Heparanase Enhances Tumor Growth and Chemoresistance by Promoting Autophagy

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Abstract

Heparanase is the only enzyme in mammals capable of cleaving heparan sulfate, an activity implicated in tumor inflammation, angiogenesis, and metastasis. Heparanase is secreted as a latent 65-kDa enzyme, heparanase rapidly interacts with cell membrane heparan sulfate proteoglycans (HSPG; i.e., syndecans; refs. 7–10), followed by internalization and processing into a highly active 50-kDa enzyme (7, 11). Notably, heparanase was shown to reside primarily within endocytic vesicles, assuming a polar, perinuclear localization and colocalizing with lysosomal markers (7, 8, 10, 12). In addition, it has been demonstrated that incubation with endosomal/lysosomal fraction, but not membrane or cytosolic prepara-

tions, leads to heparanase processing and activation (13). Likewise, heparanase processing was blocked by chloroquine and bafilomycin A1, which inhibit lysosomal proteases by raising the lysosome pH (11). Subsequent studies using site-directed mutagenesis, gene silencing, and pharmacological inhibitors have identified cathepsin L as the primary lysosomal protease responsible for heparanase processing and activation (14–16). Despite its localization in a highly active protein degradation environment such as the lysosome, heparanase appears stable (12) and exhibits a half-life of about 30 hours (17), relatively long compared with a 1/2 of 2 to 6 hours and 25 minutes of transmembrane and GPI-anchored HSPGs, respectively (18). Residence and accumulation of heparanase in lysosomes may indicate that the enzyme functions in the normal physiology of this organelle but such a function has not been described yet.

Autophagy is an evolutionarily conserved catabolic pathway through which cytoplasmic components, including macromolecules such as proteins and lipids as well as whole organelles, are sequestered into double-membrane vesicles called autophagosomes. Autophagosomes are subsequently fused with lysosomes, where the intracellular material is degraded and recycled. This process occurs in every cell at a basal level and is required to remove unfolded proteins and damaged organelles, thus maintaining cellular homeostasis. Autophagy is induced significantly by starvation and stress, promoting cancer cells survival by providing their metabolic needs (19, 20). Here, we provide evidence that heparanase enhances autophagy. Moreover, we show that enhanced tumor growth and chemoresistance exerted by heparanase are mediated, in part, by its proautophagic function, as demonstrated in tumor xenograft models of human cancer and through use of inhibitors of the lysosome (chloroquine) and heparanase (PG545), both alone and in combination. Notably, heparanase-overexpressing cells were more resistant to stress and chemotherapy in a manner associated with increased autophagy, effects that were reversed by chloroquine treatment. Collectively, our results establish a role for heparanase in modulating autophagy in normal and malignant cells, thereby conferring growth advantages under stress as well as resistance to chemotherapy.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cells and cell culture

Control (Mock) and heparanase-overexpressing human FaDu pharyngeal carcinoma, U87 glioma, and rat C6 glioma cells...
have been described previously (8, 21–23). SIHN-013 laryngeal carcinoma cells were kindly provided by Sue Eccles (Institute of Cancer Research, Sutton, Surrey, UK; ref. 24). Cells were grown in DMEM (Biological Industries) supplemented with 10% FBS and antibiotics. Mouse embryonic fibroblasts (MEF) of control and heparanase knockout (KO) mice have been described elsewhere (25). Cells were passed in culture no more than 2 months after being thawed from authentic stocks.

Antibodies and reagents

Anti-phospho-p70S6 kinase, anti-p70S6 kinase, anti-PTEN, and anti-mTOR antibodies were purchased from Cell Signaling. Anti-LC3 and anti-actin monoclonal antibodies were purchased from Sigma. The heparanase inhibitor PG545 was kindly provided by Progen Pharmaceuticals (26). Cisplatin and doxorubicin were obtained from the Oncology Department, Rambam Health Care Campus (Haifa, Israel). LysoTracker was purchased from Molecular Probes (Life Technologies); Torin was purchased from Tocris Bioscience.

Cell lysates and immunoblotting

Preparation of cell and tissue extracts and immunoblotting were carried out essentially as described (10). Following induction of autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (LC3-II), which is recruited to autophagosomal membranes, serving as a most acceptable mean to measure autophagy levels. Immunoblots were subjected to densitometry analyses and the relative intensity of bands (i.e., fold change) is presented underneath the gel.

Immunocytochemistry

Immunofluorescent staining of methanol-fixed cells and analysis by confocal microscopy was carried out essentially as described (10). To better detect LC3 by immunofluorescence or electron microscopy, autophagy was initiated by depriving the cells of amino acids for 3 hours, in the presence of chloroquine (Chl, 50 μg/mL; Sigma).

Colony formation in soft agar

DMEM (3 mL) containing 0.5% low-melt agarose (Bio-Rad) and 10% FCS was poured into 60-mm Petri dishes. The layer was covered with cell suspension (2 × 10⁶ cells) in 1.5-mL DMEM containing 0.3% low-melt agarose and 10% FCS, followed by addition of 2-mL DMEM containing 10% FCS without or with PG545 (25 μg/mL), chloroquine (50 μg/mL), or both. Medium was exchanged every 3 days. Colonies were visualized and counted under a microscope 2 to 5 weeks after seeding, as described previously (27).

MTT assay

The number of viable cells was evaluated by thiazolyl blue tetrazolium bromide (MTT; Sigma) that measures the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, yielding a purple color. Cells (5 × 10³/well) were grown in 96-well plate and subject to various treatments as indicated. MTT (100 μg) was then added to each well for 2 to 3 hours, followed by centrifugation. The cell pellet was resuspended in 150 μL of isopropanol and absorbance was measured at 570 nm using an ELISA plate reader, as described (27).

Tumorigenicity and immunohistochemistry

U87 cells were detached with trypsin/EDTA, washed with PBS, and brought to a concentration of 5 × 10⁶ cells/mL. Cell suspension (5 × 10⁶/0.1 mL) was inoculated subcutaneously at the right flank of 6-week-old female SCID mice (n = 7). Mice were administrated with the heparanase inhibitor PG545 (20 mg/kg; once weekly), chloroquine (50 mg/kg; every day), or both, and xenografts size was determined by externally measuring tumors in 2 dimensions using a caliper. At the end of the experiment, mice were sacrificed; tumor xenografts were removed, weighed, and fixed in formalin. Paraffin-embedded 5-μm sections were subjected to immunostaining applying anti-LC3, anti-phospho-p70S6K, or anti-glial fibrillary acidic protein (GFAP; Dako) antibodies using the Envision Kit according to the manufacturer’s (Dako) instructions, as described previously (28).

Statistical analysis

Data are presented as means ± SE. Statistical significance was analyzed by the 2-tailed Student t test. Values of P < 0.05 were considered significant. Datasets passed D’Agostino–Pearson normality (GraphPad Prism 5 utility software). All experiments were repeated at least 3 times with similar results.

Results

Heparanase augments autophagy

We first examined the possibility that heparanase is localized within autophagosomes. To this end, latent heparanase (1 μg/mL) was added exogenously to HeLa cells stably expressing LC3-GFP gene construct and cells were left untreated (Con) or were deprived of amino acids (AA) in the presence of chloroquine. The latter is a lysosome inhibitor that allows the formation and accumulation of autophagosomes but prevents their activity. LC3-GFP fluorescence was evident only in conditions that stimulate autophagy (AA + Chl; Fig. 1A, top, green), co-localizing with heparanase (Fig. 1A, top, bottom, yellow). A similar colocalization of endogenous LC3 and heparanase was noted in SIHN-013 laryngeal carcinoma cells overexpressing heparanase following autophagy stimulation (Fig. 1B). We further found that LC3-II level was reduced in MEF derived from Hpa-KO mice that lack heparanase activity (Fig. 2A, bottom; ref. 25) versus control MEF (Fig. 2A, top, Con) and even greater reduction in LC3-II level was evident by immunoblotting (Fig. 3A, bottom) of heparanase-overexpressing MEF. A similar reduction of amino acids (AA) in the presence of chloroquine. The latter is a lysosome inhibitor that allows the formation and accumulation of autophagosomes but prevents their activity. LC3-GFP fluorescence was evident only in conditions that stimulate autophagy (AA + Chl; Fig. 1A, top, green), co-localizing with heparanase (Fig. 1A, top, bottom, yellow). A similar colocalization of endogenous LC3 and heparanase was noted in SIHN-013 laryngeal carcinoma cells overexpressing heparanase following autophagy stimulation (Fig. 1B). We further found that LC3-II level was reduced in MEF derived from Hpa-KO mice that lack heparanase activity (Fig. 2A, bottom; ref. 25) versus control MEF (Fig. 2A, top, Con) and even greater reduction in LC3-II level was quantified after treatment with chloroquine (Fig. 2A, top, Chl). Reduced LC3-II level in Hpa-KO MEF was also evident by immunofluorescent staining (Fig. 2A, middle). Similarly, we observed reduced autophagy levels (i.e., LC3-II) in the lung, kidney, and mammary gland of Hpa-KO mice (Fig. 2B, KO), whereas increased level of autophagy was observed in the lung, mammary gland, and pancreas tissues of transgenic mice (Hpa-Tg; ref. 29) overexpressing heparanase (Fig. 2B, Tg). Increased autophagy in the pancreas of Hpa-Tg mice versus control was further evident by electron microscopy (EM; Fig. 2C, top, P = 0.0003). Collectively, these results suggest that heparanase is localized within autophagosomes and is involved in the regulation of autophagy.

Even higher increase of autophagy was observed following heparanase overexpression in tumor-derived cells. Autophagy (i.e., LC3-II) was markedly increased under resting conditions (Con, Fig. 3A–C, top), and further increase in LC3-II levels was noted following chloroquine treatment (Fig. 3A and B; Chl, top), evident by immunoblotting (Fig. 3A–C, top) and immunofluorescent staining (Fig. 3A, bottom) of heparanase-overexpressing...
cells treated with PG545 and chloroquine (PG
kDa) forms of heparanase was detected in the culture medium of
even higher accumulation of the latent (65 kDa) and active (50
icking the effect of heparan/heparan sulphate (7). Unexpectedly,
accumulation of heparanase in the culture medium of U87-Hepa
treated with this compound (PG; Fig. 3D, top), thus mim-
overexpressing heparanase (Hepa; Fig. 3C, right). LC3-II induction comparable in magnitude was evident also
by U87 glioma cells overexpressing heparanase (Hepa; Fig. 3C, Con); notably, autophagy induction was prevented by the hepar-
anase inhibitor PG545 (PG; Fig. 3C) and was associated with
accumulation of heparanase in the culture medium of U87-Hepa
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ing the effect of heparan/heparan sulphate (7). Unexpectedly,
even higher accumulation of the latent (65 kDa) and active (50
kDa) forms of heparanase was detected in the culture medium of
cells treated with PG545 and chloroquine (PG + Chl; Fig. 3D, top).
In PG545-treated cells, and to a lesser extent PG545 plus chloro-
quine-treated cells, accumulation of heparanase in the culture medium was accompanied by decreased levels of intracellular heparanase (Fig. 3D, second and bottom; Supplementary Fig. S1C), implying that PG545 attenuates autophagy also by decreasing the content of intracellular heparanase.

Protumorigenic function of heparanase involves induction of
autophagy

We next examined whether induction of autophagy underlies the protumorigenic function of heparanase. We have shown previously that U87 glioma cells overexpressing heparanase are endowed with higher proliferation rate and form more and bigger colonies in soft agar (21, 22, 30). Applying the MTT assay we found that chloroquine treatment reduces U87 cell viability compared with control untreated cells (Fig. 4A, Chl), decrease that was most pronounced when combined with pharmacol-
ically relevant concentrations (8 μmol/L; ref. 31) of the hepar-
anase inhibitor PG545 (Fig. 4A, Chl + PG; **, P = 6.2 × 10−10 for Chl vs. PG + Chl). A similar synergistic effect of chloroquine and PG545 was observed with C6-HePa rat glioma cells (Fig. 4B; **, P = 0.01 for PG vs. PG + Chl). Notably, colony formation in soft agar was markedly reduced by chloroquine treatment (Fig. 4C, Chl), synergizing with PG545 in terms of colony number (Fig. 4C and D; **, P = 0.003 for Chl vs. PG + Chl) and size (Fig. 4C). We have reported previously that overexpression of heparanase in U87 cells enhanced the growth of tumor xenografts (21, 30). Immunohistochemistry revealed that the growth advantage of these tumor xenografts is associated with increased staining of LC3 (Fig. 5A, Hepa). To further reveal the role of autophagy in this system, mice were inoculated subcutaneously with U87 cells and treated with chloroquine (50 mg/kg, every
day), PG545 (20 mg/kg; once a week), or both. Tumor growth was
attenuated by chloroquine (P = 0.02 for Con vs. Chl) and PG545
(P = 0.0004 for Con vs. PG) as single agents and was most evident when the two compounds are combined (Fig. 5B and C; **, P = 0.006 for PG + Chl and P = 0.003 for Chl vs. PG + Chl), resembling the in vitro results (Fig. 4A, C, and D). In addition, we found that U87 tumor xenografts from treated mice exhibit higher levels of GFAP immunoreactivity (Fig. 5D), suggesting that these

Figure 1. Heparanase colocalizes with LC3-II. A, GFP-LC3. Heparanase (1 μg/mL) was added exogenously to HeLa cells stably expressing a GFP-LC3 gene construct for 24 hours. Cells were then deprived of amino acids in the presence of chloroquine (50 μg/mL; AA + Chl) for 3 hours or were incubated under serum-free conditions as control (Con). Cells were then fixed with methanol and subjected to immunofluorescent staining applying anti-heparanase (middle, red) antibody. Colocalization of heparanase and GFP-LC3 appears yellow (bottom right). B, SIHN-013 laryngeal carcinoma cells overexpressing heparanase were starved for amino acids for 3 hours in the presence of chloroquine and were then fixed with methanol and subjected to immunofluorescent staining using anti-heparanase (middle, green) and anti-LC3 (top, red) antibodies. Note colocalization (bottom, yellow) of heparanase and endogenous LC3 in autophagosomes. Original magnifications, ×60.

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lesions are not only smaller but also assume higher level of differentiation. In the skin, LC3-II levels are undetectable under normal conditions in control, Hpa-Tg, or Hpa-KO mice (not shown). Exposing the skin tissue to 2-step 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) skin carcinogenesis model resulted in increase in the number and size of tumor lesions in Hpa-Tg compared with control mice (32). Notably, the increased tumorigenicity in Hpa-KO MEF was quantified (bottom numbers) by densitometry analyses compared with control MEF in each culture conditions. B, autophagy levels in Hpa-Tg and Hpa-KO mouse tissues. Extracts were prepared from the indicated tissues of control (C), Hpa-Tg (Tg), and Hpa-KO (KO) mice overexpressing or lacking heparanase, respectively, and lysate samples were subjected to immunoblotting, applying anti-LC3 and anti-actin antibodies. C, electron microscopy. Pancreas tissues from control (Con) and Hpa-Tg mice were fixed in glutaraldehyde and processed for electron microscopy. Shown are representative images at ×10,000 magnification. Quantitation of autophagosomes (*) is shown graphically at the bottom. P = 0.0003 for Con versus Hpa-Tg.

Figure 2. Endogenous heparanase levels reflect autophagy extent. A, MEFs were isolated from control (Con) and Hpa-KO (KO) mice and examined for heparanase activity (bottom) and LC3 levels by immunofluorescent staining (green, middle). Nuclei counterstaining is shown in blue. Original magnifications, ×60. Control (C) and KO MEF were incubated under serum-free conditions without (Con) or with chloroquine (Chl; 50 μg/mL) for 3 hours. Cell lysates were then subjected to immunoblotting, applying anti-LC3 (top) and anti-actin (second panel) antibodies. Note, decreased levels of LC3 in Hpa-KO MEF.

Mechanistically, autophagy induction by heparanase appears to involve the mTORC1. This nutrient-sensing kinase acts as a master negative regulator of autophagy because during
starvation, mTOR1 is inhibited and this induces autophagy (33). mTOR1 activity can be assessed by the phosphorylation status of p70S6K, a specific downstream substrate of mTOR1. In cells overexpressing heparanase, we observed reduced phospho-p70S6 kinase levels (Fig. 6A, Con, top), in agreement with increased autophagy (Fig. 3). Moreover, a substantial increase in p70S6K phosphorylation level was observed in Hpa-KO MEF versus control (WO; Fig. 6B), in agreement with reduced autophagy in these cells (Fig. 2A). In accordance with these results, we observed increased p70S6K phosphorylation levels in tumor xenografts following treatment with inhibitors of heparanase (PG545) or autophagy (chloroquine; Fig. 6C; Supplementary Fig. S1D). The alterations in p70S6K phosphorylation in relation to heparanase levels were associated with the cellular localization of mTOR1. In control MEF, mTOR1 was localized within scattered vesicles but appeared diffusely distributed in Hpa-KO MEF (Fig. 6D, WO). p70S6K phosphorylation was reduced markedly in MEF treated with the heparanase inhibitor PG545 without (PG; 25 μg/mL) or with chloroquine (PG + Chl) for 20 hours, and lysate samples were subjected to immunoblotting for LC3 and actin. Note a marked increase in LC3-II levels by heparanase overexpression that is prevented by PG545. The extent of increased LC3-II was quantified by densitometry analyses of heparanase-overexpressing cells (Hepa) compared with control cells in each culture conditions (bottom numbers). D, accumulation of heparanase in the culture medium of cells treated with PG545. Control (Mock) and heparanase-overexpressing (Hepa) U87 cells were cultured without (Con) or with PG545 (PG; 25 μg/mL), chloroquine (Chl; 50 μg/mL), or both for 20 hours. Cell-conditioned medium (top) and cell lysates (middle) were subjected to immunoblotting, applying anti-heparanase and anti-actin (bottom) antibodies. U87-Hepa cells cultured without (Con) or with PG545 (PG) were similarly subjected to immunofluorescent staining with anti-heparanase antibody (bottom). Original magnifications, ×60.

Figure 3.
Heparanase overexpression enhances autophagy. A and B, head and neck carcinoma-derived cells. Control (Mock) and heparanase-overexpressing (Hepa) SIHN-013 laryngeal carcinoma (A) and FaDu pharyngeal carcinoma (B) cells were cultured without (Con) or with chloroquine (Chl) for 3 hours. Lysate samples were then subjected to immunoblotting, applying anti-LC3 (top) and anti-actin (bottom) antibodies. Control (Mock) and heparanase-overexpressing SIHN-013 cells were subjected to immunofluorescent staining, applying anti-LC3 (top, red) and anti-heparanase (middle right, green) antibodies. Merged images are shown at the bottom. Electron microscopy, control (Mock) and heparanase-overexpressing FaDu cells were deprived of amino acids for 3 hours in the presence of chloroquine, fixed with glutaraldehyde, and processed for electron microscopy. Shown are representative images at ×10,000 magnification (B; bottom). Note increased abundance and size of autophagic vacuoles followings heparanase overexpression. C and D, PG545 treatment. C, control (Mock) and heparanase-overexpressing (Hepa) U87 glioma cells were left untreated (Con) or were incubated with the heparanase inhibitor PG545 without (PG; 25 μg/mL) or with chloroquine (PG + Chl) for 20 hours, and lysate samples were subjected to immunoblotting for LC3 and actin. Note a marked increase in LC3-II levels by heparanase overexpression that is prevented by PG545. The extent of increased LC3-II was quantified by densitometry analyses of heparanase-overexpressing cells (Hepa) compared with control cells in each culture conditions (bottom numbers). D, accumulation of heparanase in the culture medium of cells treated with PG545. Control (Mock) and heparanase-overexpressing (Hepa) U87 cells were cultured without (Con) or with PG545 (PG; 25 μg/mL), chloroquine (Chl; 50 μg/mL), or both for 20 hours. Cell-conditioned medium (top) and cell lysates (middle) were subjected to immunoblotting, applying anti-heparanase and anti-actin (bottom) antibodies. U87-Hepa cells cultured without (Con) or with PG545 (PG) were similarly subjected to immunofluorescent staining with anti-heparanase antibody (bottom). Original magnifications, ×60.
phosphorylation to an extent 7-fold lower than in control MEF (Supplementary Fig. S2C).

**Autophagy induction by heparanase-endowed cells with chemoresistance**

While the protumorigenic properties of heparanase are well documented, its function in chemoresistance has not been so far explored. To examine this aspect, we first exposed control (Mock) and heparanase-overexpressing (Hepa) U87 glioma cells to amino acid starvation without (AA; Fig. 7A) or with chloroquine (AA + Chl; Fig. 7A) and cell viability was evaluated by MTT assay as described in Materials and Methods. U87 cells were cultured in soft agar and were left untreated (Con) or were incubated with chloroquine (Chl; 50 μg/mL), PGS45 (PG; 25 μg/mL), or both (PG + Chl). After 3 weeks, dishes were photographed (C) and the number of colonies was counted (D).

**Discussion**

Heparanase expression is increased in many types of tumors and this elevation is often associated with more aggressive disease and poor prognosis (1, 2, 34), but the role of heparanase under normal conditions has not been resolved thus far. Here, we describe for the first time a role of lysosomal heparanase in modulating autophagy, an evolutionary conserved mechanism that delivers intracellular proteins, lipids, and organelles to the lysosomal compartment for degradation and recycling (20).

Residing for relatively long period of time in the lysosome led us to hypothesize that heparanase may be found within autophagosomes. Indeed, double immunofluorescent staining clearly shows that heparanase colocalizes with GFP-labeled and endogenous LC3-II (Fig. 1). LC3-II is a convenient and widely used marker of autophagy because it forms and remains associated with the autophagosome even after fusion with lysosomes. Moreover, LC3-II is the only known protein that specifically associates with autophagosomes and not with other vesicles and thus its level reflects the amount of autophagic vacuoles (35). Our results indicate that heparanase does not only reside in autophagosomes but also modulate autophagy. This is concluded because reduced LC3-II levels are found in MEF and tissues obtained from heparanase KO mice (Fig. 2B).
Likewise, LC3-II level was enhanced in transgenic mice that overexpress heparanase (Fig. 2B, Tg; ref. 29).

Even higher induction of autophagy was evident in head and neck carcinoma (SIHN-013, FaDu) and glioma (U87, C6) cells overexpressing heparanase (Fig. 3A–C). These cell systems were preferred because previous results provided a strong preclinical and clinical significance of heparanase in the progression of these malignancies (21–23, 30, 36–38). A noticeable 3- to 8-fold increase in LC3-II levels was observed under control, serum-free conditions (Fig. 3, Con) and following treatment with chloroquine (Fig. 3, Chl), revealed by biochemical measures and electron microscopy. Interestingly, electron microscopic analyses of FaDu cells overexpressing heparanase revealed not only a higher number of autophagic vacuoles (Supplementary Fig. S1B, bottom, asterisks) but also abundant release of vesicles, possibly exosomes, from the cell surface (Supplementary Fig. S1B, bottom, arrow). This supports the notion that heparanase enhances exosome secretion that contributes to tumor growth (39, 40).

The mechanism underlying autophagy induction by heparanase is not entirely clear but likely involves mTOR1 that plays a pivotal role in nutrient-sensing and autophagy regulation (41). mTOR1 activity inhibits autophagy but under starvation, its activity is repressed, leading to autophagy induction. Similarly, mTOR1 inhibitors such as rapamycin induce autophagy (42). Interestingly, we found that heparanase overexpression...
Figure 6.
Heparanase modulates p70S6K phosphorylation. A, immunoblotting. Control (Mock) and heparanase (Hepa)-overexpressing SHN-013 (left) and U87 (right) cells were cultured under serum-free conditions without (Con) or were deprived of amino acids for 3 hours. Cell lysates were subjected to immunoblotting, applying anti-phospho-p70S6K (p-P70; top), p70S6K (P70; second panels), and anti-actin (bottom) antibodies. B, MEF obtained from control (Con) and heparanase KO mice were incubated without (WO) or with Torin (mTOR inhibitor; 500 μmol/L, 3 hours). Cell lysate samples were then subjected to immunoblotting applying anti-phospho-p70S6K (p-P70; top), p70 S6-kinase (P70; middle), and anti-actin (bottom) antibodies. C, mice were inoculated with U87 cells and were administered with PBS (Con) or with chloroquine (Chl, 50 μg/mL), PG545 (PG, 20 μg/mL), or both. At termination, tumor lysates were subjected to immunoblotting, applying anti-phospho-p70 (top) and anti-actin (bottom) antibodies. D, MEF obtained from control (Con) and heparanase KO mice were incubated without (WO) or with Torin (500 μmol/L, 3 hours) and were then subjected to immunofluorescent staining, applying anti-mTOR antibody. E and F, mTOR localization. E, control (Mock) and heparanase (Hepa)-overexpressing U87 cells were subjected to immunofluorescent staining (applying) anti-mTOR (top, red) and anti-heparanase (middle, green) antibodies. Merged images are shown in the bottom. Note diffused, scattered distribution of mTOR in control cells compared with perinuclear staining that colocalized with heparanase (bottom right) in transfected cells. F, cells were similarly stained for mTOR (top, red) and LysoTracker (middle, green). Nuclei counterstaining appear in blue. Merged images are shown in the lower panels.
associates with reduced mTOR1 activity, evident by decreased levels of p70S6K phosphorylation, an mTOR1 substrate (Fig. 6A, top; Supplementary Fig. S2B), whereas heparanase-KO MEF are endowed with increased p70S6K phosphorylation levels (Fig. 6B). Similarly, heparanase inhibition by PG545 results in increased mTOR1 activity and p70S6K phosphorylation (Fig. 6C; Supplementary Fig. S1D). The reason for mTOR1 inhibition by heparanase may be related to alteration of mTOR1 localization within the cell evident in three independent experimental settings. First, vesicular mTOR1 appeared scattered in control MEF but accumulate at perinuclear regions following treatment with mTOR inhibitor (Torin, Fig. 6D, left). Similarly, mTOR1 appeared more diffusely scattered in control cells, whereas in cells with high content of heparanase, mTOR1 is found mostly in perinuclear regions, colocalizing with heparanase (Fig. 6E, Hepa) and LysoTracker (Fig. 6F, Hepa) that labels acidic lysosomal vesicles. Third, in heparanase KO, MEF mTOR1 do not reside in vesicles but rather diffusely distributed in the cytoplasm (Fig. 6D), associated with increased p70S6K phosphorylation (Fig. 6B, WO). This agrees with the notion that activation of mTOR1 by nutrients is associated with peripheral lysosomes, whereas starvation leads to perinuclear clustering of lysosomes and decreased mTOR1 activity (43). Thus, Torin, and to a lesser degree heparanase, results in accumulation of mTOR1 at perinuclear regions, leading to reduced mTOR1 activity and increased autophagy (43, 44).

Localization of mTOR1 to LysoTracker-positive acidic vesicles appeared unique because no such colocalization was evident for PTEN or TSC2 (Supplementary Fig. S3), intrinsic components of the PI3K signaling pathway. This is not surprising because we used nutrient starvation to induce autophagy, which, unlike growth factors, is not regulated by the PTEN/Akt/TCS pathway but rather involves as yet uncharacterized cytoplasmic sensor (45, 46).

Compelling evidence has shown that heparanase is upregulated in various primary solid tumors (i.e., carcinomas and sarcomas) and hematologic malignancies (2, 34, 47). The consequence of heparanase induction most often associates with disease progression and bad prognosis, thus encouraging the development of heparanase inhibitors (26, 48, 49).
molecular mechanism(s) exerted by heparanase to promote tumor initiation and progression is still incompletely understood. The results of the current study imply that autophagy induction contributes to the protumorigenic function of heparanase. This emerges from in vitro and in vivo experiments using inhibitors of autophagy (chloroquine) and heparanase (PG545) alone or in combination. The heparanase inhibitor PG545 significantly reduces U87 cell survival and colony number (Fig. 4A and C), resembling the effect of PG545 on pancreatic carcinoma cells (31). Notably, chloroquine treatment resulted in a comparable effect and even more effective inhibition is obtained by combining PG545 and chloroquine in vitro (Fig. 4, PG + Chi) and, moreover, in a tumor xenograph model in vivo, resulting in significantly smaller and more differentiated tumors (Fig. 5A–D). Increased cell differentiation (i.e., elevation of E-cadherin) was also noted in pancreatic carcinoma cells treated with PG545 (31), suggesting that heparanase activity drives cancer cell dedifferentiation as part of its protumorigenic properties.

Immunostaining further revealed that enhanced tumor growth in Hpa-Tg mice exposed to 2-step skin carcinogenesis model (DMBA/TPA;32) is associated with increased LC3 staining, which is reduced substantially by PG545 (Fig. 5E, left), along with decreased tumor burden (32), thus adding another route by which this compound attenuates tumor growth (50). In a subsequent experiment, the number and size of tumor lesions developed by Hpa-Tg mice exposed to the DMBA/TPA regimen were not affected by chloroquine (not shown). Pathologic examination revealed, nonetheless, that chloroquine treatment resulted in less aggressive tumors indicated by exophytic growth pattern and less invasive capacity toward the underlying skin tissue compared with untreated lesions (Fig. 5E, middle and right), suggesting that autophagy dictates skin cancer severity.

Equally important is the ability of heparanase overexpression to confer resistance to stress and chemotherapy (Fig. 7D and Supplementary Fig. S2A) mediated, at least in part, by enhancing autophagy (Fig. 7D). Accordingly, heparanase KO MEF were more sensitive to mTOR inhibitor than control MEF (Torin; Fig. 6B and Supplementary Fig. S2C). Moreover, in cell invasion assay, heparanase KO MEF exhibit lower invasion capacity and were more sensitive to chloroquine than control MEF (Supplementary Fig. S2D). This adds mechanistic view to previous results demonstrating resistance of heparanase-overexpressing cells to inhibitors of EGFR (22, 51) and the established role of autophagy as a survival pathway. Indeed, diverse classes of anticancer drugs induce autophagy, thus attenuating tumor cell elimination, whereas autophagy inhibitors overcome chemoresistance (42, 52). On the basis of this concept, chloroquine is currently evaluated in several clinical trials including head and neck carcinoma and glioma in combination with different classes of chemotherapeutic agents (42).

Interestingly, bacterial heparanase III was noted to enhance autophagy in endothelial cells, and autophagy was further enhanced when heparanase III was combined with oxidized low-density lipoprotein, a mechanism thought to allow endo-

theelial cell survival during stress (53). Heparanase is an endoglucuronidase that cleaves heparan sulphate in a far more selective manner than heparinase III, yet cleavage and/or clustering of heparan sulphate within lysosomes may still exert a proautophagy effect by heparanase, although nonenzymatic function (1, 30) cannot be excluded.

In addition to neutralizing heparanase enzymatic activity, PG545 has recently been shown to inhibit signaling properties of heparanase, attenuating Akt phosphorylation (32). PG545 similarly prevented autophagy induction by heparanase (Fig. 3C, Con vs. PG), associating with accumulation of heparanase extracellularly and decreased intracellular content of heparanase (Fig. 3D). Thus, PG545 emerges as a broad-spectrum heparanase inhibitor, neutralizing its enzymatic, signaling, and autophagic properties. Combining PG545 with inhibitors of autophagy such as chloroquine may yield even higher efficacy in cases that more strictly depend on autophagy for tumor growth.

Disclosure of Potential Conflicts of Interest
E. Hammond has ownership interest as an inventor on the PG545 patent. No potential conflicts of interest were disclosed by the other authors.

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References

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