

## The Heparanase Inhibitor PG545 Attenuates Colon Cancer Initiation and Growth, Associating with Increased p21 Expression<sup>1</sup>



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### Abstract

Heparanase activity is highly implicated in cellular invasion and tumor metastasis, a consequence of cleavage of heparan sulfate and remodeling of the extracellular matrix underlying epithelial and endothelial cells. Heparanase expression is rare in normal epithelia, but is often induced in tumors, associated with increased tumor metastasis and poor prognosis. In addition, heparanase induction promotes tumor growth, but the molecular mechanism that underlines tumor expansion by heparanase is still incompletely understood. Here, we provide evidence that heparanase down regulates the expression of p21 (WAF1/CIP1), a cyclin-dependent kinase inhibitor that attenuates the cell cycle. Notably, a reciprocal effect was noted for PG545, a potent heparanase inhibitor. This compound efficiently reduced cell proliferation, colony formation, and tumor xenograft growth, associating with a marked increase in p21 expression. Utilizing the APC Min<sup>+/-</sup> mouse model, we show that heparanase expression and activity are increased in small bowel polyps, whereas polyp initiation and growth were significantly inhibited by PG545, again accompanied by a prominent induction of p21 levels. Down-regulation of p21 expression adds a novel feature for the emerging pro-tumorigenic properties of heparanase, while the potent p21 induction and anti-tumor effect of PG545 lends optimism that it would prove an efficacious therapeutic in colon carcinoma patients.

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### Introduction

Heparanase is a mammalian endoglucuronidase that cleaves heparan sulfate (HS) side chains of proteoglycans at specific sites, generating fragments of considerable size (2.5–10 kDa) and biological activity [1,2]. By cleaving HS side chains, heparanase modifies the structural integrity of the extracellular matrix (ECM) that underlies epithelial and endothelial cells, thus facilitating cellular invasion and associated tumor metastasis, angiogenesis, and inflammation. In addition, heparanase activity releases a wide range of HS-bound growth factors, cytokines, chemokines and enzymes that can profoundly affect the tumor and its microenvironment (including endothelial cells and tumor associated macrophages) [3–6]. Expression of heparanase is rare in normal tissues, but is frequently induced in tumors, correlating

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with increased tumor metastasis and poor prognosis [7–9]. Likewise, heparanase is associated with colon cancer progression and its expression was shown to be increased substantially in colon carcinomas at the mRNA and protein levels. Elevation of heparanase was accompanied by higher TNM stage, more frequent blood and lymph vessels infiltration, and reduced patient survival [10–12]. Subsequent studies revealed that heparanase function is not limited to tumor metastasis but rather promotes tumor initiation and growth. For example, over expression of heparanase in cancer-derived cells including, among others, HT29 colon carcinoma cells, resulted in larger tumor xenografts [13–16]. Furthermore, the incidence and severity of colitis-associated colon tumorigenesis was markedly increased in transgenic mice that over express the enzyme [17].

Here, we utilized a heparanase inhibitor, PG545 [18], to further examine the significance of heparanase in colon cancer. We provide evidence that cell proliferation and tumor growth are markedly attenuated by PG545. We further utilized a genetic approach and show reduced polyps' initiation and growth in APC Min<sup>+/-</sup> mice treated with PG545. Notably, in all the above in vitro, in vivo, and genetic settings, PG545 treatment was associated with a substantial increase in p21 (WAF1/CIP1) expression, a well-known cyclin-dependent kinase (CDK) inhibitor that attenuates the cell cycle [19,20]. Conversely, heparanase over expression or its exogenous addition reduces p21 levels. Mechanistically, we show that p21 down-regulation requires heparanase enzymatic activity, and involves toll-like receptors (TLRs) and NFκB signaling. Thus, heparanase inhibitors are valuable tools that uncover previously unrecognized mechanisms that underlie the pro-tumorigenic properties of heparanase.

## Materials and Methods

### Cells, Cell Culture and Cell Cycle Analysis

Human SW480 and HCT116, and mouse CT26 colon carcinoma cells were grown in DMEM supplemented with 10% FCS and antibiotics. For cell cycle analysis, cells ( $2 \times 10^5$ ) were seeded into 60 mm dishes and were grown to reach 30% to 40% confluence. Cells were subsequently treated with the indicated concentrations of PG545 under serum-free conditions for 18 or 36 h. Cells were then collected by trypsinization and fixed in 70% ethanol at 4°C for 18 h; The fixed cells were washed twice with cold PBS and suspended in 0.5 ml staining buffer containing RNase (10 μg/ml), propidium iodide (Sigma; 20 μg/ml) and triton X-100 (0.1%). Samples were incubated for 45 min in the dark and acquired on fluorescence-activated cell sorting (FACS) BDLSRFortessa (Becton Dickinson, San Jose, CA, USA). The fluorescent signal was detected through the FL2 channel and DNA content present in the various phases of cell cycle was analyzed using ModfitLT software (version 3.3.11; Verity Software House, Topsham, ME, USA). Colony formation in soft agar was performed essentially as described [14,21]. The human SW480 and HCT116 cell lines were authenticated by the short tandem repeat (STR) profile of 15 loci plus amelogenin for sex determination (X or XY) method according the manufacturer's (Promega) instructions.

### Antibodies, Reagents, and Heparanase Activity Assay

Anti-p21 (sc-6246), anti-p27 (sc-528), anti-VEGF (sc-504) antibodies and the small molecule heparanase inhibitor OGT 2115 [22] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody and the IKK inhibitor Bay11-7082 were

purchased from Sigma (St. Louis, MI); rat anti-mouse CD31 was purchased from Dianova (Hamburg, Germany). The MyD88 peptide inhibitor was purchased from InvivoGen (San Diego, CA). PG545 was kindly provided by Zucero Therapeutics (Darra, Australia) [18]. Preparation of dishes coated with sulfate labeled ECM and determination of heparanase enzymatic activity (ie, release of labeled HS degradation fragments) were carried out essentially as described previously [13,14]. Briefly, sulfate labeled degradation fragments were analyzed by gel filtration on Sepharose CL-6B column. Fractions were eluted with PBS and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains are eluted from Sepharose 6B at  $0.5 < K_{av} < 0.8$  (fractions 15-30) [13,14]. Each experiment was performed at least three times and the variation in elution positions ( $K_{av}$  values) did not exceed  $\pm 15\%$ .

### Real Time-PCR

Real time-PCR analyses were performed using ABI PRISM 7000 Sequence Detection System employing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), essentially as described previously [14,23]. The following primers were used:

Human p21-F-5'-TGTCCGTCAGAACCCATGC;  
R-5'-AAAGTCGAAGTTCCATCGCTC.  
Mouse p21-F- 5'-CCTGGTGATGTCCGACCTG;  
R-5'-CCATGAGCGCATCGCAATC.  
Human p27-F-5'-TAATTGGGGCTCCGGCTAACT;  
R-5'-TGCAGGTCGCTTCCATTATTC.  
Mouse p27-F-5'-TCAAACGTGAGAGTGTCTAACG;  
R-5'-CCGGGCCGAAGAGATTTCTG.  
Human heparanase-F-5'-CCCTTGCTATCCGACACCTT;  
R-5'-CACCACCTTCTATTCCCATTCG.  
Mouse heparanase- F-5'-GGGGTTTCGTAGTAACGCATTT  
AG-3', R: 5' GCACCTACTCAAGAAGCTCAG-3'.  
Human actin-F-5'-CGCCCCAGGCACCAGGGC; R-5'-GCT  
GGGGTGTGAAGGT.  
Mouse actin-F-5'-ATGCTCCCCGGGCTGTAT;  
R-5'-CATAGGAGTCCTTCTGACCCATTC.

### Tumorigenicity and Immunostaining

Cells were detached with trypsin/EDTA, washed with PBS, and cell suspension was inoculated subcutaneously at the right flank of 6-week-old SCID/NOD (SW480;  $5 \times 10^6$ ) or Balb/C (CT26;  $1 \times 10^5$ ) mice. PG545 was administrated (i.p.; 20 mg/kg, once weekly) once tumors became palpable. 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 with the indicated antibody using the Envision kit according to the manufacturer's (Dako) instructions, as described previously [23,24].

APC Min<sup>+/-</sup> mice were obtained from Jackson Laboratories (Bar Harbor, ME). In a tumor prevention experiment, PG545 (20 mg/kg; once weekly) was administrated to APC Min<sup>+/-</sup> mice (n = 8) starting at 4 weeks of age. In a treatment setting, PG545 was given starting at 9 weeks of age (n = 10). In both settings, experiments were terminated at 19 weeks of age. Mice were then sacrificed, the colon and small bowel were exposed, and the number and size of polyps was evaluated. Polyps were immediately homogenized for RNA and protein extraction, or were fixed in formalin for histological and immunostaining analyses as described above.

### Statistics

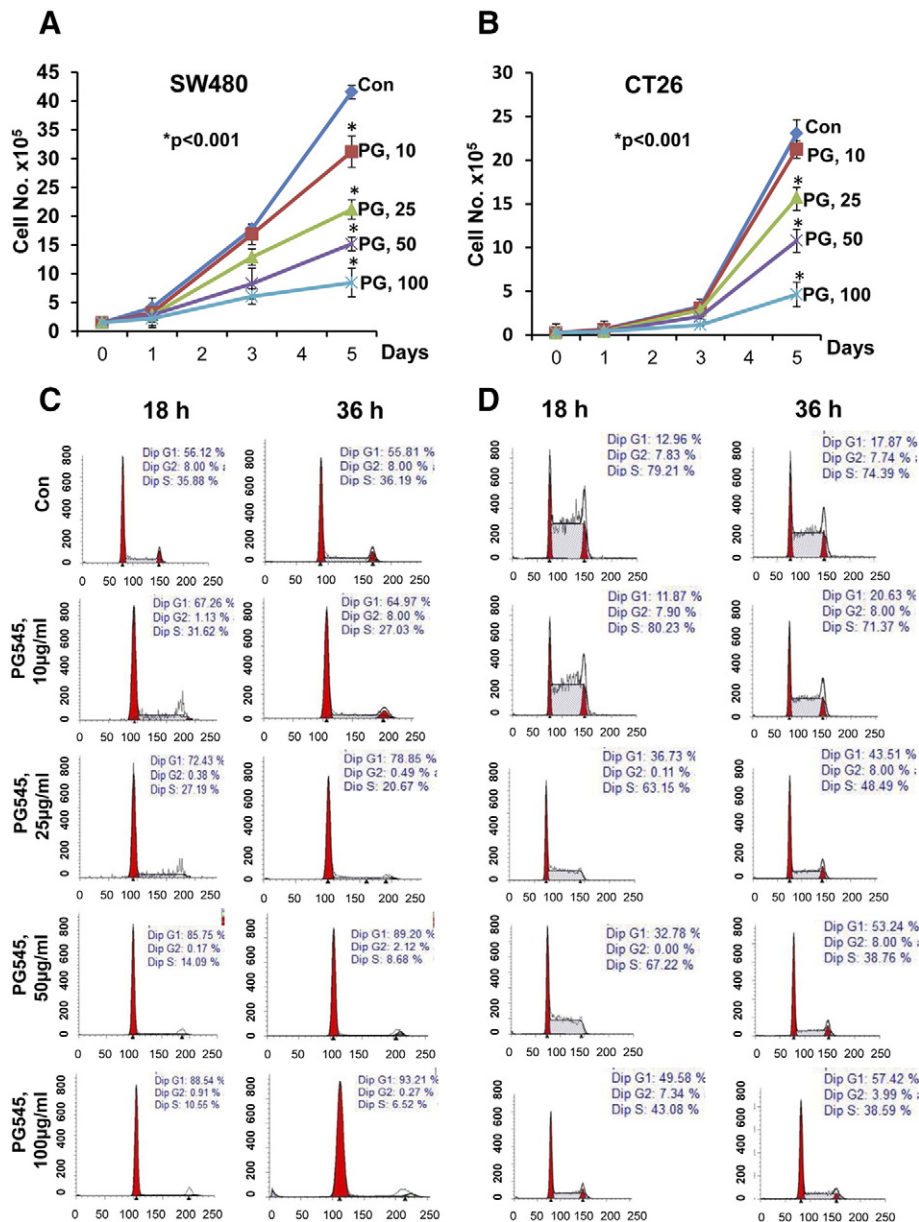
Data are presented as means  $\pm$  SE. Statistical significance was analyzed by 2-tailed Student's *t* test or  $\chi^2$  test. Values of  $P < .05$  were considered significant. Data sets passed D'Agostino-Pearson normality (GraphPad Prism 5 utility software). All experiments were repeated at least 3 times with similar results.

### Results

#### PG545 Arrests the Proliferation of Colon Carcinoma Cells

Heparanase is strongly implicated in tumor metastasis, and the heparanase inhibitor PG545 attenuated the spontaneous metastasis of HT29 colon carcinoma cells [18], but its capacity to restrain the initiation or growth of colon carcinoma tumors has not been so far

reported. In order to examine the role of heparanase in colon tumorigenesis, we added escalating doses of PG545 to colon carcinoma cells and cell number was quantified over time. We found that PG545 reduces the proliferation of SW480 (Figure 1A), CT26 (Figure 1B) and HCT116 (Suppl. Figure 1A) colon carcinoma cells, in a dose-dependent manner. Thus, cell numbers were significantly reduced by PG545 at concentrations of 25  $\mu\text{g/ml}$  ( $\sim 10 \mu\text{M}$ ) and higher (Figure 1, A and B; Suppl. Figure 1A). Notably, cell cycle analyses revealed a consistent accumulation of cells at the G1 phase following PG545 treatment, concomitant with decreased DNA synthesis (S phase; Figure 1, C and D; Suppl. Figure 1B; Table 1, Suppl. Table 1), suggesting that PG545 elicits cell cycle arrest of colon carcinoma cells.



**Figure 1.** PG545 arrests colon carcinoma cells in G1 phase, attenuating cell proliferation. A-B. Cell proliferation. Human (SW480; A) and mouse (CT26; B) colon carcinoma cells ( $2 \times 10^5$ ) were incubated with the indicated concentration of PG545, and cells were counted 1, 3, and 5 days thereafter. PG545 was added once at Day 0, and growth medium was not changed during the 5 days experiment. C-D. Cell cycle analyses. SW480 (C) and CT26 (D) cells were incubated with PG545 (50  $\mu\text{g/ml}$ ) for 18 and 36 h. Cells were then harvested and subjected to cell cycle analysis as described under 'Materials and Methods'. Note accumulation of cells at the G1 phase, and decreased cell number at the S phase (see also Table 1).

**Table 1.** Cell-Cycle Arrest Following Treatment of Colon Carcinoma Cells with PG545

PG ( $\mu\text{g/ml}$ )	G1	G2	S	G1	G2	S
SW480, 18 h				CT26, 18 h		
0	56	8	35	12.9	7.8	79.2
10	67	1	31	11.8	7.9	79.2
25	72	0.4	27	36.7	0.1	63.1
50	85	0.2	14	32.7	0	67.2
100	88	0.9	10	49.5	7.3	43.0
SW480, 36 h				CT26, 36 h		
0	55	8	36	17.8	7.7	74.3
10	65	8	27	20.6	8	71.4
25	78	0.5	20	43.5	8	48.4
50	89	2	8	53.2	8	38.7
100	93	0.3	6.5	57.4	3.9	38.5

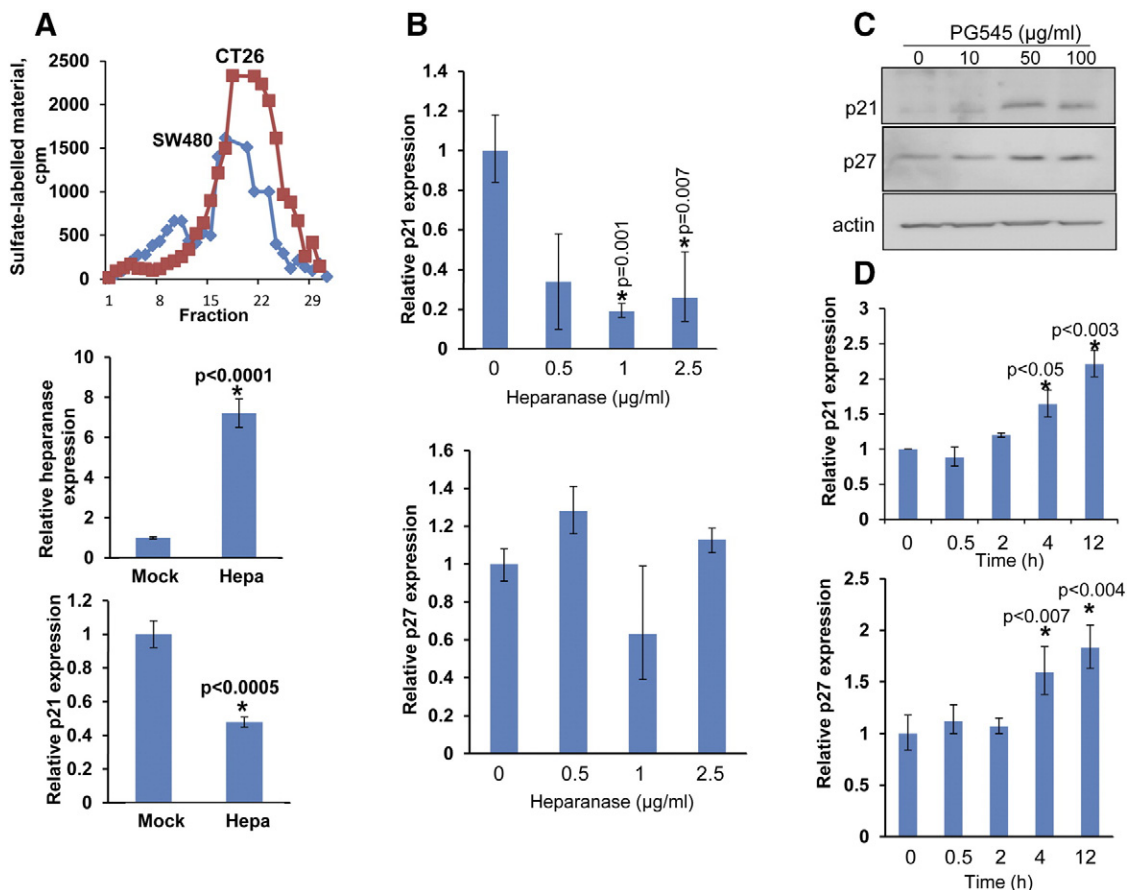
Interestingly, over expression of heparanase in SW480 cells endowed with relatively low levels of heparanase activity (Figure 2A, upper panel) was accompanied by reduced expression of p21, a cyclin-dependent kinase inhibitor (Figure 2A, lower panel). More-

over, p21 expression was significantly reduced following exogenous addition of heparanase (Figure 2B, upper panel). Accordingly, treatment of SW480 cells with the heparanase inhibitor PG545 resulted in a comparable induction of p21 expression at the protein (Figure 2C, upper panel) and mRNA (Figure 2D, upper panel) levels. Moreover, expression of p27, a p21-related CDK inhibitor, was also induced by PG545, albeit to a lesser degree (Figure 2D, lower panel). This confirms, and further expands, a previous report describing an inverse correlation between heparanase and p27 levels [25], and suggests that attenuation of the cell cycle by PG545 treatment (Figure 1; Table 1; Suppl. Table 1) is due, at least in part, to induction of p21/p27 expression.

Notably, PG545 profoundly reduced the size and number of colonies formed by colon carcinoma SW480 and HCT116 cells in soft agar (Figure 3A), an experimental system thought to closely reflect tumor growth.

### PG545 Attenuates the Growth of Colon Carcinoma Tumor Xenografts

In order to examine the significance of heparanase for tumor growth, human SW480 and mouse CT26 colon carcinoma cells were inoculated subcutaneously in SCID and Balb/c mice, respectively,

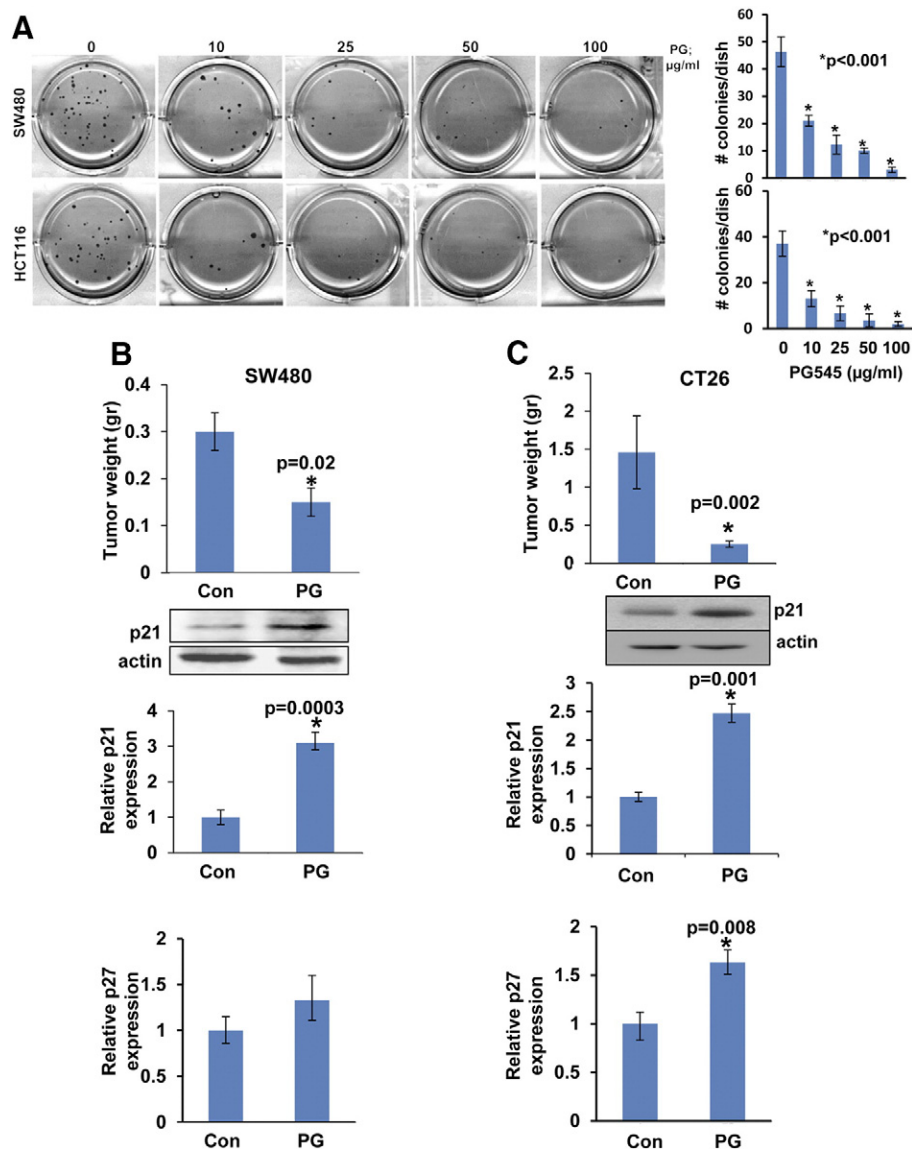


**Figure 2.** Heparanase down-regulates p21 expression. SW480 and CT26 cells ( $2 \times 10^6$ ) were subjected to three freeze/thaw cycles and heparanase activity was evaluated as described under 'Materials and Methods' (A, upper panel). SW480 that exhibit lower levels of activity were infected with control (Mock) or heparanase gene constructs and heparanase (A, middle panel) and p21 (A, lower panel) expression were quantified by real-time PCR. Note decreased p21 expression by cells over expressing heparanase. B. Exogenous addition. SW480 cells were left untreated (0) or were incubated with the indicated concentrations of recombinant heparanase under serum-free conditions for 18 h. RNA was then extracted and subjected to quantitative real-time PCR applying primer sets specific for p21 (upper panel) and p27 (lower panel). C. PG545 treatment. SW480 cells were left untreated (0) or were incubated with the indicated concentration of PG545 for 18 h. Cell extracts were then prepared and subjected to immunoblotting applying anti p21 (upper panel), anti p27 (second panel) and anti-actin (lower panel) antibodies. D. Real-time PCR. SW480 cells were incubated with PG545 (50  $\mu\text{g/ml}$ ) for the time indicated and p21 (upper panel) and p27 (lower panel) expression was quantified by real-time PCR. Data is presented as expression (fold-increase) relative to control cells (0) set arbitrarily to a value of 1.

and PG545 was administrated (i.p.; 20 mg/kg; once weekly) when tumors became palpable. The growth of SW480 tumor xenografts was decreased two-fold by PG545 ( $0.3 \pm 0.04$  vs.  $0.15 \pm 0.03$  g for control vs. PG545, respectively;  $P = .02$ ) (Figure 3B, upper panel), and an even greater, 6-fold decrease in tumor weight was noted in CT26 tumors treated with PG545 ( $1.46 \pm 0.48$  vs.  $0.25 \pm 0.04$  gr. for control vs. PG545, respectively) (Figure 3C, upper panel), differences that are statistically highly significant ( $P = .002$ ). Importantly, tumor growth inhibition by PG545 was associated with a 3-fold increase in p21 expression (Figure 3, B and C, middle panels), that is in agreement with the in vitro results (Figure 2), while p27 induction by PG545 was evident only in CT26 tumors (Figure 3, B and C, lower panels).

### PG545 Decreases Polyp's Initiation and Growth

In order to further explore the significance of heparanase for colon cancer initiation, we utilized the well-established APC Min<sup>+/-</sup> mouse model. These mice carry a truncation mutation at codon 850 of the *Apc* gene and can develop up to 100 polyps in the small intestine within several weeks after birth [26]. We found that heparanase expression (Figure 4A) and enzymatic activity (Figure 4B) are significantly elevated in polyp vs. adjacent normal tissue, suggesting that heparanase is relevant in this pre-malignant setting. We next administrated PG545 to APC Min<sup>+/-</sup> mice (20 mg/kg; once weekly), starting at 4-weeks and 9-weeks of age, representing a tumor prevention (i.e., prior to polyp initiation) and treatment (i.e., polyps are already present) settings, respectively. We found that PG545



**Figure 3.** Inhibition of tumor growth by PG545 involves induction of p21 expression. A. PG545 attenuates colony formation in soft agar. SW480 (upper panels) and HCT116 (lower panels) cells ( $5 \times 10^3$ ) were mixed with soft agar and cultured for 3 weeks in the absence (0) or presence of the indicated concentration of PG545. Shown are representative scans of dishes at original size. Quantification of colony numbers is shown graphically at the rightmost panels. B, C. Tumor xenografts. Human SW480 ( $5 \times 10^6$ ; B) and CT26 mouse ( $1 \times 10^5$ ; C) colon carcinoma cells were implanted subcutaneously in SCID and Balb/c mice, respectively. PG545 was administrated (i.p.; 20 mg/kg, once weekly) when tumor became palpable. At termination, tumors were excised, weighed and portions were taken for RNA and protein extractions. Shown are tumor weights in PG545 treated vs. control mice (B-C, upper panels) and corresponding p21 (B-C, middle panels) and p27 (B-C, lower panels) expression. Data is presented as expression (fold-increase) relative to control tumors set arbitrarily to a value of 1.

effectively reduced polyp number in both settings. Thus, while control, untreated mice, carried  $45 \pm 7$  polyps in average, only  $11 \pm 3$  polyps were counted in the small bowel of mice treated with PG545 starting at 4-weeks of age (Figure 4C, Con vs. PG-4 W), differences that are statistically highly significant ( $P = .00001$ ). Reduced polyp number was also evident once PG545 treatment was begun at 9-weeks of age ( $45 \pm 7$  vs.  $32 \pm 10$  for control and PG545, respectively;  $P = .05$ ) (Figure 4C). Importantly, not only the number but also the size of the polyps was reduced by the heparanase inhibitor. In control, untreated mice, lesions size was distributed equally between small (1-2 mm) and larger (3-4 + 5-7 mm) polyps (Figure 4D, Con; Table 2). In contrast, most polyps that developed in PG545-treated mice in prevention (starting at 4 weeks of age; Figure 4D, PG) and treatment settings (Table 2), appeared small (1-2 mm), differences that are statistically highly significant ( $P = .004$ ). Importantly, the decrease in polyp number and size following PG545 treatment, especially in the prevention mode (i.e., starting at 4 weeks of age) was associated with a marked increase in p21 and p27 levels, evident by immunoblotting of polyp extracts (Figure 5A, upper and middle panels) and immunostaining of polyp tissues (Figure 5B, upper panel). In addition, the level of VEGF was decreased following

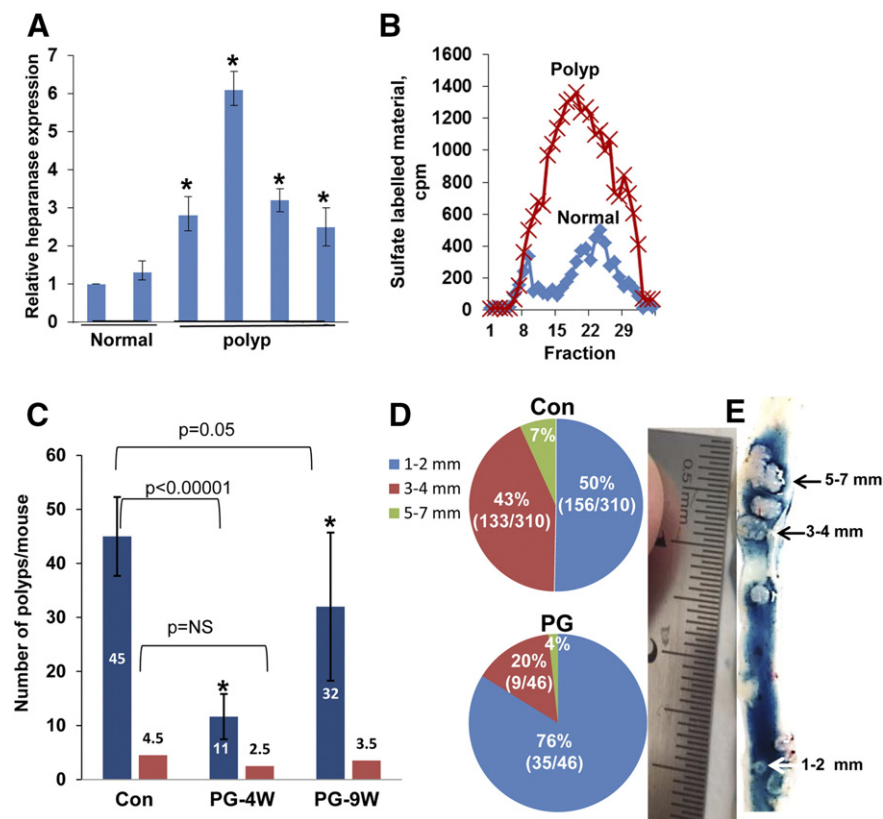
PG545 treatment (Figure 5B, middle panels), without noticeable changes in vascular density (Figure 5B, lower panels).

### p21 Down-Regulation Requires Heparanase Enzymatic Activity and Involves TLRs and the NF $\kappa$ B Pathway

In order to reveal the mechanism underlying p21 down-regulation, we first incubated cells with heparanase in the absence (Hepa, Figure 5C) or presence of heparin, a potent inhibitor of heparanase enzymatic activity (Hepa + heparin; Figure 5C) [27]. Notably, p21 repression was abrogated by heparin and moreover, by a small molecule heparanase inhibitor OGT2115 (Figure 5D, Hepa + OGT), suggesting that heparanase enzymatic activity is required for p21 down regulation. Furthermore, p21 repression by heparanase was prevented upon inhibition of the NF $\kappa$ B pathway (Figure 5C, Hepa + Bay) or TLRs signaling (Figure 5E, Hepa + MyD inh). This suggests that the heparanase-generated HS fragments-TLRs- NF $\kappa$ B axis not only stimulates [28], but also represses the expression of target genes.

### Discussion

Heparanase expression is increased in many types of tumors and this elevation is often associated with more aggressive disease and poor prognosis due to advanced local and distant metastases [7,9,29–31].



**Figure 4.** APC Min<sup>+/-</sup> mouse model. A-B. Heparanase expression and activity are increased in small bowel polyps. RNA was extracted from polyps developed in APC Min<sup>+/-</sup> mice and adjacent normal tissue and subjected to quantitative real-time PCR analysis applying primer set specific for mouse heparanase (A). Corresponding normal and polyp tissue extracts were evaluated for heparanase activity (B). C-D. PG545 decreases polyp number and size. APC Min<sup>+/-</sup> mice were treated with PG545 (20 mg/kg once weekly) starting at 4 (n = 8) and 9 (n = 10) weeks of age. Control mice were administrated with vehicle (PBS) alone. Experiments were terminated when mice reached the age of 19 weeks. Mice were then sacrificed, their colon and small bowel were exposed, and the number (C) and size (D) of polyps were quantified. Shown are average number  $\pm$  SD of polyps in the small bowel (blue) and colon (red) of untreated (Con) and PG545-treated mice starting at 4 weeks (PG-4 W) and 9 weeks (PG-9 W) of age (C). Polyp size was categorized as small (1-2 mm), medium (3-4 mm) and large (5-7 mm) and is shown as percent of total polyps counted in control and mice treated with PG545 starting at 4 weeks of age (D) (see also Table 2). Representative images of small (1-2 mm), medium (3-4 mm) and large (5-7 mm) polyps are shown in (E).

**Table 2.** Reduced Tumor Size in APC Min Mice Treated with PG545

Tumor size (mm)	1-2 (%)	3-4 (%)	5-7 (%)	Total
Control	156 (50)	133 (43)	21 (7)	310
PG545-4 W	35 (76)	9 (20)	2 (4)	46
PG545-8 W	244 (84)	42 (14)	5 (2)	291

*P* = .004.

In addition, heparanase up regulation in primary human tumors correlates in some cases with tumors larger in size and enhanced micro vessel density [7,9]. Likewise, cells engineered to over-express heparanase are endowed with a more rapid expansion of tumor xenografts [15,29,30,32,33], whereas heparanase silencing results in lower tumor burden [33]. Thus, while the role of heparanase in tumorigenesis is emerging, and anti-heparanase inhibitors are being evaluated in clinical trials as anti-cancer therapeutics [34,35], the mechanism by which heparanase promotes tumor initiation, growth and chemoresistance is still incompletely understood. Heparanase enzymatic activity releases HS-bound growth factors stored in the ECM as reservoir and thereby promotes tumor growth and angiogenesis [3,6]. Heparanase can also facilitate the survival and proliferation of tumor cells by activation of signaling molecules such as Akt, EGFR, Src, HGF, and STAT [7,21,36–40], independent of its enzymatic activity. Moreover, heparanase induces the transcription of pro-angiogenic (i.e., *VEGF-A*, *VEGF-C*, *COX-2*), pro-thrombotic (i.e., *TF*), mitogenic (*HGF*), and osteolytic (*RANKL*) genes [40–43] that orchestrate a strong pro-tumorigenic effect, and down-regulate the expression of tumor suppressors [i.e., *CXCL10*; [14]]. Here, we provide evidence that heparanase down-regulates also the expression of p21 in colon carcinoma cells. This was evident in cells over expressing heparanase (Figure 2A), and even more prominent down regulation of p21 was observed following exogenous addition of heparanase (Figures 2B, 5, C-E).

p21 is a potent inhibitor of all cyclin-dependent kinases, leading to a G1 arrest [19,20]. It was first identified as a gene induced by wild type p53 tumor suppressor protein, and its expression is lost in most cancers [44,45]. In colorectal cancer, decreased p21 level was associated with more advanced disease stage, poor survival, and p53 aberrations [46,47].

Furthermore, targeted inactivation of the p21 gene enhances APC-initiated tumor formation [48], and even stronger pro-tumorigenic effects were reported for p27-null mice [20]. Utilizing the APC Min<sup>+/-</sup> mouse model, thought to recapitulate the human disease, we found that heparanase expression and activity are increased in small bowel polyps vs. adjacent normal tissue (Figure 4). This is in agreement with previous reports showing that heparanase expression is induced already at the early stages of human colon neoplasia (adenoma), followed by gradual increase during subsequent progression from severe dysplasia through well-differentiated to poorly- differentiated colon carcinoma [10]. Likewise, polyp initiation (prevention setting) and growth (treatment setting) were significantly reduced by the heparanase inhibitor PG545 (Figure 4, C and D; Table 2), and this decrease in small bowel neoplasia by PG545 was associated with a marked induction in p21 expression (Figure 5). PG545 was developed as a dual heparanase and angiogenesis inhibitor, because it can also interfere with VEGF- and FGF-induced angiogenesis [49]. However, reduced small bowel neoplasia by PG545 in the APC Min<sup>+/-</sup> mouse model does not seem

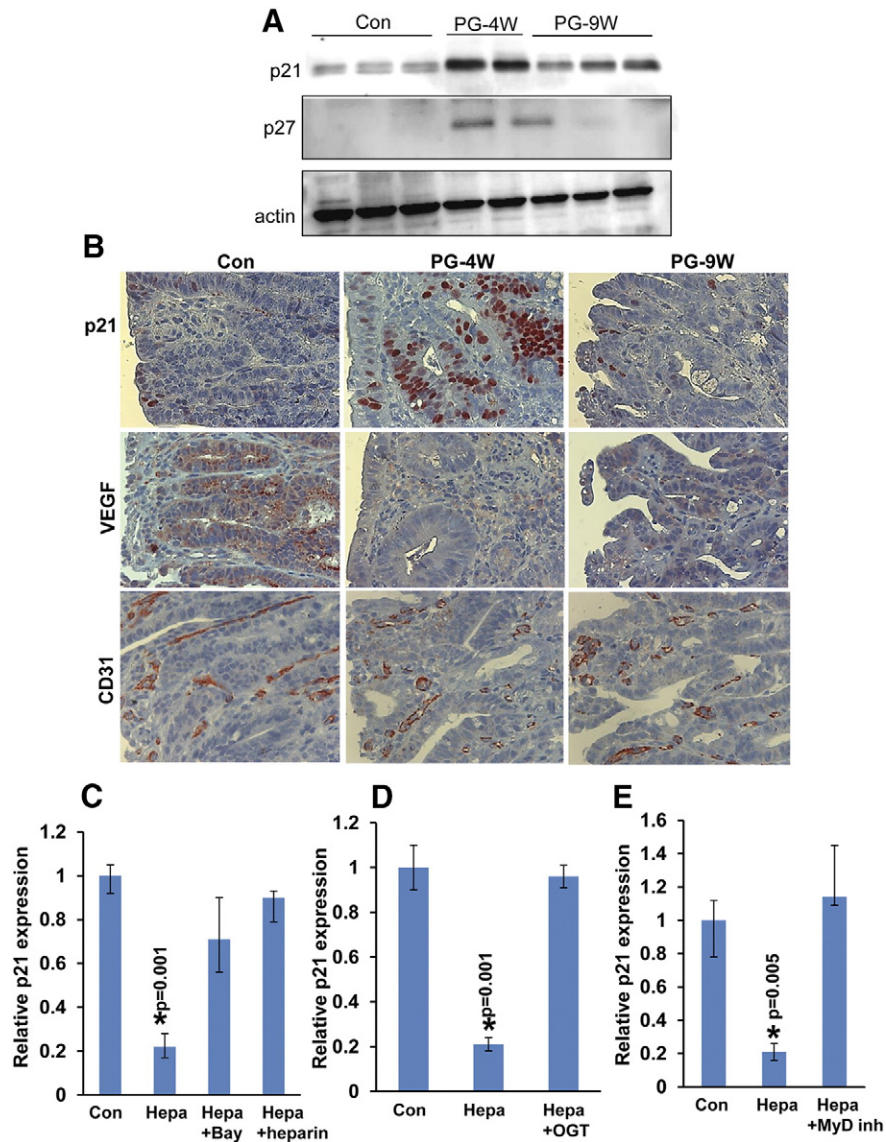
to involve angiogenesis as blood vessel density appeared comparable in treated vs. untreated polyps (Figure 5B, lower panel), suggesting that p21 induction is a leading cause responsible for polyp inhibition by PG545.

Induction of p21 by PG545 was not restricted to the APC Min<sup>+/-</sup> mouse model but was rather in common with our in vitro and in vivo experiments. p21 expression was induced in tumor xenografts produced by SW480 cells and treated with PG545, in parallel with reduced tumor growth (Figure 3A). Even greater inhibition of tumor growth was observed in tumors produced by CT26 cells and treated with PG545 (Figure 3C). In this setting, not only p21 but also p27 expression was significantly induced, resulting in tumors that were 6-fold smaller than control (Figure 3C). This may suggest that simultaneous induction of p21 and p27 by PG545 results in a most effective anti-tumor effect. Notably, p21/p27 induction was also observed in cultured cells treated with PG545 in a dose-dependent manner (Figure 2C), closely associating with marked inhibition in cell number and seemingly G1 arrest, typical of p21/p27 function (Figure 1, Table 1, Suppl. Table 1). This supports the notion that the predominant anti-tumor effect of PG545 is due to its ability to induce p21 expression.

The mechanism by which heparanase ensures p21 down-regulation is not entirely clear, but points to the TLRs-NFκB pathway in a manner that requires heparanase enzymatic activity. This is concluded because p21 down-regulation was eliminated by the inclusion of heparin, a potent heparanase inhibitor [27], together with heparanase (Figure 5C). Heparin, as well as PG545, however, not only inhibits the enzymatic activity of heparanase but also its interaction with cell-membrane HS [24,50]. We therefore used, in addition, a small molecule inhibitor (OGT2115) that is thought to target the enzyme active site [22]. Indeed, the small molecule inhibitor was as effective as heparin in eliminating p21 repression by heparanase (Figure 5D), suggesting that p21 down-regulation is elicited by heparanase-generated small HS fragments. Notably, OGT2115 did not affect heparanase activity within live SW480 cells (Suppl. Figure 1C), in agreement with our previous report [51]. This critically suggests that p21 regulation is ensued by extracellular heparanase activity. Several studies have shown that TLRs can get activated by soluble HS [52,53] and HS fragments [28], pointing to the possible involvement of TLRs in our experimental setting. Indeed, we found that p21 down-regulation by heparanase is eliminated by MyD88 inhibitor that blocks TLRs signaling (Hepa + MyD inh; Figure 5E) and by IKK inhibitor that target the NFκB signaling pathway (Hepa + Bay; Figure 5C). This cascade resembles the molecular mechanism that drives heparanase-mediated cytokine induction in mononuclear cells [28], suggesting that the HS fragments-TLRs-NFκB axis can lead to induction or repression of target genes.

Of note, it was recently reported that Smad4 suppresses neuroblastoma tumorigenesis through repressing the expression of heparanase [54]. Similarly, over-expression of Smad4 in colon cancer cells suppresses their growth in vivo [55], possibly via induced expression of p21 as was demonstrated in breast and pancreatic cancer [56]. Whether such a dual anti-tumorigenic effect of Smad4 (i.e., down regulation of heparanase and up-regulation of p21) takes place in the Min<sup>+/-</sup> model remains to be elucidated.

Taken together, the results support and significantly expand the role of heparanase in the initiation and growth of colon carcinoma. We further describe a novel manner by which heparanase promotes tumor growth namely, down regulating p21 expression. A reciprocal effect is described for the heparanase inhibitor PG545, associating with a marked decrease in cell proliferation in vitro, tumor xenograft



**Figure 5.** Reduced polyp initiation and growth are associated with p21 induction. A, Immunoblotting. Extract of untreated (Con) and PG545-treated colonic polyps were subjected to immunoblotting applying anti-p21 (upper panel), anti-p27 (second panel) and anti-actin (lower panel) antibodies. B, Immunostaining. Five-micron sections of control and PG545-treated colonic polyps were subjected to immunostaining applying anti-p21 (upper panels), anti-VEGF (second panels) and anti-CD31 (lower panels) antibodies. Note increased p21 levels in polyps treated with PG545 (original magnification  $\times 80$ ). C-E. Mode of action. SW480 cells were incubated without (Con) or with heparanase in the absence (Hepa) or presence of heparin (50  $\mu\text{g}/\text{ml}$ ) or the IKK inhibitor, Bay11-7082 (10  $\mu\text{M}$ ) for 24 h under serum-free conditions. Cells were then harvested, and p21 expression was quantified by real-time PCR. Data is presented as expression (fold-increase) relative to control cells set arbitrarily to a value of 1 (C). SW480 cells were similarly incubated without (Con) or with heparanase in the absence (Hepa) or presence of a small molecule heparanase inhibitor, OGT2115 (10  $\mu\text{g}/\text{ml}$ ; D, Hepa + OGT) or MyD88 inhibitory peptide (100  $\mu\text{M}$ ; E, Hepa + MyD Inh) for 24 h under serum-free conditions. Cells were then harvested and p21 expression was analyzed as above. Note reversal of p21 down-regulation by heparanase in the presence of heparin, OGT2115, Bay11-7082, and MyD88 inhibitors.

growth, and polyp initiation and expansion. Thus, inhibitors are valuable tools to expose previously unrecognized properties of heparanase. Attenuation of polyps' growth also in a treatment setting lends hope that PG545 would prove efficacy for colon carcinoma patients.

## Conclusions

Heparanase down-regulates p21 expression in colon carcinoma cells;

The heparanase inhibitor PG545 attenuates the tumorigenicity of colon carcinoma cells;

Polyp initiation and growth are markedly inhibited by PG545 in APC Min<sup>+/-</sup> mice;

PG545 prominently induces p21 expression.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2016.12.001>.



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## References

- [1] Vlodavsky I and Friedmann Y (2001). Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J Clin Invest* **108**, 341–347.
- [2] Sanderson RD, Yang Y, Kelly T, MacLeod V, Dai Y, Theus A (2005). Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: growth regulation and the prospect of new cancer therapies. *J Cell Biochem* **96**, 897–905.
- [3] Elkin M, Ilan N, Ishaï-Michaeli R, Friedmann Y, Papo O, Pecker I, Vlodavsky I (2001). Heparanase as mediator of angiogenesis: mode of action. *FASEB J* **15**, 1661–1663.
- [4] Xu D and Esko JD (2014). Demystifying heparan sulfate-protein interactions. *Annal. Rev Biochem* **83**, 129–157.
- [5] Billings PC and Pacifici M (2015). Interactions of signaling proteins, growth factors and other proteins with heparan sulfate: mechanisms and mysteries. *Connect Tissue Res* **56**, 272–280.
- [6] Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I (1988). Heparin-binding angiogenic protein–basic fibroblast growth factor–is stored within basement membrane. *Am J Pathol* **130**, 393–400.
- [7] Ilan N, Elkin M, Vlodavsky I (2006). Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol* **38**, 2018–2039.
- [8] Vlodavsky I, Beckhove P, Lerner I, Pisano C, Meirovitz A, Ilan N, Elkin M (2012). Significance of heparanase in cancer and inflammation. *Cancer Microenviron* **5**, 115–132.
- [9] Vreys V and David G (2007). Mammalian heparanase: what is the message? *J Cell Mol Med* **11**, 427–452.
- [10] Friedmann Y, Vlodavsky I, Aingorn H, Aviv A, Peretz T, Pecker I, Pappo O (2000). Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma. Evidence for its role in colonic tumorigenesis. *Am J Pathol* **157**, 1167–1175.
- [11] Nobuhisa T, Naomoto Y, Ohkawa T, Takaoka M, Ono R, Murata T, Gunduz M, Shirakawa Y, Yamatsuji T, Haisa M (2005). Heparanase expression correlates with malignant potential in human colon cancer. *J Cancer Res Clin Oncol* **131**, 229–237.
- [12] Sato T, Yamaguchi A, Goi T, Hirono Y, Takeuchi K, Katayama K, Matsukawa S (2004). Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. *J Surg Oncol* **87**, 174–181.
- [13] Arvatz G, Barash U, Nativ O, Ilan N, Vlodavsky I (2011). Post-transcriptional regulation of heparanase gene expression by a 3' AU-rich element. *FASEB J* **24**, 4969–4976.
- [14] Barash U, Zohar Y, Wildbaum G, Beider K, Nagler A, Karin N, Ilan N, Vlodavsky I (2014). Heparanase enhances myeloma progression via CXCL10 downregulation. *Leukemia* **28**, 2178–2187.
- [15] Cohen I, Pappo O, Elkin M, San T, Bar-Shavit R, Hazan R, Peretz T, Vlodavsky I, Abramovitch R (2006). Heparanase promotes growth, angiogenesis and survival of primary breast tumors. *Int J Cancer* **118**, 1609–1617.
- [16] Doviner V, Maly B, Kaplan V, Gingis-Velitski S, Ilan N, Vlodavsky I, Sherman Y (2006). Spatial and temporal heparanase expression in colon mucosa throughout the adenoma-carcinoma sequence. *Mod Pathol* **19**, 878–888.
- [17] Lerner I, Hermano E, Zcharia E, Rodkin D, Bulvik R, Doviner V, Rubinstein AM, Ishaï-Michaeli R, Atzmon R, Sherman Y (2011). Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice. *J Clin Invest* **121**, 1709–1721.
- [18] Dredge K, Hammond E, Handley P, Gonda TJ, Smith MT, Vincent C, Brandt R, Ferro V, Bytheway I (2011). PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models. *Br J Cancer* **104**, 635–642.
- [19] Abbas T and Dutta A (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* **9**, 400–414.
- [20] Abukhdeir AM and Park BH (2008). p21 and p27: roles in carcinogenesis and drug resistance. *Expert Rev Mol Med* **10**, e19.
- [21] Cohen-Kaplan V, Doweck I, Naroditsky I, Vlodavsky I, Ilan N (2008). Heparanase augments epidermal growth factor receptor phosphorylation: correlation with head and neck tumor progression. *Cancer Res* **68**, 10077–10085.
- [22] Li Y, Liu H, Huang YY, LJ P, Zhang XD, Jiang CC, Jiang ZW (2013). Suppression of endoplasmic reticulum stress-induced invasion and migration of breast cancer cells through the downregulation of heparanase. *Int J Mol Med* **31**, 1234–1242.
- [23] Gross-Cohen M, Feld S, Doweck I, Neufeld G, Hasson P, Arvatz G, Barash U, Naroditsky I, Ilan N, Vlodavsky I (2016). Heparanase 2 attenuates head and neck tumor vascularity and growth. *Cancer Res* **76**, 2791–2801.
- [24] Shteingauz A, Boyango I, Naroditsky I, Hammond E, Gruber M, Doweck I, Ilan N, Vlodavsky I (2015). Heparanase enhances tumor growth and chemoresistance by promoting autophagy. *Cancer Res* **75**, 3946–3957.
- [25] Nagler R, Ben-Izhak O, Cohen-Kaplan V, Shafat I, Vlodavsky I, Akris S, Ilan N (2007). Heparanase up-regulation in tongue cancer: tissue and saliva analysis. *Cancer* **110**, 2732–2739.
- [26] Yamada Y and Mori H (2007). Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci* **98**, 6–10.
- [27] Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z, Vlodavsky I (1987). Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. *Blood* **70**, 551–557.
- [28] Goodall KJ, Poon IK, Phipps S, Hulett MD (2014). Soluble heparan sulfate fragments generated by heparanase trigger the release of pro-inflammatory cytokines through TLR-4. *PLoS One* **9**, e109596.
- [29] Arvatz G, Shafat I, Levy-Adam F, Ilan N, Vlodavsky I (2011). The heparanase system and tumor metastasis: is heparanase the seed and soil? *Cancer Metastasis Rev* **30**, 253–268.
- [30] Barash U, Cohen-Kaplan V, Doweck I, Sanderson RD, Ilan N, Vlodavsky I (2010). Proteoglycans in health and disease: new concepts for heparanase function in tumor progression and metastasis. *FEBS J* **277**, 3890–3903.
- [31] Vlodavsky I, Ilan N, Naggi A, Casu B (2007). Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des* **13**, 2057–2073.
- [32] Fux L, Ilan N, Sanderson RD, Vlodavsky I (2009). Heparanase: busy at the cell surface. *Trends Biochem Sci* **34**, 511–519.
- [33] Lerner I, Baraz L, Pikarsky E, Meirovitz A, Edovitsky E, Peretz T, Vlodavsky I, Elkin M (2008). Function of heparanase in prostate tumorigenesis: potential for therapy. *Clin Cancer Res* **14**, 668–676.
- [34] Hammond E, Khurana A, Shridhar V, Dredge K (2014). The role of heparanase and sulfatases in the modification of heparan sulfate proteoglycans within the tumor microenvironment and opportunities for novel cancer therapeutics. *Front Oncol* **4**, 195.
- [35] Rivara S, Milazzo FM, Giannini G (2016). Heparanase: a rainbow pharmacological target associated to multiple pathologies including rare diseases. *Future Med Chem* **8**, 647–680.
- [36] Barash U, Cohen-Kaplan V, Arvatz G, Gingis-Velitski S, Levy-Adam F, Nativ O, Shemesh R, Ayalon-Sofer M, Ilan N, Vlodavsky I (2010). A novel human heparanase splice variant, T5, endowed with protumorigenic characteristics. *FASEB J* **24**, 1239–1248.
- [37] Fux L, Feibish N, Cohen-Kaplan V, Gingis-Velitski S, Feld S, Geffen C, Vlodavsky I, Ilan N (2009). Structure–function approach identifies a COOH-terminal domain that mediates heparanase signaling. *Cancer Res* **69**, 1758–1767.
- [38] Cohen-Kaplan V, Jrbashyan J, Yanir Y, Naroditsky I, Ben-Izhak O, Ilan N, Doweck I, Vlodavsky I (2012). Heparanase induces signal transducer and activator of transcription (STAT) protein phosphorylation: preclinical and clinical significance in head and neck cancer. *J Biol Chem* **287**, 6668–6678.
- [39] Riaz A, Ilan N, Vlodavsky I, Li JP, Johansson S (2013). Characterization of heparanase-induced phosphatidylinositol 3-kinase-AKT activation and its integrin dependence. *J Biol Chem* **288**, 12366–12375.
- [40] Ramani VC, Yang Y, Ren Y, Nan L, Sanderson RD (2011). Heparanase plays a dual role in driving hepatocyte growth factor (HGF) signaling by enhancing HGF expression and activity. *J Biol Chem* **286**, 6490–6499.
- [41] Cohen-Kaplan V, Naroditsky I, Zetser A, Ilan N, Vlodavsky I, Doweck I (2008). Heparanase induces VEGF C and facilitates tumor lymphangiogenesis. *Int J Cancer* **123**, 2566–2573.
- [42] Nadir Y, Brenner B, Zetser A, Ilan N, Shafat I, Zcharia E, Goldshmidt O, Vlodavsky I (2006). Heparanase induces tissue factor expression in vascular endothelial and cancer cells. *J Thromb Haemost* **4**, 2443–2451.

- [43] Yang Y, Ren Y, Ramani VC, Nan L, Suva LJ, Sanderson RD (2010). Heparanase enhances local and systemic osteolysis in multiple myeloma by upregulating the expression and secretion of RANKL. *Cancer Res* **70**, 8329–8338.
- [44] Ropponen KM, Kellokoski JK, Lipponen PK, Pietilainen T, Eskelinen MJ, Alhava EM, Alhava EM, Kosma VM (1999). p22/WAF1 expression in human colorectal carcinoma: association with p53, transcription factor AP-2 and prognosis. *Br J Cancer* **81**, 133–140.
- [45] Erstad DJ, Tumusiime G, Cusack Jr JC (2015). Prognostic and predictive biomarkers in colorectal cancer: implications for the clinical surgeon. *Ann Surg Oncol* **22**, 3433–3450.
- [46] Viale G, Pellegrini C, Mazzarol G, Maisonneuve P, Silverman ML, Bosari S (1999). p21WAF1/CIP1 expression in colorectal carcinoma correlates with advanced disease stage and p53 mutations. *J Pathol* **187**, 302–307.
- [47] Holland TA, Elder J, McCloud JM, Hall C, Deakin M, Fryer AA, Elder JB, Hoban PR (2001). Subcellular localisation of cyclin D1 protein in colorectal tumours is associated with p21(WAF1/CIP1) expression and correlates with patient survival. *Int J Cancer* **95**, 302–306.
- [48] Yang WC, Mathew J, Velcich A, Edelmann W, Kucherlapati R, Lipkin M, Yang K, Augenlicht LH (2001). Targeted inactivation of the p21(WAF1/cip1) gene enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosal. *Cancer Res* **61**, 565–569.
- [49] Dredge K, Hammond E, Davis K, Li CP, Liu L, Johnstone K, Handley P, Wimmer N, Gonda TJ, Gautam A (2010). The PG500 series: novel heparan sulfate mimetics as potent angiogenesis and heparanase inhibitors for cancer therapy. *Invest New Drugs* **28**, 276–283.
- [50] Gingis-Velitski S, Zetser A, Kaplan V, Ben-Zaken O, Cohen E, Levy-Adam F, Bashenko Y, Flugelman MF, Vlodaysky I, Ilan N (2004). Heparanase uptake is mediated by cell membrane heparan sulfate proteoglycans. *J Biol Chem* **279**, 44084–44092.
- [51] Gutter-Kapon L, Alishekevitz D, Shaked Y, Li JP, Aronheim A, Ilan N, Vlodaysky I (2016). Heparanase is required for activation and function of macrophages. *Proc Natl Acad Sci U S A* **113**, E7808–7817.
- [52] Johnson GB, Brunn GJ, Kodaira Y, Platt JL (2002). Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J Immunol* **168**, 5233–5239.
- [53] Brennan TV, Lin L, Huang X, Cardona DM, Li Z, Dredge K, Chao NJ, Yang Y (2012). Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation. *Blood* **120**, 2899–2908.
- [54] Qu H, Zheng L, Jiao W, Mei H, Li D, Song H, Fang E, Wang X, Li S, Huang K, et al (2016). Smad4 suppresses the tumorigenesis and aggressiveness of neuroblastoma through repressing the expression of heparanase. *Sci Rep* **6**, 32628.
- [55] Schwarte-Waldhoff I and Schmiegel W (2002). Smad4 transcriptional pathways and angiogenesis. *Int J Gastrointest Cancer* **31**, 47–59.
- [56] Hunt KK, Fleming JB, Abramian A, Zhang L, Evans DB, Chiao PJ (1998). Overexpression of the tumor suppressor gene Smad4/DPC4 induces p21waf1 expression and growth inhibition in human carcinoma cells. *Cancer Res* **58**, 5656–5661.