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14. ABSTRACT
Enhanced heparanase expression correlates with metastatic potential, tumor vascularity and reduced postoperative survival of cancer patients. These observations, the anti-cancerous effect of heparanase gene silencing (ribozyme, siRNA) and of heparanase-inhibiting oligosaccharides, peptides and antibodies, as well as the unexpected identification of a single functional heparanase, suggest that the enzyme is a promising target for anti-cancer drug development. Our studies focused on the regulation of heparanase gene expression (i.e., promoter methylation, action of sex steroids, p53) and effect of augmented levels the enzyme on malignant behavior of prostate cancer cells. We designed effective inhibitory strategies, based on recently created chemical and molecular tools (chemically modified heparin species, siRNA-expressing vector), as well as on better understanding of biochemical aspects of heparanase proenzyme activations (inhibitory peptide approach), toward future development of effective anti-cancer therapeutic modalities.

15. SUBJECT TERMS
Heparanase, metastasis, siRNA, promoter regulation, gene silencing

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INTRODUCTION

Heparanase is an endo-β-D-glucuronidase involved in cleavage of heparan sulfate (HS) and hence participates in ECM degradation and remodeling (1, 2). Heparanase activity has been traditionally correlated with the metastatic potential of tumor-derived cell types. Similarly, heparanase has been shown to facilitate cell invasion associated with autoimmunity, inflammation, and angiogenesis. Heparanase is preferentially expressed in human tumors and its over-expression in tumor cells confers an invasive phenotype in experimental animals (1, 2). Heparanase also releases angiogenic factors from the ECM and thereby induces an angiogenic response *in vivo*. A link between heparanase expression and prostate tumorigenesis was clearly indicated by our previous studies. Two lines of evidences supported this link: **i)** correlation between the levels of heparanase and human prostate carcinoma progression, and **ii)** involvement of known mediators of prostate tumor growth (i.e., androgen, p53) in transcriptional activity of the heparanase promoter. The series of experiments described in the present report provide direct evidence for causal involvement of the heparanase enzyme in prostate tumor growth and metastasis. This report describes experimental systems (i.e., highly metastatic heparanase over-expressing prostate carcinoma cells) and molecular tools (i.e., electroporation- and lentivirus-assisted delivery of heparanase-siRNA expressing vectors), developed in our laboratory during the previous year, that will allow to proceed to the culminating point of the heparanase/prostate cancer project, namely recruitment of the most effective heparanase-inhibiting technologies toward development of comprehensive anti-heparanase based therapeutic modalities for prostate cancer.

BODY

Task # I. Involvement of heparanase, expressed by the tumor and stromal compartments, in PC progression. As stated in our previous report (submitted Feb. 2005), homozygous TRAMP mice were cross-mated with *hpa*-transgenic mice, however, only few double homozygous male mice were identified and all dyed 3-4 weeks after birth, much earlier than the control TRAMP mice. Because of this and an almost complete loss of breeding capacity in our *hpa*-tg colony, the system did not allow consisted measurements of tumor size, vascularity and heparanase expression pattern, as originally proposed. We therefore decided to abandon the TRAMP/ *hpa*-tg system and to adopt a different experimental system (PC bone to lung metastasis).

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i). PC3 cells express relatively low levels of the heparanase enzyme (Fig. 1) and, upon intraosseous injection in mice, do not metastasize to lung or any other distant organs (3). We determined whether over-expression of heparanase would confer a more malignant and metastatic behavior on these cells. For this purpose, PC3 cells were stably transfected with empty pCDNA3 expressing vector (PC3-Vo), vector encoding for human heparanase (PC3-hpa), or for a secreted form of heparanase (PC3-Sp). The cells were first tested for heparanase activiy *in vitro* and, as expected, increased enzymatic activity was detected in lysates prepared from both PC3-hpa and PC3-Sp cells, as compared to PC3-Vo cells (Fig. 1A). Heparanase activity was also easily detected in medium conditioned by PC3-Sp, but not PC3-hpa cells (Fig. 1B), confirming secretion of heparanase by PC3-Sp. Secretion and cell surface localization of heparanase in PC3-Sp cells was also demonstrated by their increased adhesion to endothelial cells, vs. the adhesive ability of PC3-hpa cells, that was similar to that of PC3-Vo cells (Fig. 2A,B). Interestingly, expression of either the secreted (PC3-Sp cells) or non-secreted (PC3-hpa cells) forms of heparanase conferred the same extent of increased ability to invade through Matrigel (a reconstituted basement membrane preparation) (Fig. 2C, D). These results emphasize the importance of extracellular localization of heparanase for a known non-enzymatic activity of this protein (i.e., adhesion), which is regarded as an integral part of the overall pro-tumorigenic action of heparanase in tumor development and progression.

ii). To evaluate the effect of heparanase on intraosseous prostate tumor growth, PC3-Vo, PC3-hpa and PC3-Sp cells were injected into the marrow cavity of the tibia of male SCID mice. Tumor growth was monitored for 6.5 weeks. PC3-Sp cells and, to the less extent, PC3-hpa cells formed well-established osteolytic tumors, while small or no tumors were detected in mice injected with control PC3-Vo cells (Fig. 3A). Large bone tumors developed already 20 days after injection of PC3-Sp cells, as compared to PC3-Vo and PC3-hpa cells that formed tumors of detectable size only by day 32 of the experiment. The statistically significant difference between PC3-Sp and either PC3-Vo or PC3-hpa bone tumors persisted throughout the duration of the experiment (Fig. 3A). Histological examination of PC3-Sp bone tumors on day 23 post injection revealed that the bone tissue was almost completely replaced by a tumor that consisted of prostate carcinoma cells, occasionally mixed with bone tissue remnants (Fig. 3B, right). In contrast, in bones injected with either PC3-Vo or PC3-hpa cells, tumor masses of limited volume were found within the bone marrow, but the size of the bone remained unchanged, no bone

destruction was noted and proper organization of the bone tissue was maintained (Fig. 3 B left). To ensure that the observed effects were not a result of a difference in the proliferative capacity of PC3-Vo, PC3-hpa, and PC3-Sp cells, we compared their growth rate and found that the 3 cell types exhibit the same rate of proliferation *in vitro* (not shown). These results reveal a novel role for heparanase in skeletal complications that accompany prostate cancer. It appears that prostate tumors having elevated levels of secreted heparanase promote bone remodeling and resorption. This is likely to enrich the bone microenvironment with growth promoting factors that may nurture the growth of metastatic prostate tumor cells. Thus, therapies designed to block heparanase function may disrupt the early progression of bone-homing tumors.

iii). To demonstrate the effect of heparanase on spontaneous metastatic dissemination of prostate carcinoma, we injected PC3-Vo, PC3-hpa, or PC3-Sp cells, genetically engineered to express the luciferase gene (LUC), into the tibia of SCID mice, and used the whole-body bioluminescent LUC imaging to monitor prostate tumor progression *in vivo* (Fig. 4C). On day 44 post injection, lung metastases became evident in both PC3-hpa- and PC3-Sp-, but not PC3-Vo-injected mice (Fig. 4C bottom). The mice in all three groups were euthanized on day 46, their lungs were stained in Bouin's solution and evaluated for the number of surface metastatic colonies. As demonstrated in figures 4 A&B, the lungs of mice injected with PC3-hpa or PC3-Sp cells were massively infiltrated with prostate carcinoma cells vs. only a few nodules detected in the lungs of PC3-Vo injected mice. To our knowledge, these data represent the first report on *in vivo* spontaneous PC3 prostate carcinoma pulmonary metastasis (3). Altogether our results clearly demonstrate a causal involvement of heparanase in prostate carcinoma progression and metastasis and emphasize the importance of extracellular localization of the enzyme in enhancement of primary tumor growth. Whole-body imaging of LUC-expressing PC3-hpa and PC3-Sp primary tumors and metastases represents a new and attractive animal model for easy screening of effective heparanase-inhibiting anti-metastatic compounds, as a function of time and without having to scarify the mice and apply histological examination at different time points.

Task # II. Regulation of heparanase promoter activity

As outlined in our previous annual report, we found that wild type (wt) p53 is capable of direct binding to heparanase promoter and inhibiting its activity in several cell types, including prostate

carcinoma. Here we report that mutant p53 variants failed to exert such an inhibitory effect (Fig. 5). Moreover, p53-H175R mutant even activated heparanase promoter activity(Fig. 5).

In an attempt to investigate molecular mechanisms responsible for repression of heparanase transcription by p53, we tested whether trichostatin A (TSA), a potent and specific inhibitor of histone deacetylases (HDAC) (4), is able to prevent this repression (association between transcriptional repression by p53 and the recruitment of HDAC to the regulatory sequences of target genes, has been previously reported (5). Trichostatin A abolished the inhibitory effect of wt p53 on heparanase expression (Fig. 6), suggesting the involvement of histone deacetylation in negative regulation of the heparanase promoter. Altogether, our results indicate that the heparanase gene is regulated by p53 under normal conditions, while mutational inactivation of p53 during cancer development leads to induction of heparanase expression, providing a possible explanation for the frequent increase of heparanase levels observed in the course of prostate and other types of tumorigenesis. Our results implicate p53 in regulating heparanase expression not only in carcinoma cells *per se*, but also in fibroblasts. A marked stimulation of heparanase gene expression and enzymatic activity has been demonstrated in mouse embryonic fibroblasts derived from p53$^{-/-}$ mice. Moreover, transcriptional (siRNA) or functional (GSE 56) inactivation of p53 in telomerase-immortalized WI-38 human fibroblasts led to increased heparanase expression (Fig. 7 and [Baraz et al., Oncogene 2005, in press]).

The critical importance in carcinogenesis of stromal elements (e.g., carcinoma-associated fibroblasts, CAFs) and their secreted factors is increasingly documented (6). Among other mechanisms, it was suggested that stromal cellular elements may contribute to the malignant potential of the tumor by producing heparanase (7, 8). While normal fibroblasts lack detectable heparanase activity (9), and our unpublished observations), elevated levels of the heparanase protein were detected in fibroblasts associated with deeply invading carcinoma (8). Recently, it becomes increasingly clear that inactivating mutations in p53 are responsible for the prostate tumor-supporting activities exerted by CAFs (10-12). Our results propose a molecular pathway through which CAFs, due to the loss of functional p53, may become an independent source of heparanase in the tumor vicinity, thus contributing to prostate carcinoma progression.

Task # IV: Inhibitory strategies

i) EGFR-heparanase interaction: Numerous studies have shown experimental evidence of a role of the EGFR family, particularly ErbB-2, in the development of prostate cancer and suggested that EGFR-targeting could be of therapeutic relevance in prostate cancer. Studying effects of EGFR tyrosine kinase inhibitors on heparanase activity in human PC lines, as proposed in our original application, we noticed that, unexpectedly, over expression of endogenous heparanase (Fig. 8), or addition of the exogenous enzyme (Fig. 9) to the cells induces EGFR phosphorylation. In an attempt to characterize a molecular mechanism of such an unexpected activity of heparanase protein, we found that enhanced EGFR phosphorylation upon heparanase over expression is Src-dependent (Fig. 10). Thus, some of the heparanase-inhibiting strategies described below (particularly, those interfering with both enzymatic and non-enzymatic activities of heparanase) may exert additional beneficial impact in PC treatment, acting to diminish EGFR effects.

ii). Low molecular weight glycol-split species of heparins: Analysis of a series of modified species of heparin led to the identification of several nonanticoagulant compounds that efficiently inhibit heparanase enzymatic activity while lacking additional, undesired activities of heparin (e.g., anticoagulation, release and activation of heparin-binding pro-angiogenic factors). A promising heparanase inhibitors are glycol-split heparin (compound ST1514) or N-acetylated glycol-split heparin (compound G-4000), identified as most effective heparanase-inhibiting species of non-anticoagulant heparin, which inhibits the enzyme *in vitro* at nanomolar concentrations (0.02–0.1 nM, not shown). Since until recently, prostate carcinoma metastasis models were not available in our lab, we have used the B16-BL6 mouse melanoma experimental metastasis model to demonstrate anti-metastatic effects these compounds, (>90% inhibition of melanoma lung colonization, Fig. 11). The effectiveness of the glycol-split heparin for suppressing the biological activity of heparanase was also judged by the inhibitory effect of ST1514 administration in experimental model of heparanase-driven biological processes, such as DTH inflammation (13). Presently, following the development of PC bone-to-lung spontaneous metastasis model *in vivo* (see Fig. 4), the effect of these compounds on prostate carcinoma dissemination is being investigated. Initially, compounds were administered intraperitoneously, but, taking into account the relatively short half-life (~2 h) of these compounds in the circulation

and in order to improve the inhibitory activity in vivo, the compounds will be administered continuously (mini-osmotic pumps; 25 mg/kg/day). Drug levels in the circulation will be monitored using fluorescinated G-4000, already prepared and found to fully retain its heparanase-inhibiting activity.

iii) Hpa gene silencing *in vivo*, as an approach to: **i)** eliminate both enzymatic and non-enzymatic activities of heparanase; and **ii)** elucidate the role of host- and tumor-derived heparanase in cancer metastasis and angiogenesis

We prepared siRNAs directed against the human and mouse heparanase mRNAs and designed anti-mouse heparanase siRNA-expressing plasmid pSi and anti-human heparanase siRNA-expressing plasmid pSH1 (13, 14). Heparanase gene silencing approach is especially important in light of the recently discovered non-enzymatic functions of heparanase such as cell adhesion and survival signals (15, 16) — functions that may be integral part of cancer promotion by heparanase, but are not sensitive to the currently available inhibitors of heparanase enzymatic activity.

a. *Electroporation assisted delivery of siRNA*. We developed a reliable system for siRNA administration by *in vivo* electroporation of siRNA expressing vectors. To verify the effectiveness of this approach for suppressing the biological activity of heparanase *in vivo* we first utilized the skin DTH inflammation model, as a prototype of heparanase-driven biological processes which enables quick and reliable monitoring of the inhibitory effect of siRNA administration (13). Following confirmation of heparanase-inhibiting ability of siRNA electroporation in vivo in the above-mentioned study, we further apply this approach for experimental PC treatment. We injected SCID mice intradermally with DU145 PC cells and, upon development of palpable tumors (~2-3 mm in diameter), electroporated the tumors with either mixture of anti-human and anti-mouse heparanase siRNA expressing plasmid, or control empty vector pSUPER. Treatment with heparanase siRNA resulted in augmented DU145 tumor regression, as compared to tumors treated with the control plasmid (Fig. 12). Using LUC-expression plasmid, we demonstrated the feasibility of electroporation-assisted delivery of expression vector into the mouse tibia *in vivo* (Fig. 13) and are now proposing to apply the same technique to deliver anti-heparanase siRNAs into DU145 carcinoma growing in the mouse tibia.

Apart of exploration of the therapeutic potential of heparanase gene silencing for PC, siRNA tools enable to dissect the contributory role of tumor- vs. host-derived heparanase in prostate carcinoma progression. It is well established that cellular components of the tumor stroma (i.e., activated fibroblasts, immune and inflammatory cells, blood vessel cells) are actively involved in modulation and promotion of carcinoma growth (17). The siRNA approach, unlike enzymatic inhibitors, offers the opportunity to silence selectively either the mouse or human heparanase gene in experimental systems of human carcinoma growing in mouse host, since anti-mouse siRNA is unable to silence the human gene (Fig. 15) and vice versa. To evaluate the precise role of host- vs. tumor-derived heparanase in tumor growth, we treat the human PC tumor growing in mouse host with either anti-mouse siRNA, anti human siRNA, a mixture of the two plasmids, or empty vector alone. The first experiment of this type was recently performed: we compared both primary tumor growth rate and spontaneous metastasis in mice bearing intraosseous DU145 tumor, treated with either anti mouse or anti human siRNA, a mixture of the two plasmids, or control vector alone, and it appears that both host- and tumor- derived heparanase are required for primary tumor growth, whereas tumor-derived enzyme is of primary importance for metastatic spread (Fig. 14).

b. *siRNA administration in vivo using lentiviral vector.* Lentivirus-based vectors enable sustained siRNA expression in the target tumor cells (18). Such a vector has been recently constructed in our lab and effective infection of various tissues and organs was demonstrated (Fig. 15). The ability of lentivirus to infect both dividing and non-dividing cells and to allow for long-term multilineage gene expression (19) is expected to extend the range of tumor cell types in which heparanase silencing will be achieved. In the upcoming series of experiments we plan to inject DU145 cells orthotopically, followed by repetitive administrations of siRNA-expressing lentivector by direct injection into the prostate, or systemic administration into the tail vein, as described (20). The effect of lentivirus-delivered heparanase siRNA on primary tumor take, angiogenic switch and growth rate, as well as metastatic potential, will be investigated.

KEY RESEARCH ACCOMPLISHMENTS.

i)   direct evidences for causal involvement of the heparanase enzyme in both prostate tumor primary growth and metastasis were obtained.

ii)   prostate tumors having elevated levels of heparanase were shown to promote bone remodeling and resorption, boosting the promise for effective therapies, designed to block heparanase function, that may disrupt the early progression of bone-homing tumors.

iii)   heparanase protein effects on EGFR phosphorlation and signaling were revealed

iv)   anti-heparanase inhibitory strategies, developed during previous year of research, were further tested and applied in proof-of-concept *in vivo* settings.

REPORTABLE OUTCOMES.

1. Edovitsky, E., Lerner, E., Zcharia, E., Vlodavsky, V., and Elkin, M. Role of endothelial heparanase in delayed-type hypersensitivity. Blood. 2005, (Epub ahead of print Jan.29, 2005).

2. Baraz, B., Haupt, Y., Elkin, M., Peretz, T., and Vlodavsky, I. Tumor suppressor p53 regulates heparanase gene expression. Oncogene, 2005, In press.

CONCLUSIONS.

The described results represent a direct continuation of studies reported in the previous annual report. In the course of the second year of research the progress has been made primarily in performance of the tasks # 1, 2 and 4 outlined in the original Statement Of Work. Importance of the heparanase enzyme in prostate tumor primary growth, bone resorption, and metastatic spread was demonstrated. The molecular mechanisms underlying heparanase regulation by p53, both in epithelial and stromal elements of tumor, were elucidated. Emerging link between heparanase and EGFR system and its relevance to PC is being investigated. The biological systems and tools established during this year were applied to provide experimental proof of concept for anti-heparanase therapeutic approach in prostate carcinoma treatment.

References

1. Parish, C. R., Freeman, C., and Hulett, M. D. Heparanase: a key enzyme involved in cell invasion. Biochim Biophys Acta, *1471:* M99-108., 2001.

2. Vlodavsky, I. and Friedmann, Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J Clin Invest, *108:* 341-347., 2001.

3. Sobel, R. E. and Sadar, M. D. Cell lines used in prostate cancer research: a compendium of old and new lines--part 2. J Urol, *173:* 360-372, 2005.

4. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem, *265:* 17174-17179, 1990.

5. Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., and George, D. L. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev, *13:* 2490-2501, 1999.

6. Bhowmick, N. A. and Moses, H. L. Tumor-stroma interactions. Curr Opin Genet Dev, *15:* 97-101, 2005.

7. Marchetti, D., Li, J., and Shen, R. Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase. Cancer Res, *60:* 4767-4770, 2000.

8. Friedmann, Y., Vlodavsky, I., Aingorn, H., Aviv, A., Peretz, T., Pecker, I., and Pappo, O. 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, 2000.

9. Nadav, L., Eldor, A., Yacoby-Zeevi, O., Zamir, E., Pecker, I., Ilan, N., Geiger, B., Vlodavsky, I., and Katz, B. Z. Activation, processing and trafficking of extracellular heparanase by primary human fibroblasts. J Cell Sci, *115:* 2179-2187, 2002.

10. Hill, R., Song, Y., Cardiff, R. D., and Van Dyke, T. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell, *123:* 1001-1011, 2005.

11. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res, *59:* 5002-5011, 1999.

12. Tuxhorn, J. A., McAlhany, S. J., Dang, T. D., Ayala, G. E., and Rowley, D. R. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. Cancer Res, *62:* 3298-3307, 2002.

13. Edovitsky, E., Lerner, I., Zcharia, E., Peretz, T., Vlodavsky, I., and Elkin, M. Role of endothelial heparanase in delayed-type hypersensitivity. Blood, 2005.

14.   Edovitsky, E., Elkin, M., Zcharia, E., Peretz, T., and Vlodavsky, I. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst, *96:* 1219-1230, 2004.

15.   Goldshmidt, O., Zcharia, E., Cohen, M., Aingorn, H., Cohen, I., Nadav, L., Katz, B. Z., Geiger, B., and Vlodavsky, I. Heparanase mediates cell adhesion independent of its enzymatic activity. Faseb J, *17:* 1015-1025, 2003.

16.   Zetser, A., Bashenko, Y., Miao, H. Q., Vlodavsky, I., and Ilan, N. Heparanase affects adhesive and tumorigenic potential of human glioma cells. Cancer Res, *63:* 7733-7741, 2003.

17.   Mueller, M. M. and Fusenig, N. E. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer, *4:* 839-849, 2004.

18.   Matta, H., Hozayev, B., Tomar, R., Chugh, P., and Chaudhary, P. M. Use of lentiviral vectors for delivery of small interfering RNA. Cancer Biol Ther, *2:* 206-210, 2003.

19.   Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet, *33:* 401-406, 2003.

20.   Iyer, M., Salazar, F. B., Lewis, X., Zhang, L., Carey, M., Wu, L., and Gambhir, S. S. Noninvasive imaging of enhanced prostate-specific gene expression using a two-step transcriptional amplification-based lentivirus vector. Mol Ther, *10:* 545-552, 2004.
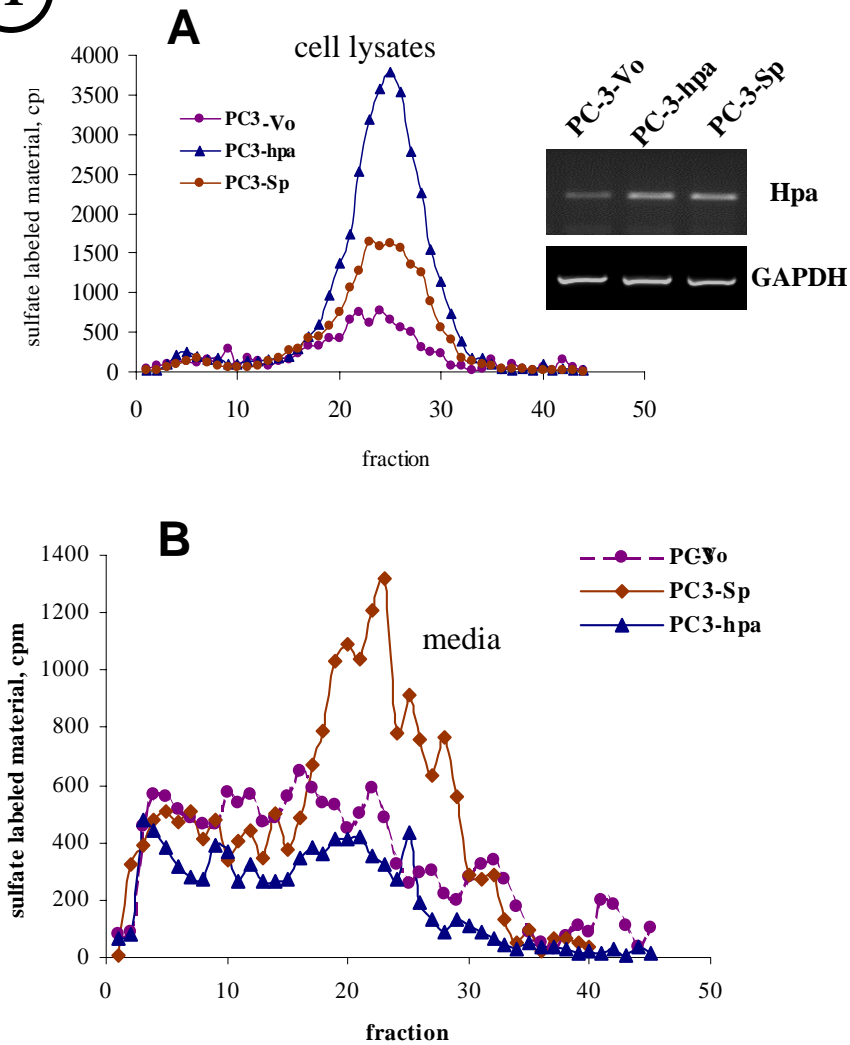
Figure 1. Heparanase enzymatic activity in transfected PC3 cells . **A**. PC3 cells stably transfected with empty vector (PC3-Vo), vector encoding human heparanase (PC3-hpa), or vector encoding a secreted form of heparanase (PC3-Sp) were lysed and tested for heparanase enzymatic activity. RNA was isolated from the 3 stable transfected cell types and subjected to RT-PCR for heparanase and GAPDH (control) (right, top). **B**. Heparanase enzymatic activity in medium conditioned by PC3-Vo, PC3-Sp and PC3-hpa cells.
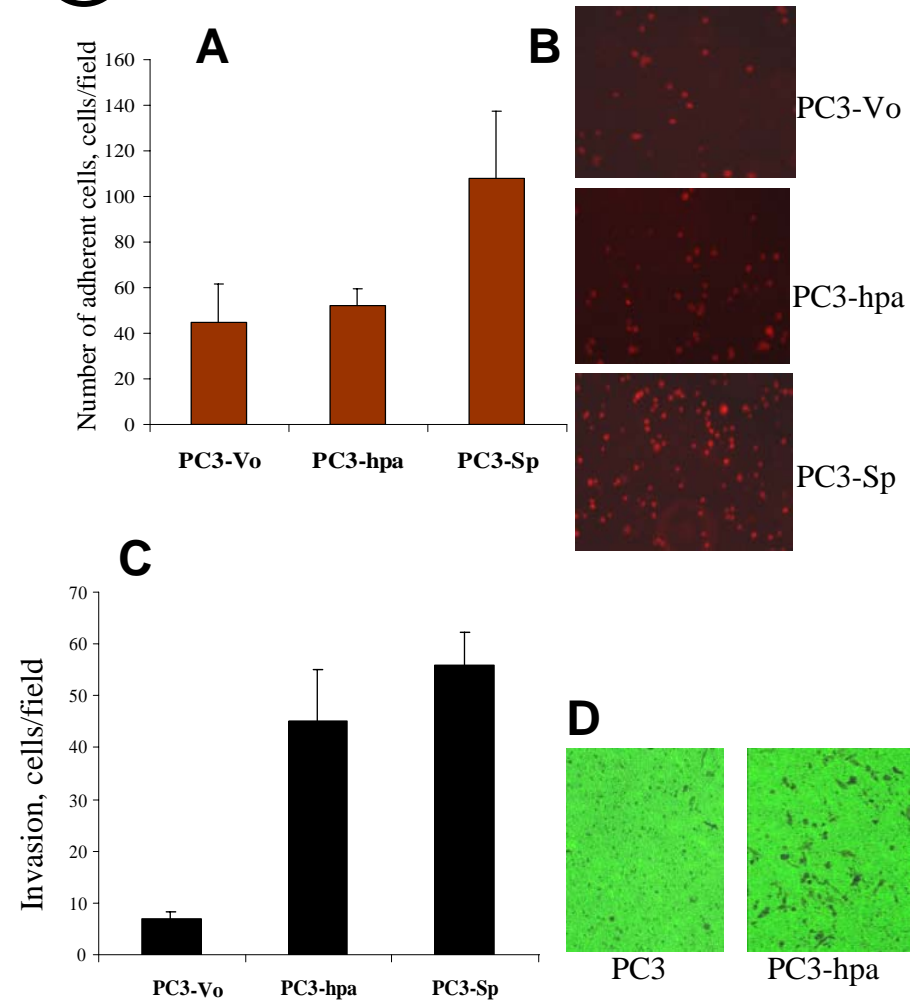

Figure 2. *In-vitro* **invasion and adhesion of heparanase transfected PC-3 cells.**
A, **B.** PC3-Vo, PC3-Sp and PC3-hpa cells were tested for their ability to firmly adhere to bovine aortic endothelial cells (BAEC). Secreted heparanase (PC3-Sp) increases PC-3 adhesion to BAEC . **C, D**. Matrigel invasion assay. PC-3 cells transfected with human heparanase(PC3-hpa) or with secreted heparanase (PC3-Sp) exhibit a marked increase in cell invasion as compared to mock transfected PC3 cells.
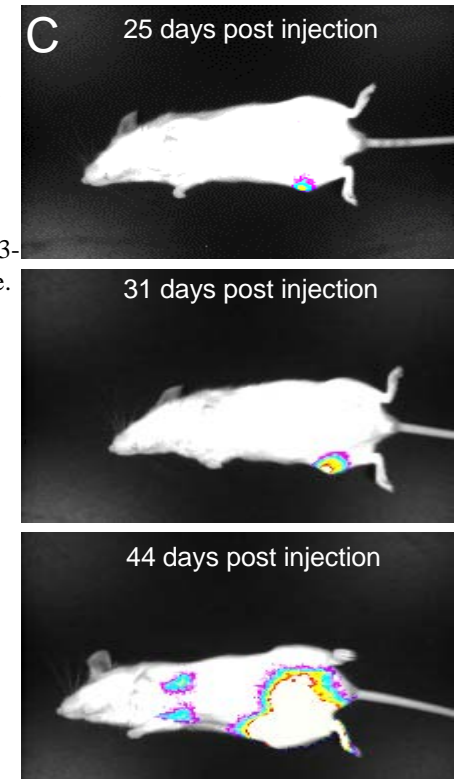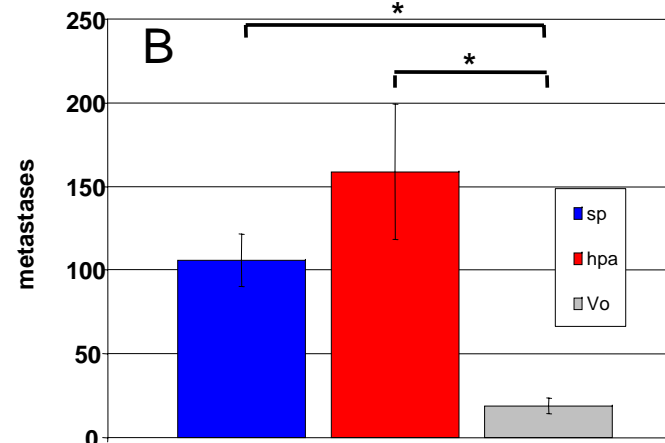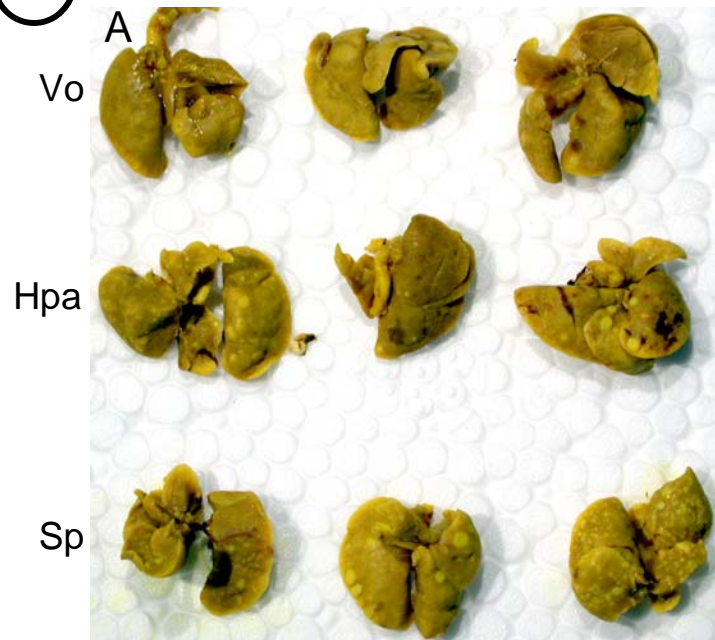
Figure 3. **Over-expression of heparanase accelerates prostate tumor formation and growth. A**. PC3 cells stably transfected with empty vector (PC3-Vo), vector encoding heparanase (PC3-hpa), or secreted form of heparanase (PC3-Sp) were injected into the right tibia of SCID mice. Tumor size was monitored for 6.5 weeks.
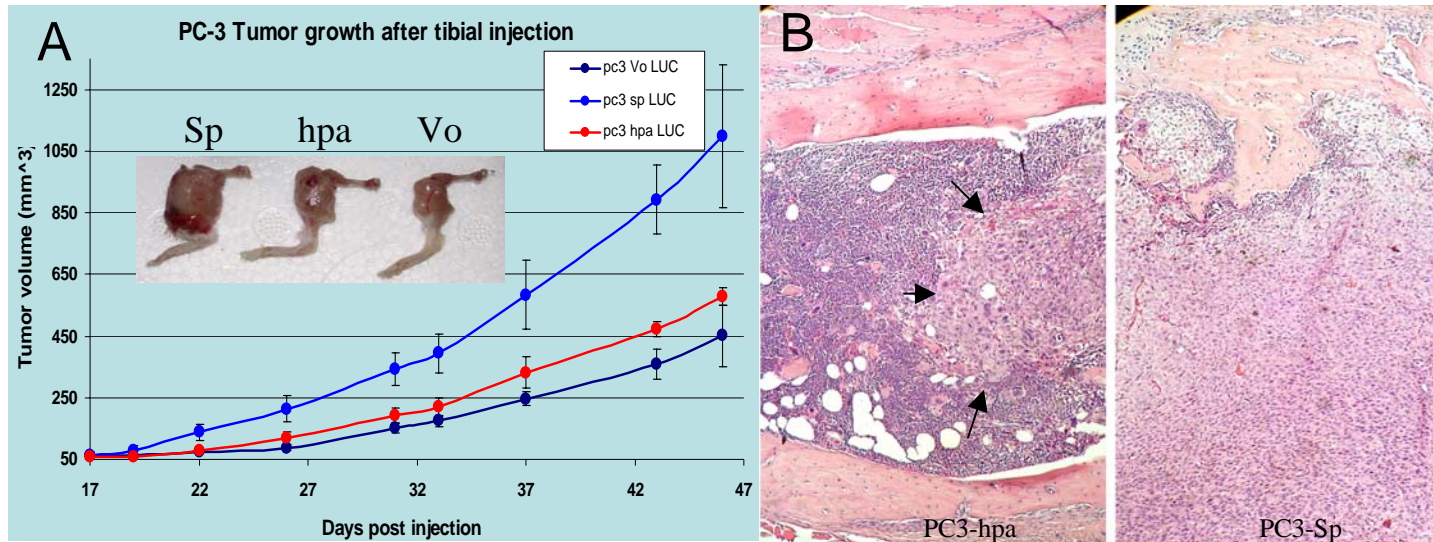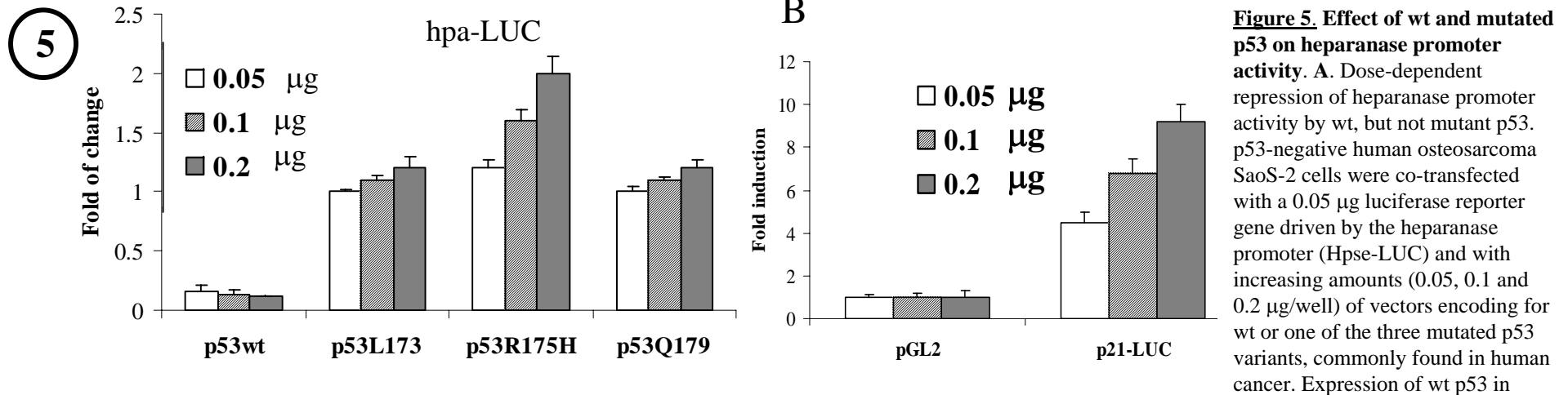
Inset: representative tumors, growing in mouse tibia on day 46 of experiment. **B, Right.** Histological examination of PC3-Sp bone tumors on day 23 post injection: the bone tissue is almost completely replaced by a tumor that consists of prostate carcinoma cells, occasionally admixed with bone tissue remnants. In bones injected with either PC3-Vo (not shown) or PC3-hpa (**B, Left**) cells, tumor masses of limited volume were found within the bone marrow (arrows), but the size of the bone has not changed. No bone destruction was noted and proper organization of the bone tissue was observed.
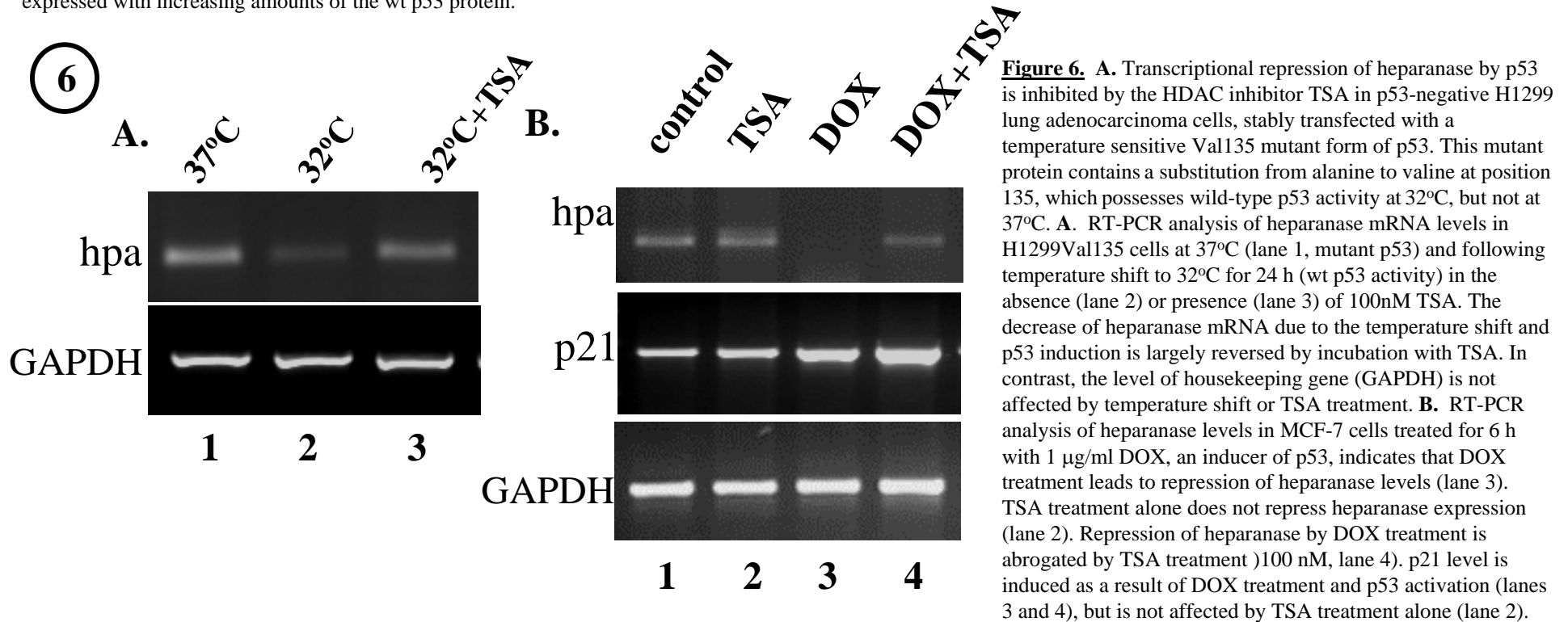
Figure 4. **Over-expression of heparanase increases pulmonary metastasis in SCID mice** . **A**. PC3 cells, expressing LUC and stably transfected with empty vector (PC3-Vo), vector encoding heparanase (PC3-hpa), or secreted form of heparanase (PC3-Sp) were injected into the right tibia of SCID mice. 47 days post injection the mice were sacrificed, the lungs isolated and photographed. **B**. metastatic nodules where counted and a statistically significant difference was observed between PC3-Sp and PC3-Vo (*P=0.019*), and between PC3-hpa and PC3-Vo (*P=0.0413*). **C**. *in-vivo* real time bioluminescence imaging of PC3-hpa prostate carcinoma growth and lung metastasis in SCID mice.

Figure 5. **Effect of wt and mutated p53 on heparanase promoter activity**. **A**. Dose-dependent repression of heparanase promoter activity by wt, but not mutant p53. p53-negative human osteosarcoma SaoS-2 cells were co-transfected with a 0.05 µg luciferase reporter gene driven by the heparanase promoter (Hpse-LUC) and with increasing amounts (0.05, 0.1 and 0.2 µg/well) of vectors encoding for wt or one of the three mutated p53 variants, commonly found in human cancer. Expression of wt p53 in SaoS-2 cells led to a dose-dependent decrease in heparanase promoter activity, as measured by the luciferase assay, reaching ~9-fold reduction in cells co-transfected with 0.2 µg of wt-p53 expressing plasmid. In contrast, none of the three tested p53 mutants displayed any repression ability. Moreover, one of the p53 mutant variants, demonstrated a slight (up to 2-fold) but consistently reproducible activation of heparanase promoter at the highest concentration.The graph represents the fold difference ±SD, as compared to control (SaoS-2 cells transfected with empty pcDNA3 vector only). Three independent experiments were performed in quadruplicates. **B.** Wt p53 does not affect SV40 promoter activity and activates the p21 promoter (p21-LUC). The experiment was performed as in A, except that pGL2 or p21-LUC plasmids, instead of Hpse-LUC, were co-expressed with increasing amounts of the wt p53 protein.
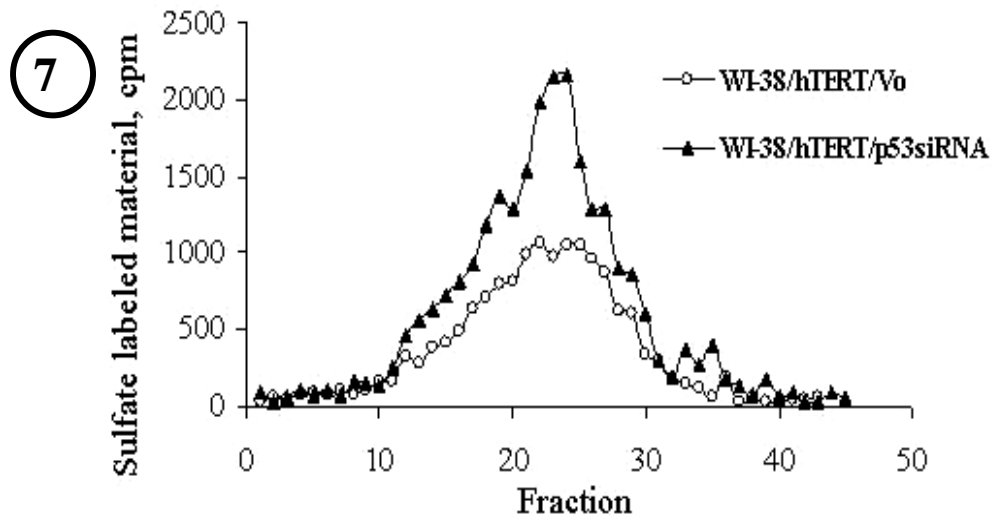

Figure 6. **A.** Transcriptional repression of heparanase by p53 is inhibited by the HDAC inhibitor TSA in p53-negative H1299 lung adenocarcinoma cells, stably transfected with a temperature sensitive Val135 mutant form of p53. This mutant protein contains a substitution from alanine to valine at position 135, which possesses wild-type p53 activity at 32ºC, but not at 37ºC. **A**. RT-PCR analysis of heparanase mRNA levels in H1299Val135 cells at 37ºC (lane 1, mutant p53) and following temperature shift to 32ºC for 24 h (wt p53 activity) in the absence (lane 2) or presence (lane 3) of 100nM TSA. The decrease of heparanase mRNA due to the temperature shift and p53 induction is largely reversed by incubation with TSA. In contrast, the level of housekeeping gene (GAPDH) is not affected by temperature shift or TSA treatment. **B.** RT-PCR analysis of heparanase levels in MCF-7 cells treated for 6 h with 1 µg/ml DOX, an inducer of p53, indicates that DOX treatment leads to repression of heparanase levels (lane 3). TSA treatment alone does not repress heparanase expression (lane 2). Repression of heparanase by DOX treatment is abrogated by TSA treatment)100 nM, lane 4). p21 level is induced as a result of DOX treatment and p53 activation (lanes 3 and 4), but is not affected by TSA treatment alone (lane 2).

Figure 7. siRNA-mediated silencing of p53 elevates heparanase expression. WI-38/hTERT cells (1x106), infected with either lentiviral vector containing p53siRNA (▲) or control vector (○) were lysed 3 days post infection, normalized for equal protein, and cell lysates were tested for heparanase enzymatic activity. **Inset**. Heparanase (Hpa) mRNA expression. RNA was isolated 72 h post infection, reverse transcribed to cDNA and subjected to comparative semiquantitative PCR.
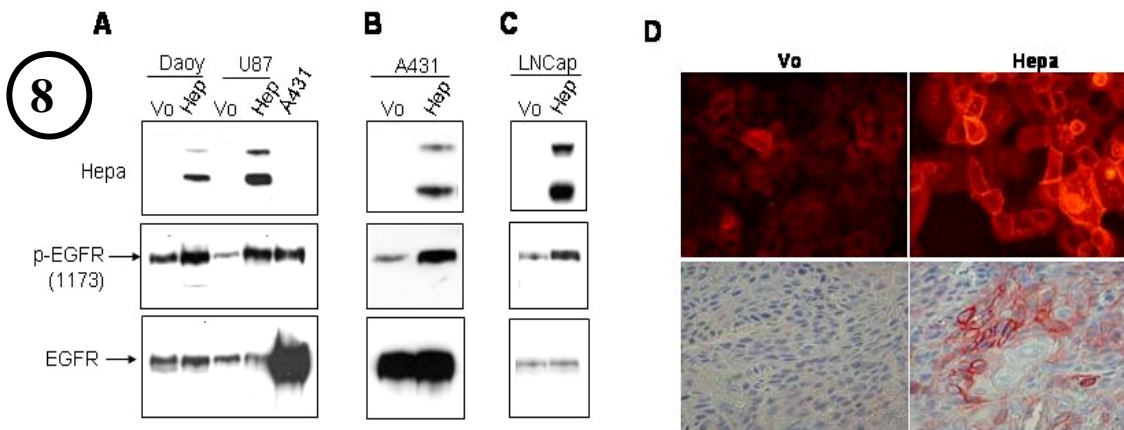

Figure 8. Heparanase over expression induces EGFR phosphorylation. **A-C**. Immunoblotting. Control (Vo) and heparanase transfected Daoy, U87 (A), A431 (B) and LnCAP (C) cell lysates were immunoblotted with anti-heparanase (upper panels), anti-phospho-EGFR (middle panels) and anti-EGFR (lower panels) antibodies. Note enhanced EGFR phosphorylation upon heparanase over expression. **D**. Immunostaining. Control, Vo, and heparanase transfected A431 cells (upper panels) and tumor xenografts sections (lower panels) were stained with anti-phospho-EGFR.
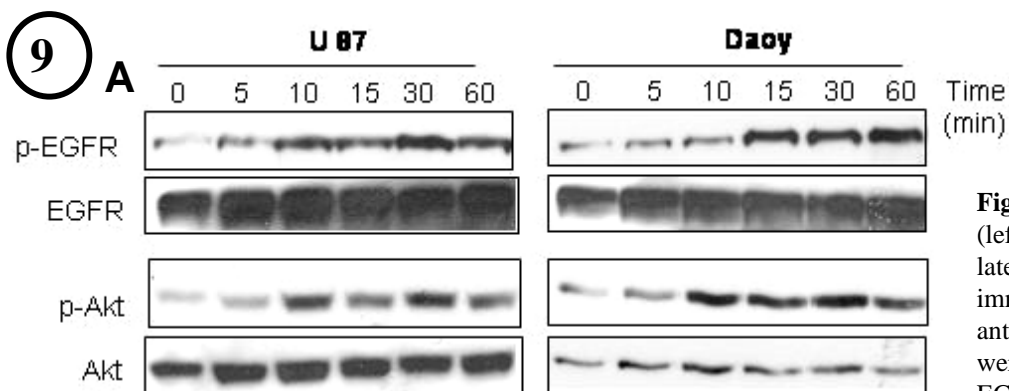

Figure 9. Exogenous addition of heparanase induces EGFR phosphorylation. **A**. U87 (left) and Daoy (right) cells were incubated without (0) or with recombinant purified latent 65 kDa heparanase (1 µg/ml) for the time indicate, and total cell lysates were immunoblotted with anti-phospho-EGFR (upper panels), anti-EGFR (second panels), anti-phospho-Akt (third panels) and anti-Akt (bottom panels) antibodies. **B**. U87 cells were left untreated (0) or incubated for the time indicated with heparanase (1µg/ml) or EGF (10ng/ml), and cell lysates were immunoblotted with anti-phospho-EGFR.
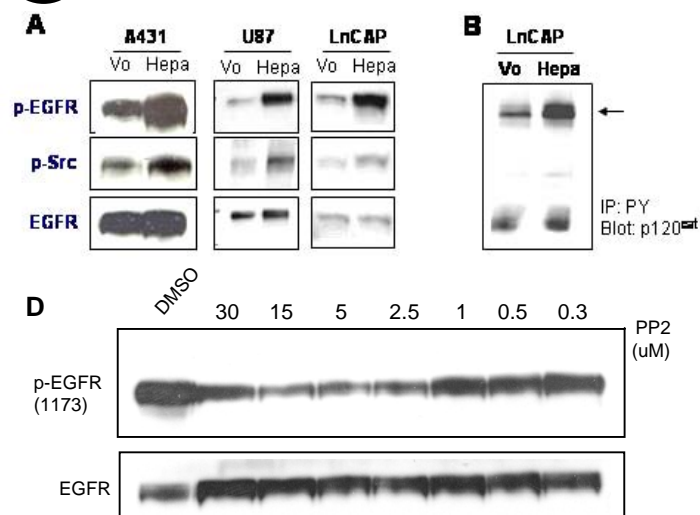
Figure 10. Enhanced EGFR phosphorylation upon heparanase over expression is Src-dependent. **A**. Src activation. Control, Vo, and heparanase transfected A431 (left), U87 (middle) and LnCAP (right) cell lysates were immunoblotted with anti-phospho-EGFR (upper panel), anti-phospho-Src (second panels) and anti-EGFR (bottom panels) antibodies. **B**. p120cat phosphorylation. Control, Vo, and heparanase transfected LnCAP cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine (PY) antibody, followed by immunoblotting with anti-p120cat antibodies. **C**. Inhibitors screen. Heparanase transfected U87 (upper two panels) and A431 (lower two panels) were incubated (18 h, 37oC) with control vehicle (DMSO), PI 3-kinase (LY-294002), Src (PP2), p38 (SB 203580), and MAPK (PD 98059) inhibitors, or with chloroquine (Chl), which inhibits heparanase processing. Cell lysates were immunoblotted with anti-phospho-EGFR (first and third panels) and with anti-EGFR (second and fourth panels). **D**. Heparanase transfected A431 cells were incubated (18 h, 37oC) with control vehicle (DMSO), or with the indicated concentrations (µM) of the Src inhibitor PP2 and total cell lysates were immunoblotted with anti-phospho-EGFR (upper panel) and anti-EGFR (lower panel) antibodies.
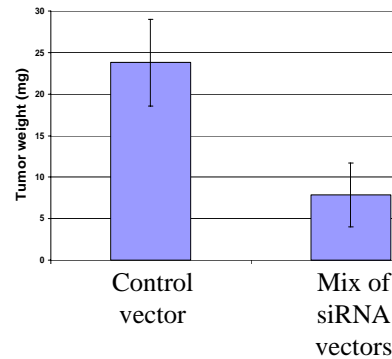

Figure 11. Inhibition of experimental metastasis by N-acetylated and glycol split heparin
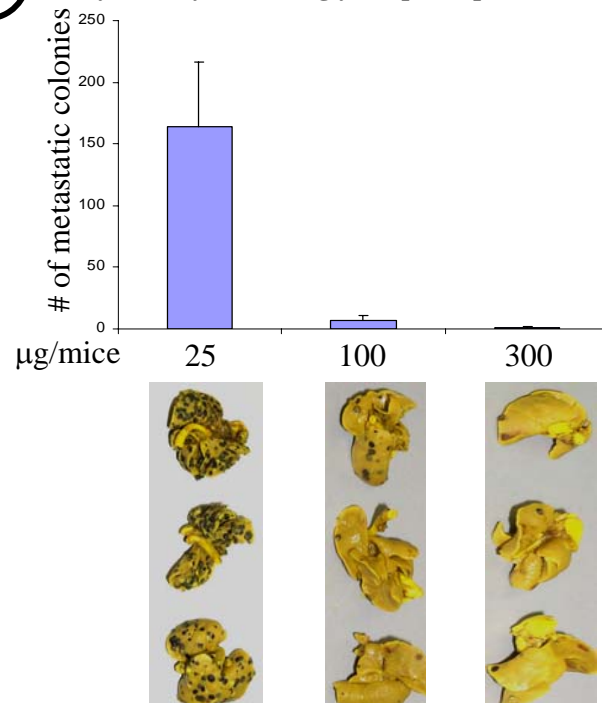
(12) Percentage of tumor-bearing mice **before** & **after** the treatment
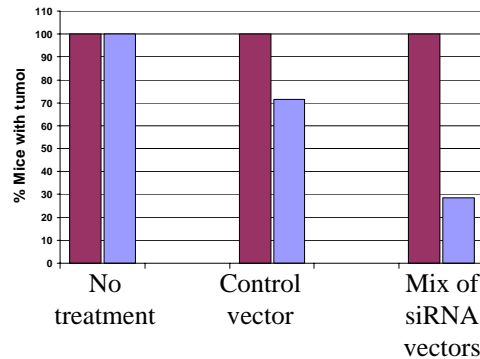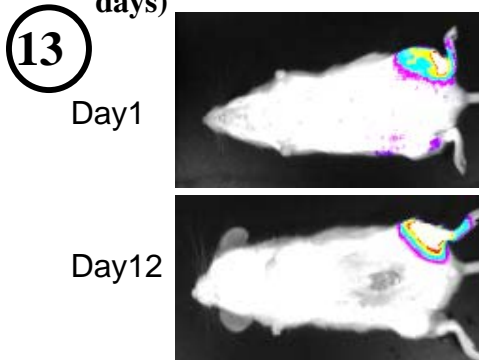

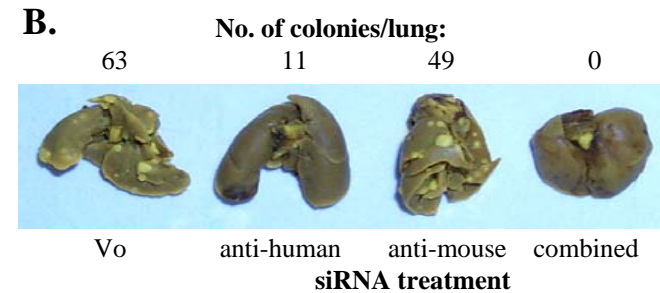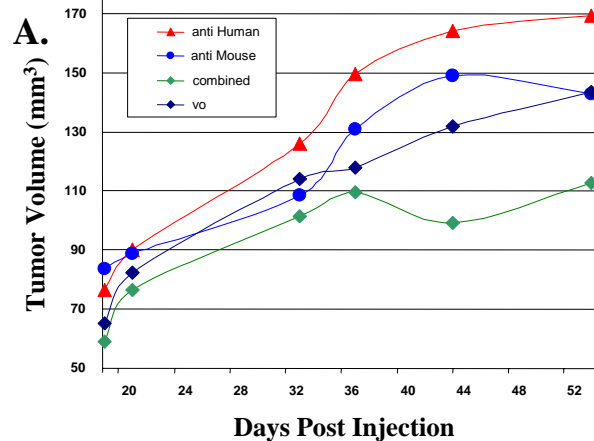
Weight of tumors after treatment

Macroscopic appearance of the DU145 tumors in mice treated with:
1. mix of siRNA vectors   2. control vector

Figure 13. Electroporation-assisted delivery of LUC-expression vector to the mouse tibia (expression lasts for 12 days)

(13)

Day1

Day12

(14) **Figure 14.** Effect of human, mouse and combined siRNA treatment on human prostate tumor growth (A), and pulmonary metastasis (B) in mouse host

A.

- anti Human
- anti Mouse
- combined
- vo

Tumor Volume (mm³)

Days Post Injection

B.

No. of colonies/lung:
63   11   49   0

Vo   anti-human   anti-mouse   combined
siRNA treatment

(15) **Figure 15.** Effective and specific action of anti-human heparanase siRNA in DU145 cells

RT-PCR

pSUPER   anti-human siRNA   anti-mouse siRNA

Hpa
L-19

Heparanase activity

- anti-mouse siRNA
- anti-human siRNA