Heparanase Cooperates with Ras to Drive Breast and Skin Tumorigenesis

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Abstract

Heparanase has been implicated in cancer but its contribution to the early stages of cancer development is uncertain. In this study, we utilized nontransformed human MCF10A mammary epithelial cells and two genetic mouse models [Hpa-transgenic (Hpa-Tg) and knockout mice] to explore heparanase function at early stages of tumor development. Heparanase overexpression resulted in significantly enlarged asymmetrical acinar structures, indicating increased cell proliferation and decreased organization. This phenotype was enhanced by coexpression of heparanase variants with a mutant H-Ras gene, which was sufficient to enable growth of invasive carcinoma in vivo. These observations were extended in vivo by comparing the response of Hpa-Tg mice to a classical two-stage 12-dimethylbenz(a)anthracene (DMBA)/12-o-tetradecanoylphorbol-13-acetate (TPA) protocol for skin carcinogenesis. Hpa-Tg mice overexpressing heparanase were far more sensitive than control mice to DMBA/TPA treatment, exhibiting a 10-fold increase in the number and size of tumor lesions. Conversely, DMBA/TPA-induced tumor formation was greatly attenuated in Hpa-KO mice lacking heparanase, pointing to a critical role of heparanase in skin tumorigenesis. In support of these observations, the heparanase inhibitor PG545 potently suppressed tumor progression in this model system. Taken together, our findings establish that heparanase exerts protumorigenic properties at early stages of tumor initiation, cooperating with Ras to dramatically promote malignant development. Cancer Res; 74(16); 4504–14. ©2014 AACR.

Introduction

Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) side chains presumably at sites of low sulfation, releasing saccharide products with appreciable size (4–7 kDa). Enzymatic degradation of HS leads to disassembly of the extracellular matrix (ECM) and is therefore involved in fundamental biological phenomena associated with tissue remodeling and cell migration, including inflammation, angiogenesis, and metastasis (1–4). The clinical significance of the enzyme in tumor progression emerged from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, in situ hybridization, RT-PCR, and real-time PCR analyses revealed that heparanase is upregulated in essentially all major types of human cancer, namely carcinomas, sarcomas, and hematologic malignancies (2, 5–7).

Notably, increased heparanase levels were most often associated with reduced patients’ survival post operation, increased tumor metastasis, and higher microvessel density (2, 7, 8), thus critically supporting the intimate involvement of heparanase in tumor progression and encouraging the development of heparanase inhibitors as anticancer therapeutics (9, 10). Importantly, heparanase upregulation in human tumors (i.e., head and neck, tongue, hepatocellular, breast, and gastric carcinomas) is associated with tumors larger in size (2, 8). Likewise, heparanase overexpression enhanced (11–14), whereas local delivery of anti-heparanase siRNA inhibited (15) the progression of tumor xenografts, altogether implying that heparanase function is not limited to tumor metastasis but is also engaged in accelerated growth of the primary lesion (12).

Although the clinical significance of heparanase is well documented and anti-heparanase compounds are being tested in clinical trials (16), the role of heparanase in the early stages of tumor development has not been sufficiently explored. Here, we utilized nontransformed human mammary epithelial cell line (breast MCF10A) and genetic (Hpa-Tg/KO mice) approaches to reveal the function of heparanase and its C-terminal domain (8C) reported to mediate signaling properties of heparanase and to promote tumor growth (17), in the early phases of tumor development.

Materials and Methods

Antibodies and reagents

Anti-smooth muscle actin (SMA) and anti-actin monoclonal antibodies were purchased from Sigma; phospho-Akt,
phospho-Met, and phospho-Src antibodies were purchased from Cell Signaling. Anti-Erk2, anti-phospho-Erk, anti-Src, anti-Akt, anti-E-cadherin, anti-vimentin, and anti-Ki67 antibodies were purchased from Santa Cruz Biotechnology. Anti-LYVE and anti-FOXO1 antibodies were from Abcam; anti-V5 epitop tag antibody was from Invitrogen, and anti-F4/80 was from Serotec. Anti-heparanase polyclonal antibody (#1453) has been described previously (18). Matrigel was purchased from BD. The heparanase and angiogenesis inhibitor PG545 was kindly provided by Progen Pharmaceuticals (9).

Cell culture, infection, immunoblotting, and xenotransplantation
MDA-MB-231 breast carcinoma and A431 epidermoid carcinoma cells were purchased from the American Type Culture Collection (ATCC) in August 2013. MCF10A cells were kindly provided by Dr. Y. Yarden (Weizmann Institute of Science, Rehovot, Israel) in December 2009 and were cultured as described (19). MCF10AT1 cells were kindly provided by Dr. A. Raz (Wayne State University, Detroit, MI; ref. 20) in January 2010. Cells were infected with control empty vector (Mock), heparanase, or its C-terminal domain (8C; ref. 17) gene constructs, selected with blasticidin (10 μg/mL; Invitrogen), and expanded and pooled. Immunoblotting was carried out essentially as described (14, 21). For xenotransplantation, cells from exponential cultures of control (Mock) and heparanase/8C-infected MCF10AT1 cells were detached with trypsin/EDTA, washed with PBS, and brought to a concentration of 1 × 10^8 cells/mL. Matrigel. Cell suspension (1 × 10^7/0.1 mL) was inoculated subcutaneously at the right flank or orthotopically at third mammary fat pad of 8-weeks old female SCID/Beige mice. Tumor lesions were harvested after 8 (subcutaneous) or 3 (mammary fat pad) months, when small lesions start to appear in Hpa-Tg mice. The Hpa-Tg mice were then divided randomly into 2 groups and treated with saline or the heparanase inhibitor PG545 once weekly (400 μg/mouse, i.p.) for 3 weeks together with twice weekly administration of TPA. All animal experiments were approved by the Animal Care Committee of the Technion, Haifa, Israel.

Histology and immunohistochemistry
Histologic examination and immunostaining of formalin-fixed, paraffin-embedded 5 micron sections was performed essentially as described (26). Alkaline phosphatase detection system (Cell Markre) was utilized to visualize phospho-Akt staining (27). Images were acquired by Nikon ECLIPSE microscope and Digital Sight Camera (Nikon) with objectives ×20 or ×40.

Statistical analysis
Data are presented as mean ± SE. Statistical significance was analyzed by 2-tailed Student t test. The value of P<0.05 was considered significant.

Results
Heparanase overexpression enhances the development of acini-like structures produced by MCF10A cells and tumor lesions produced by MCF10AT1 cells
To investigate the role of heparanase in the early stages of cancer development, we infected nontransformed MCF10A human breast cells with heparanase or its C-terminal domain (8C) gene constructs (Supplementary Fig. S1A) and high level of expression was confirmed by immunoblotting, activity assay, and immunofluorescent staining (Supplementary Fig. S1B and S1C). Akt phosphorylation was not enhanced by heparanase/8C overexpression in MCF10A cells grown on tissue culture plastic dishes (Supplementary Fig. S1D, left). Plating the cells on reconstituted basement membrane (Matrigel) resulted in substantial increase in Erk phosphorylation by all cell types, as would be expected (Supplementary Fig. S1D, right third panel). In striking contrast, Akt phosphorylation was increased markedly only by MCF10A cells overexpression heparanase/8C (Supplementary Fig. S1D, right top), in agreement with previous results relating heparanase and increased Akt phosphorylation (17, 23, 28, 29). Once plated on top, and overlaid with Matrigel, control (Mock) transfected MCF10A cells formed typical spherical acini-like structures composed of a single layer of epithelial cells and a hollow lumen (Fig. 1A, left). Notably, MCF10A cells overexpressing heparanase or the 8C variant produced significantly higher acini that seemed disorganized and lacked a lumen (Fig. 1A, middle and right).

To examine these growth-advantage features of heparanase in vivo, we utilized MCF10AT1 cells that acquired the ability for xenograft growth after transfection with T24 H-Ras (30). MCF10AT1 cells were similarly infected with control or heparanase/8C gene constructs and expression levels comparable to those observed in MCF10A cells were
Overexpression of heparanase or the 8C variant resulted in increased cell migration (Supplementary Fig. S2C, third panel; Supplementary Fig. S2D, left) and cell invasion (Supplementary Fig. S2C, fourth panel; Supplementary Fig. S2D, right) compared with control cells. Palpable lesions were not detected even 8 months after subcutaneous implantation of the MCF10AT1 cells. Small yet visible lesions were observed, however, after the mice were sacrificed and the implantation site exposed. Lesions formed by control (Mock), heparanase, or 8C gene constructs and inoculated into SCID/beige mouse mammary fat pad (n = 6). Xenografts were harvested 12 weeks after cell transplantation and formalin-fixed, paraffin-embedded 5-μm sections were subjected to histologic analyses. Shown are representative images of whole sections scanned by 3DHISTECH Pannoramic MIDI System attached to HITACHI HV-F22 color camera (3dhitech kft). C, immunostaining. Xenografts produced by MCF10AT1-infected cells were stained with hematoxylin and eosin [left; arrows, invasive carcinoma (vs. DCIS)], anti-Ki67 (second left), anti-SMA (middle), anti-E-cadherin (second right), and anti-vimentin (right) antibodies. Original magnifications, left and right, ×40; middle, ×10. Quantification of Ki67-positive cells is shown graphically in D. *P = 0.01 for Mock versus heparanase and Mock versus 8C.

Figure 1. Heparanase promotes the formation of disorganized acinar structures by MCF10A cells and tumorigicity of MCF10AT1 cells. A, acinar structures formation. Control (Mock), heparanase (Hepa), and 8C-infected MCF10A cells were plated on, and overlaid with Matrigel for 10 days. Formation of 3-dimensional acini-like structures was evaluated by fluorescent confocal imaging, applying DAPI nuclei counterstaining. B, tumorigenicity of MCF10AT1 cells. MCF10AT1 cells were infected with control (Mock), heparanase, or 8C gene constructs and inoculated into SCID/beige mouse mammary fat pad (n = 6). Xenografts were harvested 12 weeks after cell transplantation and formalin-fixed, paraffin-embedded 5-μm sections were subjected to histologic analyses. Shown are representative images of whole sections scanned by 3DHISTECH Pannoramic MIDI System attached to HITACHI HV-F22 color camera (3dhitech kft). C, immunostaining. Xenografts produced by MCF10AT1-infected cells were stained with hematoxylin and eosin [left; arrows, invasive carcinoma (vs. DCIS)], anti-Ki67 (second left), anti-SMA (middle), anti-E-cadherin (second right), and anti-vimentin (right) antibodies. Original magnifications, left and right, ×40; middle, ×10. Quantification of Ki67-positive cells is shown graphically in D. *P = 0.01 for Mock versus heparanase and Mock versus 8C.

In contrast, lesions developed by MCF10AT1-overexpressing heparanase were diagnosed as invasive carcinoma (Supplementary Fig. S3B, top; Hepa).

Implanting the MCF10AT1 cells in the mammary fat pad produced palpable lesions, and yielded noticeable differences. Overexpression of heparanase or its 8C variant resulted in far greater amount of highly cellular lesions (Fig. 1B, middle and right). Most importantly, although control cells developed into atypical hyperplasia/DCIS (Fig. 1C, top left; Mock), lesions formed by heparanase- or 8C-overexpressing MCF10AT1 cells were diagnosed as invasive carcinoma (Fig. 1C, left, arrows). These lesions exhibited higher levels of cell proliferation as indicated by staining for Ki67 (Fig. 1C, second left; Fig. 1D), decreased staining for SMA (Fig. 1C, middle) and E-cadherin (second right), and increased expression of vimentin (right)
that signifies epithelial–mesenchymal transition (EMT). Collectively, these results imply that heparanase and its signaling properties (8C) promote tumor expansion, cooperating with Ras.

**Heparanase cooperates with Ras to drive skin cancer**

To substantiate the cooperation observed between Ras and heparanase, we exposed Hpa-Tg and control Balb/c mice to 2-steps DMBA/TPA skin carcinogenesis model because more than 90% of skin cancer initiated by DMBA contained Ha-Ras activating mutations (32). Hpa-Tg mice were far more sensitive to DMBA/TPA treatment. Noticeable lesions were developed by Hpa-Tg mice already after 9 weeks of DMBA/TPA treatment whereas control mice did not show tumor lesions at this time (Supplementary Fig. S4A). At termination, 19 weeks after DMBA exposure, Hpa-Tg mice developed a significantly higher number and bigger tumor lesions compared with control mice (Fig. 2A; Supplementary Fig. S4B). Thus, an average of 10.2 tumor lesions were counted per Hpa-Tg mouse compared with 1.6 in control mice (Fig. 2B, left; $P = 1.5 \times 10^{-7}$), exhibiting a combined average tumor weight of 0.63 g per Hpa-Tg mouse compared with an average of 0.07 g per control mouse (Fig. 2B, right; $P = 1.5 \times 10^{-5}$). Histologically, lesions developed in control mice were diagnosed as a proliferative, hyperkeratosis process, whereas tumors developed by Hpa-Tg mice were diagnosed as atypical papilloma suspected early squamous cell carcinomas (Supplementary Fig. S5A). Heparanase was highly expressed in the tumor lesion and adjacent skin tissues of Hpa-Tg mice compared with control mouse tissues as indicated by immunoblotting (Fig. 2C) and immunostaining (Fig. 2D, left). This high level of heparanase expression was associated with increased cell proliferation as indicated by Ki67 staining in the tumor and skin tissues of Hpa-Tg mice (Fig. 2D, right and E), in agreement with accelerated tumor development in Hpa-Tg mice.

We next examined the phosphorylation levels of signaling components that are associated with accelerated tumor development in Hpa-Tg mice. Immunostaining for phospho-Erk yielded low reactivity in the skin tissue adjacent to the control tumor lesion (Fig. 3A, top left). In striking contrast, strong phospho-Erk staining was observed in the skin tissue of Hpa-Tg mice (Fig. 3A, top right; $P = 3.8 \times 10^{-7}$). Histologically, lesions developed in control mice were diagnosed as a proliferative, hyperkeratosis process, whereas tumors developed by Hpa-Tg mice were diagnosed as atypical papilloma suspected early squamous cell carcinomas (Supplementary Fig. S5A). Heparanase was highly expressed in the tumor lesion and adjacent skin tissues of Hpa-Tg mice compared with control mouse tissues as indicated by immunoblotting (Fig. 2C) and immunostaining (Fig. 2D, left). This high level of heparanase expression was associated with increased cell proliferation as indicated by Ki67 staining in the tumor and skin tissues of Hpa-Tg mice (Fig. 2D, right and E), in agreement with accelerated tumor development in Hpa-Tg mice.

Increased Akt activity is further revealed by reduced FOXO1 levels in Hpa-Tg tumor lesions (Fig. 3B, upper and second panels). Likewise, Akt phosphorylation is increased in Hpa-Tg lesions compared with control lesions, localizing primarily to cell junctions and mirroring phospho-Met localization (Fig. 3B, third panels). Increased Akt activity is further revealed by reduced FOXO1 levels in Hpa-Tg tumor lesions (Fig. 3B, fourth levels) because Akt-dependent phosphorylation reduces the DNA-binding activity of FOXO1 and elicits its export from the nucleus to the cytoplasm. Cytoplasmic Akt-phosphorylated FOXO1 interacts with ubiquitin ligases (Skp2, MDM2) and is targeted for proteasomal degradation (33, 34). These results imply that increased Erk phosphorylation in Hpa-Tg skin is associated with tumor initiation whereas increased Met and Akt phosphorylation contributes to accelerated tumor progression.

**Tumor development is severely attenuated in heparanase-KO mice**

To further reveal the role of heparanase in tumor initiation and progression, we next applied the DMBA/TPA protocol in heparanase knockout (Hpa-KO) and control C57/BL6 mice. Tumor formation seemed faster in the background of C57/BL6 compared with Balb/c mice, with lower incidence. By 19 weeks, control C57/BL6 mice developed 4.25 tumor lesions in average per mouse (Fig. 4A, Con and Supplementary Fig. S4C). In striking contrast, Hpa-KO mice failed to develop noticeable lesions; in 2 of 8 Hpa-KO mice very small dysplastic lesions were observed (Fig. 4A, KO, arrow and Supplementary Fig. S4C, arrows; $P = 3.8 \times 10^{-7}$). Histologic examination nonetheless identified the lesions developed in control and Hpa-KO mice as papilloma suspected early squamous cell carcinoma (Supplementary Fig. S5B). Surprisingly, phospho-Erk staining similar in magnitude was detected in the skin and tumor lesion of control and Hpa-KO mice (not shown). In contrast, phospho-Akt and phospho-Met staining were markedly reduced in the skin and tumor lesions, respectively, of Hpa-KO mice compared with control mouse (Fig. 4C, left and middle) whereas FOXO1 levels were increased (Fig. 4C, right), further implying reduced Akt activity in Hpa-KO lesions. Moreover, the phosphorylation of Src in tumor extracts correlated with heparanase levels. Thus, a 4-fold increase in Src phosphorylation was quantified in lesions developed in Hpa-Tg compared with control mouse (Fig. 4D, left), whereas a marked decrease in Src phosphorylation was observed in the very small lesions developed by Hpa-KO mice (Fig. 4D, right), in agreement with previous results relating heparanase and Src phosphorylation (14, 21, 35).

**Tumor development is attenuated by heparanase inhibitor, PG545**

To better appreciate the protumorigenic function of heparanase in the DMBA/TPA model system and the underlying molecular mechanism, we have next utilized a heparanase inhibitor, PG545 (9). By 15 weeks, when small tumor lesions start to appear (Supplementary Fig. S6A, top), Hpa-Tg mice were divided into 2 groups and PG545 was administrated once a week at 20 mg/kg (~400 µg/mouse, i.p.; ref. 9) for 3 weeks. As in previous experiments, Hpa-Tg mice were far more sensitive to DMBA/TPA treatment (Fig. 5A) and developed 13.3 lesions per mouse in average whereas no tumor lesions were seen in control mice (Fig. 5B). The number and size of tumors was decreased markedly in Hpa-Tg mice treated with PG545, reaching an average of 1.5 lesions per mouse, differences that are statistically highly significant ($P = 3 \times 10^{-7}$; Fig. 5A and B and Supplementary Fig. S6A), and associating with decreased Akt phosphorylation (Fig. 5C, left) and activity as indicated by increased FOXO1 levels (Fig. 5C, right). In addition, recruitment of macrophages to the tumor lesions was markedly attenuated by PG545 treatment (Fig. 5D, top) without noticeable change in macrophages content in the underlying skin tissue (Fig. 5D, bottom). Histologic examination diagnosed...
these lesions as papillomas, and revealed decreased downward epithelial proliferation in Hpa-Tg lesions treated with PG545 compared with untreated Hpa-Tg mice (not shown).

Prominent inhibition of tumor development in Hpa-Tg mice treated with PG545 suggests that heparanase enzymatic activity predominates, yet heparanase signaling, executed by the 8C variant, seems sufficient for tumor development by MCF10AT1 cells (Fig. 1B). We hypothesized that PG545 not only inhibits heparanase enzymatic activity but also its signaling capacity. Overexpression of heparanase in MDA-MB-231 breast carcinoma cells resulted in a 2-fold increase in Akt phosphorylation (Fig. 6A, Hepa, top). Akt phosphorylation was reduced markedly in cell treated with PG545 (Fig. 6A, +PG, top) in a dose-dependent manner (Fig. 6B), whereas Erk phosphorylation was not affected (Fig. 6A, bottom). Akt phosphorylation was similarly elevated in A431 epidermoid carcinoma cells following exogenous addition of heparanase (Fig. 6C, left), and reduced Akt phosphorylation was observed in A431 cells treated with PG545 (Fig. 6C, right), suggesting that this reagent neutralizes both enzymatic and signaling properties of heparanase.

Discussion

Compelling evidence tie heparanase with human cancer, but the timing of its induction and the significance of heparanase in the early phases of tumor initiation and development are largely obscure. In several examples (i.e., colon and esophagus; refs. 36 and 37), heparanase expression is induced already at the very early stages of tumor initiation. Results of this study suggest that elevated levels of heparanase early on have a prominent impact on tumor expansion and aggressiveness. This is exemplified by bigger, asymmetrical, and disorganized...
(i.e., lacking hollow lumen) acinar structures developed by MCF10A cells overexpressing heparanase or the 8C variant (Fig. 1A), and was most evident in MCF10AT1 cells. Overexpression of heparanase or the 8C variant in MCF10AT1 cells noticeably enhanced the expansion of tumor xenografts developed in the mammary fat pad (Fig. 1B). These lesions, as well as lesions developed subcutaneously (Supplementary Fig. S3B, top), progress into invasive carcinoma compared with DCIS developed by control lesions (Fig. 1C, left and Supplementary Fig. S3B, top). Xenograft expansion by heparanase/8C overexpression was associated with increased cell proliferation, marked reduction in the levels of SMA and E-cadherin, and elevation of vimentin expression (Fig. 1C), collectively signifying EMT that is considered a key process in cancer cell metastasis and acquired resistance to apoptosis (38–40). This, and the development of lymphatic vasculature within the tumor lesion (Supplementary Fig. S3B, bottom), strongly signify a progressive disease. The prometastatic function of heparanase thus emerges to uniquely combine cell invasion, activation of EMT program, and increased vascular and lymphatic vessel density that mobilize metastatic cells to distant organs (1, 2). This is accomplished by enzymatic (invasion and ECM remodeling) and nonenzymatic (cell migration, EMT) properties of heparanase, the latter critically exemplified by the 8C variant.

Because MCF10AT1 cells were generated by overexpression of active H-Ras in nontumorigenic MCF10A cells, we rationalized that heparanase cooperates with Ras to promote tumor progression. This notion is critically established by utilizing the DMBA/TPA 2-step skin carcinogenesis model system. By itself, heparanase does not function as an oncogene and its overexpression by essentially all tissues and cell types of the Hpa-Tg mice does not lead to tumor initiation over the life span of a mouse (ref. 25 and data not shown). However, combining heparanase overexpression with Ras activation by applying DMBA/TPA led to a 10-fold increase in the number and size of tumor lesions (Fig. 2A and B, Fig. 5A and B, and Supplementary Figs. S4A and S4B and S6). Moreover, lesions developed by Hpa-Tg mice seemed more advanced histologically (Supplementary Fig. S5), and exhibited higher proliferative capacity in the tumor lesion and adjacent skin tissue as indicated by Ki67 staining (Fig. 2D and E). As striking was the remarkable decrease in tumor development observed in Hpa-KO mice subjected to the DMBA/TPA treatment (Fig. 4A and B, and Supplementary Fig. S4C). Tumor lesions were hardly noticed by gross examination of the Hpa-KO mice (KO; Fig. 4A, arrow). Moreover, histologic examination of skin tissue away from noticeable lesions (Fig. 4A, rectangles) revealed tumor initiation in control but not KO mice (Supplementary Fig. S6B), further indicating low tumor incidence in the absence of

Figure 3. Accelerated tumor development in Hpa-Tg mice is associated with increased Erk, Met, and Akt phosphorylation. A, phospho-Erk. Five-micrometer sections of tumor lesions (Lesion) and adjacent skin tissue (Adj. Skin) of control (Con, top) and Hpa-Tg mice (Tg, bottom) were subjected to immunostaining, applying anti-phospho-Erk antibody. B, tumor lesions of control (Con, left) and Hpa-Tg (Tg, right) mice were similarly stained with anti-phospho-Met (top and second panels), anti-phospho-Akt (third panels), and anti-FOXO1 (bottom) antibodies. Original magnification, top and bottom panels, ×20; middle panels, ×40.
heparanase. Noteworthy, DMBA alone did not elicit premalignant or malignant process in control or Hpa-Tg mice even 6 months after its administration (Supplementary Fig. S7A, left). Similarly, TPA applied without prior DMBA application was not sufficient to drive tumor formation in Hpa-Tg mice (Supplementary Fig. S7A, right). This may suggest that heparanase cooperation with Ras is a major driving force in this skin cancer model, yet other mechanisms promoted by TPA cannot be ignored. Most relevant are inflammation induction and protein kinase C (PKC) activation by TPA. The essential contribution of inflammation to tumor development and progression has gained increasing acceptance (41, 42). TPA elicits a strong inflammatory reaction that likely facilitates the development of skin tumors (43). Given the cooperation of heparanase and inflammation in the aggravation of colon cancer (44), it is conceivable that a similar mechanism also occurs in the skin. TPA is also well known for its ability to activate PKC, a group of at least 10 related isoforms that are highly implicated in cancer progression (45, 46). Unlike the transient activation of PKC by the endogenous second messenger diacylglycerol (DAG) owing to its fast metabolism, the action of TPA is sustained, leading to prolonged activation of PKCs. The possible cooperation of heparanase with inflammation and PKCs in this model system is feasible, but awaits further in-depth investigation.

To appreciate the molecular mechanism underlying the tumor-promoting function of heparanase, we applied

**Figure 4.** Tumor development is severely attenuated in Hpa-KO mice. Control C57/BL6 (n = 8) and Hpa-KO mice (n = 8) were exposed to DMBA/TPA treatment as described in Materials and Methods for 19 weeks. At termination, mice were photographed (A), and the number of tumor lesions per mouse was counted and plotted as average number of lesions per mouse (B, *P* = 3.8 × 10⁻⁶). Representative images of the whole sections of tumor lesions developed by control (Con) and Hpa-KO (KO) mice scanned by 3DHISTECH Pannoramic MIDI System attached to HITACHI HV-F22 color camera are shown in A (right). Tumor lesions and adjacent skin tissues were fixed in 4% PFA for histologic evaluation or subjected to protein extraction and immunoblotting analyses. C, immunostaining. Five-micrometer sections of tumor lesions (middle and right) and adjacent skin tissue (left) of control (Con, top) and Hpa-KO (KO, bottom) mice were subjected to immunostaining, applying anti-phospho-Akt (pAkt, left), anti-phospho-Met (pMet, middle), and anti-FOXO1 (right) antibodies. Original magnification, left and middle, × 40; right, × 20. D, immunoblotting. Extracts of tumor lesions developed by control (Con) or Hpa-Tg (Tg; left) mice, and by control (Con) or Hpa-KO mice (KO; right) were subjected to immunoblotting, applying anti-phospho-Src (pSrc, top), anti-Src (middle), and anti-actin (bottom) antibodies. It should be noted that tumors developed faster and more aggressively in C57/BL6 than Balb/c mice. Thus, staining for signaling components seems most often stronger in control lesions developed in C57/BL6 (Fig. 4C) than Balb/c mice (Fig. 3B; i.e., pMet). Rectangles in A indicate skin tissue away from noticeable tumor lesion.
immunohistochemical analyses, examining the tumor lesion, immediate adjacent skin, and skin tissue without a noticeable tumor lesion (Fig. 4A, rectangles). Erk phosphorylation was increased in the skin of Hpa-Tg mice treated with TPA alone compared with control mice (Supplementary Fig. 7B, bottom). Erk phosphorylation was also markedly increased in the skin tissue adjacent to the tumor lesion in Hpa-Tg compared with control mice (Fig. 3A, left). Surprisingly, Erk phosphorylation seemed equally low in the tumor lesions developed in Hpa-Tg and control mice (Fig. 3A, right), suggesting that Erk phosphorylation is essential for the tumor initiation and promotion phases but to a lesser extent in the later stages of tumor progression in this model (47). Instead, we found that Met phosphorylation is increased in the skin tissue adjacent to the tumor lesion in Hpa-Tg compared with control mice (Fig. 3A, left). Surprisingly, Erk phosphorylation seemed equally low in the tumor lesions developed in Hpa-Tg and control mice (Fig. 3A, right), suggesting that Erk phosphorylation is essential for the tumor initiation and promotion phases but to a lesser extent in the later stages of tumor progression in this model (47). Instead, we found that Met phosphorylation is increased in the tumor lesions developed by Hpa-Tg mice compared with control mice (Fig. 3B, top two panels), suggesting that this pathway possibly mediates tumor expansion. Furthermore, we found that Akt phosphorylation is increased in lesions developed by Hpa-Tg mice compared with control lesion (pAkt; Fig. 3B, third panels), yet, the result that distinguished most markedly between the two types of lesions was staining for the Akt substrate, FOXO1 (Fig. 3B, bottom). FOXO1 is a direct substrate of Akt; phosphorylation of FOXO1 by Akt results in exclusion of FOXO1 from the nucleus and its translocation to the cytoplasm where it is subjected to proteasomal degradation following ubiquitination (33, 34). Indeed, although FOXO1 is readily detected in lesions developed in control mice, localizing to the cell nuclei, its levels are reduced substantially in tumor lesions developed in Hpa-Tg mice (Fig. 3B), thus indirectly reflecting increased Akt activity by heparanase. This is in agreement with previous results showing decreased FOXO1 levels in tumor xenografts produced by U87 glioma cells over expressing heparanase (27). Met phosphorylation and Akt activity seem most relevant to tumor promotion by heparanase because these signaling pathways are inhibited considerably in lesions developed by Hpa-KO mice (Fig. 4C; Supplementary Fig. S6B). Moreover, Akt phosphorylation was reduced and FOXO1 levels were increased following treatment with the heparanase inhibitor PGS545 (Fig. 5).
properties of heparanase and thus govern its potency in vivo. PG545 (Fig. 5C). We also found that PG545 inhibits Akt phosphorylation. A, MDA-MB-231 breast carcinoma cells were infected with Hepa or a Mock and were left untreated or incubated for 20 hours with PG545 (10 μg/mL; + PG). Lysate samples were then subjected to immunoblotting applying anti-phospho-Akt (top), anti-Akt (second), anti-phospho-Erk (third), and anti-Erk (fourth) antibodies. B, dose response. MDA-MB-231 cells were incubated without (0) or with the indicated concentration of PG545. Lysate samples were then subjected to immunoblotting, applying anti-phospho-Akt (top) and anti-Akt (second) antibodies. C, latent heparanase was added to A431 epidermoid carcinoma cells for the time indicated, and cell lysates were subjected to immunoblotting, applying anti-phospho-Akt (top), anti-Akt (second), anti-phospho-Erk (third), and anti-Erk2 (bottom) antibodies (left). A431 cells were left incubated without (Con) or with PG545 (20 hours, 10 μg/mL; PG), and lysate samples were subjected to immunoblotting, applying anti-phospho-Akt (top) and anti-Akt (bottom) antibodies (right).

PG545 (Fig. 5C). We also found that PG545 inhibits Akt phosphorylation in vitro (Fig. 6), suggesting that this compound neutralizes both enzymatic activity and signaling properties of heparanase and thus governs its potency in vivo. Inhibition of Akt phosphorylation by PG545 seemed more prominent in breast MDA-MB-231 cells that carry K-Ras mutation compared with A431 cells that expresses normal Ras (48, 49), suggesting that patients that are diagnosed with high levels of heparanase and also carry Ras mutation may benefit the most from PG545 treatment. Studies examining this aspect in breast and skin/melanoma cell lines are currently in progress.

We were unable to obtain a reproducible staining for phospho-Src. In immunoblotting, nonetheless, Src phosphorylation is increased 4-folds in tumors developed in Hpa-KO mice whereas a comparable decrease was observed in lesions developed in Hpa-Tg mice (Fig. 4D), collectively positioning Akt and possibly Src phosphorylation as major determinants underlying the protumorigenic capacity of heparanase.

Marked decrease in tumor-associated macrophages following treatment with PG545 (Fig. 5D) is in agreement with previous results in a model of pancreatic cancer (50) and provides additional mechanism for attenuated tumor expansion by heparanase inhibitors.

Cooperation of heparanase and Ras described above in preclinical tumor models is likely to be clinically relevant because mutant forms of Ras function as driver oncogenes in approximately one third of human cancers (51). Most recent example is the frequent mutations in K-Ras, N-Ras, and B-Raf identified in subclonal myeloma cell populations (52). Heparanase promotes myeloma growth, dissemination, and angiogenesis (53, 54), and heparanase inhibitor (Roneparstat = SST0001) is being tested in phase I clinical trial in patients with myeloma. The aggressive phenotype exerted by heparanase in myeloma is largely attributed to increased syndecan-1 expression and shedding because this proteoglycan is considered critical determinant of myeloma cell survival and growth (55–57). Cooperation with Ras may provide another mechanism for the protumorigenic function of heparanase in myeloma and possibly other hematologic and solid malignancies carrying Ras mutation (58).

Taken together, our results indicate that heparanase plays a decisive role in tumor development. Mediated in part by activation of Erk, Akt, Src, and Met phosphorylation, the heparanase inhibitor PG545 potently attenuates tumor progression in a model system that seems relevant to the development of human cancer (i.e., exposure to carcinogens, acquired mutations, activation of oncogenes), and seems to neutralize both enzymatic and signaling properties of heparanase, lending hope for a favorable outcome in the clinic.

Disclosure of Potential Conflicts of Interest

E. Hammond is an inventor on a PG545 patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: N. Ilan, I. Vlodavsky
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Boyango, U. Barash, J.-P. Li, N. Ilan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Boyango, U. Barash, I. Naroditsky, N. Ilan
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Other (discussion with senior author): J.-P. Li

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