variants assoc. hypertension ↑[83, 84]
Testisin	Cervix	Expression ↑[85]; maspin interaction[86]; KD=colony formation↓[85,] apop. ↑[85,] chemo sensitivity ↑[86]	Graft + PrAg-PCIS treatment = tumor size, prolif. ↓[87]	Expression ↑[86]
	Gastric	N/A	N/A	Pt. low corr. Survival ↑[88]
	Ovary	Expressed in epithelial cultured cells [85, 89, 90]	N/A	Expressed in tissue[89, 91]
		Expression ↑[89]; KD = apop. ↑[85] OE = vascular leakiness markers ↓[90]	RE graft = tumor volume ↑[85] OE graft = metastasis ↓[90]	Expression ↑[89]
	Testes	Expressed in sperm[92-95]; fertilization[92, 96]; ZP penetration[96, 97]; ZP-protein complexing[93] *Prss21* deficiency = motility↓, decapitation ↑[92]	Expressed in elongated spermatids[95, 98, 99]; *Prss21* deficiency = curled tails[92]	Expressed in spermatozoa[92, 94,95, 100]
		Expression↓[94, 101]; ♀♀♀[100]; RE=anchorage dependent growth↓[100]	RE graft = tumor burden ↓[100]	Expression ↓[94]; ♀♀♀[100]
	Vascular	Expressed in endothelial cell culture[102]; KD = cell migration, tubular reorganization ↓, permeability ↑[103]; Fibrin formation[104]; uPA activation[104]	*Prss21*-deficient ovaries = hemorrhagic phenotype[103]	Expressed in eosinophils[91]

All results presented are compiled from cell culture models, animal studies, and patient samples. Arrows (↑↓) indicate increases or decreases, respectively. Normal tissue: blue background, cancerous tissue: red background. Expression is decreased or completely lost (Expression ↓). Re-expression in cell culture models (RE), overexpression in cell culture models (OE), knockdown in cell lines for cell culture models or grafts (KD), conditional knock-out in mouse models (cond. KO), cell culture models not expressing prostasin (loss), prostasin-low or testisin-low patient tissue samples (Pt. low). Epithelial-to-mesenchymal transition (EMT), apoptosis (apop.), proliferation (prolif.), blood pressure (BP), E–, N-cadherin (cad), inflammation (inflam.), promoter region methylation (♀♀♀), cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF), Wild-type (WT), High-fat diet (HFD), chemoresistance (chemo), modified anthrax toxin protein C inhibitor derived sequence (PrAg-PCIS), zona pellucida (ZP). For more in-depth summaries and analyses, refer to text sections. Key to cited literature: [1]Chen et al. 2009; [2]Chen et al. 2006; [3]List et al. 2007; [4]Chen and Chai 2002; [5]Bergum et al. 2012; [6]Murray et al. 2020; [7]Planès et al. 2010; [8]Myerburg et al. 2008; [9]Tarran et al. 2006; [10]Yu et al. 1994; [11]Yu et al. 1995; [12]Raghu et al. 2018; [13]Gao et al. 2022; [14]Ma et al. 2017; [15]Bao et al. 2016b; [16]Sakashita et al. 2008; [17]Buzza et al. 2010; [18]Netzel-Arnett et al. 2012; [19]Buzza et al. 2013; [20]Holt-Danborg et al. 2019; [21]Huang et al. 2024; [22]Frateschi et al. 2012; [23]Sugitani et al. 2020; [24]Bao et al. 2016a; [25]Selzer-Plon et al. 2009; [26]Bao et al. 2019; [27]Buzza et al. 2017; [28]Vallet et al. 1997; [29]Vuagniaux et al. 2000; [30]Andreasen et al. 2006; [31]Essigke et al. 2021; [32]Bruns et al. 2007; [33]Tuyen et al. 2005; [34]Wakida et al. 2006; [35]Narikiyo et al. 2002; [36]Wang et al. 2003; [37]Ehret et al. 2022; [38]Ehret et al. 2023; [39]Olivieri et al. 2005; [40]Koda et al. 2009; [41]Uchimura et al. 2014; [42]Sekine et al. 2021; [43]Zhang et al. 2016; [44]Ashida et al. 2017; [45]Yamamoto et al. 2021; [46]Yamamoto et al. 2018; [47]Mok et al. 2001; [48]Costa et al. 2009; [49]Tamir et al. 2016; [50]Yan et al. 2014; [51]Ma et al. 2014; [52]Cai et al. 2021; [53]Ishii et al. 2023; [54]Camerer et al. 2010; [55]Szabo et al. 2012; [56]Hummler et al. 2013; [57]Szabo et al. 2016; [58]Szabo and Bugge 2018; [59]Szabo et al. 2009; [60]Szabo et al. 2007; [61]Ma et al. 2009; [62]Chen et al. 2001a; [63]Yu et al. 1996; [64]Takahashi et al. 2002; [65]Takahashi et al. 2003; [66]Chen et al. 2004; [67]Chen et al. 2007; [68]Lai et al. 2016a; [69]Leyvraz et al. 2005; [70]Hayashi et al. 2002; [71]Spacek et al. 2010; [72]Panteleyev and Christiano 2001; [73]Frateschi et al. 2011; [74]Friis et al. 2016; [75]Lindner et al. 2000; [76]Crisante et al. 2014; [77]Friis et al. 2013; [78]Peters et al. 2014; [79]Lee et al. 2018; [80]Chang et al. 2020; [81]Shamseldin et al. 2023; [82]Maekawa et al. 2009; [83]Zhu et al. 2008, 21; [84]Li et al. 2011; [85]Tang et al. 2005; [86]Yeom et al. 2010; [87]Martin et al. 2015; [88]Li et al. 2022; [89]Shigemasa et al. 2000; [90]Conway et al. 2019; [91]Inoue et al. 1998; [92]Netzel-Arnett et al. 2009; [93]Swegen et al. 2019; [94]Hooper et al. 1999; [95]Honda et al. 2002; [96]Yamashita et al. 2008; [97]Kawano et al. 2010; [98]Nakamura et al. 2003; [99]Scarman et al. 2001; [100]Manton et al. 2005; [101]Hooper et al. 2000; [102]Aimes et al. 2003; [103]Peroutka et al. 2020; [104]Buzza et al. 2023.

therefore appears to be dependent on the genetic background of the mouse model. Interestingly, knock-in mice expressing catalytically inactive prostasin (*Prss8*$^{Ki/Ki}$) displayed normal prenatal and postnatal survival indicating that prostasin supports embryonic survival independent of its proteolytic activity (Szabo et al. 2016).

2.3 Regulation of the prostasin-matriptase pathway by HAIs in embryonic development

It is clear that the prostasin and matriptase proteases and their inhibitors HAI-1 and HAI-2 (each inhibits both prostasin and matriptase) constitute a tightly controlled complex system that is functionally essential in multiple aspects of embryonic development and postnatal epithelial homeostasis (Buzza et al. 2013, 2010; Friis et al. 2016, 2013; Netzel-Arnett et al. 2006; Peters et al. 2014; Szabo and Bugge 2018; Szabo et al. 2009, 2016, 2007, 2014). In early embryonic ectoderm formation, a prostasin-matriptase cell surface protease cascade exists whose activity must be suppressed by HAI-1 and HAI-2 for placental morphogenesis and neural tube closure to properly occur. Thus, loss of either HAI-1 or HAI-2 is associated with embryonic lethality in mice, which can be rescued by simultaneous mutations in matriptase or prostasin (Szabo et al. 2009, 2007, 2012). Crossing HAI-deficient mice into *Prss8* V170D mutant mice, a prostasin hypomorphic model (see Section 3), restored placentation and normal development of HAI-1-deficient embryos and rescued early embryonic lethality and neural tube abnormalities in HAI-2-deficient embryos (Szabo et al. 2012). A similar rescue of HAI-2 deficient embryos was observed with concomitant expression of zymogen-locked (prostasin R44Q activation site mutant) knock-in alleles (Szabo and Bugge 2018). For review on the prostasin/matriptase/HAI functional interplay in development see (Szabo and Bugge 2020).

3 Role in epidermis and gastrointestinal tract

3.1 Prostasin in neonatal survival, epidermal barrier function, and adult epidermal homeostasis

Mice carrying one *Prss8* null allele, one floxed *Prss8* allele (Rubera et al. 2002), and a keratin (K)14-Cre transgene were generated to assess the role of prostasin in the epidermis (Leyvraz et al. 2005). These mice were born alive at the expected Mendelian frequency but died within 60 h after birth due to defects in the epidermal barrier leading to fatal dehydration (Leyvraz et al. 2005). At the cellular and molecular levels, the pups presented with abnormal cornified layers of the epidermis including impaired corneocyte differentiation and lipid matrix formation, and impaired tight junction formation and function. Incomplete processing of the key epidermal polyprotein, profilaggrin, into filaggrin monomers was also observed (Leyvraz et al. 2005). In mice with *Prss8* gene ablation in the epiblast as described in Section 2, gene-inactivation occurred from E6.5 onwards in the embryo proper and pups displayed the same phenotypes as the epidermis-ablated mice indicating that a major and central role of prostasin in perinatal development is in proper maturation of the outermost layers of the skin (Hayashi et al. 2002; Hummler et al. 2013).

Spontaneous mutations in *Prss8* have were also identified in mice and rats (Paul et al. 2008; Spacek et al. 2010). Mice carrying the frizzy (*fr)* allele have a missense mutation in the *Prss8* gene causing a V170D amino acid substitution. Mice bred to homozygosity displayed a wavy coat, curly vibrissae, and a disorganized and compacted cornified layer (Spacek et al. 2010). The 'hairless' (*fr*^{CR}) rats have a 12-bp deletion in the third exon in *Prss8*, leading to a ^{54}GQWP^{57} (G54-P57) fragment deletion, and display a thick and dense layer of corneocytes and abnormal keratinization of the hair shaft causing their hairless appearance (Panteleyev and Christiano 2001; Spacek et al. 2010). In a detailed molecular study of the mutant mouse and rat prostasin proteins, Frateschi and colleagues described changes in both their structure and glycosylation state (Frateschi et al. 2012). Activity studies of recombinant V170D prostasin have shown a profound reduction in proteolytic function compared to the wildtype protease (Netzel-Arnett et al. 2006; Szabo et al. 2012). Functionally, co-expression of V170D or G54-P57 prostasin and ENaC in *Xenopus* oocytes revealed an overall reduction in the activation of ENaC compared to the wildtype protease (Frateschi et al. 2012). *In vivo* analyses demonstrated that both *fr* and *fr*^{CR} homozygous animals displayed decreased embryonic viability, histological changes to the interfollicular and follicular epidermis, and a significant decrease in ENaC activity in the distal colon compared with control littermates. Additionally, homozygous young adult rats had a significant reduction in bodyweight and displayed increased dehydration from both skin and intestine (Frateschi et al. 2012). Recently, mutations in *PRSS8* were identified in humans with autosomal recessive ichthyosis. Two families had multiple members with congenital ichthyosis presenting with dry scaly skin (ichthyosis) and brittle hair (Shamseldin et al. 2023). Histologically, skin biopsies showed hyperkeratosis and parakeratosis, and the phenotypes were described

as strikingly similar to those in prostasin-deficient animal models. One variant involved a canonical splice site and was associated with reduced levels of the normal transcript, while the other was a missense mutation causing a V60D substitution (Shamseldin et al. 2023). In addition to the prostasin loss-of-function models, the protease was studied in gain-of-function mouse models. Transgenic expression of wildtype prostasin under the K14-promotor caused epidermal hyperplasia, ichthyosis, and abnormal hair growth with functional impairment of epidermal barrier function (Frateschi et al. 2011). Dermal inflammation with increased transcription levels of the interleukins IL-1α, IL-1β, thymic stromal lymphopoietin, and matrix metalloproteinase-9 (MMP-9) relative to littermate controls were also observed (Frateschi et al. 2011). To study a potential functional relationship between prostasin and PAR-2 in the epidermis, the K14-prostasin mice were crossed to mice with global PAR-2 deficiency (*PAR2*$^{-/-}$), with the latter not exhibiting any signs of disease or skin abnormalities under pathogen-free conditions (Lindner et al. 2000). In K14-prostasin transgenic mice with concomitant PAR-2 null status, both macroscopic, histologic, and functional phenotypes were rescued, indicating that PAR-2 is an essential mediator of prostasin-induced pathogenesis (Frateschi et al. 2011). In a follow-up study, it was demonstrated that transgenic expression of catalytically inactive prostasin under the K14 promoter in the epidermis caused similar skin disorders as wildtype prostasin (Crisante et al. 2014). Again, the phenotypes were rescued in a PAR-2 null background, suggesting that the effects of prostasin-mediated signaling through PAR-2 in this overexpression model are independent of its catalytic activity. In a study by Friis and colleagues, similar severe skin phenotypes were observed in transgenic mice expressing wildtype or catalytically inactive prostasin under the control of a keratin-5 promoter (Friis et al. 2013). Additionally, they generated zymogen-locked mutant (R44Q) transgenic mice which showed phenotypes that were indistinguishable in outward and histological appearance from mice overexpressing wildtype prostasin or the catalytically inactive form (Friis et al. 2013). It was further shown that zymogen-locked and catalytically inactive prostasin stimulated matriptase activation and PAR-2 activation in a matriptase-dependent manner in HEK293 cells (Friis et al. 2013). Generation and characterization of two different *Prss8* knock-in models enabled further detailed genetic and molecular analysis pertaining to the physiological functions of the endogenously expressed protease. First, mice homozygous for a point mutation in the *Prss8* gene were generated. This mutation substituted the catalytic triad active sight serine (S238) with alanine, rendering endogenous prostasin catalytically inactive (*Prss8*$^{Cat-/Cat-}$ mice) (Peters et al. 2014). These mice developed a functional epidermal barrier, survived to adulthood, and were generally healthy albeit with significantly reduced bodyweight beginning at 2–3 weeks of age (Peters et al. 2014). Trans-epidermal water loss in *Prss8*$^{Cat-/Cat-}$ pups was marginally increased compared to control littermates. This was in contrast to *Prss8* null mice, that lost water at a rapid pace, leading to fatal dehydration in agreement with previous studies (Leyvraz et al. 2005). At the molecular level, analysis of epidermal extracts from newborn *Prss8*$^{Cat-/Cat-}$ pups showed that prostasin-mediated profilaggrin processing did not require the enzymatic activity of prostasin, although the levels of processed filaggrin monomers were reduced in *Prss8*$^{Cat-/Cat-}$ epidermis (Peters et al. 2014). These observations demonstrated that epidermal differentiation and epidermal barrier formation require prostasin, but not its enzymatic activity. Catalytic activity of prostasin does appear to play a role in hair formation since subtle defects in vibrissae and pelage hair were apparent in *Prss8*$^{Cat-/Cat-}$ mice (Peters et al. 2014). The second knock-in model published in 2016 by the same group expressed only the zymogen-locked (R44Q) endogenous prostasin (Friis et al. 2016). These *Prss8*$^{zym/zym}$ mice showed expression of the zymogen-locked protein at levels similar to wildtype mice. Knock-in mice displayed normal interfollicular epidermal development and postnatal survival but had defects in vibrissae and pelage hair formation. Based on this study combined with the finding in *Prss8*$^{Cat-/Cat-}$ mice described above, it appears that two distinct *in vivo* functions of epidermal prostasin exist: one for the interfollicular epidermis, where proteolytic activation and activity of prostasin is not required, and the second for the follicular epidermis where proteolysis-dependent functions are necessary for normal hair development (Friis et al. 2016).

3.2 Prostasin-matriptase pathway in epithelial homeostasis

Multiple studies suggest a critical functional relationship between prostasin and matriptase in the epidermis and intestinal epithelium. The first indication was the finding that matriptase- and prostasin-null mice displayed virtually identical phenotypes with perinatal death and defects in epidermal differentiation, barrier formation, and hair growth (Leyvraz et al. 2005; List et al. 2002, 2003). Initially, it was proposed that a unidirectional pathway existed, where matriptase activates the prostasin zymogen. This was based on the observation that the epidermis of matriptase-null mice lacked detectable two-chain active prostasin and displayed accumulation of one-chain prostasin zymogen (Netzel-Arnett et al. 2006). It has since been shown in cell

culture and mouse models that prostasin can act upstream of matriptase and that prostasin can stimulate matriptase activity in cell culture as well as in tissues as a co-factor that is not strictly dependent on its enzymatic activity (Buzza et al. 2013; Camerer et al. 2010; Friis et al. 2013, 2016; Szabo et al. 2012). While prostasin and matriptase function in the same pathway in normal tissue development and homeostasis, as well as certain diseases including colitis (see below), the same might not be the case in cancer. In the majority of malignancies studied, matriptase displays tumor-promoting properties (reviewed in Martin and List 2019) while prostasin predominantly exerts tumor-suppressive properties, (either dependent or independent of proteolytic activity) and it is possible that the two proteases act through different pathways in some pathological settings (see Section 6). For review on the matriptase-prostasin proteolytic cascade in non-cancerous dermatologic diseases see (Touati et al. 2020).

3.3 Prostasin in intestinal barrier function and homeostasis

A study in 2012 first implicated prostasin as a significant regulator of intestinal homeostasis (Frateschi et al. 2012). The *fr*^{CR}/*fr*^{CR} *Prss8* mutant rats presented with reduced bodyweight and diarrhea (Frateschi et al. 2012). As mentioned above, a significant decrease in the amiloride-sensitive rectal potential difference was detected in both *fr/fr* V170D mutant mice and *fr*^{CR}/*fr*^{CR} rats compared with their control littermates, reflecting a reduced ENaC activity in the distal part of the colon (Frateschi et al. 2012). In a follow-up study using the mutant rat model to study the consequences of prostasin loss-of-function in experimental colitis, the animals were challenged with a 7-day treatment of dextran sodium sulfate (DSS) to induce inflammation, followed by 7 days of recovery (Keppner et al. 2016). The *fr*^{CR}/*fr*^{CR} rats displayed a profound loss of goblet cells and developed ulcerations and edemas that were not present in the heterozygous or wildtype prostasin littermates. These defects led to exacerbated weight loss, increased disease activity index, and diarrhea severity accompanied by rearrangement of the lamina propria, and increased infiltration of neutrophils, eosinophils, and macrophages (Keppner et al. 2016). In a conditional *Prss8* knock-out mouse model (*Prss8*$^{\Delta IEC}$) with colon-specific deletion using *Prss8* floxed mice crossed with Villin-Cre mice, unchallenged animals lacking intestinal prostasin did not show any overt phenotypes (Sugitani et al. 2020). However, in DSS-induced colitis *Prss8*$^{\Delta IEC}$ mice displayed exacerbated disease with increased weight loss and more severe inflammatory changes, including epithelial disruption and inflammatory cell infiltration (Sugitani et al. 2020). At the molecular level, Toll-like receptor 4 (TLR4) levels in colonic epithelial cells increased in DSS-treated *Prss8*$^{\Delta IEC}$ mice compared to DSS-treated control mice. Furthermore, NF-κB activation was elevated and increased mRNA expression levels of the inflammatory cytokines TNFα, IL-1β, IL-6, and CXCL1 were detected, suggesting that lack of prostasin led to continual inflammation (Sugitani et al. 2020). Treatments with broad-spectrum antibiotics suppressed the exacerbation of DSS-colitis in *Prss8*$^{\Delta IEC}$ mice, which made the authors propose that commensal microflora-stimulated TLR4 signaling may be important for the exacerbation of DSS-colitis in prostasin-deficient mice. Notably, the mRNA levels of tight junction proteins, as well as mucosal permeability assessed using FITC-dextran, were comparable between prostasin-deficient and sufficient mice, indicating that the intestinal barrier function was not significantly perturbed in the intestinal epithelium of this mouse model (Sugitani et al. 2020). In human tissue samples, prostasin mRNA and protein expression levels were significantly decreased in the active mucosa of both ulcerative colitis and Crohn's disease patients compared to normal or inactive mucosa indicating a link between prostasin and human intestinal disease. This connection could be causal and/or correlational (Sugitani et al. 2020). In a study from 2013, Buzza and colleagues proposed that prostasin, together with matriptase, comprise a single common proteolytic pathway that is required for barrier formation in the intestine (Buzza et al. 2013). The same group had previously shown that matriptase plays a critical role for intestinal barrier function and protection against DSS-induced inflammatory colitis using mice with low systemic matriptase expression (*St14* hypomorphic mice) (Buzza et al. 2010; Netzel-Arnett et al. 2012). Expanding their studies to include the prostasin-matriptase pathway, they found that addition of recombinant prostasin to the basal side of cultured polarized Caco-2 intestinal carcinoma cells stimulated barrier-forming changes that required expression of endogenous matriptase (Buzza et al. 2013). Furthermore, siRNA-mediated silencing of prostasin in Caco-2 cells inhibited barrier function, measured by transepithelial electrical resistance (TEER), and increased paracellular permeability to macromolecular FITC-dextran, similarly to that observed in loss-of-function matriptase models (Buzza et al. 2013). Depletion of prostasin was accompanied by a significant decrease in active matriptase and elevated levels of claudin-2, a "leaky" tight junction protein that forms cation-selective and water permeable paracellular channels. The underlying mechanism was proposed to be driven by matriptase-mediated claudin-2 protein turnover and signaling through atypical protein kinase C (PKC) ζ (Buzza et al. 2010, 2013). Prostasin was proposed to work

upstream from matriptase acting as a co-factor for matriptase zymogen activation, in a manner that is independent of the proteolytic activity of prostasin (Buzza et al. 2013). In a 2017 mouse study by the same group, mRNA and protein expression of both matriptase and prostasin were rapidly down-regulated in the initiating inflammatory phases of DSS-induced experimental colitis, and the loss of these proteases preceded the onset of clinical symptoms (Buzza et al. 2017). Similarly, decreased levels of matriptase and prostasin mRNA were detected in samples from patients with ulcerative colitis or Crohn's disease compared to healthy colon (Buzza et al. 2017). Mechanistically, the Th2-type cytokines, IL-4 and IL-13 down-regulated matriptase and prostasin during disruption of polarized epithelial barriers in colonic T84 cells. Regulation was proposed to occur via phosphorylation of the transcriptional regulator STAT6 and inhibition of STAT6 with suberoylanilide hydroxamic acid (SAHA), which among many functions inhibits IL-13-induced STAT6 phosphorylation (Rosen et al. 2011), restored protease expression and reversed cytokine-induced barrier dysfunction (Buzza et al. 2017). SAHA treatment also led to a reduction in cytokine-induced claudin-2 upregulation. Since it has been shown that IL-4 and claudin-2 expression increases in ulcerative colitis (Inoue et al. 1999b; Randall et al. 2016), it was proposed that the inflammatory cytokine-mediated down-regulation of the prostasin-matriptase proteases and upregulation of claudin-2 may contribute to disease susceptibility and progression (Buzza et al. 2017).

3.4 Regulation of the prostasin-matriptase pathway by HAI-2 in intestines

The prostasin-matriptase proteolytic pathway is tightly regulated by HAI-1 and HAI-2 during embryonic development as described in Section 2. Similarly, regulation of proteolysis by HAI-2 was described to be important for intestinal homeostasis. In humans, genetic mutations in the *SPINT2* gene encoding HAI-2 play causal roles for development of syndromic congenital sodium diarrhea (SCSD) and congenital tufting enteropathy (CTE) (Heinz-Erian et al. 2009; Holt-Danborg et al. 2019; Salomon et al. 2014). Szabo and colleagues investigated the functional relevance of prostasin and HAI-2 in mouse intestine by generating *Prss8*$^{zym/zym}$ (prostasin zymogen-locked) knock-in mice with concomitant HAI-2 deficiency (Szabo and Bugge 2018). HAI-2 deficient *Prss8*$^{zym/zym}$ embryos displayed no defects in prenatal development and were born in the expected Mendelian ratios, but died 4–7 days after birth due to severe intestinal defects including widespread villous atrophy, tufted villi, loss of goblet cells and colonic crypt structure, and bleeding (Szabo and Bugge 2018). These phenotypes were similar to those in patients with CTE and the authors proposed that matriptase activity plays an important role in CTE pathogenesis. At the cellular level, Holt-Danborg and colleagues reported that *SPINT2* missense variants within the second Kunitz domain are associated with SCSD, and reduced the ability of HAI-2 to inhibit prostasin, but not matriptase (Holt-Danborg et al. 2019). In an independent study, recombinant HAI-2 with SCSD mutations found in humans displayed loss of inhibitory activity towards prostasin in Caco-2 cells (Huang et al. 2024). Huang and colleagues proposed that excessive prostasin proteolysis plays a role in disease progression. Together, prostasin-matriptase and HAI-2 constitutes a complex protease-inhibitor triad that must be finetuned to support normal epithelial function.

3.5 Evolutionary conservation of prostasin-matriptase proteolysis

The aforementioned studies performed in mammalian animal and cell culture models showed a close functional relationship between prostasin and matriptase and their endogenous inhibitors in epithelial function. Interestingly, studies in *Drosophila* suggest that the prostasin-matriptase proteolytic cascade is evolutionarily functionally conserved (Drees et al. 2019). Thus, the *Drosophila* protease Notopleural (Np) acts as a functional homologue of matriptase, and Tracheal-prostasin (Tpr) mediates prostasin functions in the *Drosophila* respiratory system (Drees et al. 2019). These functional prostasin-matriptase homologues are critically involved in morphogenesis and physiology including extracellular matrix formation and barrier function in tracheal tubes, highlighting their importance for both invertebrates and vertebrates.

4 Vascular homeostasis, coagulation, and fibrinolysis

4.1 Testisin in vascular homeostasis

Testisin mRNA was detected in human microvascular endothelial cells (HMVECs) undergoing reorganization and tube-like formation in angiogenesis assays using artificial reconstituted basement membrane and during pre-capillary morphogenesis on 3-D fibrillar type I collagen (Aimes et al. 2003). In functional cell culture studies, siRNA-mediated knockdown of testisin was performed using primary human

dermal microvascular endothelial cells (HMVEC-d), an immortalized derivative of HMVEC-d (HMEC-1 cells), and primary human umbilical vein cells (HUVEC) (Peroutka et al. 2020). Impaired cell migration and tubular reorganization was observed in all three cell lines upon testisin silencing, with no effect on cell viability (Peroutka et al. 2020). Furthermore, testisin depletion increased vascular permeability in HMEC-1 monolayer culture accompanied by loss of the adherens junction protein vascular endothelial (VE)-cadherin and diminished β-catenin (Peroutka et al. 2020). The latter associates with VE-cadherin to stabilize endothelial cell barriers (Cattelino et al. 2003). Testisin function *in vivo* was studied using a murine model of rapid physiological angiogenesis during corpus luteal development. In this model, angiogenesis after gonadotropin-induced ovulation was compared in testisin-deficient and sufficient female mice and revealed a hemorrhagic phenotype in testisin-deficient ovaries. This phenotype was associated with a functional defect in vascular integrity as evidenced by increased vascular permeability and leakiness (Peroutka et al. 2020). VE-cadherin protein visualization at the inter-endothelial junctions in the neovasculature of corpora lutea revealed less intense staining in testisin-deficient mice compared to control mice. It is plausible that loss of VE-cadherin protein during angiogenesis contributes to the increased vascular permeability and fragility (Peroutka et al. 2020).

4.2 Testisin in coagulation and fibrinolysis

To maintain a well-functioning circulatory system, a balance between bleeding cessation and clearance of coagulating products must be tightly regulated. Testisin has been implicated in pericellular hemostasis with a potential role in acceleration of both thrombin-dependent fibrin polymerization and in subsequent fibrinolysis (Buzza et al. 2023). A cell culture model using ectopic expression of testisin in the ES-2 cell line isolated from the ovary of a cancer patient and exhibiting fibroblast-like morphology was used to measure fibrin generation on the cell surface. In adherent ES-2 cells provided with fibrinogen and prothrombin, the conversion of fibrinogen to fibrin in testisin-expressing cells caused a rapid increase in turbidity, a readout for fibrin gel formation, compared to control cells. This was followed by a rapid decrease, indicating cell-mediated dissolution of the formed fibrin polymers (Buzza et al. 2023). Catalytically active cell-surface testisin was required for fibrin polymerization; however, testisin did not activate thrombin zymogen directly, but stimulated prothrombin activation and fibrin generation through a pathway promoting FXa activation. Testisin-mediated acceleration of fibrin degradation was plasmin(ogen)-dependent but did not directly activate plasminogen. Instead, testisin directly activated the inactive proform of urokinase plasminogen activator (pro-uPA) into active uPA, which converts plasminogen to plasmin (Buzza et al. 2023). Testisin-deficient mice displayed no detectable defects in hemostasis when observed under unchallenged conditions and the physiological role of testisin in processes that challenges hemostasis e.g., injury or inflammation awaits further studies.

5 Prostasin in kidney, bladder, liver, pancreas, and lung

5.1 Prostasin in the kidney

ENaC, the amiloride-sensitive sodium channel of the collecting ducts and connecting tubules in the kidney, plays a critical role in the renal regulation of sodium excretion and blood pressure. The mineralocorticoid aldosterone and its corresponding nuclear receptor are important for ENaC activation and sodium reabsorption (Tsilosani et al. 2022). Prostasin mRNA was first detected in proximal tubules of the human kidney (Yu et al. 1995). In 1997, Vallet and colleagues discovered a protein that induced a 3-fold increase in sodium current generated by ENaC in *Xenopus* oocytes termed xCAP1. Confirmation of tissue expression revealed that xCAP1 transcript was expressed in the kidneys of *X. laevis* (Vallet et al. 1997). A mammalian full-length CAP1 homologue in mice (mCAP1) was discovered in murine kidneys with expression in proximal tubules and in intact and cultured collecting duct cells (Vuagniaux et al. 2000). Co-expression of ENaC and mCAP1 increased amiloride-sensitive current, a similar finding to that observed with xCAP1 in *Xenopus* oocytes (Vuagniaux et al. 2000). Aldosterone treatment in the mouse cortical collecting duct cell line M–1 increased prostasin expression in a time-dependent manner and stimulated amiloride-sensitive uptake of sodium determined by tracer activity of $^{22}Na^{+}$ (Narikiyo et al. 2002). These findings were confirmed *in vivo,* where aldosterone administration in rats increased the presence of prostasin in urine in a time-dependent manner, suggesting release of prostasin from the apical membrane of the kidney tubules (Narikiyo et al. 2002). Aldosterone-treated rats also exhibited hypertension, hypokalemia, and metabolic alkalosis relative to controls (Narikiyo et al. 2002). Urinary prostasin protein was proposed to be a candidate marker for ENaC activation in humans, and a positive correlation was reported between urinary prostasin concentration and primary aldosteronism, an endocrine disorder characterized by high levels of aldosterone.

Additionally, a negative correlation between urinary prostasin excretion and Na/K ratios was reported (Olivieri et al. 2005; Pizzolo et al. 2017). Treatment with the diuretic spironolactone increased urinary Na^+/K^+ ratios and decreased urinary prostasin in subjects in whom the renin/aldosterone axis was activated by a low Na^+ intake; however, the drug was ineffective in individuals with high Na^+ intake (Olivieri et al. 2005). Koda and colleagues also reported a significant positive correlation between urinary prostasin and urinary aldosterone levels (Koda et al. 2009). A 2017 study concluded that albuminuria was associated with increased prostasin in urine while aldosterone had no direct effect on urine and kidney tissue levels of prostasin (Oxlund et al. 2017).

To experimentally study the functional interplay between aldosterone, produced in the cortex of the adrenal glands, and prostasin expressed in multiple tissues, the effect of systemic tail vein administration of adenovirus carrying human prostasin cDNA in rats was interrogated. The transgenic transcript was detected in rat liver, adrenal gland, heart, lung, kidney, and aorta and protein was detected in the circulation and urine (Wang et al. 2003). The rats displayed increased blood pressure accompanied by elevated plasma aldosterone levels and reduced plasma renin activity, which correlated with the expression of human prostasin transgene. Prostasin expression also significantly reduced urinary K^+ excretion but increased urinary Na^+ and kallikrein excretion (Wang et al. 2003). Elevated renal kallikrein levels promote natriuresis (Majima and Katori 1995), which may prevent further increases of blood pressure upon transgenic prostasin expression. Based on these results, the authors proposed that prostasin participates in blood pressure and electrolyte homeostasis by regulating the renin-angiotensin-aldosterone and kallikrein-kinin systems (Wang et al. 2003). Ko and colleagues set out to identify the mechanism by which prostasin in the systemic circulation could increase plasma levels of aldosterone and found that wildtype prostasin increased aldosterone synthesis and elevated aldosterone synthase mRNA expression in the human adrenocortical cell line H295R (Ko et al. 2010). The elevated aldosterone levels persisted in the presence of the broad-spectrum serine protease inhibitor camostat mesilate. Furthermore, catalytically inactive prostasin had similar effect as wildtype prostasin on aldosterone levels, together suggesting a prostasin-mediated stimulation of aldosterone synthesis independent of catalytic activity (Ko et al. 2010). TGF-β1 has been shown to antagonize aldosterone-mediated sodium transport in rat inner medullary collecting duct primary cells (Husted et al. 2000). It was investigated whether TGF-β1 regulated kidney prostasin levels, and thereby affected sodium reabsorption, and prostasin promoter activity in M–1 cells was decreased upon TGF-β1 treatment in a time- and dose-dependent manner accompanied by decreased prostasin mRNA and protein (Tuyen et al. 2005). Consequently, the decrease in prostasin protein was associated with a decrease in the amiloride-sensitive $^{22}Na^+$ uptake. Mechanistically, the authors proposed that TGF-β1-induced inhibition of prostasin expression may act through NF-κB/Rel and IkBα pathways (Tuyen et al. 2005). As a follow-up to this study, and with the identification of PN-1 as an inhibitor of prostasin (Chen et al. 2004), it was investigated whether inhibition of prostasin by PN-1 affected sodium reabsorption (Wakida et al. 2006). Expression of PN-1 in *Xenopus* oocytes already expressing ENaC and prostasin led to a significant decrease in the amiloride-sensitive sodium current. Interestingly, ENaC and PN-1 co-expression without prostasin also resulted in a marked reduction in sodium current compared to ENaC alone, suggesting that PN-1 may regulate ENaC function independent of prostasin inhibition (Wakida et al. 2006). Regulation of PN-1 by TGF-β1 and aldosterone was also investigated in M–1 cells where the addition of TGF-β1 decreased ENaC mRNA levels, but increased mRNA and protein levels of PN-1. Aldosterone decreased PN-1 mRNA and protein levels in M–1 cells, and transient siRNA-mediated gene silencing of PN-1 increased equivalent current relative to controls. It was therefore proposed that PN-1 serves as a regulator of ENaC activity in the kidney (Wakida et al. 2006).

Multiple mechanistic studies reported on catalytic and non-catalytic prostasin functions. Point mutations were introduced at all three residues of the catalytic triad of mouse prostasin to investigate the non-catalytic function of prostasin in the *Xenopus* oocyte model (Andreasen et al. 2006). As expected, co-expression of wildtype mouse prostasin and rat ENaC in oocytes by cRNA injection led to a 10-fold increase in sodium current compared to oocytes injected with rat ENaC cRNA alone (Andreasen et al. 2006). Interestingly, co-expression of rat ENaC and prostasin with any one or all the three residues of the catalytic triad mutated, had no lesser effect on ENaC suggesting non-catalytic functions of prostasin. The presence of an intact GPI anchor, however, was required (Andreasen et al. 2006). Proteolytic cleavage of ENaC occurs in its extracellular domains and involves a furin-dependent cleavage site in the α subunit and an additional site in the γ subunit (Bruns et al. 2007; Hughey et al. 2004). In addition to furin, other channel activating proteases have been identified to increase sodium currents when co-expressed with ENaC (Adachi et al. 2001; Vallet et al. 1997; Vuagniaux et al. 2000, 2002). Bruns and colleagues reported prostasin-mediated cleavage of the γ subunit at a site distal to the furin cleavage site and that dual

cleavage of the γ subunit was predicted to release a 43-amino acid peptide. ENaC with a γ subunit lacking this 43-residue tract was shown to have increased activity due to a high open probability (Bruns et al. 2007). A synthetic peptide mimicking the released fragment acted as a reversible inhibitor of endogenous ENaC in mouse cortical-collecting duct $mpkCCD_{cl4}$ cells and in primary cultures of human airway epithelial cells. The authors proposed a model where prostasin promotes maturation and cleavage of the ENaC γ subunit and, in concert with furin-dependent cleavage, releases an inhibitory peptide domain leading to increased ENaC activity (Bruns et al. 2007). Essigke and colleagues utilized zymogen-locked and catalytically inactive prostasin to further investigate non-proteolytic functions of prostasin in ENaC activation and subsequent sodium reabsorption in *Xenopus* oocytes and mice (Essigke et al. 2021, 2022). Full-length mouse ENaC α, β, and γ cRNA were co-injected with mouse wildtype, catalytically inactive, or zymogen-locked prostasin cRNA into *Xenopus* oocytes. ENaC with wildtype or catalytically inactive prostasin induced maximal proteolytic ENaC activation in oocytes (Essigke et al. 2021). Interestingly, ENaC was only partially activated in oocytes co-expressing zymogen-locked prostasin and this mutant caused a concomitant reduction in trypsin-like proteolytic activity at the cell surface measured by cleavage of a fluorogenic substrate. Subsequent mouse studies included wildtype and genetically modified knock-in mice carrying corresponding mutations in the *Prss8* gene: $Prss8^{+/+}$, $Prss8^{Cat-/Cat-}$, and $Prss8^{zym/zym}$ (characterized in Friis et al. 2016; Peters et al. 2014) to investigate ENaC activation and renal sodium handling. Mice were initially fed a normal diet for two days, where aldosterone levels were comparable between all groups. Once switched to a low sodium/normal potassium (LS/NK) diet, all genotypes showed an increase in plasma aldosterone levels after two days. The levels of aldosterone in $Prss8^{+/+}$and $Prss8^{Cat-/Cat-}$ mice returned to normal after 5 days on LS/NK, whereas aldosterone levels persisted in $Prss8^{zym/zym}$ mice and led to hyperaldosteronism. To investigate ENaC-mediated sodium transportation, the pharmacologic ENaC inhibitor triamterene was given for four days. Initially, all genotypes had increased sodium/potassium ratios in the urine and decreased bodyweights after the first day of triamterene (Essigke et al. 2021). While natriuresis and bodyweight stabilized in $Prss8^{+/+}$and $Prss8^{Cat-/Cat-}$ mice, the $Prss8^{zym/zym}$ mice continued to lose weight, showed severe dehydration, and subsequent death or termination of the experiment with signs of acute renal insufficiency and profound increases in plasma aldosterone concentrations (Essigke et al. 2021). Lastly, under triamterene treatment, the γ-subunit ENaC cleavage was impaired in $Prss8^{zym/zym}$ mice. The authors proposed a model where cleavage at the R44 activation site in wildtype and catalytically inactive prostasin induces a shift in the tertiary structure of prostasin that enables the recruitment of another, yet unknown, serine protease capable of activating ENaC by proteolysis, independent of proteolytic activity of prostasin. The scaffold function is impaired in zymogen-locked prostasin leading to abrogated recruitment of the other serine protease, causing impaired ability to promote ENaC activity (Essigke et al. 2021). The same group expanded these knock-in mouse experiments to include nephrotic syndrome to assess whether prostasin is involved in ENaC-mediated sodium retention in doxorubicin-induced nephrotic syndrome mice (Essigke et al. 2022). After doxorubicin treatment, mice of all genotypes developed proteinuria, and when these nephrotic mice were treated with triamterene, natriuretic responses and bodyweight changes were comparable between mice of all genotypes. Additionally, cleavage of α, β, and γ subunits of ENaC were similar between all genotypes. These findings indicated that proteolytic ENaC activation was independent of the proteolytic activity of prostasin in this disease model (Essigke et al. 2022). In 2022, Ehret et al. employed an inducible Pax8 promoter driven, renal-tubule specific *Prss8* conditional knockout mouse to investigate the effects on ENaC activation (Ehret et al. 2022). Surprisingly, prostasin-deficiency did not impair ENaC-mediated Na^+ homeostasis and there were no observable differences in ENaC cleavage between prostasin wildtype and knockout mice, indicating that ENaC was undergoing activation cleavage by an alternative mechanism. Since urinary Na^+/K^+ ratios were not different between wildtype and knockout mice, plasma aldosterone concentrations in mice on various salt diets were measured. On a low sodium diet, the knockout mice had lowered aldosterone levels compared to controls (Ehret et al. 2022). Furthermore, when on a low sodium diet, *Prss8* knockout mice showed an increase in plasma renin activity compared to the controls. Finally, when mice on low sodium diets were treated with losartan, an angiotensin II type 1 receptor blocker, plasma aldosterone concentrations decreased in treated wildtype mice compared to untreated wildtype mice. There was no change in aldosterone in knockout mice with losartan treatment. The results indicated that prostasin was not required for ENaC activation and that upon sodium deprivation, prostasin deficiency resulted in an aldosterone-independent activation of ENaC (Ehret et al. 2022). In a 2023 study, Ehret and colleagues expanded their studies further and reported that matriptase, prostasin, and the α subunit of ENaC are co-expressed in distal tubules in wildtype mice by RNA-scope detection (Ehret et al. 2023). In inducible, renal tubule specific, Pax8 promoter driven matriptase (*St14*)

knockout and *Prss8/St14* double-knockout (DKO) mice they detected an increase of ENaC subunit β and γ protein levels, and an increase of α subunit levels in matriptase knockout kidney lysates while cleavage patterns remained consistent. No differences in ENaC mRNA levels were detected (Ehret et al. 2023). Interestingly, when matriptase knockout mice were on a low sodium diet, there was a significant reduction of the phosphorylated sodium chloride co-transporter (pNCC) in the kidneys, however, the sodium balance remained stable. In *Prss8/St14* DKO mice with dietary sodium deprivation, pNCC protein levels were significantly reduced compared to wildtype controls indicating that loss of both proteases affected NCC activity, however mice still maintained sodium homeostasis. Matriptase knockout and DKO mice on low sodium diets did not display any changes in natriuretic response when treated with diuretics or benzamil, an inhibitor of ENaC, when compared to control mice. Overall, these findings led the authors to conclude that neither prostasin nor matriptase are essential for ENaC activation, but that matriptase is important for regulating levels of ENaC subunit proteins (Ehret et al. 2023). These results warrant further study to fully delineate the catalytic and non-catalytic function(s) of prostasin in the complex molecular mechanisms regulating sodium retention in the kidney.

5.2 Prostasin in hypertension

Several studies have reported an association between genetic variations of the prostasin gene and hypertension (Li et al. 2011; Zhu et al. 2008). Reported functional studies include treatment of the mouse cortical collecting duct cell line (M–1) and of rats with camostat mesilate, a synthetic serine protease inhibitor, to test its effect on sodium transport in M–1 cells and on blood pressure in Dahl salt-sensitive rats (Maekawa et al. 2009). Treatment decreased equivalent current in M–1 cells and inhibited the protease activity of recombinant prostasin in a cell-free system. RNAi-mediated silencing of prostasin also reduced equivalent current in M–1 cells. Oral administration of camostat mesilate to Dahl rats fed a high-salt diet resulted in a significant decrease in blood pressure with elevation of the urinary Na^+/K^+ ratio (Maekawa et al. 2009). The authors proposed that camostat mesilate may have beneficial effects on both hypertension and kidney injury in Dahl salt-sensitive rats due to its prostasin-inhibitory properties. It should be noted that camostat mesilate inhibits a wide array of serine proteases that may contribute to the observed effects of the drug.

5.3 Prostasin in the bladder

Prostasin was detected in the normal bladder epithelial cells of mice and humans (Chen et al. 2009, 2006; List et al. 2007). In mice injected intraperitoneally (i.p.) with bacterial lipopolysaccharide (LPS), bladder prostasin mRNA expression was decreased (Chen et al. 2006). To investigate the potential role of prostasin during bladder inflammation, transgenic mice expressing human prostasin driven by the RSV LTR promoter, were challenged with LPS. Transgenic mice displayed a reduction of LPS-induced nitric oxide synthase (iNOS) mRNA levels compared to control mice. Cyclooxygenase-2 (COX-2), TNF-α, IL-1β, and IL-6 expression levels were not altered (Chen et al. 2006). In an alternative prostasin expression mouse model, the effects of liposome-mediated delivery of prostasin-expressing plasmids into the bladder via transurethral injection were assessed (Chen et al. 2006). Wildtype prostasin caused attenuation of LPS-induced iNOS expression, while not affecting COX-2 or cytokine induction. Catalytically inactive prostasin did not elicit any of these effects and it was therefore proposed that active prostasin participates in bladder inflammatory signaling in this model through attenuation of iNOS expression (Chen et al. 2006). In a follow-up study, the same group showed that the non-steroidal anti-inflammatory drug (NSAID) ibuprofen increased prostasin expression at the mRNA and protein levels in the normal human bladder urothelial cell line UROtsa in a dose and time-dependent manner (Chai et al. 2015). COX-2 expression was also upregulated in the UROtsa cells by ibuprofen but was not required for up-regulating prostasin expression (Chai et al. 2015).

5.4 Prostasin in the liver, pancreas, and role in diabetes

In a 2014 study by Uchimura and colleagues, the focus was to establish the role of prostasin for hepatic insulin sensitivity in high fat diet (HFD)-induced insulin resistance (Uchimura et al. 2014). Prostasin in the livers of wildtype mice on a normal or HFD upon 16 h of fasting was undetectable by western blot analysis. This finding was in agreement with previous reports that prostasin protein was not detectable or detectable at very low levels by IHC under basal conditions (Chen 2023; List et al. 2007). However, refeeding mice with a high-sucrose diet for 16 h led to high levels of prostasin in mice previously on a normal diet and detectable low level prostasin expression in mice previously fed a high fat diet (Uchimura et al. 2014). This observation led the investigators to propose that a HFD triggered the suppression of prostasin

expression by inducing endoplasmic reticulum (ER) stress accompanied by increased TLR4 levels in the liver. Furthermore, treatment with 4-phenyl butyric acid, a chemical chaperone and inhibitor of ER stress, restored hepatic prostasin levels and improved insulin resistance (Uchimura et al. 2014). To further test whether the reduction in hepatic prostasin contributed to the insulin resistance phenotype in HFD fed mice, a genetic mouse model with prostasin deletion in the liver using an albumin promoter-driven Cre-recombinase was generated. The liver-specific prostasin knockout mice displayed a significant elevation in blood glucose, serum insulin, and TLR4 levels under fasting and refeeding conditions when compared to prostasin-expressing control mice under the same conditions (Uchimura et al. 2014). After i.p. injection of LPS, prostasin-deficient mice displayed a marked increase in the inflammatory cytokines IFNγ, IL-1β, and IL-6 in the liver and aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in the serum compared to control mice challenged with LPS. This finding was corroborated in cell culture studies using the human hepatoma cell line HepG2 with siRNA-mediated prostasin knockdown. Additionally, in HepG2 cells, prostasin modulated TLR4 signaling through cleavage of the TLR4 ectodomain, which caused attenuation of inflammatory signaling through TLR4. In an additional *in vivo* experiment, liver-specific prostasin knockout mice were tail vein injected with adenovirus vectors containing human prostasin or LacZ as a control. The LPS-induced inflammatory response observed in prostasin liver knockout mice was decreased after adenovirus mediated re-expression of human prostasin, as measured by mRNA levels of IFNγ, IL-1β, and IL-6 (Uchimura et al. 2014). Re-expression of prostasin in the liver by adenovirus led to decreased hepatic TLR4 and amelioration of insulin resistance due to decreased hepatic TLR4 (Uchimura et al. 2014). Importantly, hepatic prostasin mRNA and protein levels were significantly reduced in diabetic *db/db* mice and the protein levels of TLR4 were reciprocally increased in the livers of *db/db* mice when compared with control mice under refeeding conditions. Forced expression of human prostasin or TLR4 depletion in *db/db* mouse livers reduced insulin resistance. Additionally, prostasin levels in serum of healthy human patients negatively correlated with Body Mass Index (BMI) and homeostasis model assessment-insulin resistance (HOMA-IR). Based on these findings, it was proposed that prostasin regulates TLR4 in the liver, thereby modulating TLR4-mediated inflammatory signaling and consequently hepatic insulin sensitivity (Uchimura et al. 2014). In a 2021 study, the role of prostasin in metabolic regulation and steatosis (fatty liver disease) was explored using a transgenic mouse model overexpressing human prostasin in the liver under the control of an albumin-promoter (LTg-prostasin mice) (Sekine et al. 2021). When LTg-prostasin mice were fed a HFD, glucose tolerance was improved, and after a 14-h fasting period, they displayed attenuated steatosis and insulin resistance compared to wildtype mice (Sekine et al. 2021). In mechanistic studies in HepG2 cells with doxycycline-inducible prostasin expression, prostasin increased activity of the matrix metalloproteinase (MMP)-14, elevated epidermal growth factor receptor (EGFR) phosphorylation, and induced downstream ERK phosphorylation which was proposed to be in an MMP-14 and EGFR dependent manner. *In vivo*, MMP-14 activity and EGFR phosphorylation levels were elevated in the liver of HFD fed LTg-prostasin mice compared to wildtype mice after 14 h fasting conditions (Sekine et al. 2021). Conversely, when liver-specific prostasin knockout mice from the previous study (Uchimura et al. 2014) were fed under the same conditions, MMP-14/EGF/ERK-mediated signaling was decreased (Sekine et al. 2021). It was also reported that human serum prostasin levels were negatively associated with the presence of type 2 diabetes (Sekine et al. 2021). A different conclusion was reached by Bao and colleagues in a large population-based prospective study measuring plasma protein where elevated levels of prostasin were associated with a higher risk for developing diabetes (Bao et al. 2022). Whether the observed changes are causally involved in hepatic lipid metabolism and insulin sensitivity await further investigation.

IHC analysis of mouse pancreatic tissue indicated that prostasin was expressed in islets and exocrine cells where it co-localized with insulin-positive β-cells (Ishii et al. 2023). To study the role of prostasin during insulin secretion, prostasin loss-of-function and gain-of-function mouse models were generated (Ishii et al. 2023). Mice with abrogation of prostasin expression in pancreatic β-cells were generated by crossing transgenic mice carrying a rat insulin promoter-driven Cre-recombinase into mice carrying floxed *Prss8* (Ishii et al. 2023). Mice with β-cell prostasin overexpression were generated by transgenic expression of human prostasin cDNA under the control of the rat insulin II promoter. Pancreatic β-cell prostasin knockout mice developed glucose intolerance with a reduction in glucose-stimulated blood insulin levels compared to wildtype mice (Ishii et al. 2023). Additionally, islets isolated from these prostasin knockout mice had significantly decreased insulin levels following stimulation with high glucose. Conversely, pancreatic islet β-cell isolated from prostasin overexpressing mice showed increased insulin secretion upon stimulation with high glucose. Under fasting and refeeding conditions, isolated islet cells from wildtype mice displayed an increase in prostasin protein levels once refed, and the authors concluded that endogenous prostasin expression was regulated by blood

glucose concentrations. This finding was confirmed in the mouse pancreatic β-cell line MIN-6, showing that glucose regulated prostasin protein levels (Ishii et al. 2023). Interestingly, there were no differences in prostasin mRNA levels at low versus high glucose concentrations. Instead, the glucose-mediated regulation of prostasin protein level was modulated by decreased prostasin protein degradation. Prostasin protein persisted longer upon treatment with the eukaryotic translational elongation inhibitor cycloheximide in cells given high glucose. Moreover, treatment with the proteasome inhibitor MG132 inhibited degradation of prostasin in the presence of glucose suggesting the prostasin turnover is mediated by the proteasome and regulated by glucose (Ishii et al. 2023). Mechanistically, prostasin silencing decreased phosphorylation of EGFR and downstream proteins (AKT, ERK) and reduced insulin secretion with high glucose treatment, whereas prostasin overexpression increased levels of phosphorylated EGFR and insulin secretion under low and high glucose conditions.

5.5 Prostasin in the lung

In the alveolar space in the lungs, sodium transport by ENaC is essential for removal of fluid and represents the main mechanism for alveolar edema resolution (Basset et al. 1987; Hummler et al. 1996; Matthay et al. 1982, 2002; Planès et al. 2010). Planès and colleagues interrogated the role of prostasin-mediated activation of ENaC in the lungs using *in vitro* and *in vivo* models (Planès et al. 2010). A transgenic mouse model using a doxycycline-inducible Cre-loxP system under the control of the surfactant protein C promoter was generated to ablate floxed *Prss8* in the alveolar epithelium (Planès et al. 2010). Cultured alveolar epithelial cells isolated from prostasin knockout mice showed a 40 % decrease in ENaC-mediated sodium currents compared to control cells. Additionally, mice lacking alveolar epithelial prostasin had reduced sodium-driven alveolar fluid clearance (AFC) due to a 48 % decrease in amiloride-sensitive AFC and were less sensitive to adrenergic β_2 receptor agonist treatment. Under stress conditions, using a hydrostatic volume-overload model of pulmonary edema, prostasin-deficient mice displayed an increased fluid accumulation in the alveolar epithelial lining compared to control mice (Planès et al. 2010). This suggested an important role of prostasin in the regulation of ENaC-mediated lung fluid homeostasis.

Prostasin has been implicated in lung diseases including cystic fibrosis (CF) (Myerburg et al. 2008) and idiopathic pulmonary fibrosis (IPF) (Gao et al. 2022; Raghu et al. 2018). In a study by Myerburg and colleagues, it was demonstrated that prostasin is regulated by the airway surface liquid (ASL) absorption in primary human airway epithelial cells (HAECs), allowing for increased activation of ENaC when the ASL volume is high (Myerburg et al. 2008). Furthermore, HAECs cultured from CF patients expressed over 50 % more prostasin protein on the epithelial surface than cells from non-CF donors (Myerburg et al. 2008). This corroborated the finding previously reported in a study where prostasin mRNA levels were increased in primary bronchial epithelial cells from CF patients compared to control donors (Tarran et al. 2006). Additionally, prostasin was detected in human CF patient sputum samples (Myerburg et al. 2008). The authors proposed that dysregulated prostasin in CF epithelia can cause excessive activation of ENaC that contributes to the Na^+ hyperabsorption characteristic of CF airway disease (Myerburg et al. 2008). Prostasin was also detected in the human nasal epithelial cell line JME/CF15 that is homozygous for the ΔF508 cystic fibrosis mutation and prostasin silencing by siRNA caused reductions in amiloride-sensitive and basal sodium currents (Tong et al. 2004). Prostasin was present in its active form on the apical surface of wildtype and CF bronchial epithelial cells (Nimishakavi et al. 2012). A study by Raghu and colleagues aimed to identify circulating biomarkers of IPF in a prospective case-controlled study and found that patients with IPF had increased prostasin serum levels relative to controls (Raghu et al. 2018). In a study by Gao et al., differentially expressed genes in explanted lung samples from IPF patients and control individuals were queried and prostasin levels were significantly decreased in IPF samples (Gao et al. 2022). The increased levels of serum prostasin (Raghu et al. 2018) and decreased levels of lung prostasin observed in IPF samples (Gao et al. 2022) warrant further investigation into the utility of prostasin as a biomarker for IPF and the functional implication of the protease in this disease.

6 Roles of prostasin and testisin in cancer

Both GPI-anchored serine proteases covered in this review have been implicated in cancer. In this section, expression and prognosis data, proposed molecular mechanisms, and potential uses in treatment are summarized.

6.1 GPI-anchored serine protease expression is lost in multiple cancer types

Over the last two decades, studies from multiple groups have reported similar findings regarding prostasin expression

during cancer progression. Thus, a loss of prostasin transcripts and/or protein during malignant progression in a wide array of cancer types were reported. A 2001 study by Chen and colleagues reported that prostasin was highly expressed in the normal human prostate epithelia, but was down-regulated in high-grade and invasive prostate tumors (Chen et al. 2001a). Additional prostate cancer studies found that expression levels were inversely correlated with tumor grade (Takahashi et al. 2002, 2003). Similar findings in patient samples were reported in bladder transitional cell carcinomas (Chen et al. 2009), oral squamous cell carcinoma (OSCC) (Yamamoto et al. 2021), and esophageal squamous cell carcinoma (ESCC) (Bao et al. 2016b). In ESCC, high epithelial prostasin expression in ESCC significantly correlated with longer overall and disease-free survival (Bao et al. 2016b).

A shared mechanism underlying the loss of prostasin expression in many of these cancers was identified as hypermethylation of the *PRSS8* promoter region in both tumor samples and cancer cell lines that could be reversed by chemical demethylation with restoration of expression. This epigenetic gene regulation mechanism of prostasin expression was first described in breast cancer cell lines where the methylation status of the promoter and first exon were examined across multiple prostasin-expressing and non-expressing cell lines by Southern blot analysis. Prostasin mRNA and protein was expressed in normal human mammary epithelial cells (NHMEC) and in the low invasive cell lines MCF7 and MDA-MB-453 but not in the highly invasive and metastatic breast carcinoma lines MDA-MB-231 and MDA-MB-435s (Chen and Chai 2002). NHMEC, MCF7, and MDA-MB-453 cell lines had a promoter methylation pattern consistent with prostasin expression, while the invasive prostasin-silenced cells MDA-MB-231 and MDA-MB-435s had a pattern indicative of gene silencing (Chen and Chai 2002). Upon demethylation in combination with histone deacetylase (HDAC) inhibition, prostasin mRNA expression was restored (Chen and Chai 2002). Similar findings were later described in prostate cancer (Chen et al. 2004), ESCC (Bao et al. 2016b), and bladder cancer (Chen et al. 2009). Urothelial carcinoma cells negative for prostasin mRNA showed consistent methylation patterns of the CpG island region −96 in the prostasin promoter and demethylation restored prostasin expression of protein, further supporting promoter methylation as an important epigenetic regulatory mechanism of prostasin in cancer (Chen et al. 2009). At the transcriptional level, nerve growth factor (NGF), that had previously been reported to reduce malignant phenotypes, induced prostasin mRNA expression in prostate cancer cells lines (Chen et al. 2004; Sigala et al. 1999, 2002).

Testisin was detected in the cytoplasm and on the plasma membrane of cells in normal testicular tissue; however, in germ cell-derived testicular tumors, there was a loss of detectable testisin mRNA (Hooper et al. 1999, 2000). In agreement, a recent study assessing testisin protein expression in healthy human testicular tissues and testicular germ cell tumor concluded that testisin is a biomarker of healthy testicular tissue (Krasic et al. 2023). Similarly to prostasin, hypermethylation of the testisin promoter suppressed expression in testicular cancer cell lines and analysis of testisin mRNA levels in human patient tissue samples revealed that testisin silencing in testicular germ cell tumors was associated with hypermethylation compared to adjacent normal tissue (Manton et al. 2005).

6.2 Tumor suppressive functions of prostasin and testisin

The observations that prostasin expression is suppressed during progression of multiple cancers prompted investigators into identifying possible causal consequences and potential tumor suppressive functions of prostasin. In cultured invasive prostate cancer cell lines that had lost prostasin expression, forced re-expression of prostasin by transfection significantly reduced invasive capability compared to controls (Chen et al. 2001a). Molecular mechanisms underlying the suppressive role of prostasin in cellular invasion were interrogated by re-expression of human wildtype or catalytically inactive prostasin in PC-3 cells which both led to reduced levels of EGFR protein and decreased levels of downstream phosphorylated Erk1/2 protein (Chen et al. 2007). Additionally, mRNA of the epithelial-to-mesenchymal (EMT) associated transcriptional repressor Slug was detected in cells re-expressing wildtype prostasin, but not in cells expressing inactive prostasin. The mRNA transcripts of urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), COX-2, and iNOS decreased upon expression of either wildtype or catalytically inactive prostasin in PC-3 cells. Catalytically inactive prostasin upregulated matriptase and the epithelial differentiation marker E-cadherin at both the mRNA and protein level in PC-3 cells, while wildtype prostasin led to a significant but comparatively lesser increase of E-cadherin mRNA and protein (Chen et al. 2007). In bladder cancer, re-expression of either wildtype or catalytically inactive prostasin in bladder cancer cells increased expression of E-cadherin (Chen et al. 2009).

In breast cancer, similar suppressive effects of re-expression of prostasin on migration/invasion were observed (Chen and Chai 2002). In breast cancer cell lines and in patient invasive ductal carcinomas samples, prostasin and matriptase were co-expressed and cells/tumors lacking prostasin and matriptase expression displayed a more

mesenchymal cellular phenotype with loss of E-cadherin (Bergum et al. 2012). In molecular and cellular studies of breast cancer, prostasin zymogen was cleaved and activated by the type II transmembrane serine protease TMPRSS13 (Murray et al. 2020). TMPRSS13 has tumor-promoting functions in breast cancer as it promoted cancer cell survival, proliferation, invasion, and resistance to chemotherapy *in vitro*. Moreover, TMPRSS13 promoted cancer progression by accelerated tumor development, growth, proliferation, and increased incidence of lung metastasis in the transgenic mouse mammary tumor virus (MMTV) Polyoma middle T (PymT) model when compared to TMPRSS13 deficient mice (Murray et al. 2020). Additionally, TMPRSS13 silencing in triple-negative breast cancer cells led to increased levels of endogenous prostasin protein, and plasmid-mediated prostasin overexpression in HCC1937 cells caused decreased cell survival relative to control cells. It is possible that regulation of prostasin levels by TMPRSS13 contributes to the progression of breast cancer (Murray et al. 2020).

Transgenic overexpression of wildtype prostasin in human non-small cell lung carcinoma (NSCLC) cells led to decreased proliferation and invasion, accompanied by an increase in E-cadherin and decrease in N-cadherin protein, suggesting a potential role of prostasin for EMT suppression in these cells (Ma et al. 2017). Furthermore, transgenic prostasin expression in lung adenocarcinoma cells led to significantly lower tumor volumes and weights compared to control cells (Ma et al. 2017). Mechanistically, prostasin overexpression suppressed the activity of the tumor promoting JAK/STAT3 signaling pathway in NSCLC cells (Ma et al. 2017). Chen and colleagues established a CRISPR/Cas9 prostasin knock-out subline of the NSCLC cell line Calu-3, followed by re-expression of wildtype, catalytically inactive, or membrane-anchor-free secreted prostasin (Chen et al. 2021). It was demonstrated that without any external stimuli, the immune checkpoint therapy target programmed death-ligand 1 (PD-L1) protein expression was induced in a null background by wild-type prostasin, but not catalytically dead or secreted prostasin suggesting that the prostasin-mediated up-regulation of PD-L1 requires its protease activity, as well as the membrane anchorage. Upon interferon (IFN)-γ-induction, PD-L1 was increased in all Calu-3 sublines with the highest increase in wildtype prostasin expressing cells. EGF/EGFR, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) participated in the prostasin-mediated up-regulation of PD-L1 expression. A Gene Set Enrichment Analysis of patient lung tumors in The Cancer Genome Atlas (TCGA) database revealed that prostasin and PD-L1 regulate common signaling pathways in both lung squamous cell carcinoma and adenocarcinoma (Chen et al. 2021). *In vivo* studies on the potential effects of prostasin for immune evasion have not yet been published.

In a 2018 study, Yamamoto and colleagues suggested that inhibition of prostasin activity by HAI-2 plays a promoting role in oral squamous cell carcinoma (OSCC) (Yamamoto et al. 2018). When *SPINT2* was abrogated in OSCC cell lines using CRISPR/Cas9 gene editing, it led to impaired proliferation, invasion, and anchorage-independent growth. Interestingly, both prostasin mRNA and protein level were elevated in HAI-2 deficient cell lines. It should be noted that it was not determined whether the observed increase of prostasin was the zymogen or active form. Functionally, prostasin silencing by siRNA in HAI-2 deficient cells recovered migratory and invasive capabilities with no detectable effect in HAI-2 sufficient cells leading the authors to conclude that prostasin was responsible for the invasion suppression caused by HAI-2 deficiency. Cellular proliferation was not recovered by prostasin silencing indicating that prostasin is primarily involved in the regulation of invasiveness upon deletion of HAI-2 in this *in vitro* model (Yamamoto et al. 2018). *In vivo*, HAI-2 deficient SAS cells formed significantly smaller subcutaneous tumors than control cells, suggesting that HAI-2 may have tumor promoting functions in OSCC (Yamamoto et al. 2018). IHC analysis of OSCC tumors showed that HAI-2 expression increased along with neoplastic progression with intense immunoreactivities in invasive OSCC cells. HAI-2 staining was observed in cancerous tissue, whereas prostasin was primarily expressed in differentiated keratinocytes in OSCC and adjacent oral epithelium tissue samples (Yamamoto et al. 2018). As observed in several other cancers, loss of E-cadherin and gain of the mesenchymal marker vimentin was observed in high grade OSCC samples. In cell culture, transgenic overexpression of prostasin in OSCC cell lines suppressed cellular migration and proliferation whereas silencing of prostasin enhanced proliferation (Yamamoto et al. 2021). A potential mechanistic role for prostasin during ESCC tumor suppression pointed to regulation of proteins involved in the cell cycle (Cyclin D1) and EMT (E-Cadherin, Snail, and Twist) (Bao et al. 2016b).

In colorectal cancer (CRC), prostasin expression was significantly reduced in colorectal adenomas and adenocarcinomas and low levels correlated with poor differentiation and shorter survival in CRC patients (Bao et al. 2016a). Transgenic overexpression of wildtype prostasin in CRC cell lines with low endogenous levels of prostasin reduced proliferation and reduced subcutaneous tumor growth accompanied by reduced protein levels of Sphingosine Kinase 1 (Sphk1), Sphingosine-1-Phosphate (S1P) receptor, phosphorylated (p-) STAT3, and *p*-AKT (Bao et al. 2016a). The Sphk1/S1P/Stat3/Akt signaling pathway was previously

linked to colitis-associated colorectal cancer (Liang et al. 2013). Inversely, knockdown of prostasin in CRC cell lines with high levels of endogenous prostasin, led to increased proliferation and Sphk1/S1P/Stat3/Akt signaling (Bao et al. 2016a). In a follow-up study, a conditional knockout mouse model with floxed *Prss8* alleles and transgenic Cre-recombinase that directs deletion of *Prss8* to the colon (*Prss8*$^{fl/fl}$, *p*-Villin-Cre^{+}) was generated and characterized (Bao et al. 2019). Mice lacking colonic prostasin exhibited a high incidence of hyperplasia in the intestine and colorectal inflammation by three months of age and a high incidence of adenomas by 6 and 9 months of age. Functionally, prostasin deficient intestines showed more proliferative cells that migrated faster to the upper villi from crypts compared to wildtype mice. Additionally, Cyclin D1 and β-catenin were elevated in small intestine and colon with prostasin deficiency. Stable re-expression of prostasin in poorly differentiated human RKO colorectal cancer cells inhibited cellular invasion, migration, colony formation, and tumor sphere formation *in vitro* and decreased primary tumor weight and volume *in vivo* (Bao et al. 2019). Furthermore, tail-vein injection of these cells yielded a decrease in liver metastases (cancer cell colonization) and colony formation in lungs compared to control cells. Using shRNA-mediated prostasin knockdown in human CRC cells that endogenously express prostasin caused formation of larger tumors, and an increased liver colonialization upon tail-vein injection compared to control cells. Additional cellular studies revealed that re-expression of prostasin inhibited cellular invasion, migration, colony formation, and tumor sphere formation in RKO cells compared to controls (Bao et al. 2019). Prostasin overexpression decreased levels of EMT-associated proteins Twist and Snail, while increasing E-cadherin protein levels, while prostasin knockdown in CRC cells led to an increase in proteins associated with the Wnt/β-catenin pathways (Bao et al. 2019). As stem cell features are important for initiation of tumors and metastasis (Lee et al. 2017), the effects of prostasin levels on the stemness-related proteins CD44, LGR5, OCT4, and KLF4 were examined in CRC cells (Bao et al. 2019). Silencing of prostasin increased protein levels of these stemness markers, whereas re-expression of prostasin in RKO cells decreased these markers, indicating an inverse correlation between prostasin levels and stemness in colorectal cancer cell lines. Moreover, mRNA expression data from TCGA revealed that prostasin transcript levels were reduced in colorectal adenocarcinomas compared to normal tissues, which was confirmed at the protein level by IHC. Loss of prostasin was associated with increased β-catenin, LGR5, c-myc, and Twist staining (Bao et al. 2019). In sum, prostasin levels were decreased in colorectal cancer and reduced expression was causally associated with tumor progression, potentially through regulation of EMT, Wnt/β-catenin signaling, Sphk1/S1P/STAT3/AKT signaling, and stem cell pathways.

In testicular cancer, re-expression of testisin in the non-expressing Tera-2 testicular cancer line led to impaired anchorage independent growth relative to empty vector and parental controls *in vitro* and to lower tumor burden in a murine orthotopic transplantation model compared to control cells, indicative of a tumor suppressive role of testisin in testicular cancer (Manton et al. 2005).

6.3 Elevated levels of prostasin and testisin in ovarian cancer

While the majority of cancers studied display loss of prostasin expression during progression as described above, increased prostasin expression in epithelial ovarian cancer was reported (Mok et al. 2001). Furthermore, prostasin levels in serum samples were higher levels in sera from ovarian cancer patients relative to controls, leading the authors to propose serum prostasin as a candidate biomarker for detection of ovarian cancer (Mok et al. 2001). Other groups also reported increases of prostasin in ovarian cancer tissues and serum samples (Costa et al. 2009; Tamir et al. 2016). Functionally, the role of prostasin in ovarian cancer chemotherapy resistance was explored based on the observation that low prostasin levels correlated with high mRNA levels of excision repair cross-complementing 1 (ERCC1), which is associated with chemoresistance (Yan et al. 2014). Forced overexpression of prostasin in the endogenous prostasin expressing paclitaxel-resistant ovarian cancer cell line O432-RP resulted in an increase in sensitivity to paclitaxel treatment *in vitro* compared to control cells. Conversely, siRNA-mediated prostasin knockdown rendered the cells less susceptible to paclitaxel treatment compared to controls. Prostasin overexpressing cells gave rise to smaller subcutaneous xenograft tumors that were more responsive to paclitaxel compared to controls (Yan et al. 2014). Mechanistically it was proposed that prostasin acts through the CASP/P21-activated protein kinase (PAK2)-p34 pathway, and thereafter JNK/c-jun and MLCK/Actin signaling pathways to regulate cell survival and chemoresistance. In a follow-up study, prostatic secretory protein 94 (PSP94), a potential biomarker of ovarian cancer and an upstream signaling mediator of prostasin, was examined (Ma et al. 2014). O432-RP cells over-expressing PSP94 showed increased prostasin protein levels, and the authors proposed that PSP94 acts as a regulator upstream of prostasin (Ma et al. 2014). PSP94 was found to be overexpressed in ovarian cancer cell lines and patients, and its expression levels significantly correlated

with prostasin levels (Ma et al. 2014). Cai and colleagues observed that prostasin was upregulated in multiple ovarian cancer cell lines, and sterol regulatory element binding factor 2 (SREBF2) was identified as a potential regulator of prostasin expression (Cai et al. 2021). The functional relationship between SREBF2 and prostasin was verified by luciferase reporter assay and chromatin immunoprecipitation (ChIP) experiments. Interaction between the sodium channel epithelial 1 alpha unit (SCNN1A), a proposed tumor promoter, and prostasin was also identified and it was proposed that SREBF2 activates the prostasin/SCNN1A axis to accelerate cell proliferation, migration, and EMT in ovarian cancer (Cai et al. 2021).

Testisin transcript was abundant in ovarian carcinoma with significantly higher levels in advanced stage (stage 2 or 3) disease compared with early stage disease (stage 1), but was not detected in normal ovary (Shigemasa et al. 2000). Subcutaneous implantation in mice of ovarian cancer cells with stable overexpression of testisin yielded significantly higher tumor volume compared to control cells, whereas transient knockdown of testisin induced apoptosis in cultured cells (Tang et al. 2005). A study aiming to identify metastasis-associated genes in ovarian serous papillary carcinomas (OSPC), the most common histological type of ovarian carcinoma, used tissue samples with hierarchic cluster gene expression analysis. While testisin was not detected in normal ovarian tissue (Shigemasa et al. 2000), and testisin was upregulated in 80–90 % of stage II and III ovarian tumors (Shigemasa et al. 2000), there was a reported decrease in testisin levels in metastases compared to primary tumors (Bignotti et al. 2007). Therefore, loss of testisin in late-stage ovarian cancer may be functionally important, which was explored experimentally by Conway and colleagues (Conway et al. 2019). Athymic nude mice were i.p. injected with metastatic clear cell carcinoma ES-2 cells, which have low to negligible levels of endogenous testisin. ES-2 cells with lentiviral-mediated over-expression of wild-type testisin displayed impaired intraperitoneal tumor seeding, colonization, and ascites formation compared to controls, whereas inactive testisin had no significant effect (Conway et al. 2019). Additionally, metastatic tumor burden at the mesenteric arteries and diaphragm was decreased and fewer metastatic foci were observed on livers compared to control mice or mice with tumors from ES-2 cells expressing inactive testisin. Furthermore, testisin localized to the cell surface and caused a loss of cell surface PAR-2, and the diminished PAR-2 signaling led to decreased levels of phosphorylated ERK 1/2 and lower IL-8 mRNA levels (Conway et al. 2019). To further investigate the role of testisin in ovarian tumor metastasis suppression, mRNA and protein levels of angiopoietin-2 (ANG2) and angiopoietin-like 4 (ANGPLT4), both of which promote vascular leakiness and edema, were measured in testisin overexpressing ES-2 cells. Interestingly, cells overexpressing active testisin had lower levels of ANG2 and ANGPLT4 mRNA and protein. In the xenograft model, clarified ascites from mice injected with wildtype testisin expressing ES-2 cells revealed a reduction of ANG2 and ANGPTL4 protein compared to cells expressing catalytically inactive testisin or vector control cells (Conway et al. 2019). Additionally, when overexpressing cells were resected from the diaphragm in mice, there was a reduction in ANG2 and ANGPTL4 mRNA compared to control or inactive testisin ES-2 tumors. The authors proposed a model where testisin activates PAR-2 to antagonize proangiogenic angiopoietins which can modulate vascular permeability and accumulation of ascites associated with ovarian tumor metastasis (Conway et al. 2019).

6.4 Increased testisin expression in gastric and cervical cancer

A study examining gene signatures to identify and validate therapeutic and prognostic targets in gastric cancer utilized comparative single-cell RNA-seq data of gastric cancer and surrounding normal epithelial cells (Li et al. 2022). Genes highly correlated with copy number variations (CNVs) through cross-validation of prognosis and risk management with RNA-seq data from the Gene Expression Omnibus (GEO) and TCGA databases, revealed that low testisin expression correlated with prolonged patient survival. Additionally, *PRSS21* was found to be positively associated with immune cell infiltration (Li et al. 2022).

A high level of testisin mRNA in the cervical adenocarcinoma HeLa cell line was detected when compared to a panel of tumor cell lines (Tang et al. 2005), and in patient samples, testisin was overexpressed in most cervical tumor samples (Yeom et al. 2010). Functionally, knockdown of testisin in HeLa cells led to decreased colony formation in soft agar and caused increased apoptosis compared to controls (Tang et al. 2005). In a yeast two-hybrid study, maspin, a non-inhibitory serpin which has been proposed to have tumor suppressive properties (Liu et al. 2004), was identified as a testisin binding partner (Yeom et al. 2010). It was confirmed by co-immunoprecipitation that endogenous maspin interacts with endogenous testisin using proteins extracted from HeLa cell lysates. Based on the previous findings that maspin sensitizes cancer cells to apoptosis (Liu et al. 2004) and that testisin reduces apoptosis (Tang et al. 2005), the effects of testisin on maspin-mediated increases in caspase-3 activity and cell death were interrogated. Maspin increased caspase-3 activity in testisin-silenced HeLa and

cervical epidermoid carcinoma MS-751 cells, however, in the presence of testisin, maspin did not display this effect (Yeom et al. 2010). Similarly, testisin impaired maspin-induced suppression of cancer cell invasiveness in HeLa and MS-751 cells. Furthermore, testisin knockdown significantly increased sensitivity to doxorubicin in HeLa cells. Together, these studies suggest that testisin acts as a tumor promoter in cervical cancer, and mechanistically, suppression of maspin functions may be involved.

6.5 Testistin-targeted treatment of ovarian and cervical cancer

Invasive and metastatic tumors are associated with dysregulation of proteases including multiple cell-surface serine proteases (Martin and List 2019; Pawar et al. 2019). Late-stage ovarian cancer patients currently have limited treatment options and therefore a method to selectively target cancer cells expressing membrane-anchored serine proteases in ovarian cancer was developed (Martin et al. 2015). Several modified anthrax toxins were previously engineered to be specifically cleaved by serine- or metallo-proteases, including uPA or MMPs for activation, internalization, and toxicity to cells (Liu et al. 2001, 2000, 2005). These modified toxins have been used in multiple cancer types to selectively target cancer cells with high expression of the chosen protease (reviewed in Alfano et al. 2008; Frankel et al. 2002; Liu et al. 2003). Using a protein C inhibitor derived sequence (PCIS), a sequence derived from SERPINA5 known to be cleaved by testisin in place of the native anthrax toxin protective antigen (PrAg) activation sequence, which is cleaved by ubiquitously expressed furin, PrAg-PCIS could be cleaved *in vitro* by testisin (Martin et al. 2015). The ovarian cancer cell lines ES-2, OVCAR3, SKOV-3, and NCI/ADR-Res showed a dose-dependent cytotoxicity when co-incubated with PrAg-PCIS and the chimeric anthrax toxin FP59, a fusion of lethal factor (LF) with the catalytic domain of *Pseudomonas aeruginosa* exotoxin A, which inhibits protein synthesis through ADP-ribosylation of eukaryotic elongation factor-2. Cell-surface proteolytic cleavage of the fusion protein allowed for cytosolic delivery of the toxin, led to cell death through ADP-ribosylation and inhibition of protein synthesis. This study provided proof of concept and rationale for developing newer generations of prodrug toxins to selectively target late-stage cancer (Martin et al. 2015). Later, additional prodrug toxins designed to be cleaved on the cell surface to facilitate delivery of a cytotoxic cargo were developed. These toxins were designed based on the eight amino acid furin activation sequence of anthrax PrAg which was mutated to the zymogen activation sequences of prostasin (PAS), testisin (TAS), and uPA (UAS) (Duru et al. 2022). The PAS zymogen activation prodrug toxin was cleaved by testisin as well as by matriptase and hepsin and was selected for further studies (Duru et al. 2022). Importantly, patients with various histological types and stages showed a significant increase of testisin, matriptase, and hepsin mRNA compared to normal tissue. Ovarian cancer cell lines ES-2 and NCI/ADR-Res were susceptible to treatment with PAS:LF and PAS:FP59 relative to cargo toxin alone. Pre-treatment of cells with the synthetic serine protease inhibitor AEBSF resulted in attenuation of toxicity in both cell lines. Due to toxicity of FP59 *in vivo*, PAS:LF was selected for further studies. *In vitro* studies using primary patient-derived tumors from ascites showed decreased viability with PAS:LF treatment compared to untreated or LF treatment controls. In an orthotopic transplantation mouse model using metastatic ES-2 and NCI/ADR-Res cells i.p. injected into athymic nude mice, PAS:LF treated mice had reduced tumor burden and prolonged survival compared to controls. Additionally, immunodeficient NOD rag gamma (NRG) mice implanted with an ovarian patient derived xenograft (PDX) tumor, showed a reduced tumor burden when treated with PAS:LF compared to LF alone. This treatment was well tolerated in mice, as evidenced by no significant differences in blood count, blood chemistry, or changes in liver, kidney, and colon histology between treated and control groups (Duru et al. 2022). The use of the modified, testisin-activated anthrax toxin (PrAg-PCIS), as described above, was also explored in the treatment of cervical cancer cells expressing high levels of testisin. This treatment was cytotoxically effective in HeLa cells expressing endogenous testisin, whereas HeLa cells with siRNA-mediated testisin knockdown were resistant to treatment. *In vivo* studies with subcutaneous HeLa xenografts showed reduced tumor size, weight, and tumor cell proliferation in mice treated with PrAg-PCIS compared to controls (Martin et al. 2015).

7 Conclusions and perspectives

In the past three decades, close to two hundred studies on prostasin and testisin have been published. Our knowledge about their biochemical and cellular properties has expanded, while also exposing gaps in our understanding of their physiological functions in the context of complex *in vivo* systems. Studies using *in vitro* models have provided many clues into protease function; however, not all substrates/pathways identified in simple systems have directly translated to animals. An example is ENaC control where the activation of the sodium-channel by prostasin-mediated proteolytic cleavage was identified in cell culture models,

whereas later studies in mice demonstrated that prostasin (or matriptase) is not required for direct proteolytic activation of ENaC. Using knock-out and knock-in genetic mouse models, the dramatic difference between the complete loss of prostasin (perinatal death) versus loss of its proteolytic activity only (viable with mild phenotypes) have demonstrated that essential non-proteolytic functions of prostasin exist. Prostasin may function as a scaffolding protein to modulate the functions of target proteins with modulation of ENaC and matriptase activation/activity being the most studied examples. Prostasin is unique because it has proteolytic activity yet can also act as a non-enzymatic modulator of biological processes under some conditions. How prostasin exerts these effects remain largely unknown and could represent a hitherto unexplored biochemical mechanism.

There are likely many more prostasin effector proteins and pathways to be discovered, and when designing unbiased screening/omics approaches, the non-proteolytic functions of prostasin should be considered. In addition to using proteomics to characterize the proteolytic cleavage landscape within a biological system, using techniques that can detect changes in signaling pathways such as phosphorproteomics and transcriptomics may be valuable in identifying novel prostasin functions. It is important to emphasize that many published studies so far identifying prostasin-mediated pathways have been performed in cell-free systems, in cell-culture, or in xenograft models using human cell lines. Verification of *in vivo* functions and candidate substrates/pathways in whole organisms are pivotal for both basic science and translational advances. The complexity of this task is exemplified by the pleiotropic functions of prostasin in the endocrine-metabolic system where prostasin is protective against glucose tolerance and hepatic steatosis shown by both loss and gain of function mouse studies. Mechanistically, it was proposed that prostasin mediates regulation of TLR4 by ectodomain cleavage and shedding from the cell surface, as well as activation of MMP-14 and EGFR; however, at this time, it is not known whether the proteolytic activity of prostasin is required, and if so, if prostasin directly cleaves these substrates/effectors *in vivo.*

While the majority of prostasin studies concluded that the protease is necessary for tissue homeostasis, and that loss causes impairment of functions in multiple organs, dysregulation leading to increased levels of prostasin may also play a role in human disease. Thus, epidermal overexpression of prostasin in mice causes hyperplasia that is independent of its proteolytic activity and dependent on matriptase-mediated PAR-2 activation. Therefore, the use of matriptase inhibitors, several types of which have been generated and published, might be suitable. This underscores the importance of identifying prostasin-mediated effectors that are targetable. In both cystic and idiopathic pulmonary fibrosis, proteases play a significant role in disease progression by damaging lung tissue, contributing to inflammation, and ENaC-mediated airway surface dehydration. While prostasin levels are increased in these lung diseases, it has not yet been confirmed experimentally that this contributes to disease severity and progression. Protease inhibition in lung fibrosis as a therapeutic option has mainly focused on neutrophil serine proteases, but if prostasin proves to play a causal role, it may be a potentially useful target.

Testisin, with its narrow expression profile, has comparatively been less studied, where initial studies focused on its essential functions in male fertility. However, in recent years, studies have expanded to vascular biology and ovarian cancer where its proteolytic activity is critical. It was discovered that testisin is involved in angiogenesis and re-establishment of microvascular endothelial barrier by promotion of inter-junctional adhesions, modulation of the PAR-2 proangiogenic signaling pathway, and directs deposition of fibrin to facilitate endothelial cell migration. Advances in these studies may provide avenues for augmenting testisin-mediated pathways to promote vascular homeostasis with implications for multiple diseases including cardiovascular disease, diabetic wound healing, and ischemia.

In cancer treatment strategies, the focus on proteases including serine proteases, has been on the development of inhibitors to target the proteolytic activity of tumor-promoting proteases. In most cancer types studied, prostasin has tumor and/or metastasis suppressing functions, and expression is frequently lost due to promoter methylation. Therefore, from a cancer treatment perspective, direct targeting of prostasin by inhibitors is not a viable option. As an alternative strategy, re-constitution of prostasin expression can be considered by delivery of exogenous protein, mRNA, or DNA. Additionally, novel techniques for editing endogenous genes by CRISPR-based approaches for targeted DNA demethylation of specific loci have emerged where activation of several genes including the MMP-2 gene was achieved in methylated and transcriptionally silenced human cells, providing a proof of principle (Xu et al. 2016). Although the approach of re-establishing tumor suppressor function in tumors as a therapeutic option is theoretically possible, technical challenges and safety precautions complicate use in patients in the near future. Therefore, understanding the mechanisms of action of prostasin as a tumor suppressor protease are important for developing therapeutic strategies to target downstream pathways e.g., with small molecule

drugs. One example is the colorectal cancer study in mice with intestinal prostasin deficiency where activation of the Wnt/β-catenin was reported. Several drugs targeting this pathway have been developed and clinical trials for Wnt/β-catenin signaling inhibitors in colorectal cancer are ongoing. Similar strategies may be applicable to chronic inflammatory illnesses, including inflammatory bowel disease, where loss of prostasin exacerbated symptoms. Additionally, bladder transgenic overexpression of prostasin in mice attenuated inflammatory cytokine signaling. In the bladder, local delivery of liposomes containing a prostasin-expressing plasmid had similar effects as genomic transgene expression. The bladder might be suitable site for direct installation of prostasin in human patients since intravesical drug delivery is already commonly used, not only for delivery of chemotherapy drugs in cancer, but also for delivery of macromolecules such as heparin and hyaluronic acid for treatment of interstitial cystitis. Testisin represents a promising target in select cancers, such as ovarian and cervical, due to its *de novo* expression in these malignancies. Options include protease inhibitors selective for testisin or, as discussed above, harnessing testisin-mediated activation of engineered bacterial toxins to guide killing of cancer cells while limiting damage to normal cells.

In conclusion, the GPI-anchored serine proteases contribute to many facets of biology showcasing their diverse roles across physiology and disease. Further understanding of the mechanisms of action, regulation and dysregulation, and downstream pathways of these proteases is crucial for the development of novel targeted therapeutics, with the hope of improving human health and patient outcomes in the future.

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