Suppression of relaxin receptor RXFP1 decreases prostate cancer growth and metastasis

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Abstract

Relaxin (RLN) is a small peptide hormone expressed in several cancers of reproductive and endocrine organs. Increased expression of RLN in prostate cancer correlates with aggressive cancer. RLN G-protein-coupled receptor (RLN family peptide receptor 1, RXFP1) is expressed in both androgen receptor (AR)-positive and -negative prostate cancers as well as in prostate cancer cell lines. RLN behaves as a cell growth factor and increases invasiveness and proliferation of cancer cells *in vitro* and *in vivo*. The objective of this study is to determine whether downregulation of RXFP1 expression using small interfering RNA (siRNA) reduces cancer growth and metastasis in a xenograft model of prostate cancer. We used two well-characterized prostate adenocarcinoma cell lines, AR-positive LNCaP cells and AR-negative PC3 cells. The tumors were established in nude male mice by s.c. injections. Intratumoral injections of siRNAs loaded on biodegradable chitosan nanoparticles led to a downregulation of RXFP1 receptor expression and a dramatic reduction in tumor growth. In LNCaP tumors, the siRNA treatment led to an extensive necrosis. In PC3 xenografts treated with siRNA against RXFP1, the smaller tumor size was associated with the decreased cell proliferation and increased apoptosis. The downregulation of RXFP1 resulted in significant decrease in metastasis rate in PC3 tumors. Global transcriptional profiling of PC3 cells treated with RXFP1 siRNA revealed genes with significantly altered expression profiles previously shown to promote tumorigenesis, including the downregulation of *MCAM*, *MUC1*, *ANGPTL4*, *GPI*, and *TSPAN8*. Thus, the suppression of RLN/RXFP1 may have potential therapeutic benefits in prostate cancer.

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Introduction

Prostate cancer is the most common type of cancer in men in the United States and the second leading cause of cancer deaths. Androgens play a key role in prostate cancer initiation and progression and androgen ablation is initially effective but once advanced to the

androgen-independent stage prostate cancer becomes nonresponsive to androgen ablation therapy. The identification of novel endogenous factors involved in the proliferation, viability, and migration of prostate cancer cells may create potential therapeutic targets for future treatment. The prostate is one of the main sites

of relaxin (RLN) expression in males (Agoulunik 2007). Originally, this peptide hormone was identified by its effects on the relaxation (hence the name) of pubic symphysis, cervical softening, and the inhibition of spontaneous myometrial contractions during pregnancy and parturition in females (Sherwood 2004). New roles for RLN in various other physiological scenarios have recently been established. These include regulation of connective tissue remodeling and fibrosis, promotion of angiogenesis, blood vessels dilation, chronotropic action in the heart, wound healing, inhibition of apoptosis, and other physiological effects (Sherwood 2004, Bathgate et al. 2006). There are two almost identical RLN genes in the genome of great apes and humans (*RLN1* and *RLN2*); all other studied mammalian species have only one copy of the orthologous gene (Klonisch et al. 2001). *RLN2* is the only circulating form of RLN detected in peripheral blood. Both the RLN genes are expressed in the prostate; however, the *RLN1* expression is significantly lower (Hansell et al. 1991). Although sharing only 48% identity at the amino acid level, mouse *RLN1* and human *RLN2* are each fully bioactive in both species (Feng et al. 2005). RLN family peptide receptor 1 (RXFP1) is a G-protein-coupled receptor expressed on the cell surface (Hsu et al. 2002). Stimulation of RXFP1 with RLN causes a cAMP increase mediated through G_s/G_i proteins and protein kinase A in some cells (Bathgate et al. 2006). In addition, the involvement of tyrosine kinases, protein kinase C zeta, phosphatidylinositol 3-kinase (PI3K), and other molecules in RLN signaling was also demonstrated (Bathgate et al. 2006). RLN regulates a number of critical molecules directly relevant to tumor progression. These molecular targets of RLN signaling include collagens and various metalloproteinases (MMPs), tissue inhibitors of MMPs, vascular endothelial growth factor, insulin-like growth factor-binding protein 1, hyaluronic acid, prolactin, laminin, nitric oxide synthase, S100A4, and others (Klonisch et al. 2007).

An increased expression of RLN has been reported in breast, endometrial, thyroid, and prostate cancers especially in patients with aggressive metastatic disease (Klonisch et al. 2007). The overall survival was shorter in patients with increased expression of RLN (Kamat et al. 2006, Feng et al. 2007). The *in vitro* invasiveness of carcinoma cells was significantly increased on incubation with RLN or in cells transfected with RLN expression constructs (Kamat et al. 2006, Silvertown et al. 2006, Feng et al. 2007). The stimulating effect on growth and angiogenesis of lentiviral-delivered RLN in the prostate carcinoma

cell line PC3 was recently demonstrated *in vivo* (Silvertown et al. 2006). Furthermore, we have demonstrated that the survival of transgenic adenocarcinoma of mouse prostate (TRAMP) mice with transgenic RLN overexpression was decreased (Feng et al. 2007). Neuroendocrine differentiation of prostate adenocarcinoma LNCaP cells and the presence of p53 gain-of-function mutant allele R273H in LNCaP cells coincide with an increase in RLN expression (Vinall et al. 2006). It was suggested that RLN might be negatively regulated by androgens *in vitro* and *in vivo*. Furthermore, PI3K/Akt signaling and components of the Wnt pathway can facilitate RLN-mediated activation of the androgen receptor (AR) pathway (Liu et al. 2008). On the other hand, small interfering RNA (siRNA)-mediated downregulation of endogenous RXFP1 expression in PC3 and LNCaP cells decreased cell invasion and proliferation and increased cell apoptosis *in vitro* (Feng et al. 2007).

Based on the available data, we hypothesized that the suppression of RLN signaling in prostate cancer cells might affect tumor growth and metastasis *in vivo*. In the present study, we analyzed the effect of RLN receptor RXFP1 downregulation on prostate cancer progression using an *in vivo* model. Tumor growth and metastasis were analyzed in AR-positive LNCaP and AR-negative prostate adenocarcinoma PC3 cells transplanted into nude mice treated with RXFP1-specific small interfering RNA (siRNA) (siRXFP1) incorporated into chitosan nanoparticles (CNNP) to inhibit RXFP1 expression. Our data indicate that RXFP1 may be a promising new target in prostate cancer therapy.

Materials and methods

Cell culture

LNCaP and PC3 cells were originally purchased from American Type Culture Collection, Inc. (Manassas, VA, USA). LNCaP cells were maintained in RPMI-1640 medium while PC3 cells were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotics mix (100 U penicillin/ml and 100 µg streptomycin/ml; Invitrogen) at 37 °C in a humidified atmosphere of 95% air with 5% CO₂.

siRNA transfection *in vitro*

In this project, we have used two siRNAs targeting *RXFP1* expression. The siRXFP1-0 GGAUGUCAA-GUGCUCUUtt (sense strand), a highly efficient siRNA was identified previously (Kamat et al. 2006,

Feng *et al.* 2007). Another one was selected after testing four HP GenomeWide siRNAs (Qiagen) targeting human RXFP1 (Hs_LGR7_1,2,3, and 5 HP siRNA) in LNCaP cells. The siRXFP1-5 siRNA, ACGUGACACGUUCGGAGAAtt, was selected for this project (Supplementary Figure 1, see section on supplementary data given at the end of this article). The siRXFP1-0 and siRXFP1-5 were synthesized by Sigma-Genosys and Qiagen respectively. Negative control siRNA (siNC) with no significant sequence similarity to human gene sequences was used as control (Qiagen). LNCaP or PC3 cells were seeded on 100 mm dishes 24 h before transfection. siRXFP1 and siNC (5 µg each) were transfected into the cells using the Cell Line Nucleofector kit V (PC3) or kit R (LNCaP) with the nucleofector device (Lonza Group Ltd, Basel, Switzerland) according to the manufacturer's instruction. Transfected cells were harvested after 48 h, and total RNA was isolated for cDNA microarray assay and quantitative real-time reverse transcription-PCR (qRT-PCR).

Incorporation of siRNA into chitosan nanoparticles

CNNP are formed spontaneously upon the addition of aqueous tripolyphosphate (TPP) solution to chitosan solution under magnetic stirring at 200 g and mixed for a further 10 min after addition of TPP. siRNA was added to the TPP solution in a 3:1 w:w ratio under constant magnetic stirring (200 g) at room temperature. The chitosan preparation was then centrifuged at 9000 g for 30 min at 5 °C. Supernatants were discarded and the pellet containing the CNNP was washed three times with distilled water to remove any residual sodium hydroxide, and finally resuspended in ultrapure water. The preparations consisted of control siRNA incorporated in CNNP (siNC–CNNP) and siRNA RXFP1 incorporated in CNNP (siRXFP1–CNNP).

Animal experiments

All experiments were conducted using the standards for human care in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Baylor College of Medicine Institutional Committee. The xenograft experiments were performed on nude mice obtained from the NCI Animal Production Program. LNCaP or PC3 cells (10^7) (per mouse/injection site) suspended in 200 µl of a 1:1 mixture of RPMI-1640 or DMEM/F12 and Matrigel (BD Biosciences, San Jose, CA, USA) were injected s.c. into the right flank of nude mice. The tumor size

was measured by a digital slide caliper and the tumor volume in cubic millimeters was calculated by the following formula: $\text{volume} = \text{width}^2 \times \text{length} / 2$. When tumors became visible at 2 weeks (PC3) and at 4 weeks (LNCaP), the animals were randomized into two groups. For experiments with LNCaP cells, 45 mice were injected and 18 developed tumors. In the xenograft experiments using PC3 cells, a total of 45 mice were used in two consecutive experiments (40 tumors). Tumor-bearing nude mice were treated with siRXFP1–CNNP and siNC–CNNP. For the LNCaP xenografts, we used an equal amount of siRNAs (RXFP1-0 and RXFP1-5), whereas PC3 tumors were treated with siRXFP1-0 only. Four micrograms of siRNA CNNP were diluted in 100 µl of 0.9% saline before injection and a final volume of 100 µl was applied directly into the tumor as a bolus injection. Each siRNA species incorporated into the CNNP was injected intratumorally twice a week. Mice with LNCaP tumors were treated for 21 days (seven injections) and were killed 2 days after the last treatment. Mice with PC3 tumors received a total of six injections and were killed 9 days after the last treatment. All mice were analyzed for the presence of macroscopic and microscopic metastasis in the abdominal organs, the thorax, and the brain. The xenograft tumors, lung, liver, kidneys, brain, and regional lymph nodes were collected and tumor weights were measured.

Histological analysis and immunohistochemistry

Xenograft tumor tissues were fixed in cold 4% paraformaldehyde (PFA) and were embedded in paraffin. Prior to the histological analysis, tissue sections (5 µm) were deparaffinized in xylene and a descending alcohol series and were stained with hematoxylin and eosin. Staining with picrosirius red was performed to examine the density of collagen in the extracellular matrix (ECM; EMS, Hatfield, PA, USA).

Tumor cell proliferation in xenograft tumors treated with RXFP1 siRNA was evaluated using Ki67 immunodetection. The expression of RXFP1 was evaluated using a monoclonal RXFP1 antibody. Briefly, tissue sections were deparaffinated and antigen retrieval was performed by boiling slides in 10 mmol/l sodium citrate buffer (pH 6.0) for 20 min. Nonspecific binding sites were blocked with 5% BSA in PBS for 1 h at room temperature. Slides were incubated with anti-rabbit Ki67 (1:200; Abcam, Cambridge, MA, USA) or RXFP1/LGR7 monoclonal antibody M01, clone 3E3 (1:200; Abnova, Taipei, Taiwan) for 1 h at room temperature and incubated for 45 min

with an appropriate HRP-conjugate/HRP-substrate stain (Pierce, Rockford, IL, USA). Slides were counterstained with hematoxylin and images were taken with a Nikon-TMS inverted microscope equipped with Olympus DP70 digital imaging system. Ki67 staining was scored by the percentage of positive tumor cells.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used for the detection of apoptosis in PC3 xenograft tumors using ApopTag *In Situ* Apoptosis Detection kit (Serologicals Corp., Norcross, GA, USA). The mouse anti-human cluster of differentiation 34 (CD34) monoclonal antibody (1:300; Dako North America, Carpinteria, CA, USA) was used to identify vascular cells in tumor sections. A vessel was defined as an open lumen lined by one or more CD34-positive cells.

Eight random fields per section were scored at $\times 400$ magnification. Scoring was performed by two independent researchers in a blind manner. In the LNCaP xenograft experiment, seven siRXFP1 and ten siNC tumors were analyzed. In the PC3 xenograft experiment, the analysis was performed with 12/15 (Ki67), 8/8 (apoptosis), and 10/10 (CD34) mice per group, siRXFP1/siNC.

To analyze an efficiency of siRNA gene knock-down *in vitro*, LNCaP cells were transfected with siRXFP1-0, siRXFP1-5, and siNC (5 μ g each) using the Amaxa Cell Line Nucleofector kit R (Lonza) according to the manufacturer's manual and were grown in poly-D-lysine-coated six-well plates. After 48 h, cells were washed with PBS and fixed for 10 min with 4% paraformaldehyde. The wells were washed twice with PBS and then incubated in 5% BSA/0.3 M glycine in 0.1% PBS-Tween for 1 h to increase cell permeability and block nonspecific protein-protein interactions. The cells were incubated with the RXFP1 antibody (1:200) overnight at $+4^{\circ}\text{C}$. Detection was performed by a Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA, USA). Cells were counterstained with Harris hematoxylin. Wells were mounted for microscopic examination.

RNA isolation and cDNA synthesis

Total RNA was isolated from PC3 and LNCaP cells transfected with siRNA or from xenografts using the RNeasy kit (Qiagen). For the expression array studies, the PC3 cell RNA was digested with the DNA-free DNase Treatment and Removal Reagents (Ambion, Austin, TX, USA) according to the manufacturer's protocol. cDNA was synthesized using an oligo(dT) primer and RETROscript kit (Ambion).

Real-time quantitative RT-PCR

Real-time qRT-PCR was performed according to a qPCR SybrGreen real-time protocol on the IQ5 iCycler (Bio-Rad). Primers for different genes are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. The *GAPDH* expression was used for normalization of SybrGreen data. RXFP1 expression level was estimated using TaqMan Gene Expression Assay Hs00222171 (Applied Biosystems, Foster City, CA, USA) in LNCaP cells or qPCR SybrGreen protocol in PC3 cells as described before ([Kamat et al. 2006](#)). For normalization of mouse *Rxfp1* expression, we used ubiquitously expressed hydroxymethylbilane synthase gene *Hmbs* (or *Pbgd*). The relative fold change in mRNA level was calculated by the comparative C_t ($2^{-\Delta\Delta C_t}$) method. Each measurement point was repeated at least in triplicate for three samples and the average and S.E.M were calculated.

Expression microarray analysis

Gene expression profiles were analyzed using the Illumina HumanRefseq-8 Expression BeadChip platform (Illumina, San Diego, CA, USA). Each profile represented RNA from pooled biological triplicate samples (two profiles per group, six biological replicates in total for each group). Two hundred nanograms of total RNA were amplified and purified using Illumina TotalPrep RNA Amplification kit (Ambion) following manufacturer's instructions. *In vitro* transcription was performed and biotinylated cRNA was synthesized during 14 h amplification with dNTP mix containing biotin-dUTP and T7 RNA polymerase. Amplified cRNA was subsequently purified and the concentration was measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 750 ng of amplified product were loaded onto Illumina Sentrix Beadchip Array Human RefSeq-8 arrays, hybridized at 58°C in an Illumina Hybridization Oven (Illumina) for 16–20 h, washed, and incubated with streptavidin-Cy3 to detect biotin-labeled cRNA on the arrays. Arrays were dried and scanned with BeadArray Reader (Illumina). Data were analyzed using BeadStudio software (Illumina). Expression of selected genes was confirmed by qRT-PCR. Expression data were quantile normalized (using software provided by [Giordano et al. \(2003\)](#) and [Saldanha \(2004\)](#)) and expression patterns were visualized as a heat map using Java TreeView ([Saldanha 2004](#)).

Statistical analysis

Student's *t*-test and ANOVA were used to assess significance of differences among the different groups. Differences were expressed as mean $\pm$ S.E.M. with *P* values smaller than 0.05 being considered as statistically significant.

Results

Selection of siRNA and analysis of RXFP1 suppression *in vitro* and *in vivo*

The expression of RXFP1 RLN receptor in LNCaP and PC3 cells has been established previously (Feng *et al.* 2007). We identified one siRNA (siRXFP1-0) capable of downregulating the expression of RXFP1 in a variety of prostate and endometrial cancer cells *in vitro* (Kamat *et al.* 2006, Feng *et al.* 2007). Additional four siRNAs obtained from Qiagen were tested in LNCaP cells (Supplementary Figure 1A). All four constructs significantly downregulated the *RXFP1* expression 48 h after transfection as determined by qRT-PCR with RXFP1-specific primers. The GAPDH expression was used for normalization. The immunohistochemistry (IHC) with specific anti-RXFP1 monoclonal antibodies confirmed the significant downregulation of the receptor in cells transfected with siRXFP1 (Supplementary Figure 1B).

We next assessed the efficiency of siRNA *RXFP1* gene targeting *in vivo* using siRNAs incorporated

into CNNP (siRXFP1–CNNP). We analyzed the effect of the siRXFP1-0–CNNP injection on *RXFP1* mRNA expression in tumor cells. PC3 cells (10^7 /injection site) were injected s.c. into the flanks of nine nude male mice. When tumors became clearly visible at 14 day, a single intratumoral injection of siRXFP1-0–CNNP or siNC–CNNP was administered. Analysis of *RXFP1* transcript levels in xenograft tumor mRNA extracts was performed by qRT-PCR and clearly showed a downregulation of gene expression in the siRXFP1 group, which reached statistical significance after 48 h as compared with siNC-treated tumor tissue ($P < 0.05$; Fig. 1). Thus, we confirmed our previous data on the dynamic stability and suppressive effects of chitosan siRNA (Lu *et al.* 2010). Based on these results, we decided to proceed with a bi-weekly injection schedule.

Effect of siRXFP1 on *in vivo* growth of LNCaP xenografts

Forty-five male nude mice received s.c. injection of 10^7 LNCaP cells per injection site. After 28 days, 18 mice developed well-defined tumors and were divided into two groups with the same average tumor volume. Tumors were injected either with siNC–CNNP complexes or with a mix of siRXFP1-0 and siRXFP1-5 siRNA–CNNP complexes twice a week (seven injections). As shown in Fig. 2A, the tumors injected with control siNC grew faster and by the end of 23 day reached the criteria for euthanasia. Tumors treated with siRXFP1 increased in volume within 3–7 days after the first injection. However, at the end of 21 day, tumor volumes were slightly lower than at the beginning of experiment. The growth curves of the tumors in this experimental group were significantly different from controls ($P < 0.0001$) and the average tumor volume at the end of experiment was 60% lower than in the control group. No animal mortality or morbidity was observed for the duration of the experiments suggesting that the treatment with siRNA–CNNP complexes was not toxic to the animals under the applied conditions.

Two days after the last injection, mice were killed and the primary tumors were extracted. Tumor weight of the siRXFP1-treated group was significantly lower than that of the siNC group (64% reduction, $P = 0.029$, Fig. 2B). Examination of the mice at both the macro- and microscopic levels did not reveal any metastases in the abdominal organs, the cervical nodes, the thorax, or the brain in control or experimental animals. Histological analysis of the majority of tumors treated with

Figure 1 Downregulation of *RXFP1* expression by siRNAs *in vivo*. Intratumoral injection of siRXFP1-0–CNNP inhibited *RXFP1* expression in PC3 xenograft tumor. *RXFP1* mRNA expression in PC3 xenograft was evaluated by qRT-PCR 24 and 48 h after siRNA injection ($n = 3$ in each group). Error bars denote $\pm$ S.E.M.

Figure 2 Suppression of *in vivo* tumor growth by siRXFP1-CNNP in androgen-sensitive LNCaP cells.

(A) LNCaP cells (10^7 cells/injection site) in 200 μ l RPMI-1640:Matrigel B (1:1) were inoculated s.c. into the right flank of nude mice. Four weeks later, the tumor-borne mice were treated with a mix of siRXFP1-0 and siRXFP1-5-CNNP ($n=8$) complexes and control siNC-CNNP ($n=10$) bi-weekly (seven injections). Tumor volumes were measured by digital caliper. The difference in the tumor volume was significant ($P<0.05$) from days 14 to 21. (B) A 64% reduction in tumor weight was observed in the siRXFP1-treated group after resection at the end of experiment ($P<0.05$). (C) A reduction in cell density was detected in siRXFP1-CNNP-treated tumors. Representative sections stained with hematoxylin and eosin are shown. (D) Small tumors in the siRXFP1-CNNP-treated group encapsulated in a thick collagen layer are shown with picrosirius red staining. Magnification, $\times 400$. Error bars denote $\pm$ S.E.M.

siRXFP1-CNNP revealed extensive vacuolated areas with granulation and evidence of necrosis. In contrast, the control siNC-CNNP-treated tumors were mostly dense and composed primarily of sheets of epithelial cells (Fig. 2C). The presence of vacuolated and necrotic areas within the tumors on treatment suggested that the siRNA-CNNP nanoparticles were taken up and likely caused the adverse cellular effects observed in close vicinity of the injection sites. The small tumors were encapsulated in a thick collagen-positive layer (Fig. 2D).

The examination of human *RXFP1* and mouse *Rxfp1* gene expressions in primary tumors was performed using qRT-PCR. The specificity of the human and mouse primers was confirmed using LNCaP RNA and the RNA isolated from mouse gubernaculum (data not shown). Treatment of the tumors with siRXFP1-CNNP complexes sharply reduced the expression of the human target gene (30% of control level), although the effect was not quite significant ($P=0.056$; Fig. 3A) perhaps due to a variability in the siRNA delivery within the tumor. There was no difference in mouse RLN receptor *Rxfp1* expression in

tumor RNA (Fig. 3A) confirming the specificity of siRXFP1. IHC with specific monoclonal anti-RXFP1 antibody revealed a significant reduction in receptor expression. There was a strong correlation of the data obtained by qRT-PCR and IHC for individual xenografts (Fig. 3B).

Treatment with siRXFP1s in this experiment did not induce classic interferon target gene 2'5'-oligoadenylate synthetase, *OSA1*, suggesting that the delivery of siRNA complexes did not trigger a substantial type I interferon response under these conditions (Bridge et al. 2003, Sledz et al. 2003, Pebernard & Iggo 2004). There was no difference in the expression of *OSA1* gene between the control and experimental group at RNA level based on results of qRT-PCR analysis (data not shown).

Figure 3 The siRXFP1 suppressed the expression of human *RXFP1* gene in LNCaP xenograft tumors. (A) Downregulation of human *RXFP1* gene expression in LNCaP tumors after seven injections with siRXFP1-CNNP1 treatment ($P=0.056$). There was no downregulation of mouse *Rxfp1* gene in the host cells in xenografts. siNC-CNNP, $n=10$; siRXFP1-CNNP1, $n=7$. Error bars denote $\pm$ S.E.M. (B) Representative sections of siRNA-CNNP-treated tumors stained with anti-RXFP1 monoclonal antibody showing a significant reduction in RXFP1 expression in siRXFP1-CNNP-treated xenografts. Magnification, $\times 400$.

Effect of siRXFP1 on *in vivo* growth of PC3 xenografts

Two independent experiments were performed with PC3 xenografts and different batches of siRXFP1-0 and chitosan-siRNA preparations; similar findings were obtained in both the experiments and, thus, the results were combined (total of 20 mice in each group). The higher tumorigenicity of PC3 cells resulted in higher tumor taking rate (89%, 40/45). Tumor volumes were measured during the course of the experiment (details see Materials and methods). As shown in Fig. 4A, tumor growth was significantly decreased 1 week after siRXFP1-0-CNNP injection and only a moderate increase in tumor size in the experimental group was observed until the last (6th) injection. Notably, the tumors in the siRXFP1-0-CNNP treatment group partially resumed the growth potential after the termination of siRXFP1 treatment indicating

Figure 4 Suppression of *in vivo* tumor growth by siRXFP1-CNNP in androgen-receptor-negative PC3 xenograft tumors. (A) Intratumoral injection of siRXFP1-0-CNNP inhibited the growth of PC3 cell xenografts. PC3 (10^7 cells/injection site) in 200 μ l DMEM/F12:Matrigel B (1:1) were inoculated s.c. into the right flank of nude mice. Two weeks later, the tumor-borne mice ($n=20$ in each group) were treated with siRXFP1-1-CNNP and control siNC-CNNP bi-weekly for 3 weeks (six injections). Tumor volumes were measured by digital caliper. The difference in the tumor volume was significant ($P < 0.05$) from days 6 to 23. (B) Reduction in tumor weight in the siRXFP1-treated group after resection at the end of experiment ($P < 0.05$). (C) Decrease in metastasis rates in siRXFP1-CNNP-treated animals. Metastases were exclusively detected in the cervical lymph nodes shown above. The difference between the siRXFP1-CNNP and siNC-CNNP groups was statistically significant ($P = 0.0186$). Yellow bars correspond to 200 μ m. (D) Small tumors in the siRXFP1-CNNP-treated group encapsulated in a thick collagen layer as shown with picosirius red staining. Magnification, $\times 400$. Error bars denote $\pm$ s.e.m.

that the RXFP1 suppression effect might be transient and reversible.

On termination of the experiments, the primary tumor weight in the siNC-CNNP group was 60% higher than that in the siRXFP1-0-CNNP-treated group ($P < 0.05$; Fig. 4B). The relative RXFP1/GAPDH expression within the tumor did not reveal significant differences between the groups (siNC-CNNP: 0.18 ± 0.03 versus siRXFP1-0-CNNP: 0.21 ± 0.08 relative arbitrary units), suggesting that the RXFP1 expression was normalized 9 days after the termination of siRNA treatment.

One of the most consistent RLN effects on cancer cells *in vitro* is an increased cell migration, matrix penetration, and cell adhesion (Klonisch *et al.* 2007). Alternatively, the suppression of RLN or RXFP1 expression in cancer cells by siRNA downregulation caused decreased cell migration and invasiveness (Kamat *et al.* 2006, Feng *et al.* 2007). We analyzed the nude mice with PC3 xenografts for signs of metastatic tissue invasion and detected metastases exclusively in the cervical lymph nodes (Fig. 4C). The incidence of metastasis in mice injected with the siRXFP1-0-CNNP was 3.6 times lower (15.89%) than in mice injected with control siNC-CNNP (57.9%, $P = 0.0186$). Furthermore, primary small tumors were more frequently observed in the siRXFP1-CNNP-treated group and were encapsulated in a thick collagen layer (Fig. 4D).

The proliferation of tumor cells in primary tumors from experimental and control siRNA-treated mice was evaluated by IHC using proliferation marker Ki67. The number of Ki67-positive cells was significantly higher in the siNC-CNNP group indicating that siRXFP1-0-CNNP treatment significantly down-regulated the proliferation of PC3 cells within the xenografts (Fig. 5A). The TUNEL assay was used to analyze cell apoptosis in xenografts. Suppression of RXFP1 by siRXFP1-0-CNNP treatment significantly increased the rates of apoptosis in these prostate cancer cells (Fig. 5B). We did not find significant differences in the vascularization between the siRXFP1-0-CNNP and siNC-CNNP-treated PC3 xenografts as determined by CD34 IHC staining (Fig. 5C). Thus, the reduced PC3 tumor growth during siRXFP1-CNNP treatment was mainly the result of decreased proliferation and enhanced apoptosis of PC3 cells.

Gene expression profiling of PC3 cells after downregulation of RXFP1

To identify the potential molecular mechanisms involved in tumor cell inhibitory effects of siRNA against RLN receptor, we performed the global gene

Figure 5 Effect of siRXFP1 treatment on tumor cell proliferation, apoptosis, and tumor vascularization in PC3 xenografts. (A) Suppression of tumor cell proliferation in siRXFP1–CNNP-treated animals revealed by the proliferation marker Ki67 (siNC–CNNP, $n=15$; siRXFP1–CNNP, $n=12$; *** $P < 0.001$). Representative photomicrographs (magnification, $\times 400$) showing immunohistochemical dark brown staining for Ki67 in tumor sections of siNC–CNNP and siRXFP1–CNNP-treated mice. (B) Increased apoptosis in tumors in siRXFP1–CNNP-treated animals detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (siNC–CNNP, $n=8$; siRXFP1–CNNP, $n=8$, * $P < 0.05$). Black arrows point to the dark brown stained apoptotic cells. (C) siRXFP1–CNNP treatment did not affect vascularization in tumor tissues. Arrows indicate blood vessels. Representative photomicrographs are shown. Black bars on all images correspond to 200 μm . Error bars denote $\pm$ S.E.M.

expression analysis of $\sim 22\,000$ RNA transcripts in PC3 cells transfected with siRXFP1-0 and siNC. This treatment did not significantly affect viability of the prostate cancer cells (Feng et al. 2007). Forty-eight hours after siRNA transfection, total RNA was isolated and qRT-PCR confirmed an 80% suppression of *RXFP1* gene expression in cells treated with siRXFP1 as compared with treatment with control siNC (data not shown). In the siRXFP1-treated group, 78 genes appeared downregulated and 21 genes were upregulated more than 1.3 fold (each RXFP1 profile was compared with each of the siNC profiles; Fig. 6A, complete gene list in Supplementary Table 2, see section on supplementary data given at the end of this article). The 1.3 cutoff fold was determined based on qRT-PCR results. The array data were then confirmed for a selected number of genes using qRT-PCR analysis (Fig. 6B). The potential target genes included a number of genes previously implicated in cancer progression. These included type 1 transmembrane protein, MUC1; melanoma cell adhesion molecule, MCAM (MUC18); tumor-associated antigen CO-029, TSPAN8; glucose phosphate isomerase, *GPI*; and angiotensin-like 4, *ANGPTL4*.

Discussion

The growth and progression of prostate cancer is influenced by an array of endogenous growth factors and hormones. We have studied the tumorigenic effect of RLN, a small peptide hormone produced both in the normal prostate and in prostate cancer (Figueiredo et al. 2005, Silvertown et al. 2006, Thompson et al. 2006, Vinall et al. 2006, Feng et al. 2007, Liu et al. 2008). Previously it has been shown that the RLN expression was increased in aggressive metastatic disease (Figueiredo et al. 2005, Thompson et al. 2006, Feng et al. 2007), and that the stimulation of prostate cancer cells with RLN accelerated their invasiveness, adhesion, survival, and decreased cell apoptosis (Feng et al. 2007). Moreover, in the TRAMP mouse model, transgenic overexpression of RLN decreased survival of the males with prostate cancer (Feng et al. 2007). In this study, we used the *in vivo* nude xenograft mouse model to analyze the effect of RLN receptor suppression on the growth of two commonly used prostate adenocarcinoma cell lines, AR-positive LNCaP cells and AR-negative PC3 cancer cells. The effect of RXFP1 downregulation on tumor spread was also evaluated in PC3 model prone to metastasis. The targeted downregulation of human RXFP1 in prostate cancer cell xenografts by means of specific siRNA against *RXFP1* coated to CNNP resulted in significantly decreased tumor growth and metastasis rate in nude mice. The downregulation of RLN receptor RXFP1 in tumor cells may be a novel strategy in prostate cancer therapy.

RLN hormone produced in prostate cells signals through G-protein-coupled receptor RXFP1 (Hsu et al. 2002). The closely related insulin-like 3 peptide receptor RXFP2 also responds to higher doses of RLN by cAMP increase in cells transfected with RXFP2 (Feng et al. 2007), however, such cross-reactivity was not detected *in vivo* (Kamat et al. 2004, Feng et al. 2006). Despite significant variations in the amino acid sequence of different mammalian RLN peptides, they all efficiently cross-activate human RXFP1 (Sherwood 2004, Bathgate et al. 2006). Recently, it was demonstrated that an analog of RLN peptide with a mutated receptor-binding domain has a moderately suppressive effect on prostate tumor when stably or transiently expressed in prostate cancer cells (Silvertown et al. 2006). With apparent limitations of an overexpression approach in mind, we decided to investigate the effect of direct RXFP1 knockdown in prostate cancer cells. The siRNA approach provided an opportunity to target RLN receptor specifically in prostate cancer cells, since the siRNA–RXFP1

Figure 6 Global transcriptional profiling of PC3 cells treated with siRXFP1 revealed genes previously shown to promote tumorigenesis. (A) Expression data matrix ('heat map') of 99 genes differentially expressed between siRXFP1 and siNC-treated PC3 cells. Each column represents a gene and each row a sample profile. The relative level of expression of each gene in each treatment group is represented using a yellow–blue color scale (black: no change; blue: low expression, bright yellow > 2-fold from control). Color bars above the matrix denote the corresponding annotation of genes using selected Gene Ontology (GO) terms. (B) RT-PCR validation of selected gene targets in PC3 cells 48 h after siRNA transfection. Expression differences between siRXFP1 and siNC were significant with $P < 0.05$ for each gene. Error bars denote $\pm$ s.e.m. Expression differences between siRXFP1 and siNC were significant with $P < 0.05$ for each gene.

construct utilized in this study had little homology with the mouse RXFP1 sequence. Indeed, the data showed specific downregulation of RXFP1 in human prostate cancer cells but had no effect on the expression of mouse RLN receptor. For siRNA delivery, we have used the recently developed approach of biodegradable CNNP (Howard *et al.* 2006, Katas & Alpar 2006, Dass & Choong 2008, Katas *et al.* 2008, de Martimprey *et al.* 2008). In these experiments we have directly injected nanoparticles into tumor tissues. Such an approach will be difficult to apply to a clinical setting; however, it allowed us to achieve maximum down-regulation of *RXFP1* target gene and provided a proof of principle that RXFP1 targeting may be clinically useful in prostate cancer. We have found that a single injection of siRXFP1 into established PC3 human prostate cancer xenografts in nude males lead to a 35 and 60% reduction in *RXFP1* mRNA 24 and 48 h after siRNA injection respectively. Bi-weekly injections of siRXFP1–CNNP into the tumors significantly slowed the growth of prostate adenocarcinoma xenografts. Already after two injections, tumors ceased to increase in volume and either shrank as in LNCaP cells or only grew slightly (PC3) by the end of third week. Previous observations had shown the *in vitro* suppressive effects

of impaired RLN signaling on migration, invasiveness, adhesion, and other characteristics affecting the dissemination of cancer cells (Klonisch *et al.* 2007). In this study, we show a significant reduction in metastasis rates in tumors treated with RXFP1–CNNP complexes in metastasis-prone PC3 cells *in vivo*. The siRXFP–CNNP group displayed a significantly reduced (42.3%) rate of cervical lymph node metastases as compared with the siNC–CNNP group, clearly implicating RLN–RXFP1 signaling pathways as important players in tumor cell metastasis in mice.

The broad range effects of RLN signaling on cancer cell proliferation, survival, apoptosis, ECM remodeling, and tumor angiogenesis are well-established (Silvertown *et al.* 2003, Klonisch *et al.* 2007). In the PC3 xenograft mouse model, suppression of RLN signaling leads to increased apoptosis and decreased cellular proliferation. The smaller tumors observed with siRXFP1–CNNP treatment were encapsulated in a thick picrosirius-positive layer indicating higher collagen deposition. We did not detect increased tumor vascularization in this model as has been reported previously in experiments that used overexpression of a mutant RLN peptide with antagonistic properties to suppress RLN signaling (Silvertown *et al.* 2007).

One possible explanation is that in our model RLN signaling was suppressed only in tumor cells but not in the host tissues as a result of the siRNA effectively and specifically depleting human *RXFP1*, with little or no effect on mouse *Rxfp1* transcripts. On the other hand, the peptide antagonist used in the previous study (Silvertown et al. 2007) might have suppressed RLN responses in both human tumor and mouse host cells and thus may have caused lower tumor vascularization and angiogenesis in that model. This may suggest the intriguing possibility of an active role of RLN signaling in modulating host cell–tumor cell interactions resulting in specific responses, including tumor progression and tumor angiogenesis.

It was shown previously that the short hairpin RNAs (shRNAs) or siRNAs may induce an interferon response in transfected cells (Bridge et al. 2003, Sledz et al. 2003, Pebernard & Iggo 2004). To exclude such nonspecific effects, the expression level of classical interferon target gene *OSAI* was measured in cells transfected with siRNA and in tumors treated with siNC/siRXFP1–CNP complexes. No differences were detected in the experiments with two cell lines. In addition, the microarray analyses performed on PC3 cells did not reveal significant upregulation in the expression of known target genes of the interferon pathway. The two siRXFP1 siRNAs did not affect the expression of host mouse *Rxfp1* gene in xenografts, indicating the absence of off-target effects.

The histological analysis of LNCaP tumor sections revealed that the majority of tumors treated with siRXFP1–CNP presented with extensive central necrosis suggesting the demise of tumor cells as a result of siRXFP1 nanoparticle uptake in close vicinity to the injection sites. Control siNC–CNP-treated tumors were mainly composed of densely packed epithelial tumor cells indicating that neither the CNP nor the siNC–CNP nanoparticles were toxic to the tumor cells. In conclusion, siRXFP1–CNP may be more effective in tumors derived from LNCaP cells causing more extensive necrosis. By contrast, PC3 cells are possibly more resistant to the actions of siRXFP1–CNP and this could explain the most subtle tumor tissue response observed in these prostate cancer cells. One of the major differences between two cell lines, PC3 and LNCaP, is the presence of active AR signaling in LNCaP cells. It was shown that RLN was acting through RXFP1 to stimulate androgen-independent growth of LNCaP cells by a mechanism that involved the activation of the AR signaling pathway. The specific mechanisms by which RLN facilitates tumor growth may be different in different cancer cells. Previously it was shown that PI3K/Akt

and the components of the Wnt pathway could be responsible for RLN-mediated activation of the AR pathway in androgen-responsive LNCaP prostate cancer cells (Liu et al. 2008). We have shown that in AR-negative PC3 cells the inactivation of the RLN receptor also caused a decrease in Ser308 Akt phosphorylation (Agoulnik AA, Feng S, unpublished data, 2009). It is likely, therefore, that the RLN effects on prostate cancer cells are mediated through both AR-dependent (Liu et al. 2008) and AR-independent pathways.

Analysis of differentially expressed genes following siRXFP1 treatment revealed several potential targets of RLN signaling in PC3 prostate cancer cells. Examples of these novel RLN-modulated genes uncovered by microarrays and confirmed by qRT-PCR in PC3 cells included several downregulated genes with well-known function in oncogenesis. This includes MUC1, a type 1 transmembrane protein, overexpressed in a nonpolarized manner in many tumor cells (Gendler 2001). MUC1 is generally thought of having anti-adhesive effects on cell–cell and cell–substrate interactions resulting in altered cell signaling, tumor growth, and metastasis (Wesseling et al. 1996, Singh et al. 2006). In prostate cancer, enhanced expression of MUC1 correlates with increased Gleason score (Burke et al. 2006). MUC1 activates PI3K/Akt signaling (Raina et al. 2004), which correlates with our observation that suppression of RXFP1 causes inhibition of Akt phosphorylation. Another interesting target is MUC18, a cell adhesion molecule of the immunoglobulin gene superfamily (Johnson et al. 1993). MUC18 has also been shown to be overexpressed in human prostate cancer (Wu et al. 2001a,b). MUC18 expression correlates with the progression of human prostate cancer and is an important mediator for the metastatic potential of human and mouse prostate cancer cells (Wu et al. 2001a,b, 2004, 2005). The TSPAN8 (tumor-associated antigen CO-029), a member of the transmembrane 4 (tetraspanin) superfamily, is associated with a poor cancer prognosis (Claas et al. 1998, Kanetaka et al. 2001, Gesierich et al. 2005, Kuhn et al. 2007). Tumor cell-derived ANGPTL4 disrupts vascular endothelial cell–cell junctions to facilitate the metastasis of tumor cells (Padua et al. 2008). GPI is thought to facilitate tumor cell invasion and metastasis (Funasaka et al. 2002). The downregulation of all these targets observed following silencing of RLN signaling further supports the potential of siRXFP1–CNP for prostate cancer therapy in men.

In summary, our data demonstrated that downregulation of RXFP1 RLN receptor by siRNA on

CNNP effectively suppressed tumor growth *in vivo* through increased apoptosis and decreased proliferation of PC3 cancer cells. Furthermore, the suppression of RLN signaling significantly reduced metastasis rates. We have identified several RLN responsive genes in prostate cancer cells with defined effects on tumor progression implicating RLN and its receptor RXFP1 as potential therapeutic targets for prostate cancer treatment.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-10-0073>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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