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Award Number: W81XWH-11-1-0302

TITLE: Yin and Yang of Heparanase in Breast Cancer Initiation

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REPORT DATE: September 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2014	Final	1 April 2011-30June 2014

4. TITLE AND SUBTITLE
Yin and Yang of Heparanase in Breast Cancer Initiation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0302

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Rush University Medical Center, Chicago, IL 60612

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
A manuscript is attached.

14. ABSTRACT Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. Here we report that sulodexide, a HPR1 inhibitor, unexpectedly stimulates breast tumorigenesis and tumor growth of polyoma virus middle T antigen (PyMT)-induced breast tumor in a somatic breast cancer models. In contrast, HRP1 knockdown in a Neutransformed breast cancer cell line derived from TVA (the receptor for sub-group A avian leucosis virus)-transgenic mice led to a significant reduction of tumor growth in a syngeneic mouse model. Consistently, TVA-transgenic mice infected with a HPR1-miRNA construct delayed the Neu oncogene-induced breast cancer development. To determine whether the HPR1 activity was disposable for its stimulatory effect on breast cancer formation, the C-terminus of HPR1 gene, which lacks the HPR1 activity (designated as RCAS-8C), and a HPR1 enzymatically-inactive, dead HPR1 (designated as RCAS-dHPR1), were cloned into a RCAS vector and tested for their potency to stimulate breast cancer formation. In vitro studies revealed that both dHPR1 and HPR1 were capable of stimulating both PI-3 and MAP kinase pathway in three cell lines, including DF-1 fibroblast cell line, a RCAS-Neu murine breast cancer cell line, and a KAT-18 thyroid tumor cell line. Mice infected with RCAS-Neu virus plus RCAS-8C developed breast cancer more rapidly than that those infected with RCAS-Neu plus a control vector encoding green fluorescence protein. Similar observations were breast cancer in TVA-transgenic mice. Consistently, full-length, enzymatic active HPR1 also accelerated the formation of RCAS-Neu and RCAS-PyMT-induced breast cancer in TVA transgenic mice. Our results collectively suggest that the C-terimus of HPR1 is capable of promoting tumor growth, and its enzymatic activity is disposable for the tumor promoting activity of HPR1.

15. SUBJECT TERMS nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

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Introduction: Heparanase-1 (HPR1) is an endoglycosidase overexpressed in many malignancies including breast cancer (1; 2). Previous studies suggest that the enzymatic activity of HPR1 can promote tumor angiogenesis and growth by degrading extra cellular matrix and releasing the growth factors. Unexpectedly, the full-length of the HPR1 gene stimulates tumor growth less effectively than the enzymatically inactive HPR1 gene, suggesting that HRP1 enzymatic activity may not be disposable for its effect on stimulating tumor growth. Since the C-terminus of HPR1 can activate the PI-3 kinase pathway and induce endothelial and tumor cell migration independent of its enzymatic activity, it is not clear whether its enzymatic activity or C-terminus or both contribute to breast tumor initiation and growth. The goal of this project is to dissect the opposing effect of HPR1 enzymatic activity and HPR1 C-terminus epitope on breast tumor initiation in a clinically relevant mouse breast cancer model. We proposed to determine if HPR1 knockdown will suppress or accelerate breast tumor initiation mediated by three oncogenes, PyMT, Neu and Wnt, and whether HPR1 C-terminus or an enzymatically dead HPR1 can stimulates breast tumor initiation, whereas full-length HPR1 has no effect or is less effective in stimulating breast tumor initiation and progression.

Key words: Heparanase; C-terminus; Breast tumor initiation; Somatic mouse model; TVA transgenic mice; Sulodexide; PyMT; Neu.

KEY RESEARCH ACCOMPLISHMENTS

1. Successfully modified a mouse model by co-infecting TVA transgenic mice with RCAS-Neu vector plus other vector encoding tumor promoting gene, thus avoiding tedious mice strain breeding. The tumor promoting effect of HPR1 and its mutation constructs is being tested for other oncogenes.
2. Demonstrated that HPR1 C-terminus was able to promote breast cancer formation.
3. Demonstrated that the enzymatically inactive HPR1 accelerated breast cancer formation induced by both RCAS-Neu and PyMT oncogenes.

Impact

1. Studies have provided convincing evidence if HPR1 and its C-terminus cooperate with an oncogene to stimulate breast tumorigenesis.

2. Heparanase enzymatic activity is disposable for its tumor promoting activity.

3. Heparanase inhibitors may not be necessarily effective in controlling breast tumor initiation or could cause adverse effect. Caution should be taken for developing HPR1 inhibitors as an anti-cancer drug in clinical patients.

Changes/Problem

1. We made a significant change in the experimental approach in specific aim 2. Our original plan was to use double transgenic mice with a TVA plus an oncogene background to determine the effect of HPR1 on promoting tumorigenesis. Since producing double-transgenic mice is very time consuming, we have changed our plan by infecting TVA-transgenic mice with an oncogene and HPR1 virus.

Products: Plasmid DNA: pcDNA6.2/HPR1-miRNA; pcDNA6.2/Ctr-miRNA; RCAS-8C, RCAS-dHPR1, RCAS-HPR1; RCAS-Neu/HPR1-miRNA 746.

Participants & Other Collaborating Organizations

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Special Reporting Requirements: N/A

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Heparanase cooperates with oncogene to stimulate breast cancer tumorigenesis in a novel somatic mouse model

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Running title: *Role of HPR1 C-terminus on breast cancer induction*

Key words: heparanase, sulodexide, TVA; Neu; PyMT;

Conflict of Interest: All authors declare no competing interest.

Abstract

Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. Prior studies suggest that HPR1 plays an important role in promoting tumor growth, its role in breast cancer tumorigenesis remains incompletely understood. In particular, whether HPR1 enzymatic activity is required for its stimulatory effect on tumor growth and initiation is not fully understood. Here we report that sulodexide, a HPR1 inhibitor, unexpectedly stimulates breast tumorigenesis and tumor growth of polyoma virus middle T antigen (PyMT)-induced breast tumor in a somatic breast cancer models. In contrast, HRP1 knockdown in a Neutransformed breast cancer cell line derived from TVA (the receptor for sub-group A avian leucosis virus)-transgenic mice led to a significant reduction of tumor growth in a syngeneic mouse model. Consistently, TVA-transgenic mice infected with a HPR1-miRNA construct delayed the Neu oncogene-induced breast cancer development. To determine whether the HPR1 activity was disposable for its stimulatory effect on breast cancer formation, the C-terminus of HPR1 gene, which lacks the HPR1 activity (designated as RCAS-8C), and a HPR1 enzymatically-inactive, dead HPR1 (designated as RCAS-dHPR1), were cloned into a RCAS vector and tested for their potency to stimulate breast cancer formation. In vitro studies revealed that both dHPR1 and HPR1 were capable of stimulating both PI-3 and MAP kinase pathway in three cell lines, including DF-1 fibroblast cell line, a RCAS-Neu murine breast cancer cell line, and a KAT-18 thyroid tumor cell line. Mice infected with RCAS-Neu virus plus RCAS-8C developed breast cancer more rapidly than that those infected with RCAS-Neu plus a control vector encoding green fluorescence protein. Similar observations were made in RCAS-PyMT-induced breast cancer in TVA-transgenic mice. Consistently, full-length, enzymatic active HPR1 also accelerated the formation of RCAS-Neu and RCAS-PyMT-induced breast cancer in TVA transgenic mice. Our results collectively suggest that the C-terimus of HPR1 is capable of promoting tumor growth, and its enzymatic activity is disposable for the tumor promoting activity of HPR1.

Introduction

Heparanase-1 (HPR1) is an endoglycosidase that is overexpressed in a variety of adenocarcinomas [1-3]. HPR1 expression in breast cancer correlates with their metastatic potential [4, 5]. HPR1 degrades heparan sulfate (HS) proteoglycans (HSPGs), a main component of the cell surface, the extracellular matrix (ECM), and the basement membrane (BM) [6-9]. Breakdown of HSPGs in the BM and ECM leads to the release of several growth factors such as FGF and VEGF that are trapped in the tumor stroma. These growth factors stimulate tumor angiogenesis by stimulating endothelial cell proliferation and migration. In addition, breakdown of the BM and ECM allows tumor cells to invade locally or metastasize to a distant site. HPR1 overexpression in human breast cancer cell lines or induction by estrogen led to increased angiogenesis and accelerated tumor growth in breast cancer xenograft models [10-13]. Suppression of HPR1 in a MDA-435 breast cancer cell line by ribozyme RNA or HPR1 siRNA reduced their in vitro invasive potential in Matrigel [14, 15].

In addition to its function as an endoglycosidase to cleave HS side chains, HPR1 exerts its many biological functions independent of its enzymatic activity. For example, HPR1 can enhance cell adhesion [16, 17], induce VEGF expression [18], induce tumor and endothelial cell migration, and induce Akt, p38, and Src phosphorylation [18, 19]. HPR1 induces EGF receptor phosphorylation and stimulates tumor cell proliferation and growth in an enzymatic activity-independent manner [20]. A conservative, hydrophobic C-terminus domain of HPR1 has been identified to mediate these diverse biological functions [21, 22]. HPR1 C-terminus functions as a ligand to bind two potential unknown receptors (130 & 170 kDa protein) to activate the PI-3 kinase pathway [22]. More interestingly, the U87 glioma cell line overexpressing HPR1 C-terminus epitope in the absence of HPR1 enzymatic activity is more effective in stimulating tumor growth than HPR1 full molecule [22]. This raises an intriguing possibility: how much does HPR1 enzymatic activity contribute to its angiogenic and tumor-promoting function? Two recent phase I/II clinical trials using a HPR1 inhibitor to treat patients with hepatacellular carcinoma or others types of cancer are ineffective [23, 24]. These observations suggest that HPR1 activity may be disposable for its tumor promoting activity. Here we report that using a somatic breast cancer model, HPR1 C-terminus is able to promote breast cancer formation in a somatic breast cancer model.

Materials and Methods

Plasmids. The C-terminus of the HPR1 gene (encoding amino acid 413-543) was cloned into a RCAS vector digested with a PacI and Cla I. An oligonucleotide containing a Myc tag sequence (atg gaa caa aaa ctt att tct gaa gaa gat ctg) fused with a sequence encoding a 8-kDa (amino acids 36-55) of HRP1 and its complementary sequence were synthesized. The 5' end of this annealed fragment had a Not I-cleaved site, whereas its 3' end contained a cleaved Pac I site. This fragment was directly ligated into Not I/PacI-digested RCAS-C. The following plasmid designated as RCAS-8C was used to transfected DF-1 cells to generate RCAS-8C virus. To prepare miRNA vectors targeting murine HPR1 (mHPR1), three pairs of oligonucleotides with a miRNA structure that targets murine HPR1 at the nucleotide 671, 746, and 796 were synthesized and ligated into a pcDNA6.2 expression vector (Invitrogene). The effectiveness of these three miRNAs to knock down the expression of mHPR1 was analyzed for the expression of mHPR1 by Western blot and FACS analysis of cell surface HPR1 in RCAS-Neu cell line after transfection. mHPR1 miRNA insert in one construct (pcDNA/mHPR1-miRNA 746) was shuttled into RCAS-Neu vector by cloning a PCR-amplified fragment digested with Cla I and Pac I enzymes. This vector designated as RCAS-Neu/mHPR1-miRNA 746 was used to transfected into DF-1 cells. The insert of miRNA targeting β-galactosidase was PCR-amplified from a control plasmid (Invitrogen) and cloned into RCAS-Neu vector as a negative control. RCAS-GFP vector was kindly provided Dr. Y. Li (Baylor Medical College, Houston, TX).

Western blot. RCAS-Neu and DF-1 cells were harvested and lysed in Nonidet P (NP)-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptinin, and 1 mM phenylmethylsulfonyl fluoride). After electrophoresis and transfer to nitrocellulose membranes, HPR1 was detected by using a rabbit anti-HPR1 antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and SuperSignal Western Pico enhanced chemiluminoscence substrate (Pierce Chemical Co., Rockford, IL). A monoclonal antibody against □-actin was purchased from Santa Cruz Biotechnology Inc., San Diego, CA.

HPR1 activity assay. Purified platelet HPR1 (50 units/μl) or serum from a pancreatic cancer patient diluted at 1:10 in HPR1 buffer (middle panel) were premixed with the indicated concentrations of HPR1 inhibitors in HPR1 assay buffer. The mixture was added to a 96-well ELISA plate precoated with Matrigel and

incubated at 37°C for 16 hr. HPR1 activity was analyzed by an ELISA method according to a novel ELISA protocol established in this laboratory [25-28].

Cell proliferation assay. RCAS-Neu tumor cell lines infected with RCAS-Neu/Ctr-miRNA or RCAS-Neu/mHPR1-miRNA 746 were seeded in 96-well plates at the density of 2,000/well. After incubation for 96 hr, cell proliferation was monitored by using an ATP-based luminescence assay (Promegan, Madison, WI) following the manufacturer's instruction.

FACS analysis of cell surface HS. RACS-Neu and DF-1 cells transfected or infected with various RCAS vectors were harvested and analyzed for cell surface HS levels by staining with an anti-HS mAb (clone HepSS) followed flow cytometry according to previous publications [27].

***In vivo* tumor induction.** TVA-transgenic mice expressing the receptor for an avian retrovirus vector, RCAS, were infected with DF-1 cells transfected with RCAS-PyMT vector, RCAS-Neu/mHPR1-miRNA, RCAS-Nue/LacZ-miRNA, RCAS-8C by intraductal injection. Mice infected with RCAS-PyMT virus were treated with sulodexide by i.p. injection. Mice were observed for breast cancer development by palpation. The differences of tumor latency between untreated and sulodexide-treated groups, RCAS-Neu/mHPR1-miRNA versus RCAS-Neu/LacZ-miRNA, RCAS-Neu plus RCAS-8C versus RCAS-Neu plus RCAS-GFP group, were statistically analyzed by using the Log-rank test. The difference of tumor growth between three groups was statistically analyzed by using the one-way repeated measure ANOVA. The p value of <0.05 was considered statistically significant.

Results

Sulodexide treatment accelerates PyMT-mediated tumorigenesis. Sulodexide is a mixture of dermatan sulfate (20%) and low-molecular-weight heparin (80%). We first examined the ability of sulodexide to inhibit HPR1 activity by using a novel ELISA method developed in my laboratory [25, 26, 28-30]. As shown in Fig. 1A (left panel), sulodexide inhibited HPR1 activity with an IC_{50} value of approximately 5 μg/ml. The IC_{50} values for heparin and PI-88 were approximately 2-3 μg/ml. Sulodexide inhibits HPR1 activity slightly better than PI-88 and heparin when a pancreatic cancer patient's serum was used as the source of HPR1 (Fig. 1B).

We next tested whether sulodexide can prevent breast tumor formation. TVA transgenic mice were infected by intraductal injection of RCAS-PyMT virus, $1x10^7$ virions/gland, 4 glands/mouse. Mice were treated with water or sulodexide at the dose of 35 or 70 mg/kg/day by gavage and monitored for tumor formation by palpation. To our surprise, administration of sulodexide accelerated breast cancer formation in a dose-dependent manner (Fig. 1C). Breast tumors were formed in untreated mice with the median latency of >78±4.1 days, whereas administration of sulodexide at 70 and 35 mg/kg/day had a median tumor latency of 23±2 days and 53±7.4 days, respectively. Log-rank test showed that sulodexide treatment at the dose of 35 mg/kg/day significantly shortened tumor latency, compared to the untreated control group ($p=0.018$). Also, Log-Rank test revealed that mice treated with sulodexide at 70 mg/kg/day had a significantly shorter tumor latency than those treated with sulodexide at the dose of 35 mg/kg/day ($p=0.002$).

Effect of HPR1 gene knockdown on breast cancer tumor growth and tumorigenesis. The role of HPR1 in breast tumor growth and tumorigenesis was first tested in a synergic tumor graft mouse model. Three miRNA constructs were prepared by using a hairpin sequence that target murine HPR1 mRNA at the nucleotide site of 746. As shown in Fig. 2A, FACS analysis revealed that cell surface heparan sulfate levels were decreased in RCAS-Neu/mHPR1-miRNA 746-infected cells, compared to that infected with RCAS-Neu/LacZ-miRNA. Western blot analysis confirmed the ability of this vector to suppress HPR1 expression in RCAS-Neu breast cancer cell lines (Fig. 2B).

In vitro study showed that knockdown of HPR1 expression in RCAS-Neu cells did not affect cell proliferation (Fig. 2C). We next examined the effect of HPR1 knockdown in tumor growth in a syngeneic mouse model. RCAS-Neu cells stably transfected with RCAS-HPR1-miRNA or the control construct RCAS-Ctr-miRNA were inoculated into the fat pad of FVB mice ($5x10^5$ cells per fat pad) (8-12 mice/group). As shown

in Fig. 3A, knockdown of HPR1 expression led to a significant suppression of the growth of RCAS-Neu tumor cells with HPR1 knockdown, compared to that transfected with RCAS-Neu/LacZ-miRNA. To examine the effect of HPR1 in breast tumorigenesis, RCAS-Neu/HPR1-miRNA and RCAS-Neu/Ctr-miRNA retroviral vectors ($1x10^7$ virions/gland) were used to induce breast cancer by intraductal injection into the mammary gland of TVA transgenic mice. As shown in Fig. 3B, mice infected with RCAS-Neu/HPR1-miRNA developed breast cancer significantly slower than those infected with RCAS-Neu/Ctr-miRNA.

The effect of the enzymatically active and inactive HPR1 on the MAP and PI-3 kinase pathway activation. To test whether HPR1 enzymatic activity is disposable for its tumor promoting effect, an enzymatic activity-dead HPR1 gene (RCAS-dHPR1, double mutations at amino acid residues 225 & 343) tagged with a Myc epitope were prepared. Western blot analysis with an anti-Myc tag antibody revealed the strong expression of HPR1 in DF-1 cells infected with RCAS-HPR1 and RCAS-dHPR1 virus. Both the full-length HPR1 and dead HPR1 were detected as a 50-kDa protein (Fig. 4A). FACS analysis revealed that RCAS-HPR1 virus-infected DF-1 cells had lower cell surface heparan sulfate levels than uninfected control and RCAS-dHPR1-infected DF-1 cells. These results confirmed that dHPR1 did not have HPR1 enzymatic activity (Fig. 4B).

Previous studied revealed that HPR1 is activated PI-3 kinase signaling in endothelial cells Ref. We therefore determined if these HPR1 constructs were functional in stimulating the PI-3 kinase pathway. KAT-18 cells (a thyroid tumor cell line), NIH 3T3 murine fibroblast cells, and DF-1 chicken fibroblast cells were pre-starved in serum-free medium overnight and then stimulated with purified HPR1 and dHPR1. Both HPR1 and dHPR1 activated the PI-3 and the MAP kinase pathways as evidenced by increased AKT and ERK phospshorylation (Fig. 4C).

The effect of the enzymatically active and inactive HPR1 on breast cancer formation. We first determined the effect of full-length, enayzmatically active HPR1 in breast cancer initiation. TVA transgenic mice infected with RCAS-PyMT plus RCAS-HPR1 vectors developed breast cancer significantly faster than mice infected with RCAS-PyMT plus a RCAS-GFP vector. The mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-GFP were 119$\pm$ 8 days; whereas the mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-HPR1 were 86±6 (Fig. 5A). Thus, TVA transgenic mice co-infected with RCAS-PyMT and RCAS-HPR1 significantly accelerated breast cancer formation.

We next determined if an enzymatically inactive HPR1 was able to accelerate breast tumor initiation. TVA transgenic mice infected with RCAS-PyMT (Fig. 5B) or RCAS-Neu (Fig. 5C) plus RCAS-dHPR1 vectors developed breast cancer much faster than mice infected with RCAS-PyMT or RCAS-Neu plus a control RCAS-vector, respectively. The mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-dHPR1 were 89±6 versus 63±5 days in TVA transgenic mice infected with RCAS-PyMT plus RCAS-GFP. The mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-dHPR1 were 95±11 versus 228±21 days in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP. These observations strongly suggested that that HPR1 enzymatic activity is disposable for its tumor promoting effect.

The effect of the C-terminus of HPR1 on breast cancer formation. We then tested whether the C-terminus of HPR1 was sufficient to stimulate breast tumor formation. The expression vector encoding a C-terminus gene fragment (RCAS-8C, with a fusion of 8-kDa and the C-terminus of HPR1 including amino acid residues from 415-543) were used to transfect DF-1 cells. Western blot analysis with an anti-Myc tag antibody revealed that HPR1 was detected as an 18-kDa protein in DF-1 cells transfected with RCAS-8C vector, whereas the full-length HPR1 was detected as 50-kDa protein (Fig. 6A). FACS analysis revealed that RCAS cell surface heparan sulfate levels in RCAS-8C-infected DF-1 cells were equivalent to those in uninfected DF-1 cells, indicating that the C terminus of HPR1 did not have HPR1 enzymatic activity (Fig. 4B).

To determine whether the C-terminus of HPR1 was able to promote tumor initiation, TVA mice were co-infected with RCAS vectors encoding the RCAS-Neu virus plus the control RCAS vector or the vector encoding RCAS-8C. As shown in Fig. 6B, mice infected with RCAS-Neu plus RCAS-8C vectors developed breast cancer much faster than those infected with a control RCAS-GFP vector, the mean and median tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-8C were 275±27 and 180±30 respectively, whereas the mean and median tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP were 480±25 and 245 respectively. We next determine whether the C-terminus of HPR1 also stimulated PyMT-oncogene-induced breast cancer. As shown in Fig. 6C, mice infected with RCAS-PyMT plus RCAS-8C vectors developed breast cancer significantly faster than mice infected with RCAS-PyMT plus RCAS-GFP vector. The mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP were 86±6 days versus 52±4 days in TVA transgenic mice infected with RCAS-PyMT plus RCAS-8C.

Discussion

Conventional transgenic mice carrying the PyMT or rat Neu proto-oncogene in their genomes are genetically predestined to overexpress Neu or PyMT in all mammary epithelial cells and to develop lethal invasive mammary carcinomas [31]. Our studies using a clinically relevant breast cancer model to investigate the role of HPR1 on tumor formation. In this mouse model, the expression of Neu or PyMT onconge is restricted to a few mammary epithelial cells. Therefore, our mouse model closely resembles a situation in patients in which Neu-positive breast cancers also originate from a few cells. Our results demonstrated the HPR1 knockdown slowed down the formation of Neu oncogene-induced breast cancer (Fig. 4), whereas co-infection with the C-terminus of HPR1 with the Neu oncogene accelerated breast cancer induction (Fig. 5). These results collectively suggest that HPR1 is able to promote tumor formation. Boyango et al. [32] recently reported that HPR1 cooperates with Ras to drive breast tumor initiation in a MCF-10A cell line transfected with HPR1 and Ras oncogene. Moreover, HPR1-transgenic mice were more sensitive, whereas HPR1 knockout mice were more resistant to skin tumorigenesis induced by a classical two-stage 12-dimethylbenz(a)anthracene (DMBA)/12-o-tetradecanoylphorbol-13-acetate (TPA).

Our studies also suggested that HPR1 enzymatic activity is disposable for its effect on breast tumor initiation. Using a HRP1 inhibitor, our study demonstrated that sulodexide was not only unable to slow down breast cancer initiation but rather could accelerate tumorigenesis initiated by a PyMT oncogene. There are several plausible explanations for these unexpected results: 1) Our recent in vitro study showed that HPR1 inhibitors are able to stimulate the proliferation of pancreatic cancer cells by increasing the expression of cell surface HPSGs and strengthening the FGF2 receptor-activated MAP kinase pathway [26]. It is possible that sulodexide may stimulate the proliferation of PyMT-transformed breast cells in vivo by a similar mechanism; 2) HSPGs function as the co-receptor for Wnt and FGF-2, both growth factors are involved in stem cell self-renewal [33, 34]. It is possible that increased cell surface HSPG levels by sulodexide may enhance the FGF signaling pathway, leading to the acceleration of breast cancer formation. These observations are consistent with previous observations that enzymatically inactive HPR1 is more efficient in stimulating tumor growth [14, 15]. Thus, these results collectively suggest that HPR1 enzymatic activity is not only disposable but rather may negatively regulate tumor growth. In contrast, Boyango et al. [32] reported that PGC455, an HPR1

inhibitor, suppresses DMBA/TPA-induced tumorigenesis. It is not clear if different tumor models or different HPR1 inhibitors leads to these polarized results.

In the present study, we also investigated the role of HPR1 in tumor growth. Using a syngeneic breast cancer model, we found that HPR1 knockdown led to a retarded tumor growth (Fig. 3). These results were not unexpected since several prior studies using xenograft mouse model demonstrated slower growth of implanted human breast cancer cell lines with HPR1 knockdown, compared to their control vector-transfected cell line controls. Interestingly, in vitro cell proliferation experiments revealed that HPR1 knockdown did not lead to an inhibition of cell proliferation. This observation is consistent with a prior study showing that HPR1 overexpression in a human breast cancer cell line MDA-MB-231 did not increase cell proliferation in vitro but increase breast tumor growth in a nude mouse breast cancer model by stimulating tumor angiogenesis[13]. It appears that HPR1 knockdown-mediated antitumor tumor effect is likely mediated by its effect on tumor angiogenesis in our mouse model. Notably, Cohen-Kaplan et al. [20] reported that HPR1 overexpression is able to increase the proliferation of a LNCaP human lung cancer cell line, whereas HPR1 knockdown leads to decreased DNA replication in a MDA-231 breast cancer cell line and U87 glioma cell line, assuming that the cell proliferation is also inhibited in these HPR1-suppressed cell lines. The discrepancy in the effect of HPR1 on cell proliferation is not clear.

We noticed that there was some discrepancy in tumor induction in different experiments. One explanation could be due to the success rate of virus injection into the mammary gland. Second possibility could be due the discrepancy in the virus stock prepared at different time and storage. One weakness in the present study is that the effect of full-length HPR1 on breast cancer formation has not been tested (data unavailable now).

In summary, our present study demonstrated that HPR1 is able to stimulate breast tumor growth and to accelerate breast cancer initiation in a clinically relevant breast cancer model. The enzymatic activity of HPR1 is disposable for the stimulatory effect of HPR1, whereas the C-terminus of HPR1 plays a critical role in promoting breast cancer initiation.

Acknowledgements

The authors thank Keryx Pharmaceuticals Inc. (New York, NY) for providing sulodexide. This project was supported U.S. Army Breast Cancer Research Program (W81XWH-11-1-0302) to X. Xu.

Figure legends

Fig. 1. Inhibition of HPR1 activity by PI-88, heparin and sulodexide. Purified platelet HPR1 (50 units/μl) (**A**) or serum (**B**) from a pancreatic cancer patient diluted at 1:10 in HPR1 buffer were premixed with the indicated concentrations of HPR1 inhibitors in HPR1 assay buffer. The mixture was added to a 96-well ELISA plate precoated with Matrigel and incubated at 37°C for 16 hr. HPR1 activity was analyzed by an ELISA method. (**C**) Sulodexide treatment accelerates PyMT tumor formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT virus by intraductal injection of $1x10^7$ virions, 4 glands per mouse. One week later, mice were treated daily with water (20 mice), sulodexide at the dose of 35 mg/kg/day (18 mice) or 70 mg/kg/day (9 mice) by gavage. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test.

Fig. 2. Effect of HPR1 knockdown on breast tumor growth and initiation. (**A & B**) Effect of HPR1 knockdown on cell surface HS levels and HPR1 expression. RCAS-Neu cells were infected with RCAS-Neu/Ctr-miRNA (left), RCAS-Neu/mHPR1-miRNA-746 (right). After incubation for 48 hr, the cells were harvested, stained with an anti-HS IgM mAb, analyzed for cell surface HS levels by FACS analysis (A) or for HPR1 expression by Western blot (B). Black line, isotype control; Green line, anti-HS IgM. (**C**) HPR1 suppression does not inhibit tumor cell proliferation. RCAS-Neu cells infected with RCAS-Neu/LacZ-miRNA or RCAS-Neu/HPR1-miRNA virus were seeded in 96-well plates (2000 cells/well) and incubated for 24 or 72 hr. Cell proliferation was analyzed by an ATP-based Cell-Glo assay and read in a 96-well plate reader. The data represents the mean standard deviation of one of three experiments in triplicate with similar results.

Fig 3. **HPR1 knockdown suppresses breast tumor growth and initiation.** (**A**) Female FVB mice (8-12 weeks old) were inoculated with RCAS-Neu/HPR1-miRNA or RCAS/LacZ-miRNA cells by fat pad injection of $5x10^5$ cells. Mice were monitored for tumor growth 3 weeks later and measured twice weekly with a caliper. The difference of tumor growth between three groups was statistically analyzed by using the one-way repeated measure ANOVA. (**C**) HPR1 knockdown delays breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-Neu/HPR1-miRNA or RCAS-Neu/LacZ-miRNA virus by intraductal injection of $1x10^7$ virions. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test ($p<0.01$).

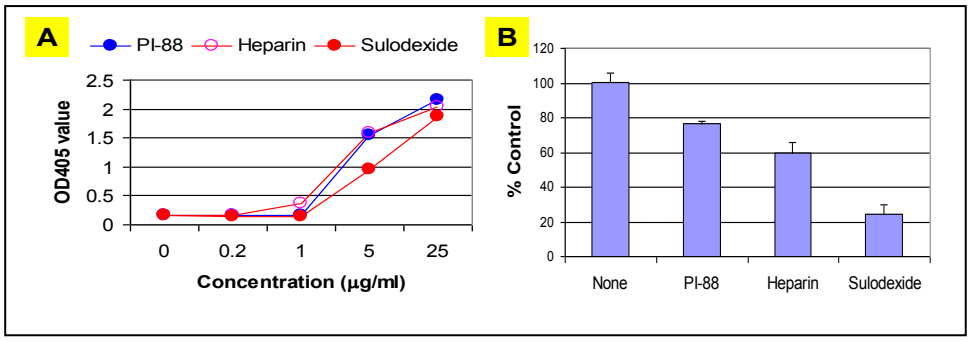
Fig. 4. Activation of the PI-3 and MAP kinase pathways by the enzymatically active and inactive HPR1. **(A)** HPR1 expression and cell surface HS levels**.** DF-1 cells were infected with RCAS-HPR1 (left), RCAS-DM-HPR1 (middle) or RCAS-HPR1-8C. After incubation for 48 hr, the cells were harvested and analyzed for HPR1 expression by Western blot with an anti-Myc epitope antibody or for cell surface HS levels by staining with an anti-HS IgM mAb followed by FACS analysis. Black line, isotype control; Green line, anti-HS IgM. **(B)** The effect of HPR1 on AKT and ERK phosphorylation. KAT-18, DF-1, and NIH-3T3 cells were starved in the medium containing 0.5% FBS overnight. The cells were then left untreated or treated for 30 min with HPR1 or enzymatically dead HPR1 (dHPR1) purified from the conditioned media of DF1 cells infected with RCAS retroviral vector. AKT and ERK phosphorylation was detected by their specific antibodies. Antibodies against total proteins were used as control.

Fig. 5. The effect of enzymatically active and inactive HPR1 on PyMT- and Neu-induced breast cancer formation. (**A**) Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT plus RCAS-dHPR1- (Black line) or RCAS-Neu plus RCAS-GFP virus (red line) by intraductal injection of $1x10^7$ virions each. (**B**) Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-Neu plus RCAS-dHPR1or RCAS-Neu plus RCAS-GFP virus (red line) by intraductal injection of $1x10^7$ virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test (p=0.006) or RCAS-Neu (right panel). (**C**) HPR1 accelerates breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT plus RCAS-HPR1 (Red line) or RCAS-PyMT plus RCAS-GFP virus (Blue line) by intraductal injection of $1x10^7$ virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test (p=0.015).

Fig. 6. Effect of HPR1 C-terminus on PI-3 kinase pathway activation and breast cancer tumor initiation. **(A)** HPR1 expression and cell surface HS levels**.** DF-1 cells were infected with RCAS-HPR1 (left), RCAS-DM-HPR1 (middle) or RCAS-HPR1-8C. After incubation for 48 hr, the cells were harvested and analyzed for HPR1 expression by Western blot with an anti-Myc epitope antibody or for cell surface HS levels by staining with an anti-HS IgM mAb followed by FACS analysis. Black line, isotype control; Green line, anti-HS IgM. **(B)** The effect of HPR1 on AKT and ERK phosphorylation. KAT-18, DF-1, and NIH-3T3 cells were starved in the medium containing 0.5% FBS overnight. The cells were then left untreated or treated for 30 min with HPR1 or

enzymatically dead HPR1 (dHPR1) purified from the conditioned media of DF1 cells infected with RCAS retroviral vector. AKT and ERK phosphorylation was detected by their specific antibodies. Antibodies against total proteins were used as control. (**C**) HPR1 C-terminus accelerates PyMT-induced breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT plus RCAS-dHPR1- (Black line) or RCAS-Neu plus RCAS-GFP virus (red line) by intraductal injection of $1x10^7$ virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test (p=0.006).

Fig. 1


A
PI-88
Heparin
Sulodexide
OD405 value
2.5
2
1.5
1
0.5
0
0
0.2
1
5
25
Concentration (µg/ml)
B
% Control
120
100
80
60
40
20
0
None
PI-88
Heparin
Sulodexide

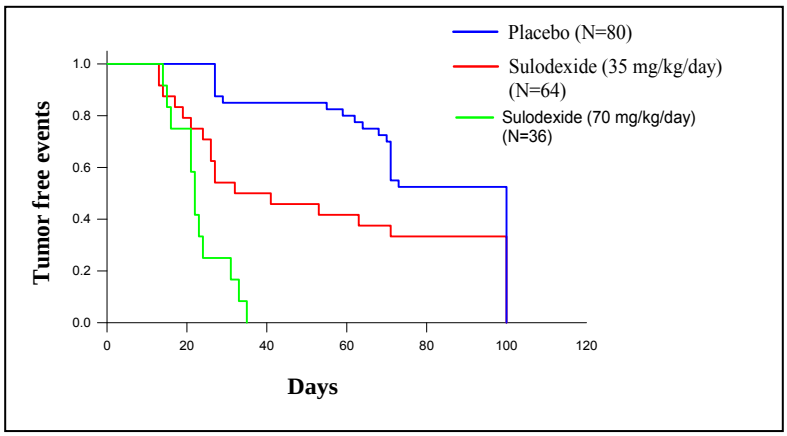
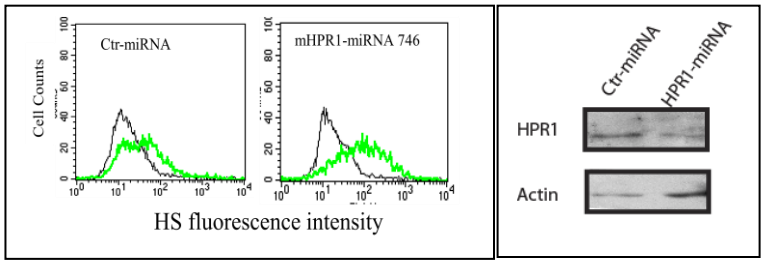

Placebo (N=80)
Sulodexide (35 mg/kg/day)
(N=64)
Sulodexide (70 mg/kg/day)
(N=36)
Tumor free events
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Days

Fig. 2.


Ctr-miRNA
mHPR1-miRNA 746
Cell Counts
HS fluorescence intensity
Ctr-miRNA
HPR1-miRNA
HPR1
Actin

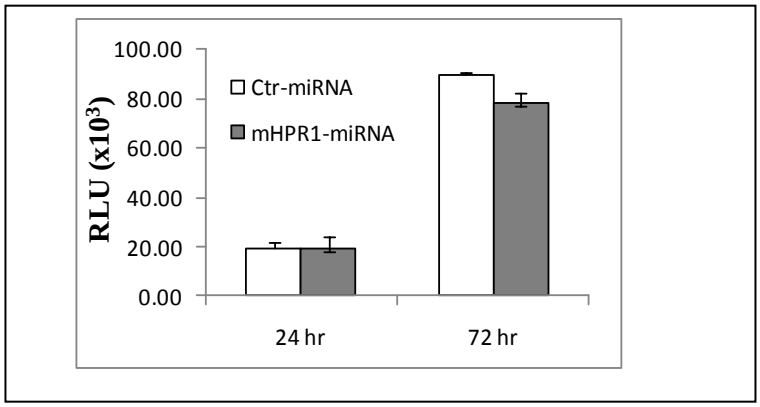

100.00
80.00
60.00
40.00
20.00
0.00
RLU (x10³)
Ctr-miRNA
mHPR1-miRNA
24 hr
72 hr

Fig. 3A

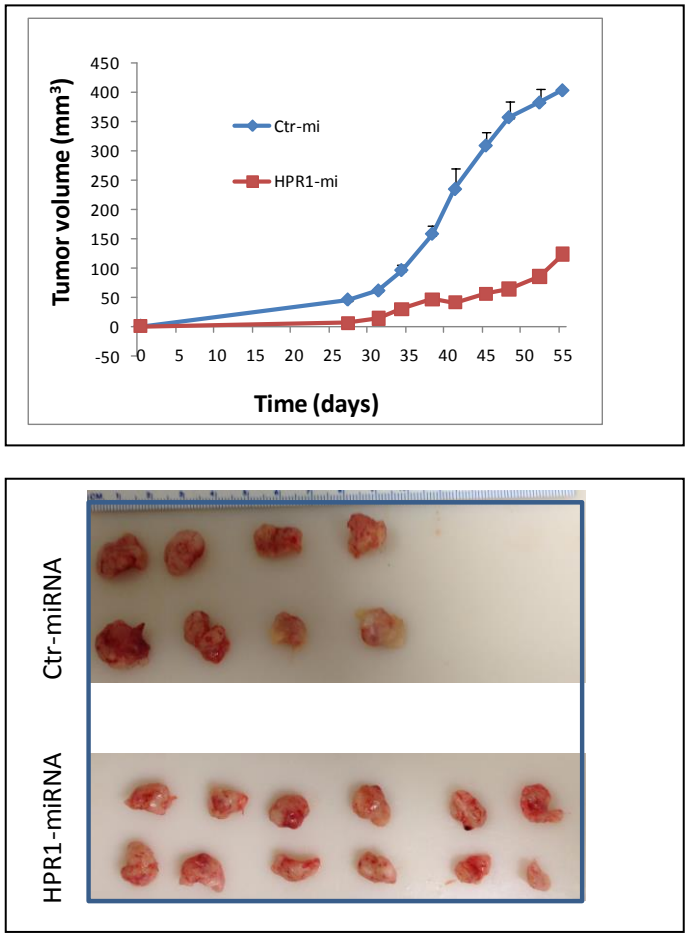

Tumor volume (mm³)
450
400
350
300
250
200
150
100
50
0
-50
0 5 10 15 20 25 30 35 40 45 50 55
Time (days)
Ctr-mi
HPR1-mi
Ctr-miRNA
HPR1-miRNA

Fig. 3C.

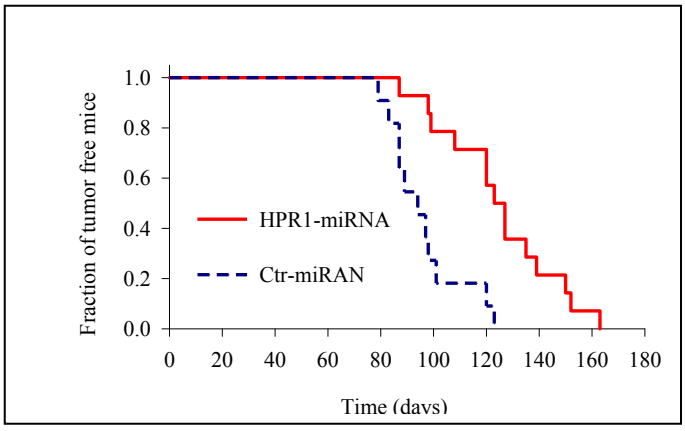
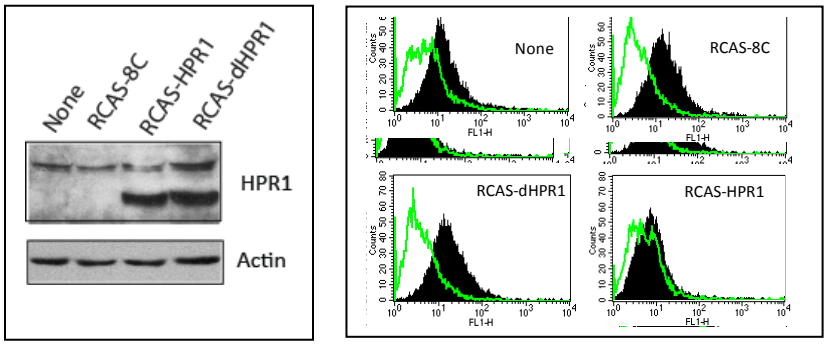
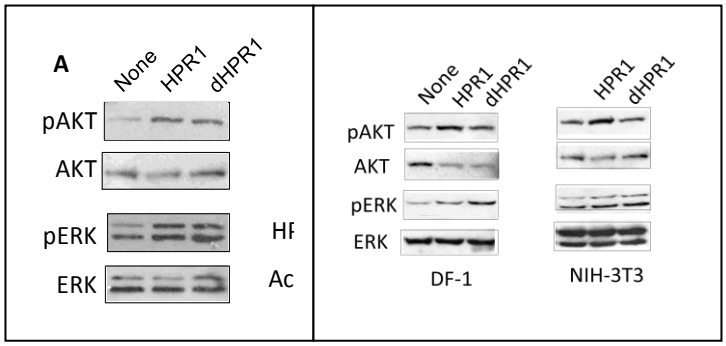

Fraction of tumor free mice
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 120 140 160 180
Time (days)
HPR1-miRNA
Ctr-miRAN

Fig. 4

A


None
RCAS-8C
RCAS-HPR1
RCAS-dHPR1
HPR1
Actin
None
RCAS-8C
RCAS-dHPR1
RCAS-HPR1
Counts
FL1-H

B


A
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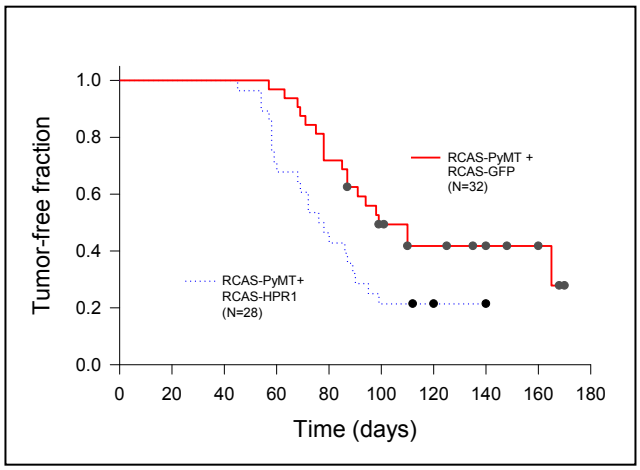
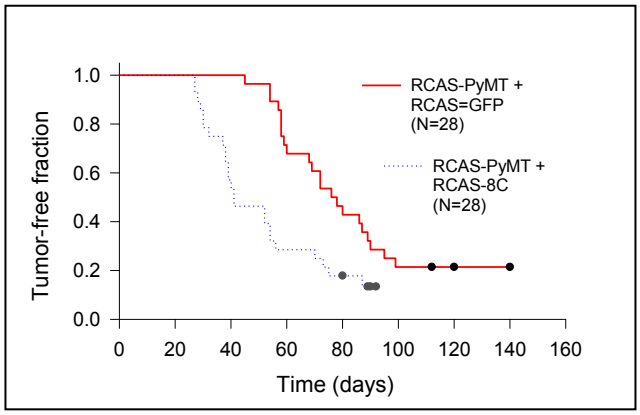
HPR1
dHPR1
pAKT
AKT
pERK
ERK
HI
Ac
None
HPR1
dHPR1
pAKT
AKT
pERK
ERK
DF-1
HPR1
dHPR1
NIH-3T3

Fig. 5

A


Tumor-free fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
180
Time (days)
RCAS-PyMT +
RCAS-GFP
(N=32)
RCAS-PyMT+
RCAS-HPR1
(N=28)

B


Tumor-free fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
Time (days)
RCAS-PyMT +
RCAS=GFP
(N=28)
RCAS-PyMT +
RCAS-8C
(N=28)

C

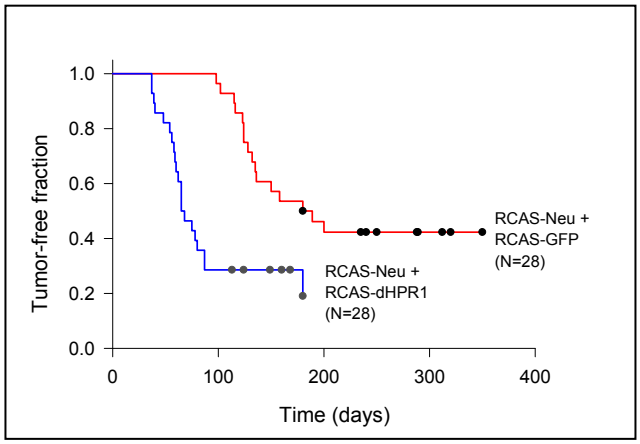

Tumor-free fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
Time (days)
RCAS-Neu +
RCAS-GFP
(N=28)
RCAS-Neu +
RCAS-dHPR1
(N=28)

Fig. 6A

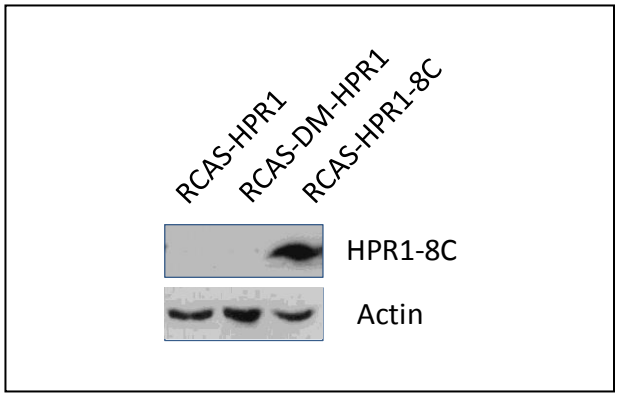


Fig. 6B

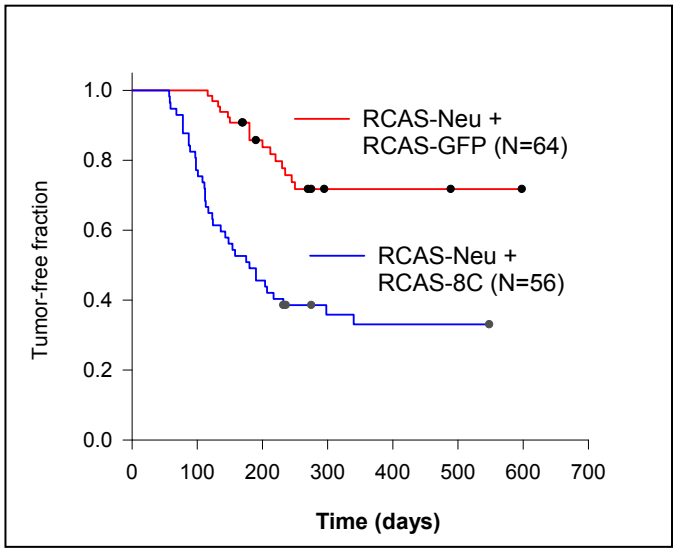
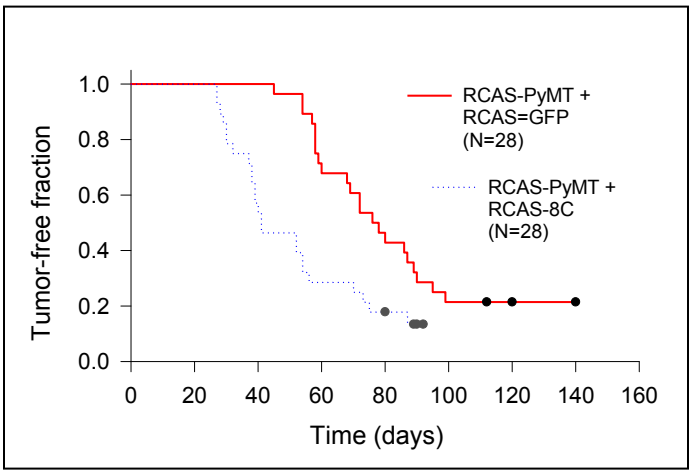


Fig. 6C



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