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REVIEW ARTICLE

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Impact of heparanase-2 (Hpa2) on cancer and inflammation: Advances and paradigms

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Abstract

HPSE2, the gene-encoding heparanase 2 (Hpa2), is mutated in urofacial syndrome (UFS), a rare autosomal recessive congenital disease attributed to peripheral neuropathy. Hpa2 lacks intrinsic heparan sulfate (HS)-degrading activity, the hallmark of heparanase (Hpa1), yet it exhibits a high affinity toward HS, thereby inhibiting Hpa1 enzymatic activity. Hpa2 regulates selected genes that promote normal differentiation, tissue homeostasis, and endoplasmic reticulum (ER) stress, resulting in antitumor, antiangiogenic, and anti-inflammatory effects. Importantly, stress conditions induce the expression of Hpa2, thus establishing a feedback loop, where Hpa2 enhances ER stress which, in turn, induces Hpa2 expression. In most cases, cancer patients who retain high levels of Hpa2 survive longer than patients bearing Hpa2-low tumors. Experimentally, overexpression of Hpa2 attenuates the growth of tumor xenografts, whereas Hpa2 gene silencing results in aggressive tumors. Studies applying conditional Hpa2 knockout (cHpa2-KO) mice revealed an essential involvement of Hpa2 contributed by the host in protecting against cancer and inflammation. This was best reflected by the distorted morphology of the Hpa2-null pancreas, including massive infiltration of immune cells, acinar to adipocyte trans-differentiation, and acinar to ductal metaplasia. Moreover, orthotopic inoculation of pancreatic ductal adenocarcinoma (PDAC) cells into the pancreas of Hpa2-null vs. wild-type mice yielded tumors that were by far more aggressive. Likewise, intravenous inoculation of cancer cells into cHpa2-KO mice resulted in a dramatically increased lung colonization reflecting the involvement of Hpa2 in restricting the formation of a premetastatic niche. Elucidating Hpa2 structure–activity-relationships is expected to support the development of Hpa2-based therapies against cancer and inflammation.

KEYWORDS

ER stress, heparanase, heparanase-2, inflammation, metastatic niche, pancreatic cancer, protective effects, tissue homeostasis, tumor microenvironment, tumor suppressor

Abbreviations: AAT, acinar-to-adipocytes-transdifferentiation; AMPK, AMP-activated protein kinase; AP, acute pancreatitis; ECM, extracellular matrix; ER, endoplasmic reticulum; HBD, heparin binding domain; Hpa1, heparanase; Hpa2, heparanase-2; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; LOX, lysyl oxidase; NLS, nuclear localization sequence; NUC, nuclear; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; UFS, urofacial syndrome; VEGF, vascular endothelial growth factor; WT, wild type.

1 | INTRODUCTION

1.1 | Heparanase-1 (Hpa1, HPSE1)

Mammalian cells express a single functional heparanase (= Hpa1, HPSE), an endoglucuronidase enzyme that degrades heparan sulfate (HS).[1,2] Cleavage of HS by Hpa1 contributes to disassembly of the ECM, thereby facilitating cancer metastasis and inflammation.[3–5] Compelling evidence strongly implies that Hpa1 functions to promote essentially all aspects of the tumorigenic process, namely tumor initiation, angiogenesis, growth, metastasis, and chemo-resistance.[2,6–11] A key venue by which Hpa1 accomplishes its multiple effects on cells and tissues is by regulating the bioavailability of HS-bound growth factors, chemokines, and cytokines that are stored in the ECM and glycocalyx, thereby priming, among other effects, the tumor microenvironment.[12–14] In this way, Hpa1 mediates tumor-host crosstalk and promotes basic cellular processes (i.e., gene transcription, signal transduction, immune responses, exosome formation, autophagy, and DNA damage)[15–18] that together orchestrate tissue remodeling, including acceleration of cancer and inflammation.[19]

1.2 | Heparanase-2 (Hpa2)

Analysis of human genomic DNA led researchers to conclude that the *HPSE1* gene is unique and that the existence of related proteins is unlikely. Based on amino acid sequence, McKenzie and colleagues nonetheless reported the cloning of the Hpa1 homolog termed heparanase-2 (Hpa2).[20] The full-length *HPSE2* gene (Genbank accession AF282887) consists of 2353 bp encoding a protein of 592 amino acids; Alignment of the coding region of Hpa1 and Hpa2 reveals an overall identity of 40% and sequence resemblance of 59%. Residues critical for Hpa1 enzymatic activity (Glu225 and Glu343) are conserved in the full-length Hpa2 at Glu260 and Glu381.[16] EMBL protein family analysis (http://pfam.xfam.org) predicts an approximately 200 amino acid glycoside hydrolase (family 79) motif spanning exons 3 to 9. Moreover, the high proportion of basic amino acids (16%), particularly in the C-terminus, and a low proportion of acidic amino acids (7%) are structural motifs in Hpa2 consistent with its binding to negatively charged glycosaminoglycans. Importantly, Hpa2 lacks intrinsic HS-degrading activity, the hallmark of Hpa1,[21] and does not undergo processing in a manner required for Hpa1 activation. The segment corresponding to the linker region and cleavage sites of pro-Hpa1 are not conserved in Hpa2.[22] Moreover, Tyr156, which is essential for removal of the Hpa1 linker by cathepsin L is not conserved in Hpa2.[22]

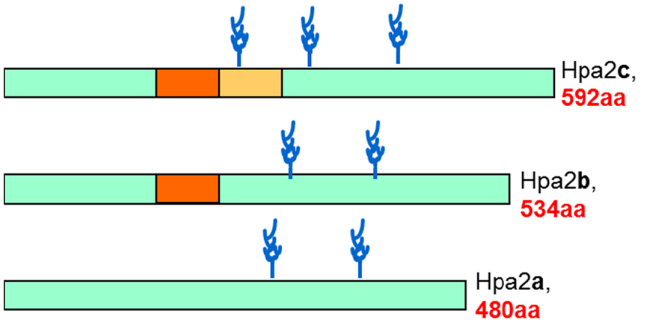

FIGURE 1 Hpa2 splice variants.[21]

In addition to the full-length Hpa2 protein (Hpa2c), several variants have been identified as a result of alternative splicing of the *HPSE2* transcript, including Hpa2a (480 aa) and Hpa2b (534 aa)[20]; Another splice variant of Hpa2, composed of only 528 amino acids, was described.[22] Hpa2c is secreted, likely due to extra glycosylation sites that are lost in the splice variants (Figure 1).[21] The biological significance and cellular localization of Hpa2 splice variants are yet to be revealed. Localization of Hpa2 splice variants to the ER implies that whereas the full-length Hpa2c protein can modulate Hpa1 activity in the cell exterior, its splice variants may modulate Hpa1 activity inside cells. Hpa2 (=Hpa2c)[20,21] accumulates in the cell-conditioned medium following the addition of heparin or HS, indicating that Hpa2 retains the capacity to interact with HS despite the lack of HS-degrading activity.[21] Notably, Hpa2 exhibits an even higher affinity toward heparin and HS than Hpa1,[21] thus competing for HS binding and thereby inhibiting Hpa1 enzymatic activity.[21] Moreover, co-immunoprecipitation studies revealed physical association between Hpa2 and Hpa1 proteins,[21] providing an additional route by which Hpa2 can inhibit Hpa1 enzymatic activity. Immuno-fluorescent staining illustrates the localization of Hpa2 on the cell surface following its exogenous addition, co-localizing with and clustering of syndecan-1 and syndecan-4 . University of Washington (Figure 2, yellow).[21] Unlike Hpa1, Hpa2 does not appear to get internalized into endocytic vesicles but rather remains on the cell surface for a relatively long time (Figure 2).[21] This result indicates that the rapid and efficient internalization of Hpa1 together with syndecans[23–25] is unique and not purely a consequence of HS-ligand binding. Given its high affinity to HS, Hpa2 attenuates Hpa1 uptake resulting in depletion of lysosomal Hpa1.[21]

1.3 | Urofacial syndrome (UFS)

Hpa2 gained attention when it was found that the *HPSE2* gene is mutated in a human disease called urofacial

syndrome (UFS).[26,27] UFS is a rare autosomal recessive disease featuring urinary voiding dysfunction and a grimace upon smiling.[28–30] The urinary tract phenotype is characterized by bladder dyssynergia manifest by incontinence of urine, and the residual urine is prone to bacterial infection. Moreover, high intravesical pressures lead to vesicoureteric reflux (VUR) which, if accompanied by urosepsis, can cause kidney infections, scarring, and end-stage kidney failure. The characteristic grimace when smiling or laughing results from an abnormal contraction of the corners of the mouth and eyes.[29,31] *HPSE2* variants have been described in around half of the families with the syndrome, all consistent with a loss-of-function mechanism.[26–28,32] Rarer individuals with classical features of UFS have biallelic variants in LRIG2 encoding a plasma membrane-associated protein called leucine-rich repeats and immunoglobulin-like domains 2.[33,34] Biallelic missense variants in LRIG2 have also been reported in rare individuals with bladder dysfunction and renal failure, but who lack the facial phenotype.[35] Like Hpa2, LRIG2 is detected in mouse pelvic ganglia,[32] and homozygous LRIG2 mutant mice have bladder dysfunction and abnormally patterned bladder nerves.[35–37]

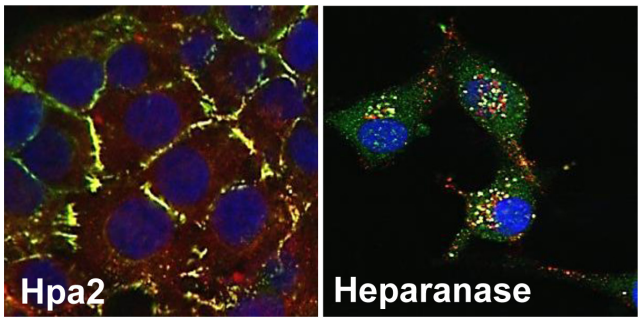

FIGURE 2 Cellular localization of exogenously added Hpa2 vs. heparanase (Hpa1).[21]

2 | HPA2 IN CANCER PROGRESSION

Hpa2 staining is evident in the normal epithelium of the human bladder, cervix, gastric, and ovarian tissues and is reduced substantially in the resulting carcinomas (Figure 3), a staining pattern typical of a tumor suppressor. Below are several examples describing the anti-cancerous effect of tumor-derived Hpa2.

2.1 | Head & neck cancer

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common neoplasm worldwide, where >900 000 new cases are projected annually.[38] The clinical relevance of Hpa2 in head & neck (H&N) cancer

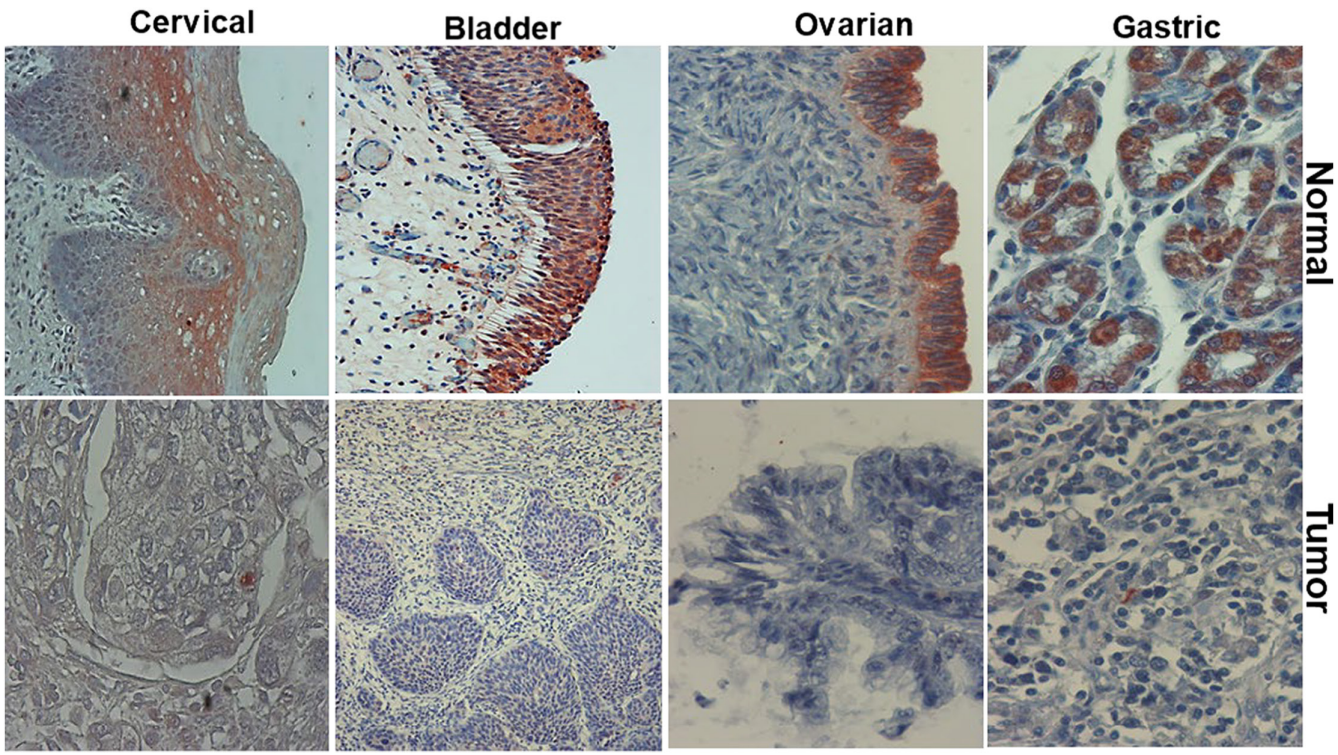

FIGURE 3 Hpa2 immunostaining appears strong in normal epithelium and is decreased substantially in the resulting carcinomas.[16]

emerges from the inverse correlation between Hpa2 staining extent, tumor cell dissemination to regional lymph nodes (patient N-stage), and prolonged time to disease recurrence (follow-up to failure).[21] Tumor metastases are common among patients with H&N cancer with uncontrolled local or regional disease, and autopsy studies revealed 25%–50% overall incidence of distant metastases.[39] An anti-metastatic feature of Hpa2 is supported by studies of the Polycomb protein EZH2, an important component of a multiprotein complex that methylates histone protein 3, leading to repression of target genes.[40] Notably, genes (e.g., *HPSE2*) repressed by EZH2, are expected to function as tumor suppressors,[41] yet research is needed to elucidate this connection in the case of Hpa2 and H&N cancer. Experimentally, restrained growth of Hpa2-high H&N tumors was associated with a prominent decrease in tumor vascularity, due, in part, to reduced Id1 expression, a transcription factor highly implicated in VEGF-A and VEGF-C gene regulation.[42] It was also noted that tumors produced by Hpa2-overexpressing cells are abundantly decorated with stromal cells and collagen deposition, correlating with a marked increase in lysyl oxidase (LOX) expression[42] and reflecting a higher degree of cell differentiation. Hpa2-null (CRISPR) Fadu pharyngeal carcinoma cells produced bigger tumors vs control cells, while rescue of Hpa2 attenuated tumor growth (Figure 4).[43] Notably, despite the well-documented pro-tumorigenic effect of Hpa1 and the poor outcomes of Hpa1-high H&N patients,[44] Hpa1 enzymatic activity was not impaired in cells overexpressing Hpa2. Likewise, the growth of Hpa2-high tumor xenografts was not affected by mAb which targets the presumed heparin-binding domain (HBD) of Hpa2, implying that Hpa2 function does not rely on Hpa1 or heparan sulfate.[42] Employing gene overexpression, gene editing (CRISPR), and silencing approaches, combined with analyses of differential gene expression profiling, Gross-Cohen et al. found that regulation of cytokeratin expression and cell differentiation by Hpa2 involves Sox2[43] which, in head and neck cancer, functions to restrain tumorigenesis. Thus, similar to Hpa2, silencing of Sox2 resulted in reduced cytokeratin 13 and E-cadherin expression; whereas overexpression of Sox2 was associated with increased cytokeratin 13 levels.[43] There was no effect of Hpa2 on the expression of Sox1, Sox3 and Sox9. In related experiments, it was found that silencing of Sox6 in Hpa2-high cells resulted in a comparable decrease in the expression of cytokeratins 4, 15, and 75. Expression of both Sox2 and Sox6 was decreased in Hpa2-null cells, but, unlike Sox2, Sox6 was restored upon the rescue of Hpa2 associated with attenuation of HNSSC tumor growth. These results indicate that the tumor suppression activity of Hpa2 is due in part to its function in maintaining the differentiation and identity of normal epithelial cells, likely via Sox2- and Sox6-regulated expression of cytokeratin family members and E-cadherin.[43]

2.2 | Gastric adenocarcinoma

Despite the use of multiple treatment modalities, including surgery, combined with radiation therapy, chemotherapy, or targeted chemo-immune therapy, gastric cancer often progresses, relapses, or metastasizes and has a 5-year survival rate of less than 35% overall, and only 2% for cases of peritoneal metastases.[45] Liu et al[46] subjected sections of gastric carcinoma to immunostaining and correlated Hpa2 immunoreactivity with clinical records, including tumor grade, stage, and patients' status. Notably, gastric tumors that were stained strongly for Hpa2 were diagnosed as low grade, whereas Hpa2-negative tumors were of high grade. Moreover, Hpa2 staining intensity correlated inversely with tumor stage and tumor metastasis to lymph nodes

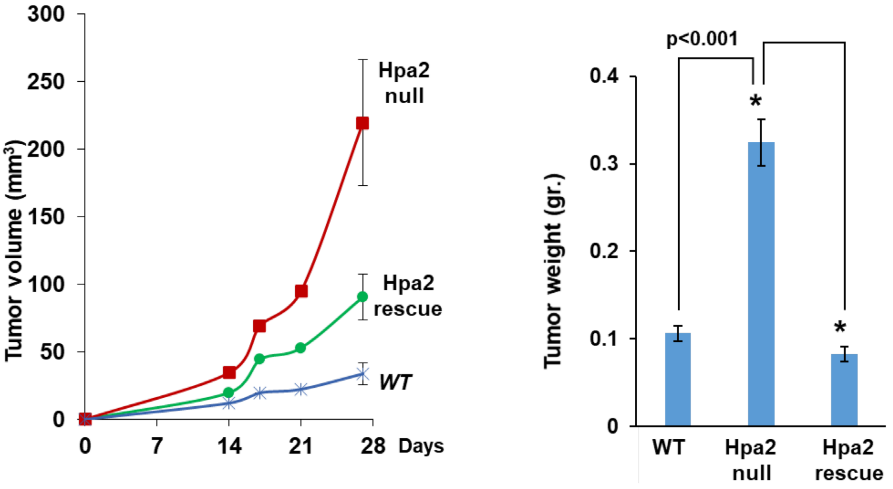

FIGURE 4 Knockdown (CRISPR) of Hpa2 in squamous cell carcinoma of the head and neck (H&N) enhances tumor growth; Rescue of Hpa2 attenuates H&N tumor growth.[43]

(N). Thus, most of the tumors that were stained strongly for Hpa2 had only a few infected lymph nodes (N1–N2), whereas patients with low levels of Hpa2 were mostly diagnosed with multiple infected lymph nodes (N3–N4). Importantly, patients who exhibited high levels of Hpa2 survived longer (median survival = 72 months) than patients with low Hpa2 levels (median survival = 23 months), indicating that Hpa2 functions to restrain gastric cancer.[46] Likewise, Zhang et al[47] reported that high expression of Hpa2 is an independent prognostic parameter for favorable survival of gastric cancer patients.

Experimentally, mice administrated with gastric carcinoma cells engineered to over-express Hpa2 produced smaller tumors and survived longer than mice administrated with control cells. This was associated with increased phosphorylation of AMP-activated protein kinase (AMPK), a kinase that is situated at the center of a tumor suppressor network, attributed to its involvement in energy sensing and cell metabolism.[48] AMPK was also reported to protect normal epithelia from oncogenic transformation.[49] Given the tumor-suppressive properties of AMPK,[50] increased AMPK phosphorylation by human gastric carcinoma cells overexpressing Hpa2 supports the notion that Hpa2 functions to attenuate tumor growth and maintain epithelial cell differentiation. Notably, metformin, a prototype of AMPK activators,[50,51] attenuated cell proliferation, migration, invasion, and colony formation by gastric carcinoma cells to an extent comparable with Hpa2,[46] suggesting that these properties of Hpa2 are mediated, at least in part, by AMPK. The mechanism by which Hpa2 promotes AMPK phosphorylation seems to involve HS because the increased phosphorylation of its substrate (acetyl CoA carboxylase) observed in Hpa2-high cells was reversed by the addition of heparin.[46]

2.3 | Other carcinomas

Zhang et al[52] investigated the function of Hpa2 in colorectal cancer (CRC) in vitro and in vivo. Cell proliferation and cell migration capabilities were significantly suppressed in HCT116 and SW480 CRC cells subjected to ectopic overexpression of *HPSE2* compared with mock-transfected cells. Consistently, xenografts with *HPSE2* overexpression showed decreased tumor volumes, attributed in part to activation of the p53/p21 signaling cascade and the resulting cell cycle arrest in the G1 phase.[52] Multivariate Cox regression analysis revealed that *HPSE2* promoter hypermethylation and gene expression are independent risk factors for shortened survival among patients with CRC, indicating that hypermethylation of *HPSE2* can predict poor prognosis of CRC patients.[52] This identifies epigenetic mechanisms that control Hpa2 gene expression.

Using immunohistochemical analysis, Gross-Cohen et al[53] found that Hpa2 is expressed in normal bladder transitional epithelium, and its levels are decreased substantially in bladder cancer. Notably, tumors that retain high levels of Hpa2 were diagnosed as low-grade and low-stage, suggesting that Hpa2 preserves cell differentiation and halts cell motility. Indeed, migration of 5637 bladder carcinoma cells was attenuated by exogenous addition of purified Hpa2, and overexpression of Hpa2 resulted in smaller tumors that were diagnosed as low grade.[53] It was also noted that tumors produced by Hpa2 overexpressing cells are abundantly decorated with stromal cells and collagen deposition evident by Masson's/Trichrome staining and correlating with a marked increase in lysyl oxidase (LOX).[53] The association between Hpa2 and LOX was further confirmed clinically because of the 16 cases that exhibited strong staining of Hpa2, 14 were also stained strongly for LOX. These results suggest that Hpa2 functions as a tumor suppressor in bladder cancer, maintaining cellular differentiation and decreasing cell motility.[53] Survival of cancer patients retrieved by the Kaplan-Meier plotter service and analyzed according to the expression levels of Hpa2 revealed that patients (i.e., cervical squamous cell carcinoma, hepatocellular carcinoma, pancreatic carcinoma) who exhibited high levels of Hpa2 survived longer than patients with low Hpa2 levels.[54]

2.4 | Sarcoma

Soft tissue sarcomas are a group of rare tumors of mesenchymal origin, with an estimated incidence of 3–4 per 100 000 people per year globally, accounting for approximately 1% of all malignant tumors and 8% of tumors developing in adolescents and young adults.[55] Despite a consistence improvement in the 5-year survival rates, 20%–40% of patients with nonmetastatic osteosarcoma at diagnosis still relapse, mostly due to the development of resistance to current treatments.[56] Employing cell lines established from osteosarcoma, fibrosarcoma, and leiomyosarcoma patients, Knani et al. consistently found that overexpression of Hpa2 resulted in strikingly smaller tumors (Figure 5), whereas bigger tumors were developed following silencing of Hpa2.[54] Overexpression of Hpa2 in sarcoma was found to elicit endoplasmic reticulum (ER) stress and to enhance tumor hypoxia and phosphorylation of JNK, the stress arm of the MAPK pathway.[54] These stress conditions, on the other hand, upregulate the expression of Hpa2, together fueling a cycle that feeds itself.[54] Briefly, Hpa2 enhances stress conditions that in turn induce Hpa2 expression in the sarcoma cells and cells that populate the tumor microenvironment, leading to tumor cell apoptosis and attenuating tumor growth.[54] Hpa2 also induced the

expression of the p53 family member, p63, which, in sarcoma, functions to attenuate tumor growth. Overexpression of Hpa2 also profoundly reduces stem cell characteristics in the sarcoma cells, most evident by failure of the Hpa2-high cells to grow as spheroids typical of cancer stem cells. Likewise, expression of CD44, a well-established cancer stem cell marker, was prominently decreased in Hpa2 cells.[54] Clinically, it was found that localization of Hpa2 in the cell nucleus was associated with low-stage tumors.[54]

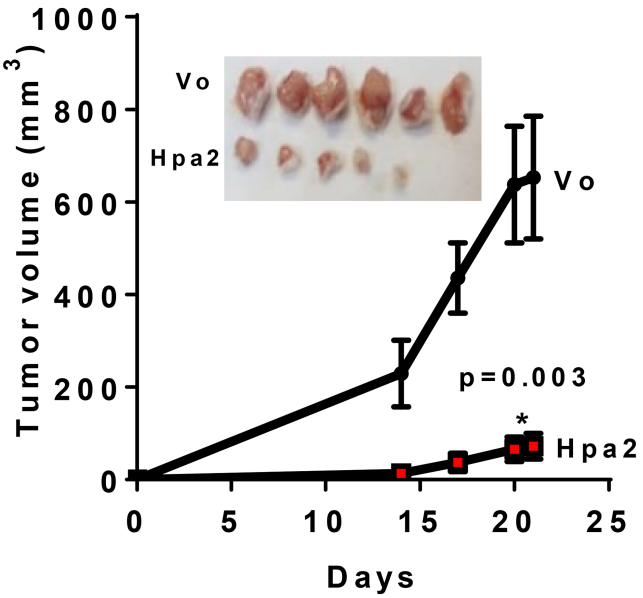

FIGURE 5 Hpa2 attenuates the growth of human osteosarcoma.[54]

3 | HEPARANASE 2 (HPA2) ATTENUATES THE GROWTH OF PANCREATIC CARCINOMA

Kayal et al[57] examined the role of Hpa2 in pancreatic cancer, a malignancy characterized by a dense fibrotic ECM associated with poor response to treatment and bad prognosis. Pancreatic ductal adenocarcinoma (PDAC) patients who exhibit high levels of Hpa2 survive longer than patients with low levels of Hpa2.[57] Strikingly, overexpression of Hpa2 in pancreatic carcinoma cells resulted in a most prominent decrease in the growth of tumors implanted orthotopically or intraperitoneally, whereas Hpa2 silencing resulted in bigger tumors (Figure 6).[57] It was further found that Hpa2 enhances endoplasmic reticulum (ER) stress response and renders cells more sensitive to external stress, associated with increased apoptosis. Notably, ER stress was found to induce the expression of Hpa2, thus forming a feedback loop, where Hpa2 enhances ER stress that, in turn, induces Hpa2 expression. This leads to increased apoptosis

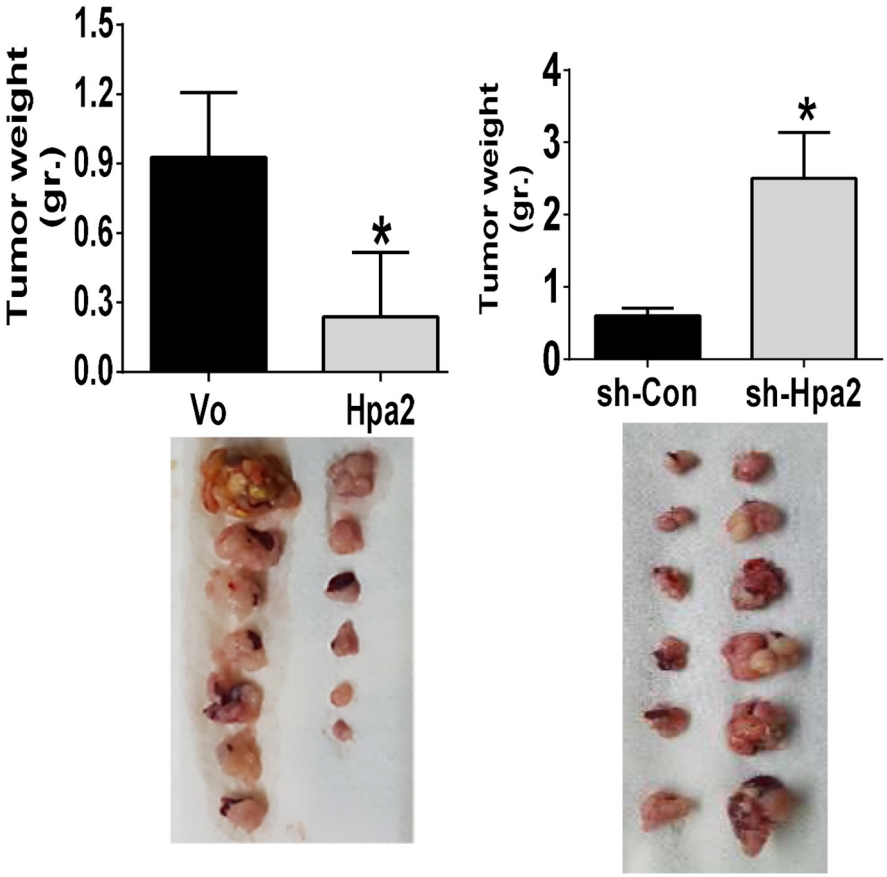

FIGURE 6 Overexpression of Hpa2 in PDAC cells attenuates the growth of tumor xenografts (left), whereas Hpa2 gene silencing results in bigger tumors (right).[57]

and attenuated tumor growth. Together, Hpa2 emerges as a powerful tumor suppressor in pancreatic cancer.[57]

3.1 | Hpa2-KO pancreas is susceptible to initiation of pancreatic neoplasia

While the above studies focused on the role of Hpa2 residing in the tumor cells,[42,43,46,53,54,57,58] the role of *host*-derived Hpa2 in tumorigenesis was not explored. To examine this aspect and given that a constitutive (CRISPR) knockout of the *HPSE2* gene is embryonic lethal (our unpublished results), Kayal et al[59] generated a *conditional Hpa2-knockout* model (cHpa2-KO) in C57BL/6j mice. To this end, exon 5 was targeted as the knockout region, and deletion of the floxed Hpa2 sequences was obtained by Cre-mediated recombination applying the tamoxifen-inducible system.[60] Notably, expression of the Cre recombinase in this model is driven by the chicken β-actin promoter thus directing deletion of the *HPSE2* gene in essentially all cells and tissues.[59] Unlike the constitutive Hpa2-KO mice, the newly generated conditional Hpa2-KO mice developed normally and deletion of the Hpa2 gene 3–4 weeks after birth did not cause noticeable effects and hence enabled tumor xenograft studies in Hpa2 null mice.[59]

To reveal the role of Hpa2 residing in the host in pancreatic tumorigenesis, mouse Panc-02 pancreatic carcinoma cells were implanted orthotopically into the pancreas of control (WT) and cHpa2-KO mice and tumor growth were inspected. Notably, as demonstrated in Figure 7, 3–5-fold bigger tumors were developed in the Hpa2-KO pancreas vs. control (*WT*) pancreas,[59] indicating that host-derived Hpa2 is critically important and functions to restrain tumor growth. Strikingly, lack of Hpa2 resulted in a dramatic decrease in the pancreas size, severe pancreatic inflammation, and profound morphological alterations of the pancreas (Figure 8), *even in the absence of tumor cell inoculation*.[59] Increased deposition of collagen and glycosaminoglycans, typical of fibrotic PDAC, was noted in the Hpa2-KO pancreas.[59] Importantly, measurements of heparanase (Hpa1) enzymatically revealed a dramatically increased activity in the pancreas of Hpa2-KO vs WT mice, suggesting a role for Hpa1 in the pro-tumorigenic and pro-inflammatory features observed in the Hpa2-KO pancreas.[59] It appears that under normal conditions, Hpa1 activity is kept at a low baseline level, likely due to its neutralization by Hpa2, highlighting the significance of maintaining a proper balance between Hpa1 and Hpa2 in dictating the severity of pancreatic cancer.

3.2 | Acinar-to-adipocyte transdifferentiation and acinar-to-ductal metaplasia

Intrapancreatic fat deposition occurs through two mechanisms—fatty replacement (i.e., replacement of acinar cells by adipocytes) and fatty infiltration, the latter mostly associated with obesity and/or metabolic syndrome.[61] Given that Hpa2-KO mice are not obese and seemingly do not exhibit metabolic syndrome(s), the massive accumulation of fat, noted primarily in Hpa2-null female pancreas (Figure 8), is most likely due to the replacement of acinar cells by fat cells through acinar-to adipocyte-trans-differentiation (AAT),[62] a form of epithelial-to-mesenchymal transition (EMT). Support for the occurrence of this mechanism in the Hpa2 null pancreas is the gradual induction of Pparɣ, which is strongly implicated in adipogenesis,[63] shortly after the administration of tamoxifen and the resulting Hpa2 knockout.[59] The massive EMT that Hpa2-KO acinar cells undergo while transforming into adipocytes strongly implies that Hpa2 functions to restrain EMT.[59]

Normally, acinar-to-ductal metaplasia (ADM) is reversible and contributes to the regeneration of acinar structures and repopulation of the pancreas after insults such as injury and stress. However, ADM may become irreversible when cells acquire oncogenic Kras mutations, persistent growth factor signaling, and/or severe inflammation, which prevent redifferentiation.[64] Under such conditions, ADM further progresses into precancerous

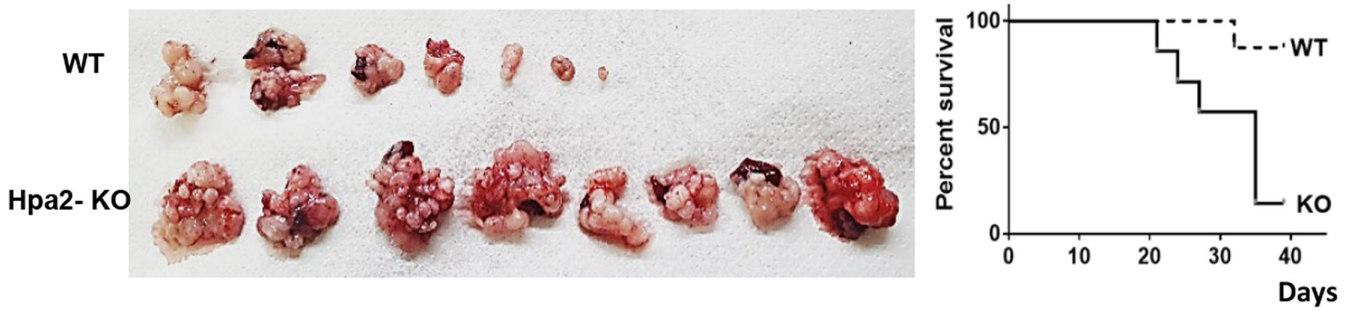

FIGURE 7 Hpa2 residing in the host suppresses pancreatic tumor aggressiveness. Panc02 PDAC tumor growth is increased in Hpa2-KO mice (left). Survival of Hpa2-KO mice inoculated with Panc02 cells is much shorter than their counterpart WT mice (right).[59]

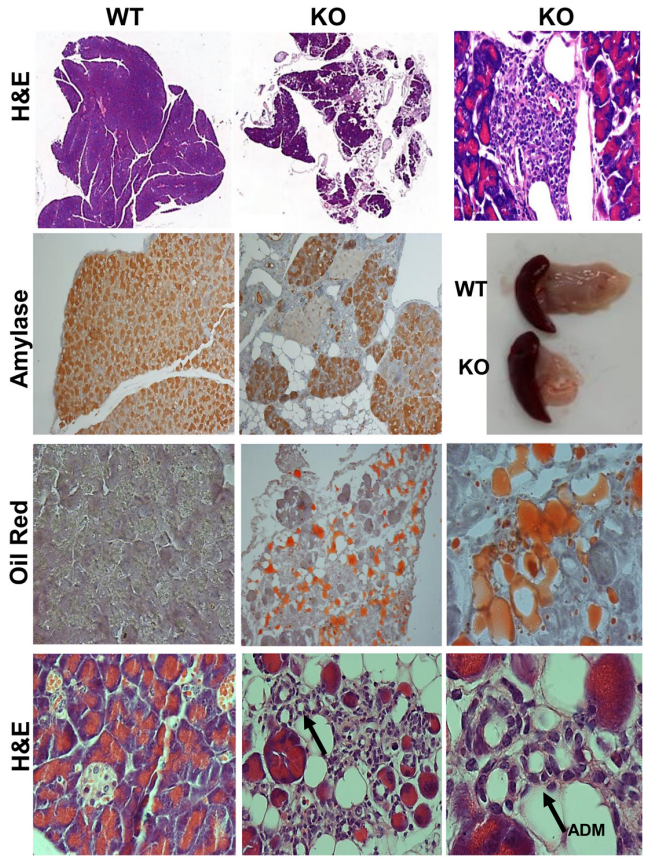

FIGURE 8 Hpa2-KO pancreas exhibits immune cell infiltration (H&E top, right), acinar-to-adipocytes-trans-differentiation (AAT) (3rd panels; Oil Red), and acinar-to-ductal metaplasia (ADM) (H&E bottom panels, ADM, arrows) together resulting in a decreased percentage of acinar cells (2nd middle panel, amylase). ADM is shown at low (bottom, middle panel) and high (bottom, right) magnification (arrows). The Hpa2-KO vs WT pancreas (second right panel) is smaller and the pancreatic tissue is distorted (top, middle panel).[59]

pancreatic intraepithelial neoplasia (PanIN).[64] Like AAT, ADM is thought to reflect a form of EMT in mouse models and human subjects.[65] The spontaneous and massive appearance of ADM noted primarily in female Hpa2-KO mice (Figure 8), implies that Hpa2 functions to maintain the epithelial identity of acinar cells and to prevent EMT.

Searching for mechanisms that may underlie the severe morphological abnormalities of the Hpa2-KO pancreas, Kayal et al[59] found that the expression of PTF1, GATA6, and MIST1, master transcription factors implicated in acinar cell differentiation,66 is significantly reduced in Hpa2-deficient pancreas. Knockdown of PTF1 is sufficient to induce ADM, potentiate inflammation, and accelerate the development of invasive PDAC by sensitizing cells to Kras-mediated transformation.[64] Moreover, knockdown of PTF1 results in apoptosis of acinar cells by activation of the ER stress pathway,[67] thus further linking Hpa2 with ER stress responses.[57] Another important player in acinar cell differentiation is GATA6. This transcription factor functions to maintain acinar cell differentiation by suppressing pro-inflammatory and EGFR signaling pathways.[68] GATA6-KO mice exhibit extensive ADM and accumulation of adipocytes, and, in the context of active Kras, accelerated tumor development.[68] Therefore, like PTF1, GATA6 functions as a tumor suppressor in the pancreas.[68] The capacity of Hpa2 to regulate the expression of key regulators of acinar cell differentiation such as PTF1, GATA6, and MIST1 grants further support for the critical role of Hpa2 in the exocrine pancreas and its tumor suppressor characteristics.[59]

3.3 | Pancreatitis-driven intraepithelial neoplasia

Unlike female Hpa2-KO mice, the male Hpa2-KO pancreas developed little or no AAT and ADM. Instead, foci of inflammation were readily detected within the Hpa2-KO pancreas of young (3-month-old) and older (8-month-old) male mice,[59] reflecting the development of acute and chronic inflammation, respectively. Subjecting pancreas extracts to antibody cytokine array and qPCR revealed a substantial increase in the expression levels of pro-inflammatory cytokines (i.e., IL-8, TNFα) by the Hpa2-KO vs *WT* pancreas. To examine the response of Hpa2-KO mice to cerulein, best recognized for its capacity to induce acute pancreatitis,[69] Kayal et al[59] used male mice in which the pancreas morphology is relatively preserved. Mice were administrated with 6 hourly injections of saline or cerulein and were sacrificed 24 h later. While *WT* C57BL/6 mice responded poorly to cerulein, cerulein profoundly affected the pancreas of Hpa2-KO mice reflected by a remarkable decrease in the number of amylase-positive acinar cells, accumulation of fat cells and recruitment of immune cells, mostly macrophages, indicating that Hpa2 functions to protect the pancreas against inflammation and pancreatitis.

Cerulein also elicited ADM in Hpa2-KO, but not *WT* pancreas as indicated by histology (H&E), alcian blue staining, cytokeratin 19, and Sox9 immunostaining. Given that fatty pancreas and ADM are considered to be pro-tumorigenic,[65,70,71] *WT* and Hpa2-KO female mice were exposed to a carcinogen (AOM) and cerulein (Cer), each alone and in combination.[72] This treatment regimen is based on the notion that combining the effect of carcinogens with chronic inflammation elicits tumor initiation and growth.[72] While, under mild conditions, AOM and cerulein alone or in combination did not elicit noticeable morphological changes in the *WT* pancreas (not shown), the combined treatment of Hpa2-KO mice resulted in atypical foci that were characterized as

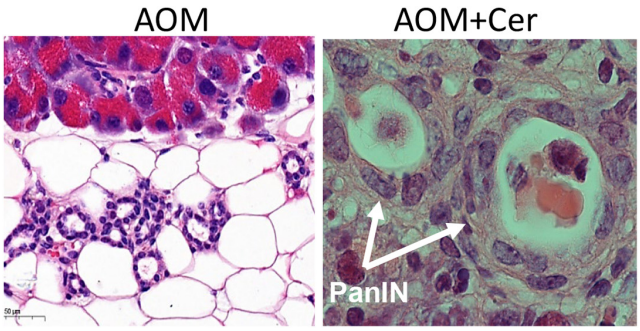

FIGURE 9 Hpa2-KO pancreas progresses to pancreatic intraepithelial neoplasia (PanIN-arrows, right panel).[59]

pancreatic intraepithelial neoplasia (PanIN) (Figure 9), considered to be a histologically well-defined precursor of invasive PDAC. These results strongly support the notion that Hpa2 functions as a tumor suppressor; in its absence, tissues become more prone to the development of premalignant and malignant lesions.[59] Collectively, the results implicate Hpa2 in acinar cell differentiation and homeostasis; deficiency of Hpa2 results in preneoplastic pancreas, which, in response to further insults, develops into pancreatic neoplasia.

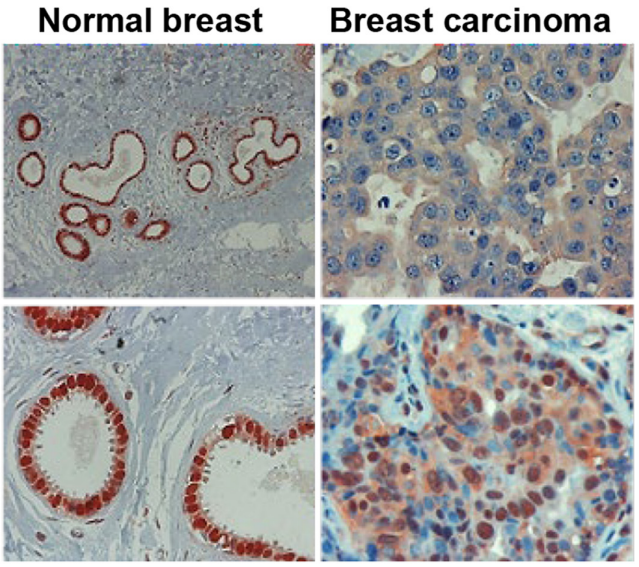

FIGURE 10 In normal breast tissue adjacent to the tumor lesion (left panels), Hpa2 localizes mainly to the cell nucleus. Shown is the same section at a low (top, left panel) and high (bottom, left) magnification. In breast carcinoma (right panels), Hpa2 staining intensity is prominently decreased and appears diffused in the cell cytoplasm or exhibiting cytoplasmic and residual nuclear staining.[73]

4 | NUCLEAR LOCALIZATION OF HPA2 ATTENUATES BREAST CARCINOMA GROWTH AND METASTASIS

Applying anti-Hpa2 antibodies, Hilwi et al[73] subjected a cohort of 61 breast carcinoma biopsies to immunostaining. Surprisingly, unlike the results with the other types of solid tumors,[46,57,58] strong staining of Hpa2 was associated with increased incidence of lymph node metastasis (N stage) and shorter overall survival of breast cancer patients.[73] An even more significant correlation was found when Hpa2 expression was retrieved from a large number ($n = 603$) of ER-positive, HER2-negative breast cancer patients subjected to analysis by publically available software. In this cohort, the overall survival of patients exhibiting high levels of Hpa2 was considerably shorter than Hpa2-low patients (198.44 vs. 99 months for Hpa2-low vs. Hpa2-high patients, respectively).[73] In addition, Hilwi et al. employed a tissue array of 150 invasive ductal breast carcinoma biopsies and correlated the staining intensity of Hpa2 with molecular parameters. Notably, strong staining of Hpa2 was associated with a high rate of cell proliferation (Ki67) and with HER2 expression levels. Importantly, patients who showed strong staining of Hpa2 in the primary lesion and the resulting metastases exhibited the lowest survival time. These clinical results suggest that in breast cancer, Hpa2 promotes, rather than inhibits, tumor progression. Careful examination of the immunostaining revealed that in normal human breast tissue, away from the tumor lesion, Hpa2 is expressed at high levels, localizing predominantly to the cell nuclei (Figure 10).[73] In breast tumors, nonetheless, Hpa2 expression is not only decreased but also lost its nuclear localization and appears diffused in the cell cytoplasm (Figure 10).[73] Scoring the tumor biopsies not only for the staining intensity but also for the cellular localization of Hpa2 revealed that patients in which nuclear localization of Hpa2 was partially retained, exhibited no lymph node metastasis, suggesting that nuclear localization of Hpa2 plays a protective role in breast cancer.

To investigate the role of nuclear Hpa2, Hilwi et al engineered Hpa2 gene construct in which the signal peptide of Hpa2 was removed, and a nuclear localization sequence (NLS) was introduced at the protein C-terminus (Hpa2-Nuc). Breast carcinoma cells were then infected with control empty vector (Vo), Hpa2, or Hpa2-Nuc gene constructs and implanted orthotopically in the mammary gland of NOD/SCID mice. Notably, over-expression of Hpa2 in MDA-MB-231 breast carcinoma cells resulted in 2–3 fold bigger tumors. Strikingly, no measurable tumors were developed by MDA-MB-231-Nuc cells after 4 weeks when the primary tumors were resected (Figure 11). Four weeks thereafter, mice were sacrificed and tumor recurrence and lymph nodes and lung metastases were examined. Five out of seven mice implanted with Hpa2 cells

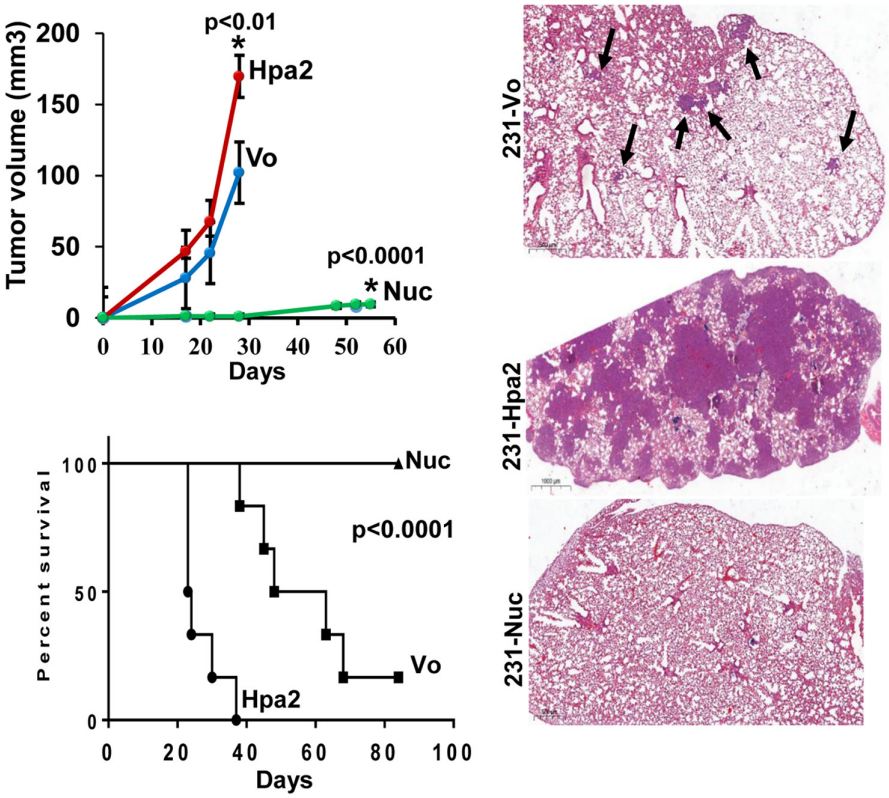

FIGURE 11 Hpa2 promotes, while nuclear Hpa2 (Nuc) attenuates mammary carcinoma (MDA-MB-231) tumor growth, mouse survival (left panels), and lung metastases (right panels).[73]

exhibited metastatic lesions also in the opposite mammary gland vs. 0/7 in the control group, implying that MDA-MB-231 cells overexpressing Hpa2 develop more aggressive and more metastatic disease. Indeed, histological examination revealed massive metastasis in the lungs of mice implanted with MDA-MB-231-Hpa2 cells vs. control (Vo) cells (Figure 11), associated with increased lymph vessel density.[73] Strikingly, no metastatic lesions were observed in the lungs of mice implanted with MDA-MB-231 Hpa2-Nuc cells (Figure 11), strongly implying that targeting Hpa2 to the cell nucleus reduces their tumorigenicity and metastatic capacities substantially. Remarkably, the survival of mice implanted with MDA-MB-231 cells overexpressing Hpa2 was markedly reduced vs control mice. In striking contrast, the survival of mice implanted with MDA-MB-231 Hpa2-Nuc cells was impressively prolonged (Figure 11).[73]

RNAseq of mammary tumors produced by control (Vo) and Hpa2-Nuc ZR-75-1 cells revealed that Hpa2-Nuc affects the expression of genes regulated by interferon, including a marked increase in STAT1 phosphorylation that mediates interferon signaling. Examination of differentially expressed genes (DEG) in the MDA-MB-231 cell model revealed that overexpression of Hpa2 or nuclear Hpa2 resulted in a distinct pattern of gene expression. Gene set enrichment analysis (GSEA) of Hpa2 transcriptome pointed to enrichment of genes induced by Myc, whereas expression of genes associated with the hallmark of Kras, beta-catenin, and TNF-alpha (via NFkB) signaling was repressed in the Hpa2-Nuc transcriptome (Figure 12). Myc is highly implicated in different aspects of breast cancer tumorigenesis, including a most prominent function in the establishment of cancer stem cells.[74] Applying the spheroid assay as an indication of cancer stem cells, it was found that MDA-MB-231 Hpa2 cells developed bigger and more spheroids vs spheroids produced by control (Vo) cells, whereas fewer spheroids were formed by Hpa2-Nuc cells.[73] Genes expression profiles affected by nuclear targeting of Hpa2 were compared in both the ZR-75-1 (hormone-positive) and MDA-MB-231 (triple-negative) cell models. Notably, genes that are associated with the tumor microenvironment emerged as most affected by Hpa2-Nuc cells. Among others, these included induction of interferon-inducible genes and cytokines such as CXCL14. CXCL14 is considered critical for upregulation of the major histocompatibility complex (MHC) class I in tumor cells.[75] Indeed, a marked increase in MHC-I immunostaining was noted in tumors produced by Hpa2-Nuc breast cancer cells. Moreover, there was a marked elevation in the number of NK cells recruited to Hpa2-Nuc tumors, and in the level of granzyme in these tumors.[73] More efficient presentation of tumor antigens in the context of MHC-I molecules, and efficient recruitment of immune cells, likely underline the better outcome of breast cancer patients that retain nuclear localization of Hpa2.

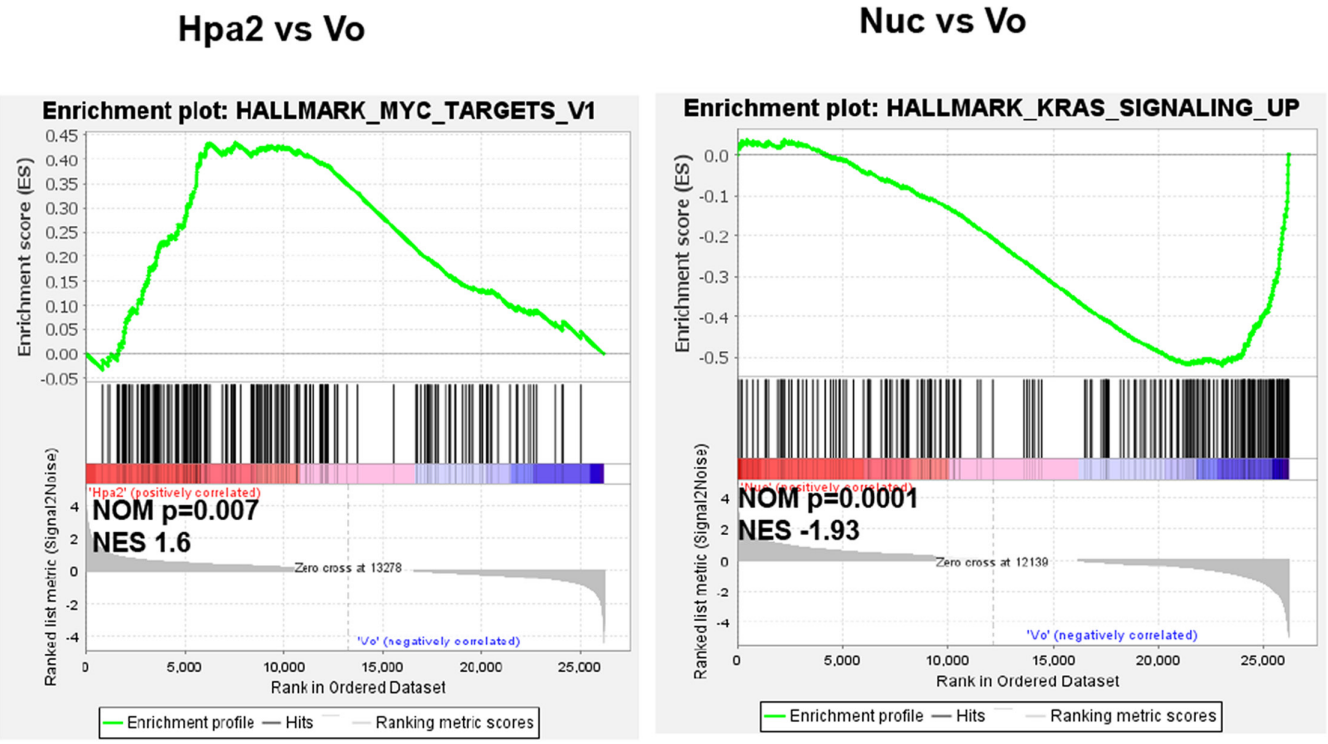

FIGURE 12 Gene set enrichment analyses (GSEA) showing induction of genes related to the Myc pathway in Hpa2 vs. Vo breast cancer tumors (left). Genes that signify the hallmark of Kras are repressed in Nuc vs. Vo tumors (right).[73]

5 | HPA2 REGULATION IN RESPONSE TO STRESS CONDITIONS

Tumor cells experience relatively high levels of stress conditions, due to their high proliferative and metabolic rates.[76] These include proteotoxic stress and ER stress, resulting from high levels of protein synthesis and secretion, as well as starvation, oxidative stress, and hypoxia.[76] Consequently, cancer cells show enhanced and continuous susceptibility to stress, leading to activation of cell death pathways and attenuation of tumor growth.[77] ER stress response is an adaptation pathway that helps cancer cells cope with changes in their microenvironment and thrive. However, when ER stress is not resolved and becomes consistent, cell-death pathways are activated.[78–81] Investigating the mode by which Hpa2 exerts its antitumorigenic effects,[46,57,58] it was found that Hpa2 enhances ER stress, evident by increased expression of ER stress markers (i.e., Bip, CHOP, ATF4) (Figure 13) and higher phosphorylation of eIF2 and PERK, typical components of the PERK arm of the ER stress pathway.[57,58] This was associated with a decrease in cell number and smaller colonies in soft agar, likely due to increased apoptosis, evident by decreased Akt phosphorylation, and higher levels of pro-apoptotic Bax, cleaved caspase 3, cleaved caspase 8, and cleaved PARP.[46,57,58]

Even higher ER stress response and apoptosis were observed when Hpa2 cells were exposed to thapsigargin,[58] known to deplete Ca^{2+} stores in the ER and thereby cause ER stress, indicating that Hpa2 renders cells more sensitive to stress conditions. Notably, while ER stress (thapsigargin, tunicamycin) and hypoxia, each alone, resulted in a 3–7 fold increase in Hpa2 expression, exposure of cells (Panc01, Hela, HT1080) to combined ER stress and hypoxia resulted in a synergistic, over 40-fold increase in Hpa2 expression (Figure 13).[58] Importantly, the increase in ER stress observed in Hpa2-high pancreatic cancer (PDAC),[57] gastric cancer,[46] and soft tissue sarcoma[54] was associated with a prominent decrease in tumor growth. Briefly, tumors that exhibit high levels of Hpa2 become more prone to stress conditions, and the consistency of such conditions likely results in cell death and decreased tumor growth.[78–80] Of note, ER stress responses can suppress tumor progression by altering the function of immune cells that coexist in the tumor microenvironment, modulating, among others, surface expression of major MHC-I molecules, increasing NK cells activity, and sensitizing tumor cells to killing by chimeric antigen receptor (CAR) T cells.[76] Importantly, ER stress induced the expression of Hpa2[57,58] making this notion even more significant and leading to a loop that feeds itself (Figure 13). This may explain the prolonged survival of PDAC[57] and gastric cancer[46] patients showing high levels of Hpa2.

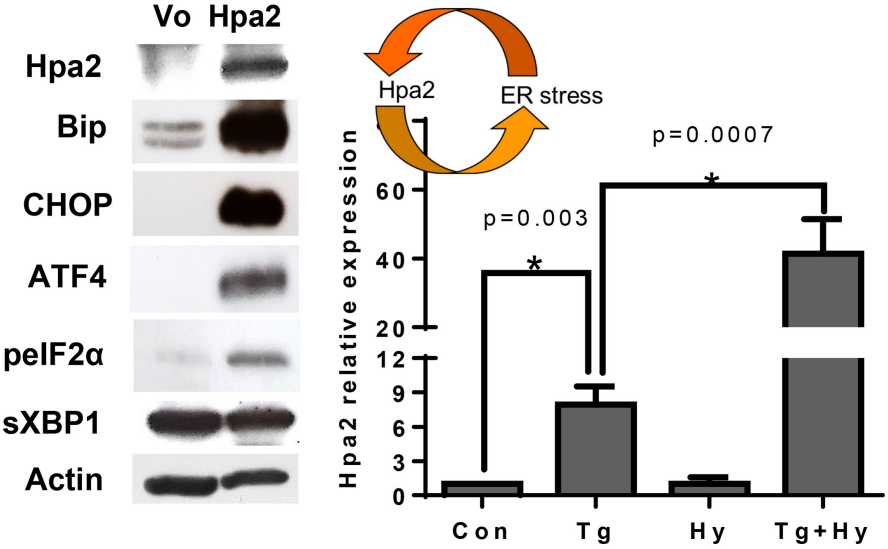

FIGURE 13 Hpa2 enhances stress response (left panel) which, in turn, induces Hpa2 expression (right panel). Hy = Hypoxia; Tg (Thapsigargin) = ER stress.[57,58]

5.1 | Induction of Hpa2 expression by stress is mediated by ATF3

To reveal molecular mechanism(s) that underlies Hpa2 gene regulation, Knani et al[58] subjected the Hpa2 promoter to site-directed mutagenesis and identified activating transcription factor 3 (ATF3) as a transcription factor that mediates Hpa2 induction by stress. ATF3 belongs to the activator protein 1 (AP-1) family of transcription factors. It is also classified as a basic leucine zipper (bZip) transcription factor and a member of the ATF/cAMP response element-binding (CREB) proteins.[82] ATF3 is an immediate early gene whose expression is highly induced in response to conditions of stress such as UV light, ER stress, chemotherapeutics, and DNA damaging agents, as well as many external stimuli (i.e., adipokines, chemokines, cytokines).[82,83] The results add *HPSE2* to the growing list of genes under ATF3 regulation. Importantly, ATF3-null cells (HEK293, MEF) failed to induce Hpa2 expression in response to ER stress, hypoxia, or chemotherapeutics. The rescue of ATF3 expression in ATF3-null cells was sufficient to restore Hpa2 expression, whereas mutant ATF3 1–100 gene construct (which does not bind DNA) failed to induce Hpa2.[58] Of note, two ATF3-binding motifs were identified in the promoter of Hpa2 and deletion of either one was sufficient for the ablation of Hpa2 induction by stress.[58] Levels of ATF3 are prominently decreased in colon, renal, endometrial, and bladder tumors vs adjacent normal tissues,[84,85] an expression pattern that resembles Hpa2 expression and is typical for tumor suppressors. Notably, Hpa2 levels are prominently decreased in hepatocellular and cervical carcinomas and high levels of Hpa2 and ATF3 predict longer survival of the respective patients.[58] Thus, it appears that stress conditions that often accompany rapidly expanding tumors lead to increased expression of ATF3 which, in turn, induces the expression of Hpa2. Given its capacity to inhibit Hpa1 activity, induction of Hpa2 by conditions of stress likely tilt the Hpa1:Hpa2 ratio in favor of Hpa2, resulting in reduced Hpa1 activity and favorable outcomes for cancer patients.

6 | IMPACT OF HPA2 ON THE FORMATION OF A METASTATIC NICHE

A suitably conducive microenvironment (premetastatic niche) must evolve for tumor cells to be able to engraft and proliferate at secondary sites.[86] We have demonstrated that intravenous inoculation of B16 melanoma cells into Hpa2-KO mice resulted in a dramatically increased lung colonization (Figure 14). Similar results were observed with mouse Luc-TC1 lung carcinoma cells (Figure 14). It is conceivable that tumor- and host-derived Hpa2 can not only attenuate the growth of the primary tumor but may also affect the nature of the premetastatic niche and thereby impact tumor cell colonization and metastatic growth. Elevated Hpa1 enzymatic activity, demonstrated, for example, in the pancreas of Hpa2-KO mice,[59] likely stimulates the bioavailability of HS-bound growth factors, cytokines, and adhesion molecules[12,13,87,88] that support tumor cell colonization and growth. These effects provide an attractive and straightforward explanation for the dramatic increase in tumor cell colonization observed in Hpa2-KO vs *WT* lung tissue (Figure 14). Given the complexity of the metastatic process, Hpa2 deficiency may stimulate lung colonization via additional multiple effects. For example, through a protective effect on the endothelial glycocalyx and cell–cell interaction,[89,90] Hpa2 appears to stabilize the capillary wall and halt the ability of blood-borne cells to extravasate. Hpa2

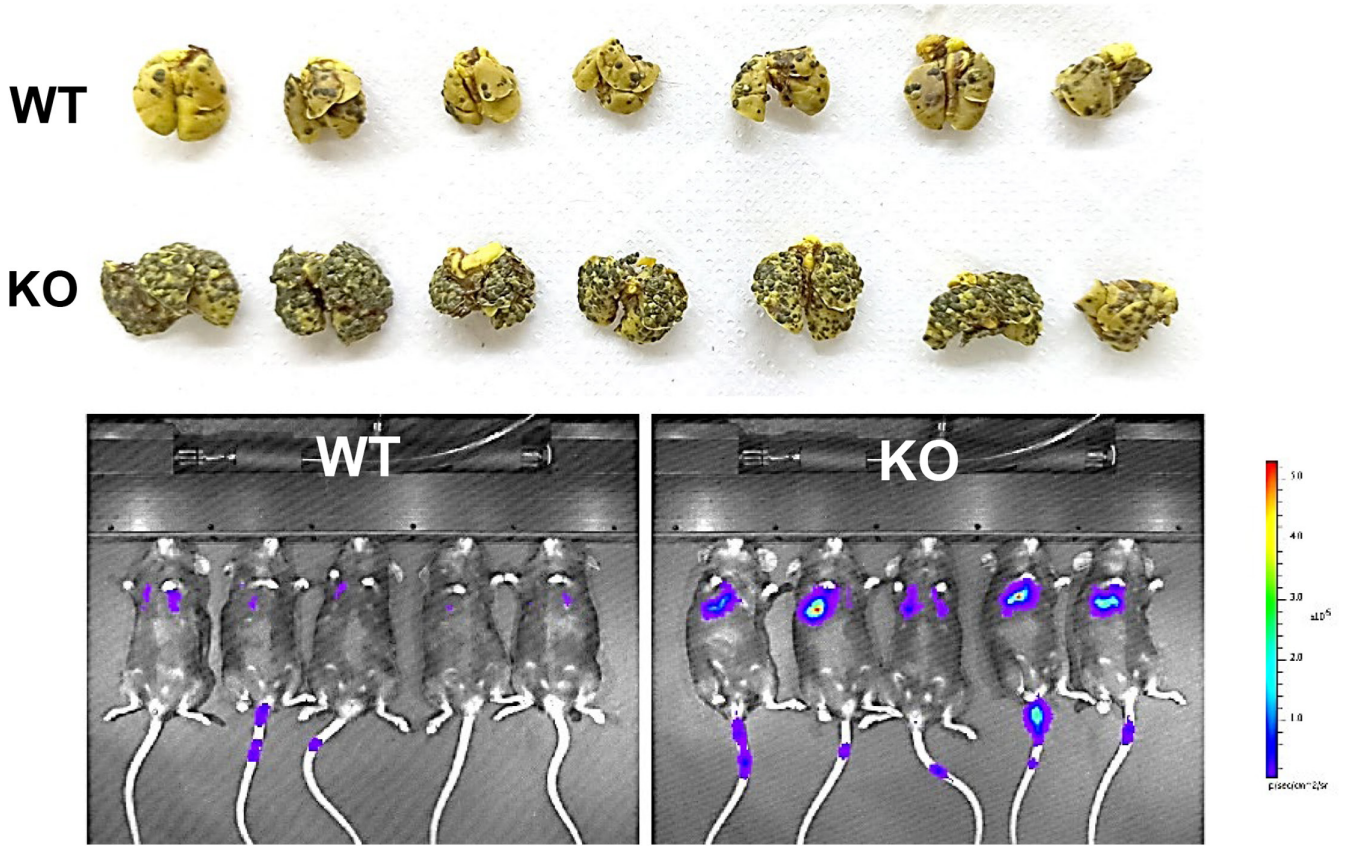

FIGURE 14 Lung colonization of B16 melanoma (top) and TC1-Luc lung carcinoma cells (bottom) is dramatically increased in Hpa2-KO mice.[59]

deficiency may also alter the content, distribution, state of activation and type of immune cells that reside in the lung tissue as well as regulate the expression of cytokines and adhesion molecules[89–91] together affecting the homing and colonization of metastatic tumor cells.

7 | PROTECTIVE EFFECTS OF HPA2 IN OTHER PATHOLOGIES

Apart from cancer, Hpa1 is involved in the pathogenesis of various human diseases including tissue fibrosis, kidney dysfunction, diabetes, diabetic nephropathy, arthritis, atherosclerosis, sepsis, and viral infections.[92–96] Hpa1 levels in cells, tissues, and body fluids must therefore be maintained at a low baseline level and under strict control, a task attributed in part to its naturally occurring antagonist, Hpa2. Studies performed by Dr. Haller et al[91] put forward the significance of the *Hpa1:Hpa2 ratio* in controlling undesirable effects of Hpa1 in inflammation, sepsis, and the cytokine storm associated with COVID-19. It appears that under normal conditions Hpa1 activity is kept at a low baseline level possibly due to its neutralization by Hpa2. The full amplitude of Hpa1 activity is expressed in the absence of Hpa2 (e.g., Hpa2-KO mice),[59] emphasizing the significance of the *Hpa1:Hpa2 ratio* in dictating the severity and aggressiveness of cancer, inflammation, and other diseases,[90,91] as discussed below.

7.1 | Endothelial glycocalyx

Vascular endothelial cells (EC) are lined with glycocalyx, consisting primarily of membrane-bound proteoglycans (e.g., syndecan-1), sialic acid-containing glycoproteins, and plasma proteins.[97] The endothelial glycocalyx (eGC) harbors chemokines, growth factors, and enzymes that contribute to a multitude of endothelial functions, including regulation of vascular tone and permeability, coagulation, leukocyte and platelet adhesion, and inflammation propagation.[97–99] The eGC is synthesized by the EC and plays a crucial role in endothelial homeostasis[98,99] and the modulation of signaling processes.[89,100] It is tightly and dynamically regulated by degradation and synthesis. While Hpa1 plays a major role in degradation of the eGC,[101,102] its antagonist Hpa2 fulfills a protective role, preventing glycocalyx loss and vascular dysfunction.[90] Focusing on tissue inflammation, Kiyan et al[89] studied whether or not Hpa2 can exert protective functions under pathological situations. Briefly, control and Hpa2-overexpressing endothelial cells were

treated with increasing concentrations of LPS. Expression of several pro-inflammatory cytokines was significantly downregulated in Hpa2-overexpressing cells, reflecting the general anti-inflammatory effects of Hpa2. Hpa2 also inhibited LPS binding to the TLR4 receptor complex.[89] Moreover, while control cells treated with LPS lost cell–cell contacts and demonstrated decreased VE-Cadherin expression, Hpa2-overexpressing cells were protected and exhibited preserved cell–cell contacts.[89,90]

7.2 | COVID-19

Stahl et al[91] found injury of the eGC in *COVID-19* and speculated that this might represent a potentially critical hallmark of later widespread endothelial injury in severe COVID-19. Reduced eGC thickness was visualized in vivo by employing sublingual SDF imaging in patients. Increased levels of syndecan-1 and sTie-2 in the blood of these patients indicated shedding of endothelial transmembrane proteins held responsible for building and maintaining the structure of the eGC. Although the key eGC sheddase Hpa1 and its enzymatic activity were not significantly increased, the protective Hpa2 was pertinently reduced in all patients with COVID-19.[91] Driven by this acquired Hpa-2 deficiency, the Hpa1:Hpa2 ratio was higher in patients with COVID-19. Together, this indicates that critically ill patients with COVID-19 suffer from an acquired Hpa2 deficiency that likely contributes to the degradation of the eGC.[91] To demonstrate that the deficiency of Hpa2 is mechanistically involved in degradation of the eGC, Stahl et al. used a microfluidic chamber with cultured endothelial cells (ECs) that synthesize an intact and stable eGC. After stimulation with COVID-19 or control serum, the eGC was visualized and its thickness was quantified by analyzing the HS-positive area.[91] Stimulation with COVID-19 was sufficient to severely damage the eGC, reflected by a marked decrease in the HS-positive area. Importantly, degradation of the eGC after perfusion with COVID-19 serum was attenuated in ECs overexpressing Hpa2, indicating that acquired Hpa2 deficiency represents a potential causative mechanism of damage to the glycocalyx integrity, which could later progress to widespread endothelial dysfunction in COVID-19.[91] The results indicate a protective role of Hpa2 against microvascular inflammation, offering new options for protection against Hpa1-mediated endothelial/eGC damage and microvascular disease.[89–91]

7.3 | Sepsis

The activity of Hpa1 is elevated in sepsis, resulting in shedding of HS.[94] LPS activates TLR-4, resulting in cytokine production and further activation of Hpa1, leading to a vicious cycle of inflammation and end-organ dysfunction such as septic cardiomyopathy and encephalopathy.[90] Kiyan et al[89] reported that Hpa2 levels are reduced in septic mice and patients, leading to an acquired imbalance between Hpa-1 and Hpa2. Administration of purified Hpa2 decreased the plasma levels of TNFα and IL-6 that were elevated in response to intravenous injections of LPS.[89] Therapeutic plasma exchange (TPE), a modality already tested in clinical practice, effectively removed injurious mediators (e.g., Hpa1, eGC degradation products), while replacing depleted protective molecules such as Hpa2.[103] In critically ill patients with septic shock, TPE restored the physiological *Hpa1:Hpa2 ratio* and attenuated eGC breakdown, resulting in a significant improvement in hemodynamic instability.[103]

7.4 | Pancreatitis

Acute pancreatitis (AP) is a common disease in gastroenterology with an increasing global incidence, accounting for about 3% of all hospitalized patients.[104] AP is a complex inflammatory syndrome that results from many etiologies of which gallstones, alcohol, and endoscopic retrograde cholangiopancreatography are the leading causes.[105] About 20% of patients who experienced their first AP attack will develop recurrent episodes and approximately one-third of the latter continue to end-stage chronic pancreatitis.[106–108] It is widely accepted that excessive stimulation of the pancreas or direct destructive insults obstruct the outflow of zymogen granules, where they are proteolytically activated in the acinar cells by lysosomal enzymes, eventually causing acute cell injury.[109] This adverse reaction is further exacerbated by neutrophilic enzymes and transcription factors, which lead to the production of various pro-inflammatory cytokines along with conversion of trypsinogen into trypsin.[110,111] In light of the unclear characterization of the mechanistic pathways responsible for AP, treatment options targeting a specific underlying cause remain elusive.[112,113] Previous studies have highlighted the keen involvement of Hpa1 (heparanase) in the pathogenesis of inflammatory diseases including AP.[114–117] Specifically, it was found that pancreatic Hpa1 expression and activity are significantly increased following cerulein-induced AP.[116] Moreover, pancreas edema and inflammation as well as the induction of cytokines and signaling molecules in response to cerulein were attenuated markedly by PG545 and SST0001, heparin/HS-like Hpa1 inhibitors,[116] implying that the enzyme plays a significant role in AP. Given that Hpa2 deficiency is associated with an inflamed pancreas (Figure 8) and that Hpa2 inhibits the enzymatic activity of Hpa1, it

is conceivable that therapeutic plasma exchange (TPE), administration of Hpa2 or Hpa2-derived peptides[118] may have a therapeutic impact, attributed to restoration of the physiological balance between Hpa1 and Hpa2.

7.5 | Glomerulonephritis and diabetic nephropathy

Diabetic nephropathy (DN) and glomerulonephritis are characterized by reduced levels of HS in the glomerular basement membrane (GBM). This reduced HS expression has been causally associated with increased glomerular expression of Hpa1 (*HPSE1*) resulting in albuminuria[119,120] and pointing to Hpa1 as a therapeutic target. Drugs that inhibit Hpa1 can be roughly divided into two categories, HS-mimetics,[121] which are drugs that structurally resemble HS, and inhibitors that directly block the HS-binding site of Hpa1.[122] Gil et al[120] previously showed that the heparin-based HS mimetic SST0001 (= Roneparstat) decreased albuminuria in experimental type 1 and 2 DN. A major drawback of heparin-based Hpa1 inhibitors is the simultaneous activation of macrophages via Toll-like receptors.[123] Given that Hpa2-deficient mice develop significant albuminuria and die within 1 month after birth[124] and that Hpa2 inhibits Hpa1 without activating macrophages, Hpa2-based therapy was suggested as a promising therapeutic strategy in glomerular diseases.[118] It was first demonstrated that Hpa2 expression is downregulated in anti-GBM and LPS-induced glomerulonephritis, streptozotocin-induced DN, and adriamycin nephropathy,[118] supporting the results of Kiyan et al[89] in which decreased levels of Hpa2 were detected in serum and kidney medullary capillaries of mice subjected to CLP-induced polymicrobial sepsis. Importantly, Buijsers et al[118] reported that both Hpa2 protein and Hpa2-derived peptides improved kidney function in LPS-induced glomerulonephritis. The two most effective peptides in Hpa1 inhibition possess a sequence of three amino acids that are annotated as HS-binding domains in Hpa1,[118] supporting the notion that Hpa2 inhibits Hpa1 activity by interfering with the interaction between Hpa1 and HS.[21]

8 | OPEN QUESTIONS, DISCUSSION, AND PERSPECTIVES

8.1 | Impact of Hpa1

Several groups of proteins that inhibit enzymes exhibit activities that are not related to their enzyme-inhibiting function. For example, tissue inhibitors of metalloproteinases (TIMPs), primarily known for their role in inhibiting the activity of matrix metalloproteinases (MMPs), have nonenzymatic functions that contribute to various physiological processes, irrespective of MMPs.[125] Cystatins, well-known for their role as inhibitors of cysteine proteases (i.e., cathepsin B), also possess nonenzymatic functions that contribute to various physiological processes.[126] Likewise, plasminogen activator inhibitor-1 (PAI-1), primarily known for its role as an inhibitor of tissue-type and urokinase-type plasminogen activators, has been found to have several nonenzymatic functions that are not related to its enzyme-inhibiting activity.[127] These nonenzymatic functions include, for example, involvement in cell signaling, cell apoptosis, cell adhesion, immune modulation, angiogenesis, and maintenance of the ECM and tissue architecture. Similarly, Hpa2, known for its Hpa1-inhibiting activity, appears to affect some physiological functions independent of its effects on the Hpa1 enzyme. In Head & Neck cancer, for example, Hpa1 enzymatic activity was not impaired in cells and tumors overexpressing Hpa2.[42] Also, the growth of Hpa2-high tumor xenografts was not affected by mAb which targets the presumed heparin-binding domain (HBD) of Hpa2,[42] implying that the tumor-suppressing function of Hpa2 does not rely on Hpa1 or heparan sulfate. The most common Hpa1-independent mechanism that appears to underlie the anti-tumoral effect of Hpa2 is a continuous induction of stress response, viciously leading to upregulation of Hpa2, resulting in tumor cell apoptosis and growth arrest.[46,57,58] Given the above considerations and the proposed lysosomal functions of Hpa1 (i.e., autophagy),[18,115] it is conceivable that an important function of Hpa2 is not necessarily to inhibit Hpa1 activity extracellularly, but rather to sequester Hpa1, attenuate its cellular uptake and thereby deplete Hpa1 from the lysosome.[115] Regardless of the mode of Hpa2 action, the studies presented in this review emphasize the importance of maintaining a proper balance between Hpa1 and Hpa2 in the control of tissue homeostasis and normal function. The significance of this concept is highlighted in Table 1 presenting some of the opposing functions of Hpa1 and Hpa2.

Surprisingly, treating Hpa2-KO mice with Hpa1 inhibitors did not reverse the abnormal morphology of the Hpa2-KO pancreas toward a more normal morphology but instead worsened it, resulting in more fat cells and a more prominent ADM.[59] While the reason for this unexpected result is not clear, it appears that deficiency of both Hpa2 (Hpa2-KO) and Hpa1 activity (Hpa1 inhibitors) is most devastating to the exocrine pancreas. Given that in Hpa1-KO mice[128] the pancreas retains normal morphology and function, it is conceivable that in the absence of Hpa1 (Hpa1-KO mice; Hpa1 inhibitors) the pancreas is protected because Hpa2 compensates for Hpa1 deficiency.

TABLE 1 Opposing functions of heparanase-1 and heparanase-2.

Hpa2	Hpa1
Protective effects	Risk effects
1. Tumor suppression[42,43,46,52–54,57–59] (Figures 3–7)	1. Tumor progression[1–11]
2. Antimetastatic[59] (Figure 1114)(Figure 14)	2. Cell invasion and metastasis[4]
3. Nonpermissive tumor niche,[59] (Figure 14)	3. Favorable pro-metastatic niche[7]
4. Antiangiogenesis[42]	4. Pro-angiogenesis[12,13,113]
5. Anti-inflammatory[89–91]	5. Pro-inflammatory (IBD, pancreatitis)[2,116]
6. Antisepsis[89,90]	6. Sepsis[94]
7. Anti-COVID19[91]	7. COVID-19/cytokine storm[90,91]
8. Anti-glomerulonephritis[118]	8. Kidney dysfunction (DN, AKI)[93,120]
9. Tissue homeostasis (pancreas, glycocalyx)[59,90]	9. Tissue damage (islets, glycocalyx)[96,101,102]
10. Antifibrosis[42,59]	10. Fibrosis[8]

Similarly, the harmful effect of the Hpa1 inhibitory compounds suggests that Hpa1 can compensate, to some extent, for Hpa2 deficiency. According to this notion, Hpa1 and Hpa2 may cooperate and compensate for one another, thus avoiding pancreatic dysfunction. For many years, an intensive effort was dedicated to revealing the role of Hpa1 in human pathologies, mainly cancer, leading to the appreciation that Hpa1 is a target for the development of anticancer therapeutics.[7,129,130] However, the role of Hpa1 in normal, non-transformed, cells is still largely unknown. Our preliminary results suggest that Hpa1 activity may play a significant role in preserving the exocrine aspect of the pancreas, cooperating with Hpa2 to maintain the differentiation state and function of acinar cells.[59] Support for this concept may arise from a double KO mouse model deficient for both Hpa1 and Hpa2, whose establishment is ongoing.

8.2 | Nuclear localization

The association between high levels of Hpa2 and poor prognosis of breast cancer patients stands in striking contrast with the majority of reports, showing that high levels of Hpa2 are associated with prolonged survival of cancer patients.[21,46,47,52,57,58] This unexpected result depends on Hpa2 cellular localization. While breast tumorigenesis was promoted by secreted Hpa2, it was markedly suppressed by nuclear Hpa2.[73] Pathways responsible for nuclear translocation of Hpa2 are presently unclear. While nuclear Hpa1 enzymatic activity enhances histone acetyltransferase activity and hence elicits gene transcription and tumor aggressiveness,[131–133] nuclear Hpa2, on the other hand, is expected to inhibit the HS-cleaving activity of Hpa1,[21,59] thus tilting the Hpa1:Hpa2 ratio in favor of Hpa2 and the resulting tumor suppression. The results indicate that targeting Hpa2 to the cell nucleus affects the expression of a gene set endowed with tumor suppression. For example, nuclear Hpa2 elicited a prominent increase in STAT1 phosphorylation, known to act as a tumor suppressor in breast cancer.[134] Notably, interferon-inducible genes that are associated with the tumor microenvironment emerged as most affected by Hpa2-Nuc, awaiting validation and in-depth mode of action studies. Also, an over 10-fold increase in the number of NK cells recruited to Hpa2-Nuc tumors was noted, likely contributing to the better outcome of breast cancer patients that retain nuclear localization of Hpa2.

8.3 | Immunological and clinical aspects

Macrophages can be polarized into classically activated (M1; pro-inflammatory/anti-tumorigenic) and alternatively activated (M2; anti-inflammatory/pro-tumorigenic) macrophages. Macrophage polarization is considered crucial for tissue repair and homeostasis, contributing to infection prevention, angiogenesis and immunomodulation. In a model of cervical cancer, we demonstrated induction of IL-6 and IL-10 (cytokines that are considered pro-tumorigenic) by Hpa2-KO macrophages. The full repertoire of mediators affected by the deficiency of Hpa2 is yet to be explored. Likewise, in a model of peritonitis, we found that Hpa2 deficiency results in decreased abundance of M1 macrophages, and increased percentage of M2 macrophages in the peritoneal fluid. Collectively, it appears that in the absence of Hpa2, macrophages are shifted from M1 toward M2 phenotype, indicating a role for Hpa2 in macrophage polarization and function.

Recently, it was reported that Hpa2 levels are reduced markedly in critically ill Covid patients,[91] in patients diagnosed with sepsis,[89] and in models of kidney disease and diabetic nephropathy.[118] Importantly, administration of purified Hpa2 relieved the symptoms of sepsis in a mouse

model[89] and improved kidney function in LPS-induced renal failure.[118] Moreover, therapeutic plasma exchange (TPE) administrated to patients with early septic shock restored Hpa2 levels and protected the vascular glycocalyx[103] thus offering, possibly, therapeutic intervention for this life-threatening condition.[90] Implementation of Hpa2-based therapy awaits in-depth investigation. The mechanism underlying the protective role of Hpa2 against sepsis, pancreatitis, viral and bacterial infection is not entirely clear but likely involves inhibition of heparanase activity[21] and glycocalyx damage[90] as well as direct effects on immune cell infiltration and function.

8.4 | Metastatic niche

Once cancer cells reach a distant organ, they need to interact with the local microenvironment, or niche, to survive and proliferate. The metastatic niche provides a supportive environment that includes interactions with surrounding cells, extracellular matrix, and various signaling molecules.[86] The success of metastasis depends on the compatibility between the disseminating cancer cells and the receptivity status of the metastatic niche. In this respect, the dramatically increased lung colonization of melanoma and lung carcinoma cells intravenously inoculated into Hpa2-KO mice,[59] suggests that Hpa2 deficiency favorably affects tumor cell colonization and metastatic growth. Focusing on the Hpa2-KO pancreas, Kayal et al[59] demonstrated a dramatic increase in Hpa1 enzymatic activity, an activity known to modify the tumor microenvironment and promote cancer cell dissemination and colonization. It is therefore conceivable that organ-tropism of cancer cells is affected by the balance between the levels of Hpa1 and Hpa2 in a given tissue. Collectively, Hpa2, by virtue of antagonizing Hpa1, appears to restrict the extent of tumor metastasis and secondary growth, thereby providing a promising new direction for investigating structural and functional alterations involved in the formation of a receptive metastatic niche.

8.5 | Embryonic development

HPSE2 knock-down model in Xenopus was the first to demonstrate an in vivo developmental role for Hpa2, supporting the notion that congenital peripheral neuropathy is a key feature of UFS.[135] The frog data are also consistent with Hpa2 modifying growth factor signaling, presumably acting as a counterbalance to Hpa1.[135] Notably, Hpa2 knockout mutant mice (gene trap approach) display a distended bladder phenotype and abnormal voiding behavior resulting in renal dysfunction, growth retardation and death within a month after birth.[124] Kayal et al applied the CRISPR/Cas9 technology and generated constitutive Hpa2-KO mice. Strickingly, all the newborn mice were either wild-type or heterozygous for Hpa2, indicating that Hpa2-KO homozygosity is embryonic lethal. Among a total of 178 newborn mice that were genotyped, none were found to be homozygous for the null mutation. The lack of viable Hpa2-KO homozygous offspring strongly indicates an essential involvement of Hpa2 in embryonic development, paving the way for extensive research on the role of Hpa2 in development.

8.6 | Structural features and clinical impact of Hpa2

Numerous molecules were developed as potent Hpa1 inhibitors, but only four sugar molecules have advanced to clinical trials. Due to their heparin-based nature, they lack specificity, vary in size and sulfation pattern, and hence elicit side effects, primarily due to residual anti-coagulation effects. Although the results in mouse tumor models were very encouraging,[3–5] the respective clinical trials were terminated or halted and none of these heparin/HS mimetics are in clinical use. Unlike Hpa1, the crystal structure of Hpa2 has not been resolved and no information is available about its functional domains (i.e., HS binding domains). McKenzie[20] noted a five amino acid motif (NHHNH), consistent with the heparin-binding consensus sequence (X-B-B-X-B-X) spanning Asn486 to Asn491, where B indicates basic and X indicates small neutral amino acids. This notion and the structural similarity between Hpa1 and Hpa2 provide insight into regions containing lysine and arginine in the presumed heparin/HS-binding domain (HBD) consensus sequences of Hpa2, annotated as HS-binding domains in Hpa1.[136] Given the high homology between Hpa1 and Hpa2, Dr. Wu (The Rosalind Franklin Institute, UK), has recently partially solved the first-ever crystal structure of Hpa2. The newly obtained structural data are of prime importance for accurately identifying the HBD regions of Hpa2 and elucidating the molecular basis of Hpa2 interactions with HS and/or other partners, including Hpa1. Electrostatic map of the Hpa2 crystal structure revealed positively charged regions on the protein surface pointing to the contributions of a few loops and an α-helix. The corresponding peptides were synthesized and are being examined for inhibition of Hpa1 enzymatic activity, attenuation of pancreatitis, diabetic nephropathy (DN), and PDAC tumor growth. Importantly, Buijsers et al. recently reported that both recombinant Hpa2 and Hpa2-derived peptides improved

kidney function in LPS-induced glomerulonephritis.[137] Given the protective effect of Hpa2 against pancreatitis[59] and the ability to rescue Hpa2 deficiency in sepsis,[89] glomerulonephritis,[118] and COVID-19,[89–91] we expect that the results will pave the way for the development of Hpa2-based therapeutic strategies directed against cancer and inflammation.

AUTHOR CONTRIBUTIONS

Israel Vlodavsky, Neta Ilan—Conception and design of the work. Israel Vlodavsky, Neta Ilan—Drafted the work and wrote and edited the manuscript. Maram Hilwi, Yasmin Kayal, Soaad Soboh—Performed the experiments. Acquisition, analysis, or interpretation of data. Maram Hilwi, Yasmin Kayal, Soaad Soboh—Acquisition, analysis, and interpretation of data. All authors have read and approved the manuscript.

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DATA AVAILABILITY STATEMENT

Not applicable.

DISCLOSURES

The authors declare that they have no competing interests; The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT

The study was approved by the hospital's Helsinki Committee. All preclinical animal studies were performed in compliance with the regulations and ethical guidelines for experimental animal studies, in accordance with the Technion's Institutional Animal Care and Use Committee (IL-078-05-21; OPRR-A5026-01).

CONSENT FOR PUBLICATION

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