NEOPLASIA
www.neoplasia.com

Volume 13 Number 7 July 2011 pp. 579–589 579

Matrix-Dependent Regulation of AKT in Hepsin-Overexpressing PC3 Prostate Cancer Cells[1,2]

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Abstract

The serine-protease hepsin is one of the most prominently overexpressed genes in human prostate carcinoma. Forced expression of the enzyme in mice prostates is associated with matrix degradation, invasive growth, and prostate cancer progression. Conversely, hepsin overexpression in metastatic prostate cancer cell lines was reported to induce cell cycle arrest and reduction of invasive growth *in vitro*. We used a system for doxycycline (dox)-inducible target gene expression in metastasis-derived PC3 cells to analyze the effects of hepsin in a quantitative manner. Loss of viability and adhesion correlated with hepsin expression levels during anchorage-dependent but not anchorage-independent growth. Full expression of hepsin led to cell death and detachment and was specifically associated with reduced phosphorylation of AKT at Ser^{473}, which was restored by growth on matrix derived from RWPE1 normal prostatic epithelial cells. In the chorioallantoic membrane xenograft model, hepsin overexpression in PC3 cells reduced the viability of tumors but did not suppress invasive growth. The data presented here provide evidence that elevated levels of hepsin interfere with cell adhesion and viability in the background of prostate cancer as well as other tissue types, the details of which depend on the microenvironment provided. Our findings suggest that overexpression of the enzyme in prostate carcinogenesis must be spatially and temporally restricted for the efficient development of tumors and metastases.

Neoplasia (2011) 13, 579–589

Introduction

Prostate cancer (PCa) is the most frequently diagnosed tumor entity in men [1]. Despite considerable advances in the identification of molecular changes [2,3], the interplay of altered genes and proteins in the progression of prostate tumors is still poorly understood. Microarray expression profiling studies identified the hepsin (*HPN*) gene as one of the most prominently overexpressed genes in PCa (see www.oncomine.org [4,5]). Hepsin belongs to the hepsin/TMPRSS/enteropeptidase subfamily within the class of type II transmembrane serine proteases [6]. The enzyme was found to cleave several biologic substrates including blood coagulation factor VII [7], pro–hepatocyte

Abbreviations: ACTB, β-actin; AKT, v-*akt* murine thymoma viral oncogene homolog; CAM, chorioallantoic membrane; ECM, extracellular matrix; FRT, Flp recombinase target; HG-PIN, high-grade prostate intraepithelial neoplasia; HPN, hepsin; ILK, integrin-linked kinase; LAMB3, laminin β_3; Ln-332, laminin-332; PCa, prostate cancer; PI3K, phosphatidylinositol-3-kinase

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[1]This study was supported by grants from the Federal Ministry of Education and Research (no. 01EZ0828 to R.H. and M.S. and no. 01GS0890 to H.S.) and the German Research Foundation (STE490/23-1 to R.S.). The authors declare no conflict of interests.
[2]This article refers to supplementary materials, which are designated by Figures W1 to W3 and are available online at www.neoplasia.com.
Received 11 February 2011; Revised 27 April 2011; Accepted 29 April 2011

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DOI 10.1593/neo.11294

growth factor [8], pro–urokinase-type plasminogen activator [9], epidermal growth factor receptor [10], as well as the β_3-subunit (*LAMB3*) of laminin 332 (Ln-332) [11], an extracellular matrix (ECM) molecule, which is part of the healthy prostate but frequently lost in PCa [12]. Tissue-specific overexpression of hepsin in prostates of transgenic mice led to matrix destabilization and reduced levels of Ln-332 [13]. In the LPB-tag mouse, a model for high-grade prostate intraepithelial neoplasia (HG-PIN) due to expression of the SV40 large T antigen in the prostate epithelium, the same genetic modification led to metastasized PCa. In an orthotopic mouse model, tumor weight and prostate-specific antigen levels correlated with hepsin expression in the transplanted cells and were reduced after administration of PEGylated Kunitz domain-1, a serine protease inhibitor with considerable specificity for hepsin [14]. Thus, hepsin cleaves a variety of tumor-relevant substrates, and its proteolytic activity is supposed to play an important role during matrix remodeling in PCa.

Interestingly, hepsin overexpression is frequently lost in PCa metastases [4]. Furthermore, forced expression of the gene in the PC3 PCa cell line, which is derived from a bone metastasis and displays low endogeneous levels of hepsin, had been shown to evoke a G_2 cell cycle arrest, which was accompanied by reduced invasiveness *in vitro* [15]. *HPN* overexpression was also found to confer growth suppressive and proapoptotic effects in endometrial and ovarian cancer cell lines [16,17]. However, others generated stable hepsin transfectants in PC3- and LnCaP-PCa cell lines without observing any growth suppressive effects [9,18], which challenged the findings cited. The reasons for the contradictory results have not been resolved to date, and functional studies on hepsin-associated signaling to resolve the "hepsin paradox" [19] are lacking. Thus, the exact role of hepsin in PCa, as well as its diagnostic and therapeutic value, remained unclear.

It has been suggested that the level of hepsin expression may influence cell viability, and the *in vivo* effects are mediated by specific hepsin substrates present in the tumor stroma [14,19]. To test this hypothesis, we used an isogenic cell line technology for inducible target gene overexpression to characterize the effects of *HPN* in both PC3 metastatic PCa and in HEK293 nontumorigenic human embryonic kidney cells. We found that hepsin overexpression led to growth suppression in both cell lines. In PCa cells, hepsin-mediated effects involve cell adhesion-associated signaling and can be modulated by expression dosage as well as by the ECM provided.

Materials and Methods

Cell Culture and Transfections

Cells were cultivated using standard conditions (37°C, 5% CO_2, >95% humidity). For stable transfections, we used GeneJammer reagent (Stratagene, Waldbronn, Germany) following the manufacturer's instructions.

RWPE1 cells (ATCC no. CRL-11609; LGC Standards, Wesley, Germany) were cultivated in K-SFM-medium supplemented with 50 µg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor. Genetically engineered derivatives of the PC3 PCa cell line (ATCC no. CRL-1435) were cultivated in Dulbecco modified Eagle medium containing high glucose, GlutaMAX (Invitrogen, Darmstadt, Germany), 10% fetal calf serum (PAA, Pasching, Austria), and penicillin (100 U/ml)/streptomycin (100 µg/ml) (PAA). The cells had been genetically engineered as follows: A vector containing a Flp recombinase target (FRT) site was stably transfected into PC3 cells for a subsequent site-specific integration of expression vectors, giving rise to the PC3L1 clone. PC3L1 had been validated for single integration of the FRT site and for inducible and homogeneous target gene overexpression after integration of a fluorescent reporter. We generated the PC3L1-HPN and -VC isogenic sublines by integration of expression constructs containing the tet repressor gene (tetR) as well as a tetracycline-responsive CMV promoter controlling the hepsin gene (accession number DQ892119) in case of PC3L1-HPN. Flp-in T-REx293 cells (Invitrogen) were cultivated as recommended. We used pcDNA5-FRT-TO and a hepsin-containing derivative for the generation of the vector control (-VC) and hepsin (-HPN) transfectant, respectively.

Preparation of ECM

PC3L1-VC cells and RWPE1 cells were grown to confluence during 6 days and subsequently removed from cell culture dishes by incubation in 20 mM EDTA for 2 hours at 37°C. ECM-coated dishes were washed twice with phosphate-buffered saline (PBS) and subsequently used for cell culture experiments. For Western blot analysis, ECM was prepared using a solubilization buffer from the Cultured Cells Acellularization Kit (Sigma, Schnelldorf, Germany) according to the manufacturer's instructions. The preparations were concentrated by acetone precipitation, and pellets were solubilized in an adequate volume of buffer containing 20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 10% glycerin, 1% Triton X-100, and 20 µl/ml Protease Inhibitor Cocktail (Sigma).

Quantitative Real-time Polymerase Chain Reaction

Cells were grown for 48 hours after induction and harvested. RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using the SuperScript III enzyme (Invitrogen) and oligo-dT primer according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) of complementary DNA was performed on the LightCycler 480 System (Roche, Mannheim, Germany). ABsolute QPCR Mix (2×; Thermo Scientific, Epsom, UK) and TaqMan Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany: HPN, Hs01056332_m1; β-actin [ACTB], Hs99999903_m1) were used with the following cycling conditions: 95°C for 15 minutes, 45 cycles of 95°C for 10 seconds, and 60°C for 1 minute. The expression level of *ACTB* was used for normalization, hepsin gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method [20] and related to the vehicle-treated PC3L1-VC sample.

Western Blot Analysis

Cells were grown until 48 to 96 hours after induction and harvested using HEPES buffer described above, which was supplemented with PhosStop phosphatase inhibitor (Roche) when appropriate. Cell lysates and ECM preparations were quantified using BCA assay (Fisher Scientific, Ulm, Germany) according to the manufacturer's instructions, subjected to SDS-PAGE, and transferred to nitrocellulose membranes (GE Healthcare, Freiburg, Germany).

Proteins were specifically detected with ab43858 (hepsin; Abcam, Cambridge, United Kingdom), sc-94 (ERK1/2; Santa Cruz), sc-7383 (p-ERK; Santa Cruz, Heidelberg, Germany), 9272 (AKT; Cell Signaling, Frankfurt, Germany), 4051 ($pAKT^{Ser473}$; Cell Signaling), 4056 ($pAKT^{Thr308}$; Cell Signaling), ab55415 (LAMB3; Abcam), ab6276 (β-actin; Abcam), ab7291 (α-tubulin; Abcam), and secondary antibodies goat-antirabbit IgG–horseradish peroxidase sc-2030 (Santa Cruz), goat antimouse IgG–horseradish peroxidase sc-2031 (Santa Cruz) using ECL Western blotting reagent (GE Healthcare).

Viability Assay

Cells were assayed for viability using the Cell Titer Blue assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 3000 to 5000 cells/well were seeded in adhesive or antiadhesive (HydroCell) 96-well microplates (Nunc, Langenselbold, Germany). Seventy-two hours after dox treatment, one-fifth volume of Cell Titer Blue reagent was added and incubated for 3 hours at 37°C. Cells were analyzed for resorufin-mediated fluorescence (excitation, 544 nm; detection, 590 nm) in a microplate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany). Cell survival was calculated as percentage of the untreated control, respectively. At least three independent experiments were performed in triplicate.

Adhesion Assay

Cells were seeded to 24-well plates (100/mm^2), treated with dox or vehicle, and incubated for 72 hours. The plates were then washed three times with PBS without Ca^{2+}/Mg^{2+}. Adhesion was determined by neutral red staining of residual cells as described previously [21] because the commonly applied crystal violet staining protocol [22] was found to interfere with ECM substrates deposited before analysis [23]. Briefly, cell cultures were incubated in an aqueous solution of neutral red (0.012%) for 2 to 3 hours and washed with isotonic saline. Neutral red was extracted using a mixture of H_2O, ethanol, and acetic acid (1:1:0.02) during shaking at room temperature. Absorbance was determined at 570 nm using the Fluostar Omega microplate reader. Cell adhesion was calculated as percentage of the untreated control, respectively. At least three independent experiments were performed in quadruplicate.

Cell Cycle Analysis

Cells were seeded in six-well plates at a density of 100/mm^2 (all PC3L1-VC populations and PC3L1-HPN untreated) or 200/mm^2 (PC3L1-HPN dox treated). Six hours after seeding, cells were treated with different amounts of dox diluted in PBS or vehicle alone, respectively. Ninety-six hours after induction, all cells were collected, washed, and fixed using ice-cold methanol. After methanol removal, DNA content was stained with 7-amino-actinomycin (Sigma; 20 μg/ml) and analyzed via flow cytometry using a FACS Calibur (BD Biosciences, Heidelberg, Germany) and WinMDI software (Version 2.9, The Scripps Research Institute, http://facs.scripps.edu/software.html).

Chicken Chorioallantoic Membrane Assay

The chorioallantoic membrane (CAM) assay was performed as described previously [24], with minor modifications. PC3L1-VC and PC3L1-HPN cells were treated with dox (100 ng/ml) or vehicle (PBS) 96 hours before transplantation. At day 8 of breeding, 700,000 cells per egg (five eggs per group) were seeded on the CAM in a total volume of 20 μl (10 μl of growth factor–reduced Matrigel [no. 356231; BD Biosciences] and 10 μl of growth medium with *vs* without dox [final concentration, 100 ng/ml]). Fresh medium was supplied every 24 hours until day 11. At day 12, tumors and surrounding CAM were sampled and fixed in PBS-buffered 4% formaldehyde solution, paraffin embedded, and cut into 5-μm sections. The sections were either stained by hematoxylin-eosin or in immunohistochemistry using a Ki67-specific antibody (Dako, Hamburg, Germany). Apoptosis was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche) according to the manufacturer's instructions. Negative controls were performed by omitting the primary antibody. Absolute numbers and percentages of (positive) cells in 22 to 25 regions of interest (ROIs) per experimental group were determined. Exemplary evaluation by a second examiner yielded similar results.

Results

Establishment and Validation of Isogenic PC3 PCa Transfectants for Quantitative Analyses of Hepsin-Mediated Effects

A PC3-clone providing dox-inducible overexpression of hepsin (PC3L1-HPN) and a corresponding isogenic vector control clone (PC3L1-VC) were generated by site-specific integration of the respective expression construct in PC3L1. PC3L1 is a genetically engineered subclone of the PC3 cell line, which had been validated for strong, homogeneous, and inducible expression of target genes inserted into a single FRT site (not shown). In the absence of dox, both clones revealed minimal hepsin expression (Figure 1) and similar growth characteristics, as determined by neutral red assay (not shown). A dose-dependent increase of hepsin mRNA (Figure 1*A*) and protein (Figure 1*B*) was observed in PC3L1-HPN but not in PC3L1-VC after dox application 48 hours after induction. Real-time PCR revealed promoter leakiness leading to a certain degree of hepsin mRNA expression in PC3L1-HPN in the uninduced state (not shown), which, however, did not result in detectable expression of hepsin protein. Full induction of hepsin protein expression was achieved at 500 pg dox/ml growth medium (Figure 1*B*).

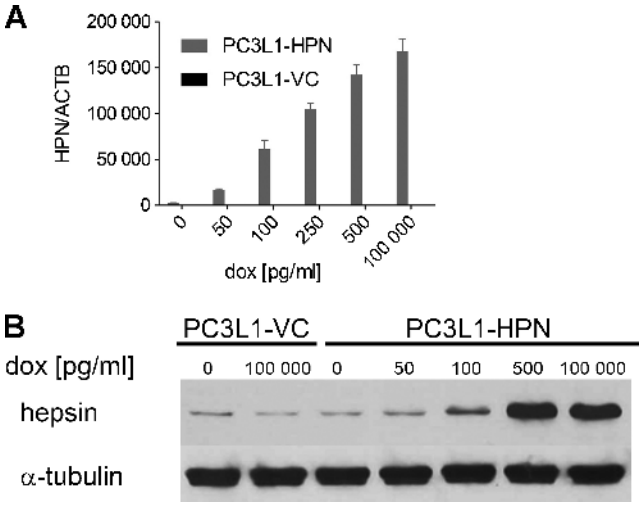

Figure 1. Hepsin expression in isogenic PC3 transfectants at the mRNA (A) and protein level (B) 48 hours after supplementation of growth medium with different amounts of dox. (A) An equivalent of 10 ng of RNA was subjected to reverse transcription and subsequently quantified in real-time PCR for HPN mRNA content using *ACTB* as a reference gene. HPN mRNA was found to correlate with the concentration of dox in PC3L1-HPN. Data are mean ± SD of three technical replicates. Calculation was performed using the $\Delta\Delta C_t$ method, with mean expression of PC3L1-VC (untreated) as a calibrator. (B) Thirty micrograms of total cell lysates was analyzed in Western blot analysis using α-tubulin as a loading control. Full expression of hepsin protein was achieved at 500 pg/ml.

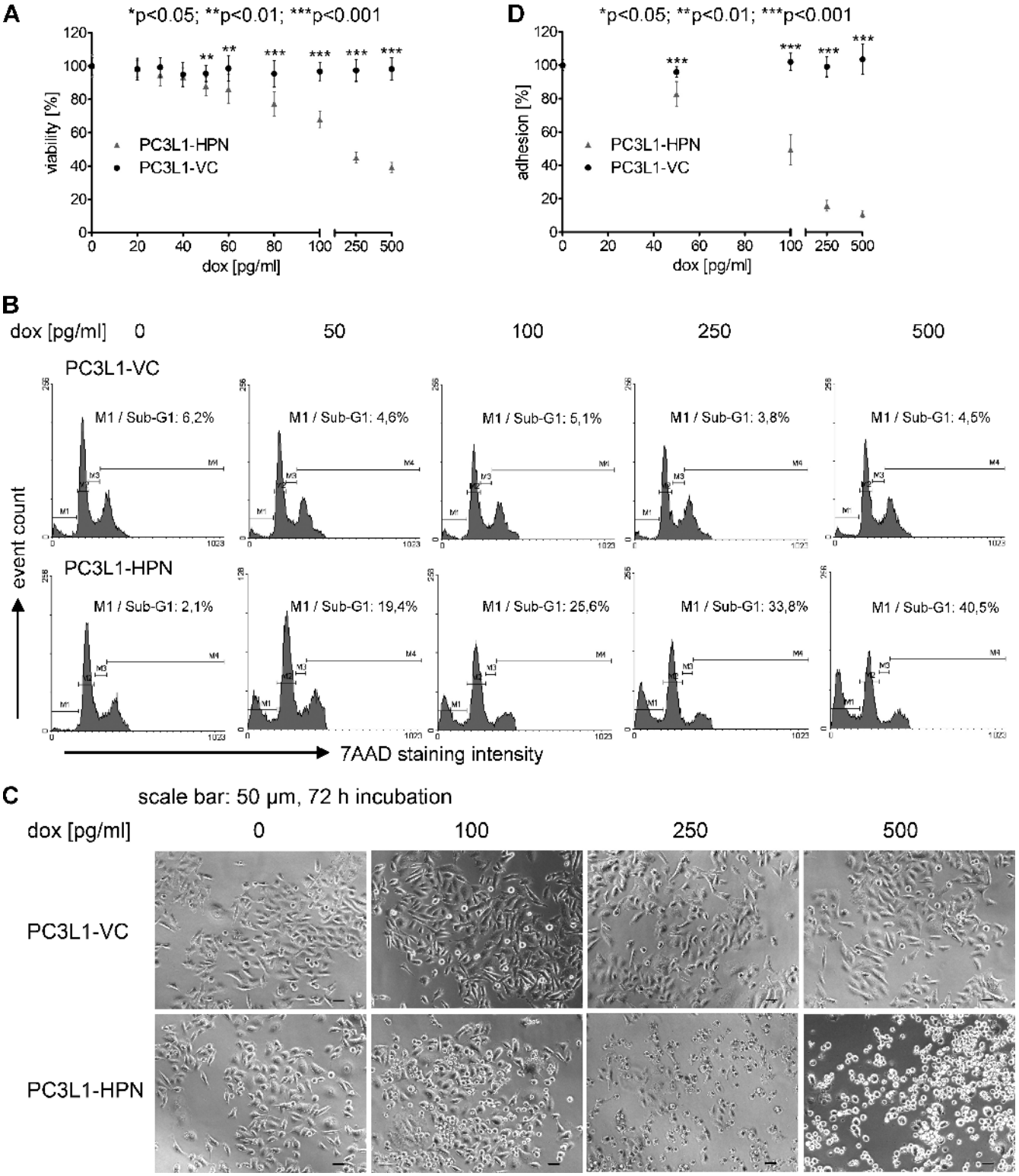

Figure 2. Viability and adhesion of PC3L1-HPN correlate inversely with hepsin expression levels 72 hours after induction: dose dependence of cellular effects. (A) Whereas viability of PC3L1-VC was unaffected by dox, viability of PC3L1-HPN was gradually suppressed with increasing concentrations of the inducer, becoming significant at 50 pg/ml (mean ± SD of four experiments performed in triplicate, two-tailed *t* test). (B) Cell cycle analysis revealed a dose-dependent increase of cell death in PC3L1-HPN 96 hours after hepsin induction, as indicated by elevated numbers of cells in sub-G_1. (C) Phase-contrast microscopy revealed a dose-dependent loss of adhesion in PC3L1-HPN but not in PC3L1-VC. Scale bar, 50 μm. (D) The number of adherent cells was significantly reduced in a dose-dependent manner in PC3L1-HPN but not in PC3L1-VC cells (72 hours after hepsin induction, median ± MAD of three experiments performed in quadruplicate, two-tailed *t* test).

Graded Overexpression of Hepsin Leads to Dose-Dependent Suppression of Viability, Loss of Adhesion, and Death of PC3 Cells

To quantitatively characterize effects of hepsin on PC3 cells, we compared PC3L1-HPN and PC3L1-VC 72 hours after treatment with different doses of dox. Whereas viability of PC3L1-VC cells was not affected by dox, we observed a dose-dependent loss of viability in PC3L1-HPN cells, which was significant at 50 pg/ml and above (Figure 2*A*). Cell cycle analysis revealed a dose-dependent increase of PC3L1-HPN cells in the sub-G_1 fraction, which is indicative for cell death in the presence of hepsin (Figure 2*B*). Loss of viability and cell death were associated with a markedly reduced adhesion of PC3L1-HPN cells after hepsin induction (as detected by phase-contrast microscopy; Figure 2*C*), which was quantified in adhesion assays using neutral red staining of cells remaining attached after rigorous washing (Figure 2*D*). A comparison of viability and adhesion data suggests that, at higher induction levels of hepsin, a percentage of nonadherent cells contributed to overall viability (Figure 2, *A* and *D*).

Anchorage-Independent Growth Is Not Affected by Overexpression of Hepsin in PC3 Cells

Under nonadhesive conditions, PC3 cells were previously reported to escape detachment-induced apoptosis (anoikis), displaying a rate of apoptotic cells in suspension culture of around 10% [25]. Srikantan et al. [15] found a reduction in anchorage-independent growth of PC3-hepsin transfectants in soft agar, when compared with vector control cells. To investigate whether cell adhesion is a prerequisite for the reduction of viability after hepsin expression, we performed assays in antiadhesive tissue culture plates. In contrast to the loss of viability in adhesive tissue culture plates, graded overexpression of hepsin did not affect viability during anchorage-independent growth (Figure 3).

Hepsin-Mediated Loss of Adhesion and Viability Correlates with Reduced Phosphorylation of AKT at Ser^{473}

PC3 cell growth and viability had been reported to critically depend on phosphatidylinositol-3-kinase (PI3K) signaling [26–28]. AKT, an effector of PI3K signaling, is phosphorylated at Thr^{308} by PDK1 in the presence of phosphatidylinositol (3,4,5)-trisphosphate, the generation of which is catalyzed by PI3K and antagonized by PTEN activity, respectively [29]. For full activation, AKT requires a second phosphorylation at Ser^{473}, which could be mediated by the mTORC2 complex [30] or, for example, by a mammalian target of rapamycin–independent protein complex containing rictor and integrin-linked kinase (ILK) [31].

We were interested in the phosphorylation state of AKT at Thr^{308} and Ser^{473}, and in the phosphorylation state of the extracellular signal–related kinases (ERK1/2), other important mediators of tumor cell viability, in response to hepsin expression in PC3 cells. Ninety-six hours after induction, cells were subjected to preparation of phosphoprotein and Western blot analysis. We found no alterations in the phosphorylation states of ERK1/2 and AKT at Thr^{308}, whereas AKT was almost completely dephosphorylated at Ser^{473} (Figure 4).

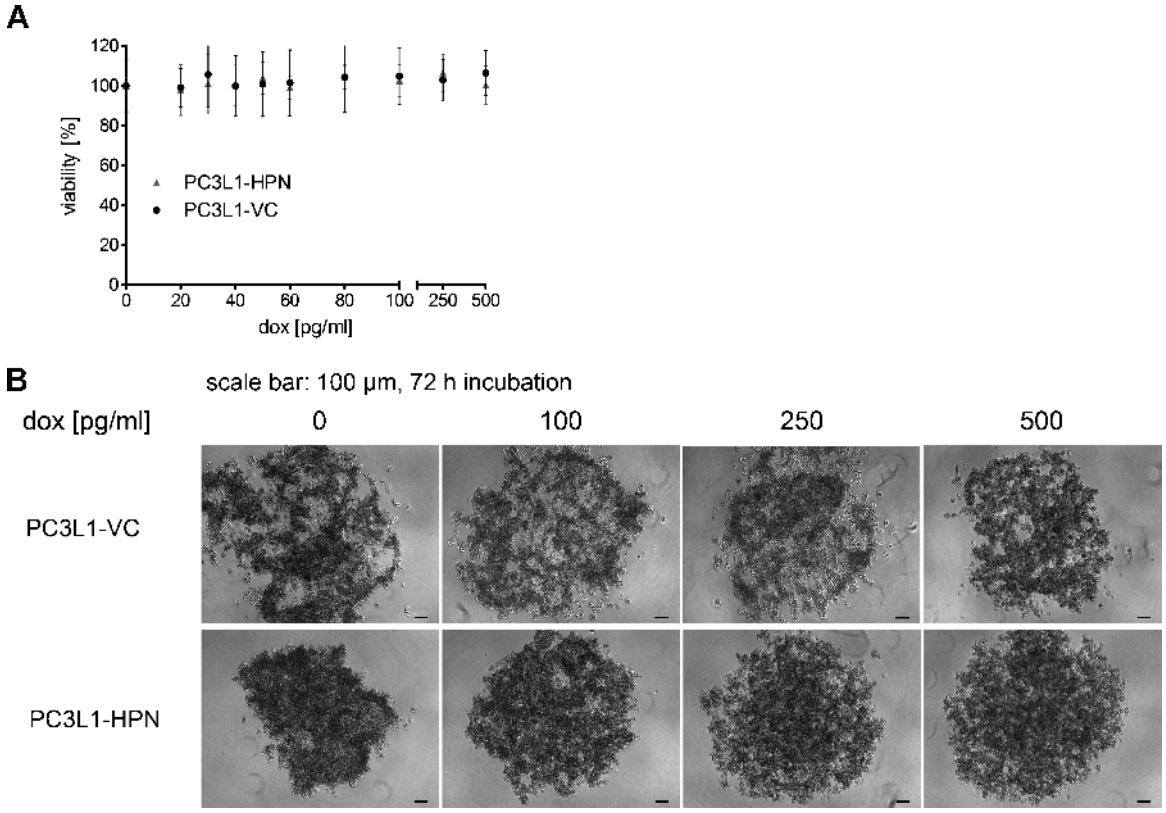

Figure 3. Hepsin expression levels do not affect anchorage-independent growth. (A) Seventy-two hours after treatment with different amounts of dox, viability was altered neither in PC3L1-HPN nor in PC3L1-VC (mean ± SD of three experiments performed in triplicate). (B) Phase-contrast microscopy revealed similar growth appearance in both cell lines, which was not affected by dox. Scale bar, 100 µm.

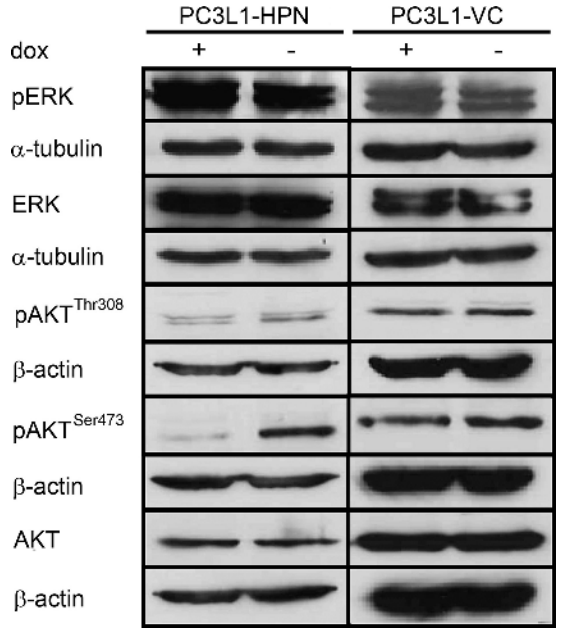

Figure 4. Overexpression of hepsin exclusively reduces phosphorylation of AKT at Ser^{473}, whereas phosphorylation of AKT at Thr^{308}, as well as phosphorylation of ERK, remained constant. Ninety-six hours after treatment with dox (100 ng/ml) or vehicle alone, PC3L1-HPN and PC3L1-VC cells were harvested and lysed. Fifty micrograms of phosphoprotein preparations was subjected to Western blot analysis for the investigation of phosphorylation states of ERK, $pAKT^{Thr308}$, $pAKT^{Ser473}$, as well as total expression of ERK and AKT. Loading controls were α-tubulin (for ERK) and β-actin (for AKT), respectively.

ECM of Prostate Cells, but Not of PCa Cells, Restores AKT Phosphorylation at Ser^{473} and Adhesion of Fully Induced PC3L1-HPN Cells

Hepsin revealed particularly strong expression in HG-PIN, which is suggested to be the precursor lesion of PCa [4]. In a mouse model, hepsin overexpression in the prostate correlated with loss of Ln-332 [13]. LAMB3, a subunit of Ln-332, was afterward identified as a hepsin substrate [11]. We therefore asked whether the ECM composition, particularly the presence of Ln-332, could affect viability and adhesion of hepsin-overexpressing PC3 cells.

We initially analyzed ECM deposited by nontumorigenic RWPE1 cells and PC3L1-VC cells for the presence of Ln-332. As expected, we found a strong signal at ~140 kDa representing LAMB3 in ECM of RWPE1- but not of PC3L1-VC cells (Figure W1). We used ECM of both cell lines deposited during 6 days of culture for comparative analyses of cell viability, adhesion, and AKT phosphorylation during forced expression of hepsin in PC3 cells. Seventy-two hours after induction of hepsin, we found elevated viability of cells grown on RWPE1-derived ECM, which was significant at dox concentrations of 60 pg/ml and above (Figure 5*A*). Microscopic examination and quantitative analysis revealed that adhesion of hepsin-overexpressing cells could be predominantly restored on RWPE1- but not by PC3L1-VC–derived ECM (Figure 5, *B* and *C*). AKT phosphorylation at Ser^{473} was largely maintained 48 hours after full induction of hepsin on RWPE1- but not on PC3L1-VC–derived ECM. Interestingly, Ser^{473} phosphorylation slightly decreased during elevated growth periods (72 and 96 hours) on RWPE1-derived ECM in the presence of hepsin, suggesting that the ECM-mediated sustainability is temporarily limited (Figure 5*D* and Figure W2).

Cleavage of LAMB3 during Growth of PC3 Cells on RWPE1-Derived Matrix

Using purified recombinant proteins, hepsin-mediated cleavage of Ln-332 had been demonstrated by Tripathi et al. [11]. To test whether the LAMB3 subunit of ECM-associated Ln-332 is specifically degraded in a dose-dependent manner by hepsin in our cell model, we compared RWPE1-deposited ECM after a 96-hour growth period of fully induced *versus* uninduced PC3L1-HPN and PC3L1-VC cells by Western blot analysis. Besides the full-length LAMB3 protein, we found cleavage fragments of LAMB3 in all ECM preparations (Figure 6). Thus, LAMB3 cleavage efficiency correlated neither with hepsin expression levels nor with the presence of dox in our model.

Hepsin Overexpression Reduces the Viability of Tumors but Does Not Prevent Invasive Growth of PC3 Cells in the CAM Xenograft Model

Srikantan et al. [15] used a Boyden Chamber assay to demonstrate that PC3 cells loose their invasive potential on forced expression of hepsin. Our previous results support the hypothesis that hepsin-mediated effects in PC3 cells are significantly influenced by the microenvironment. To test whether this holds true for invasive growth, we used the CAM *in vivo* xenograft model for invasion and angiogenesis [24] to compare untreated *versus* dox-treated PC3L1-HPN and PC3L1-VC cells. Tumors (n = 5 for each cell line) were well accepted and supported by the CAM, which is indicative for invasive growth and blood supply by the host tissue. Hepsin-overexpressing tumors revealed an altered appearance, which was characterized by a more nodular growth and extended areas of connective tissue when compared with more superficially growing tumors with high cell density from untreated PC3L1-HPN and PC3L1-VC (Figure 7*A*). Immunohistochemical analysis revealed a significantly reduced mitotic index (as determined by Ki67 staining, $P = 1.1e - 04$) and cell density ($P = 4.7e - 07$), as well as a slightly higher density of apoptotic cells (as determined by TUNEL staining, P = .0057) in dox-treated *versus* untreated PC3L1-HPN–derived tumors (Figure 7*B*). Surprisingly, we also detected a significantly reduced rate of apoptotic cells in PC3L1-VC–induced *versus* uninduced tumors (P = .0019), which demonstrates that daily administration of dox alone does not favor apoptosis in our model system. In summary, the CAM assay reflected cellular characteristics observed after hepsin overexpression *in vitro* but did not support the hypothesis that overexpression of hepsin suppresses invasive growth of PC3 cells.

Hepsin Suppresses Viability and Adhesion in Human Embryonic Kidney HEK293 Cells

To assess whether the phenotype of hepsin-overexpressing PC3 cells is prostate- (cancer-) specific or rather represents a tissue-independent general response, we constructed inducible hepsin transfectants (-HPN) and an isogenic vector control (-VC) in the Flp-In T-Rex 293 cell line, a commercially available HEK293 derivative allowing dox-inducible expression of a target gene. At 48 hours after dox administration, Flp-in T-Rex 293-HPN cells expressed hepsin, as determined by Western blot analysis (Figure 8*A*). Cell viability of Flp-In T-Rex 293-HPN was considerably lower than viability of Flp-In T-Rex 293-VC in the absence of dox (not shown). It was further reduced significantly during graded induction of hepsin, whereas we found no suppression of viability in the empty vector control cell line (Figure 8*B*). Similar to PC3L1-HPN, Flp-In T-Rex 293-HPN cells lost adhesion after

induction of hepsin (Figure 8*C*). Full expression of hepsin led to reduced AKT phosphorylation at Ser^{473} (Figure 8*D*). In summary, the effects observed during hepsin overexpression in HEK293 cells closely resembled those observed in PC3 cells, which suggests a rather general, cell-type–independent response.

Discussion

Several groups established recombinant cancer cell lines overexpressing hepsin, which were generated in a conventional manner by randomized insertion of one or several copies of the gene into the cell line's genome, followed by antibiotic selection and proof of hepsin

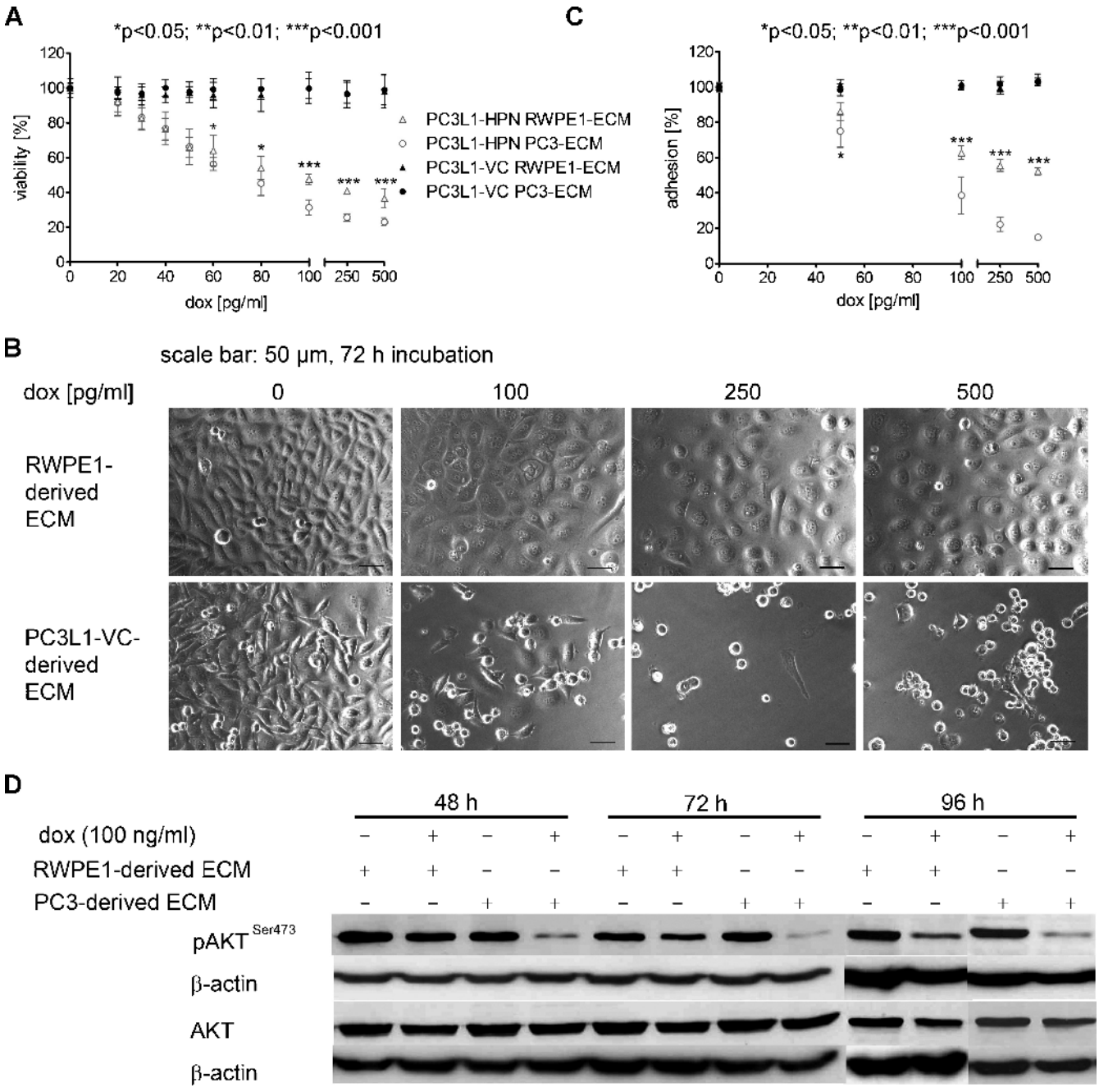

Figure 5. The ECM composition regulates viability, adhesion, and AKT phosphorylation of hepsin-overexpressing PC3 cells. (A) For PC3L1-HPN, growth on RWPE1-derived ECM confers significantly enhanced viability when compared with PC3-derived ECM at dox concentrations of 60 pg/ml and above (72 hours after hepsin induction, mean ± SD of three experiments performed in triplicate, two-tailed *t* test). (B) Phase-contrast microscopy revealed a dose-dependent loss of adhesion of PC3L1-HPN on PC3-derived matrix but not on RWPE1-derived matrix (72 hours after hepsin induction) Scale bar, 50 μm. (C) PC3L1-HPN adhered significantly stronger on RWPE1-derived matrix compared with PC3-derived matrix for all dox concentrations tested (72 hours after hepsin induction, median ± MAD of three experiments performed in quadruplicate, two-tailed *t* test). (D) RWPE1-derived matrix restored AKT-phosphorylation at Ser^{473} in hepsin-overexpressing PC3L1 cells. At 48, 72, and 96 hours after treatment with dox or vehicle alone, cells were harvested and lysed. Fifty micrograms of phosphoprotein preparations was subjected to Western Blot analysis for the investigation of AKT-phosphorylation at Ser^{473}, as well as total AKT protein. β-Actin was used as a loading control.

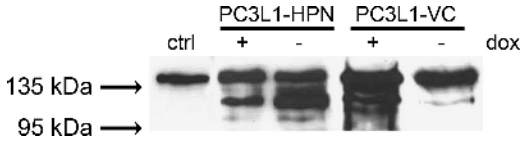

Figure 6. Analysis of LAMB3-cleavage in ECM preparations. RWPE1-derived ECM was deposited during 6 days of growth to confluence and subsequently used as a substrate for 96 hours of growth of PC3L1-HPN and PC3L1-VC cells (with 100 ng/ml *vs* without dox) to assess the impact of hepsin expression levels on LAMB3 cleavage. After the incubation period, matrix was prepared, and 5 μg of each preparation was subjected to Western blot analysis, together with 100 ng of rat Ln-332 serving as a positive control (ctrl). The presence of the ~140-kDa band (corresponding to uncleaved LAMB3) as well as additional smaller bands suggested a partial cleavage of LAMB3 during growth of all PC3 cell populations on RWPE1-derived ECM, irrespective of hepsin expression levels or the presence of dox.

expression [9,15–18]. Some groups reported a suppression of viability and other tumor-relevant functions during growth *in vitro* [15–17], whereas others found no hepsin-associated adverse effects. The inconsistency of these data suggested a dosage-dependent effect because the expression levels in the recombinant cell lines were not comparable and potential viability-suppressing effects of hepsin counteracted the generation of strongly expressing recombinant clones. To circumvent this drawback, we constructed cell lines with strong, homogeneous, and inducible expression of hepsin, as well as isogenic vector controls, for a thorough analysis of quantitative effects of hepsin overexpression in PC3 metastatic PCa as well as HEK293 nontumorigenic human embryonic kidney cells. We found that hepsin overexpression caused a dose-dependent loss of adhesion and viability in both cell types during growth in conventional tissue culture plates, which most probably interfered with the selection of strongly expressing recombinant clones in previous approaches. Loss of adhesion and

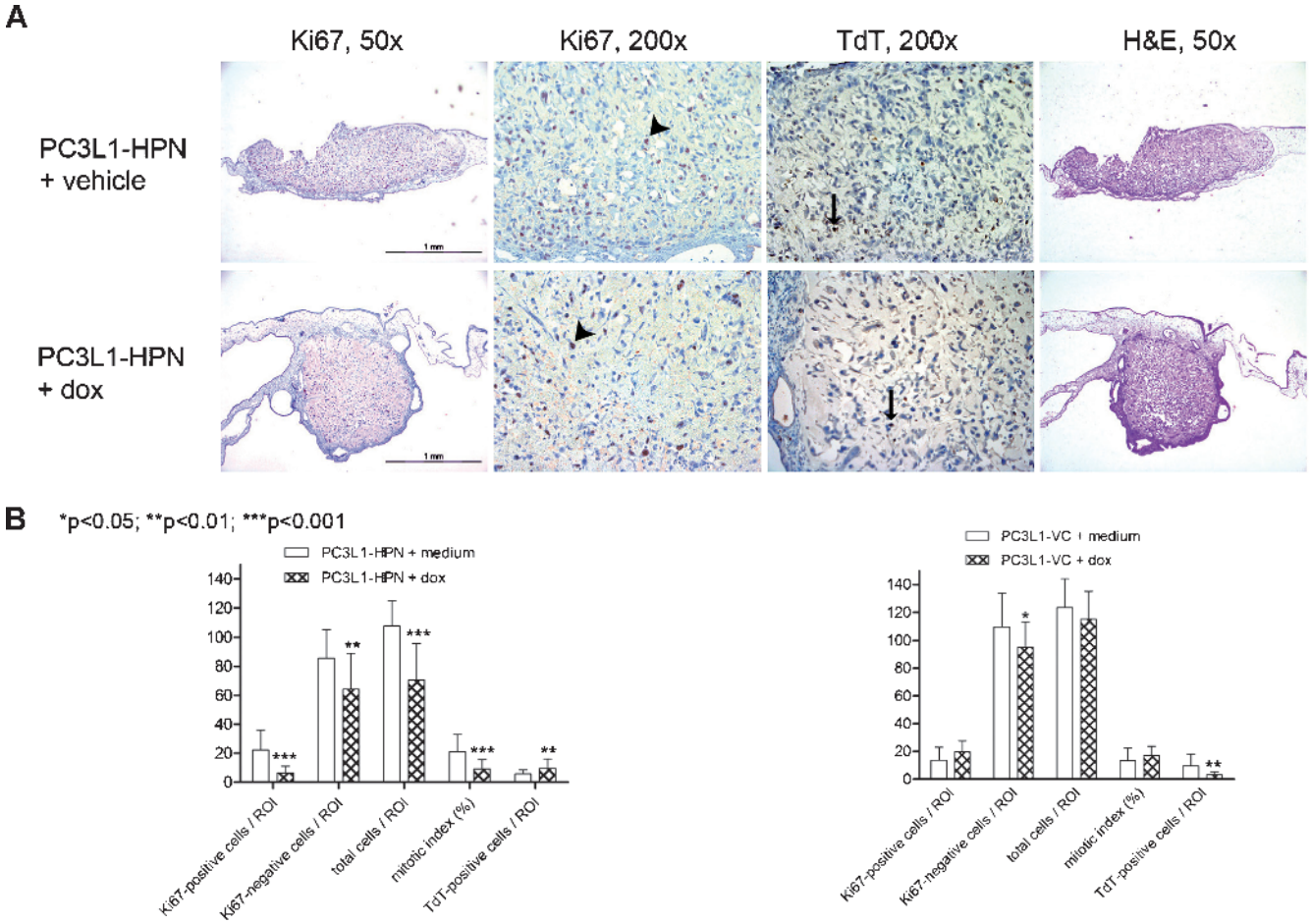

Figure 7. Full induction of hepsin expression reduces the mitotic index and elevates the density of apoptotic cells in tumors but does not prevent invasive growth of PC3L1-HPN on the CAM. PC3L1-HPN and PC3L1-VC cells were grown for 96 hours in conventional cell culture dishes with *versus* without dox (100 ng/ml) to ascertain full expression of hepsin before transplantation. At day 8 of breeding, cells were seeded on the CAM (700,000 cells/egg) and further treated daily with dox or vehicle. At day 12 of breeding, transplants were fixated and subjected to histologic examination. (A) Representative microscopic images of tumors derived from PC3L1-HPN treated with vehicle alone (upper panel) or dox (lower panel). Hepsin-overexpressing tumors (lower panel) revealed extensive penetration of the CAM, along with a high content of connective tissue and low cell density within tumor nodules. Ki67-positive nuclei are marked by arrowheads; TdT-positive nuclei are marked by arrows. (B) ROI analyses of tumors (n = 22-25 per experimental group) revealed a significant reduction in Ki67-based mitotic index and total number of cells, as well as a significantly higher absolute number of TdT-positive cells/ROI in hepsin-overexpressing tumors.

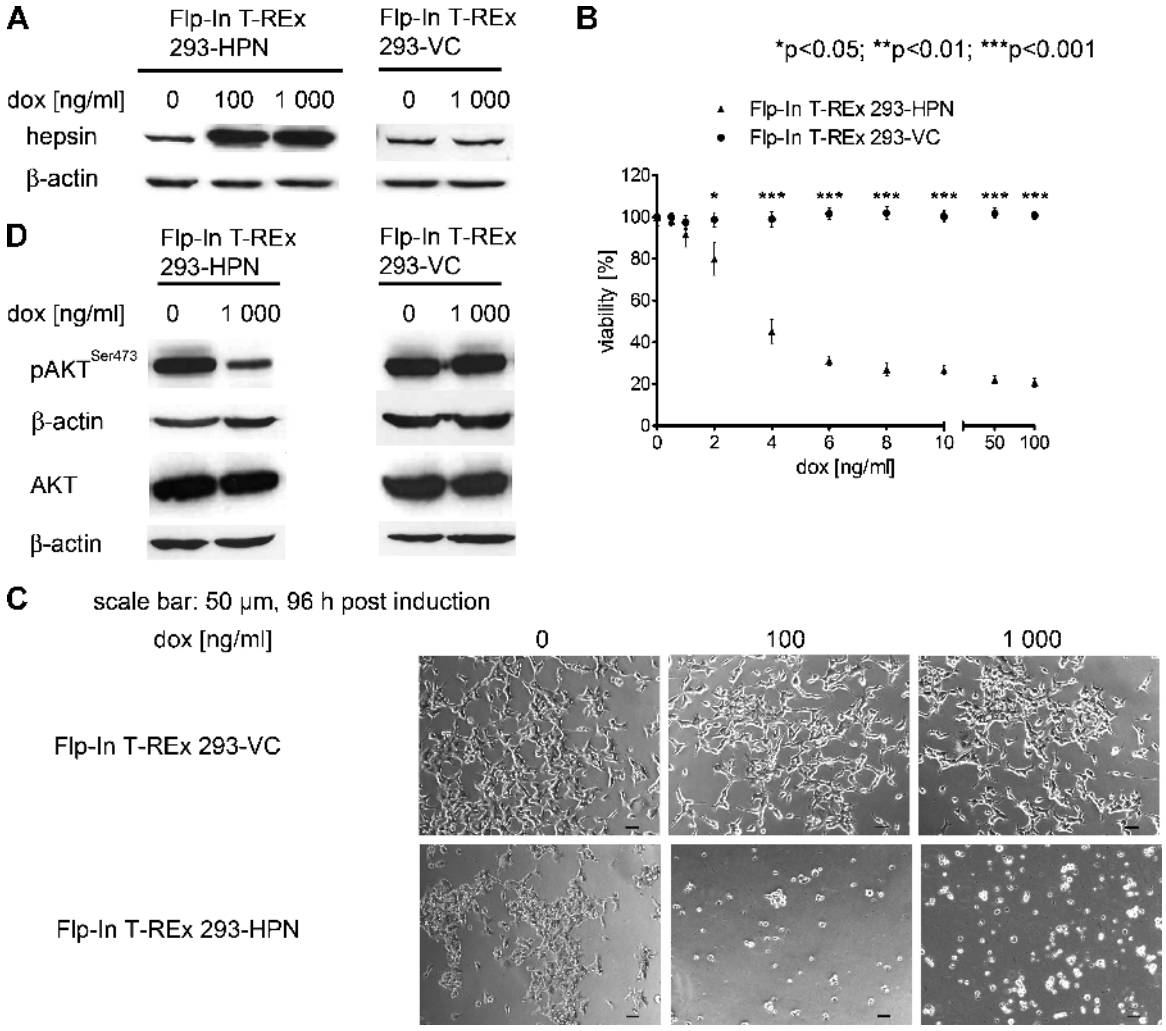

Figure 8. Hepsin overexpression suppresses viability, adhesion, and phosphorylation of AKT at Ser^{473} in human embryonic kidney 293 cells. (A) Flp-in T-Rex 293-HPN and -VC cells were grown in the presence of the indicated amounts of dox for 96 hours. Thirty micrograms of total cell lysates was analyzed in Western blot analysis using β-actin as a loading control. Flp-in T-Rex 293-HPN revealed inducible overexpression of hepsin, which was saturated at 100 ng/ml of dox. (B) Whereas viability of Flp-in T-Rex 293-VC was unaffected by dox, viability of Flp-in T-Rex 293-HPN was gradually suppressed with increasing concentrations of the inducer, becoming significant at 1 ng/ml (mean ± SD of three experiments performed in triplicate, two-tailed *t* test). (C) On dox induction, phase-contrast microscopy revealed a loss of adhesion in Flp-in T-Rex 293-HPN but not in Flp-in T-Rex 293-VC (96 hours after treatment). Scale bar, 50 µm. (D) Ninety-six hours after treatment with dox or vehicle alone, Flp-In T-Rex 293-HPN and Flp-In T-Rex 293-VC cells were harvested and lysed. Fifty micrograms of phosphoprotein preparations was subjected to Western blot analysis for the investigation of AKT phosphorylation at Ser^{473} as well as total AKT protein. β-Actin was used as a loading control.

viability were associated with a loss of AKT phosphorylation at Ser^{473}, which may at least explain the phenotype observed in PC3 PCa cells, as these cells critically depend on PI3K signaling [26–28]. In PC3 cells, AKT phosphorylation and cell adhesion could be specifically restored by growth on ECM deposited by nontumorigenic RWPE1 prostate epithelial cells.

The hepsin paradox as introduced by Valery Vasioukhin [19] describes the discrepancy between elevated levels of hepsin expression in PCa on the one hand, and the growth suppressive effects of hepsin overexpression in metastatic cancer cell lines on the other. Klezovitch et al. [13] established transgenic mice, which revealed constitutive prostate-specific overexpression of hepsin and demonstrated a marked disorganization of the prostate ECM. The latter was characterized by loss of Ln-332, diffuse organization of hemidesmosomes, and a perturbed structure of the borderline separating the stromal and epithelial compartment of the organ. Furthermore, when crossed with the LPB-tag mouse, a model for HG-PIN, double-transgenic mice developed disruption of the epithelial structure in the prostate, adenocarcinoma, and metastatic cancer. However, compared with LBP-tag mice, the double-transgenic animals revealed no differences with regard to Ki67-based mitotic index in the prostate. TUNEL staining revealed a fourfold increase in apoptosis in double-transgenic animals. The authors suggested that timing, microenvironment, and the level of hepsin expression may be critical for the correct course of proteolytic events during PC progression.

Basically, our data support the suggestions of Klezovitch et al. In contrast to results of Srikantan et al. [15], who described a loss of invasive growth of hepsin-overexpressing PC3 metastatic PCa cells *in vitro*, we detected invasive growth of hepsin-overexpressing PC3 cells when transplanted on the CAM, although this was accompanied

by reduced cell growth and density, as well as elevated apoptosis in the xenografts. The conflicting data could originate from the different microenvironments provided in the respective model systems. Most available data, including our own, indicate that irrespective of the cellular background, excessive overexpression of hepsin seems incompatible with adherent cell growth, unless cells receive a specific (extracellular) signal for adhesion and survival. We suggest that this signal is provided by noncancerous ECM, for example, from prostate epithelial cells. ECM remodeling during prostate carcinogenesis could then execute a selective pressure on hepsin-overexpressing cells through loss of this specific survival signal, resulting in reduced activity of AKT. Our hypothesis explains the protein expression patterns observed by Dhanasekaran et al. [4] in immunohistochemistry, who found that a strong overexpression of hepsin is mainly restricted to HG-PIN lesions (i.e., in the presence of "normal" prostate matrix), whereas advanced stages of PCa revealed predominantly weak or moderate staining intensities. Also, it implies that a thorough functional characterization of the role of hepsin in the different stages of PCa requires both an authentic genetic/posttranslational regulation and microenvironment.

The data presented here suggest that elevated levels of hepsin interfere with a pathway, which regulates adhesion and results in AKT phosphorylation. Persad et al. [32] demonstrated a dependence of AKT phosphorylation at Ser^{473} on the activity of ILK in PTEN-deficient PCa cells, including PC3. This mechanism was further specified by the identification of a molecular cooperation between ILK and rictor, which was required for both regulation of AKT phosphorylation and cancer cell survival [31]. A major function of ILK is the transmission of extracellular signals through focal adhesion complexes [33]. It is thus conceivable that the presence and/or the proteolytic activity of hepsin directly affect this pathway through an interaction with integrins or ECM ligands on the cell surface. Interestingly, interrogation of expression data revealed a mutually exclusive pattern of HPN and ILK expression in PCa, which is indicative for a functional role of ILK in hepsin-mediated cellular effects [3].

Ln-332 represents a candidate ECM substrate for hepsin-mediated proteolysis [11], which is downregulated in advanced prostate carcinoma [12] and was found in RWPE1- but not in PC3-derived matrix. Ln-332 provides adhesion and survival signals via focal adhesion complexes and AKT signaling [34] and is an integral component of adhesion contacts in epithelial cells [35]. Ln-332 as well as several of its cleavage products was found to modulate relevant properties of cancer cells [36,37]. Therefore, we speculated that the LAMB3 subunit could be a specific substrate of hepsin in our model system, the cleavage of which could be required for the ECM-associated cellular effects observed. Whereas we demonstrated a partial LAMB3 cleavage during growth of PC3 cells on RWPE1-deposited ECM, we found neither a specificity for hepsin-overexpressing cells nor a correlation with hepsin expression levels for this reaction. Instead, LAMB3 was also cleaved efficiently by uninduced PC3L1-HPN and PC3L1-VC, probably by matriptase, which was reported to be expressed in PC3 [38] and recently found to cleave LAMB3 *in vitro* [39]. Thus, although Ln-332 presence and/or LAMB3 cleavage may be critical for AKT phosphorylation at Ser^{473}, adhesion, and viability of hepsin-overexpressing PC3 cells on RWPE1-ECM, we could not provide evidence for any relationship between LAMB3 cleavage and the effects observed during hepsin overexpression in our model system.

While we established a relation between hepsin levels and adhesion-dependent growth of PCa cells, detailed mechanisms for the effects observed remain elusive. Surprisingly, application of the broad spectrum serine protease inhibitor 4-(2-aminoethyl)-benzensulfonylfluoride (AEBSF) did not restore viability and adhesion of PC3L1-HPN after hepsin induction but instead caused a significant loss of viability even in PC3L1-VC (Figure W3), which suggests protease-independent growth- and adhesion-suppressive effects in the presence of hepsin. However, we were not able to determine the degree of hepsin inhibition in our experiment, which was a function of (i) hepsin expression dynamics during induction, (ii) expression levels and dissociation constants of other serine proteases affected by AEBSF, and (iii) stability of AEBSF under cell culture conditions. Future studies using a more specific, stable, and nontoxic hepsin inhibitor and/or functional analysis of a protease-dead mutant in the (isogenic) PC3L1 background will help to determine whether the observed effects depend on the proteolytic activity of the enzyme.

Understanding the exact mechanism of hepsin-mediated growth suppression and cell death may reveal new therapeutic approaches for PCa, as the presence and/or activity of the enzyme suppresses PI3K/AKT signaling when placed in an adequate microenvironment. A comprehensive knowledge of hepsin interactors or substrates at the cell surface, as well as a delineation of the ubiquitous signaling axis, which is affected by hepsin may offer the opportunity for a synthetic lethal therapeutic intervention basing on the modulation of survival signaling in hepsin-overexpressing tumors.

Acknowledgments

The authors thank Felicitas Genze and Andrea Schäfer for expert technical assistance, Jan Mollenhauer for assignment of cells and vectors, and Rainer Küfer for expert advice in the initialization phase of the project.

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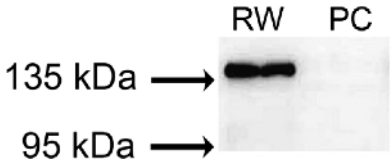

Figure W1. Western blot analysis of 5 μg of ECM deposited by RWPE1 and PC3L1-VC cells during 6 days of growth to confluence revealed the presence of a ~140-kDa band corresponding to LAMB3 in RWPE1- (RW) but not PC3L1-VC– (PC) derived ECM.

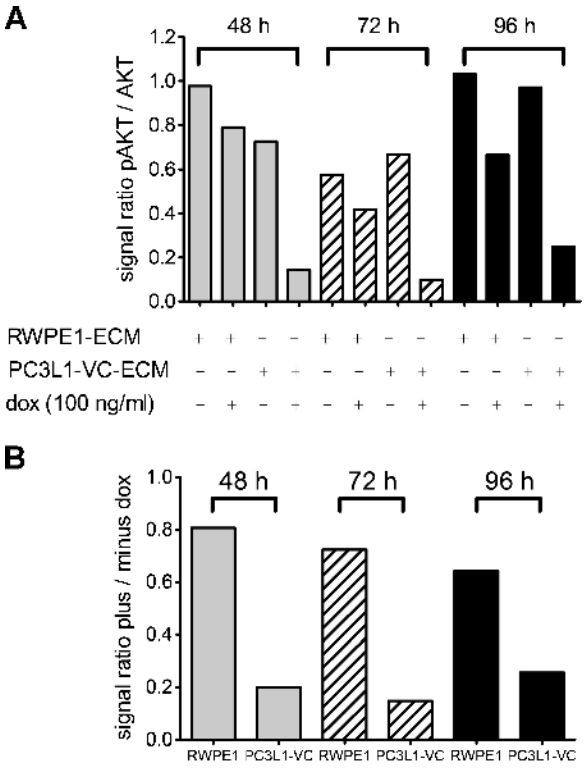

Figure W2. Semiquantitative analysis of changes in AKT phosphorylation at Ser^{473} in PC3L1-HPN after full hepsin induction on different ECM substrates over time. An image file of the Western blot depicted in Figure 5*D* was subjected to band intensity analysis using GeneTools Software (Syngene, Cambridge, United Kingdom). For each blot, a ROI was fitted to band size, multiplied and used for densitometry of individual bands. Values were background-corrected using an automatic algorithm implemented in the software package. (A) For the individual cell populations, we initially calculated signal intensity ratios of pAKT^{Ser473} *versus* AKT, which reflect relative AKT phosphorylation states at Ser^{473} in the respective samples. (B) To determine alterations in phosphorylation states after hepsin induction on the different ECM substrates, we calculated ratios of Ser^{473} phosphorylation in the induced *versus* uninduced state.

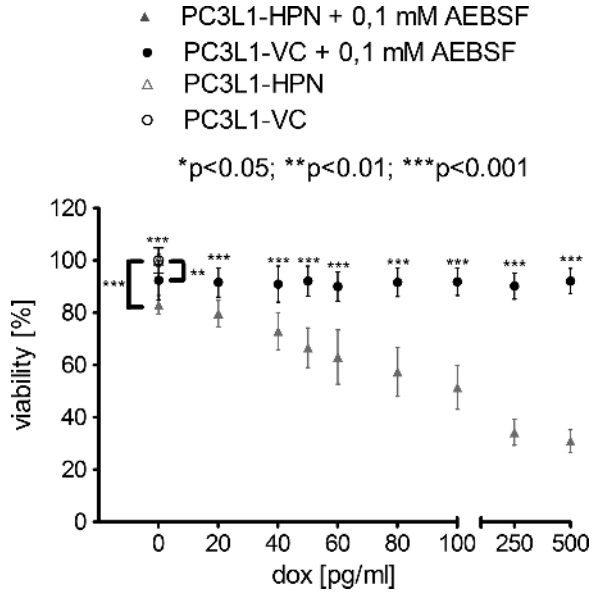

Figure W3. As previously demonstrated by others, the broad-spectrum serine protease inhibitor AEBSF suppresses proteolytic activity in cell culture at a concentration of 100 μM [1]. We used AEBSF at 100 μM to examine whether inhibition of gross serine protease activity abolishes the effects exerted by the graded overexpression of hepsin in PC3 cells. A total of 5000 cells/well were seeded in 96-well plates and treated with AEBSF and various concentrations of dox as indicated. AEBSF did not restore viability after graded overexpression of hepsin but instead already caused a significant loss of viability in PC3L1-HPN ($P = 3.0e - 12$) and PC3L1-VC ($P = .007$) cell populations, which were not induced by dox. Viability was measured 72 hours after drug treatment; cell survival was calculated as percentages of the untreated controls, respectively (mean ± SD of four experiments performed in triplicate, two-tailed *t* test).

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