Suppression of Relaxin Receptor RXFP1 Decreases Prostate Cancer Growth and Metastasis

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Short Title: siRNA targeting of RXFP1 in prostate cancer

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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. Relaxin G protein-coupled receptor (RXFP1) is expressed in both androgen receptor 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 was 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, androgen receptor (AR) positive LNCaP cells and AR-negative PC3 cells. The tumors were established in nude male mice by subcutaneous injections. Intratumoral injections of siRNAs loaded on biodegradable chitosan nanoparticles led to a downregulation of RXFP1 receptor expression and a dramatic reduction of 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 of 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 down-regulation of MCAM, MUC1, ANGPTL4, GPI, and TSPAN8. Thus, the suppression of relaxin/RXFP1 may have potential therapeutic benefits in prostate cancer.
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 expression in males (Agoulnik 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 relaxin in various other physiological scenarios have recently been established. This includes 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 (Bathgate, et al. 2006; Sherwood 2004). There are two almost identical RLN genes in the genome of great apes and humans (RLNI 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 RLN genes are expressed in 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). Relaxin receptor RXFP1 (relaxin family peptide receptor 1) 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 Gs/Gi proteins and protein kinase A in some cells (Bathgate et al. 2006). In addition, the involvement of tyrosine kinases, protein kinase C zeta, PI3 kinase 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 (TIMPs), vascular endothelial growth factor (VEGF), IGF-BP1, hyaluronic acid, prolactin, laminin, nitric oxide synthase (NOS), 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 (Feng, et al. 2007; Kamat, et al. 2006). The in vitro invasiveness of carcinoma cells was significantly increased upon incubation with RLN or in cells transfected with relaxin expression constructs (Feng et al. 2007; Kamat et al. 2006; Silvertown, et al. 2006). 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 TRAMP (transgenic adenocarcinoma of mouse prostate) 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 coincides with an increase in RLN expression (Vinall, et al. 2006). It was suggested that relaxin might be negatively regulated by androgens in vitro and in vivo. Further, PI3K/Akt signaling and components of the Wnt pathway can facilitate relaxin-mediated activation of the androgen
receptor (AR) pathway (Liu, et al. 2008). On the other hand, siRNA-mediated
downregulation of endogenous RXFP1 expression in PC3 and LNCaP cells decreased cell
invasion and proliferation and increased cell apoptosis \textit{in vitro} (Feng et al. 2007).

Based on the available data, we hypothesized that the suppression of relaxin signaling in
prostate cancer cells might affect tumor growth and metastasis \textit{in vivo}. In the present study,
we analyzed the effect of relaxin receptor RXFP1 downregulation on prostate cancer
progression using an \textit{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 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.

\textbf{Materials and methods}

\textbf{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, Carlsbad, CA, USA) 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.

\textbf{siRNA transfection \textit{in vitro}}

In this project we employed two siRNAs targeting \textit{RXFP1} expression. The siRXFP1-0
GGAUGUCAAGUGCUCCCUUtt (sense strand), a highly efficient siRNA was identified
previously (Feng et al. 2007; Kamat et al. 2006). Another one was selected after testing four HP GenomeWide siRNAs (Qiagen, Valencia, CA, USA) 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 (Supplemental Fig. 1). The siRXFP1-0 and siRXFP1-5 were synthesized by Sigma-Genosys (Woodlands, TX, USA) 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 in 100 mm dishes 24 hrs before transfection. siRXFP1 and siNC (5 µg each) were transfected into cells using the Cell Line Nucleofector Kit V (PC3) or Kit R (LNCaP) with Nucleofector device (Lonza Group Ltd, Switzerland) according to the manufacturer’s instruction. Transfected cells were harvested after 48 hrs, total RNA was isolated for cDNA microarray assay and quantitative real time RT-PCR (qRT-PCR).

**Incorporation of siRNA into chitosan nanoparticles**

Chitosan nanoparticles are formed spontaneously upon the addition of aqueous tripolyphosphate (TPP) solution to chitosan solution under magnetic stirring at 1200 rpm and mixed for a further 10 minutes after addition of tripolyphosphate. siRNA was added to the TPP solution in a 3:1 w:w ratio under constant magnetic stirring (1200 rpm) at room temperature (RT). The chitosan preparation was then centrifuged at 9000g for 30 min at 5°C. Supernatants were discarded and the pellet containing the chitosan nanoparticles 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 chitosan nanoparticles (siNC-CNNP) and siRNA RXFP1 incorporated in CNNP (siRXFP1-CNNP).
Animal experiments

All experiments were conducted using the standards for humane 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 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 subcutaneously 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: volume = width\(^2\) × 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, 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 chitosan nanoparticles 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 CNNP was injected intratumorally twice a week. Mice with LNCaP tumors were treated for 21 days (7 injections) and sacrificed two days after the last treatment. Mice with PC3 tumors received a total of 6 injections and were killed 9 days after the last treatment. All mice were analyzed for the presence of macroscopic and microscopic metastasis in 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% PFA and embedded in paraffin. Prior to histological analysis, tissue sections (5μm) were deparaffinized in xylene and a descending alcohol series and stained with hematoxylin and eosin (HE). Staining with picrosirius red was performed to examine the density of collagen in the extracellular matrix (ECM) (EMS, Hatfield, 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 done by boiling slides in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 min. Nonspecific binding sites were blocked with 5% bovine serum albumin in PBS for 1 hr at RT. 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 hr at RT and incubated for 45 min with an appropriate HRP-conjugate/HRP-substrate staining (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). The CD34 mouse anti-human 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 1 or more CD34-
positive cells.

Eight random fields per section were scored at x400 magnification. Scoring was performed
by two independent researchers in a blind fashion. In LNCaP xenograft experiment 7
siRXFP1 and 10 negative control siNC tumors were analyzed. In 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 µg each) using the Amaxa Cell Line Nucleofector
Kit R (Lonza) according to the manufacturer’s manual and grown in poly-d-lysine-coated 6
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 /
0.3M glycine in 0.1% PBS-Tween for 1h to increase cell permeability and block non-specific
protein-protein interactions. The cells were incubated with the RXFP1 antibody (1:200)
overnight at +4°C. Detection was performed by using a Vectastain ABC kit. 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 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 quantitative RT-PCR (qRT-PCR) was performed according to a qPCR SybrGreen real time protocol on the IQ5 iCycler (BioRad, Hercules, CA, USA). Primers for different genes are listed in Supplemental Table 1. 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) [20]. 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 standard deviation 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 kit instructions. *In vitro* transcription was performed and biotinylated cRNA was synthesized during 14 hr 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, 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 hr, washed and incubated with streptavidin-Cy3 to detect biotin-labelled 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 ref. (Giordano, et al. 2003; Saldanha 2004) and expression patterns were visualized as a heat map using Java TreeView (Saldanha 2004).

Statistical analysis

Student \( t \)-test and ANOVA were used to assess significance of differences among the different groups. Differences were expressed as mean±S.E.M. with p-values smaller than 0.05 considered as statistically significant.

Results

Selection of siRNA and analysis of RXFP1 suppression \textit{in vitro and in vivo}

The expression of RXFP1 relaxin 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 \textit{in vitro} (Feng et al. 2007; Kamat et al. 2006). Additional 4 siRNAs obtained from Qiagen were tested in LNCaP cells (Supplemental Fig. 1A). All four constructs significantly downregulated the \textit{RXFP1} expression 48 hrs 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 (Supplemental Fig.1B).

We next assessed the efficiency of siRNA RXFP1 gene targeting in vivo using siRNAs incorporated into chitosan nanoparticles (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 subcutaneously into the flanks of nine nude male mice. When tumors became clearly visible at 14 days, a single intratumoral injection of siRXFP1-0-CNNP or siNC-CNNP was performed. 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 hr as compared to 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 (Mangala, Han, Lu, Ali-Fehmi, Munkarah, Spannuth, Armaiz-Pena, Nick, Lee, Shahzad, Ozturk, Sanguino, Denkbas, Birrer, Lopez-Berestein, Sood, unpublished data). 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 subcutaneous injection (sc) of 10^7 LNCaP cells per injection site. After 28 days, eighteen mice developed well-defined tumors and were divided into two groups with the same average tumor volume. Tumors were injected either with negative control siNC-CNNP complexes or with a mix of siRXFP1-0 and siRXFP1-5 siRNA-CNNP complexes twice a week (7 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 was 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 in siNC group (64% reduction, P=0.029. Fig. 2B). Examination of the mice at both the macro- and microscopic level did not reveal any metastases in the abdominal organs, cervical nodes, the thorax, or the brain in control or experimental animals. Histological analysis of the majority of tumors treated with 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 upon 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 and mouse RXFP1 gene expression in RNA isolated from primary tumors was performed using qRT-PCR. The specificity of human and mouse primers was confirmed using LNCaP RNA and the RNA isolated from mouse gubernaculum (data
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 siRNA delivery within the tumor. There was no difference in mouse relaxin receptor Rxfp1 expression in tumor RNA (Fig. 3A) confirming the specificity of siRXFP1. IHC with specific monoclonal anti-RXFP1 antibody revealed a significant reduction of 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; Pebernard and Iggo 2004; Sledz, et al. 2003). There was no difference in expression of OSA1 gene between control and experimental group at RNA level based on results of qRT-PCR analysis (data not show).

**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 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 one 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 that the RXFP1 suppression effect might be transient and reversible.

Upon termination of the experiments, the primary tumor weight in the siNC-CNNP group was 60% higher than 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 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 relaxin effects on cancer cells in vitro is an increased cell migration, matrix penetration, and cell adhesion (Klonisch et al. 2007). Alternatively, the suppression of relaxin or RXFP1 expression in cancer cells by siRNA downregulation caused decreased cell migration and invasiveness (Feng et al. 2007; Kamat et al. 2006). 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 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 siNC-CNNP group indicating that siRXFP1-0-CNNP treatment significantly downregulated 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 with 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 relaxin receptor, we performed the global gene expression analysis of ~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 to treatment with control siNC (data not shown). In the siRXFP1 treated group, 78 genes appeared down-regulated and 21 genes were up-regulated more than 1.3 fold (each RXFP1 profile was compared to each of the siNC profiles) (Fig. 6A, complete gene list in Supplemental Table 2). The 1.3 cut-off fold was determined based on qRT-PCR results. The array data was 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 angiopoietin-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 relaxin, a small peptide hormone produced both in the normal prostate and in prostate cancer (Feng et al. 2007; Figueiredo, et al. 2005; Liu et al. 2008; Silvertown et al. 2006; Thompson, et al. 2006; Vinall et al. 2006). Previously it has been shown that the relaxin expression was increased in aggressive metastatic disease (Feng et al. 2007; Figueiredo et al. 2005; Thompson et al. 2006), and that the stimulation of prostate cancer cells with relaxin accelerated their invasiveness, adhesion, survival and decreased cell apoptosis (Feng et al. 2007). Moreover, in the TRAMP mouse model transgenic overexpression of relaxin 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 relaxin receptor suppression on the growth of two commonly used prostate adenocarcinoma cell lines, androgen receptor positive LNCaP cells and androgen receptor negative PC3 cancer cells. The effect of RXFP1 down-regulation on tumor spread was also evaluated in PC3 model prone to metastasis. The targeted down-regulation of human RXFP1 in prostate cancer cell xenografts by means of specific siRNA against RXFP1 coated to chitosan nanoparticles resulted in significantly decreased tumor growth and metastasis rate in nude mice. The down-regulation of relaxin receptor RXFP1 in tumor cells may be a novel strategy in prostate cancer therapy.

Relaxin 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 relaxin by cAMP increase in cells transfected with RXFP2 (Feng
et al. 2007), however such cross-reactivity was not detected \textit{in vivo} (Feng, et al. 2006; Kamat, et al. 2004). Despite significant variations in the amino acid sequence of different mammalian relaxin peptides, they all efficiently cross-activate human RXFP1 (Bathgate et al. 2006; Sherwood 2004). Recently it was demonstrated that an analogue of relaxin 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 knock-down in prostate cancer cells. The siRNA approach provided an opportunity to target relaxin receptor specifically in prostate cancer cells, since the siRNA-RXFP1 construct utilized in the current 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 relaxin receptor. For siRNA delivery we used the recently developed approach of biodegradable chitosan nanoparticles (Dass and Choong 2008; de Martimprey, et al. 2008; Howard, et al. 2006; Katas and Alpar 2006; Katas, et al. 2008). In these experiments we 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 downregulation of RXFP1 target gene and provided a proof of principle that RXFP1 targeting may be clinically useful in prostate cancer. We 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 h 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 relaxin signaling on migration, invasiveness, adhesion, and other characteristics affecting the dissemination of cancer cells (Klonisch et al. 2007). Here, 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 to the siNC-CNNP group, clearly implicating relaxin-RXFP1 signaling pathways as important players in tumor cell metastasis in mice.

The broad range effects of relaxin signaling on cancer cell proliferation, survival, apoptosis, ECM remodeling, and tumor angiogenesis are well-established (Klonisch et al. 2007; Silvertown, et al. 2003). In the PC3 xenograft mouse model, suppression of relaxin 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 which employed over-expression of a mutant relaxin peptide with antagonistic properties to suppress relaxin signaling (Silvertown, et al. 2007). One possible explanation is that in our model relaxin 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 relaxin 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 relaxin signaling
in modulating host cell - tumor cell interactions resulting in specific responses, including
tumor progression and tumor angiogenesis.

It was shown previously that the shRNAs or siRNAs may induce an interferon response in
transfected cells (Bridge et al. 2003; Pebernard and Iggo 2004; Sledz et al. 2003). To exclude
such non-specific effects, the expression level of classical interferon target gene OSA1 was
measured in cells transfected with siRNA and in tumors treated with siNC/siRXFP1-CNNP
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.

Histological analysis of LNCaP tumor sections revealed that the majority of tumors treated
with siRXFP1-CNNP 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-CNNP treated tumors were mainly composed of densely packed epithelial
tumor cells indicating that neither the CNNP nor the siNC-CNNP nanoparticles were toxic to
the tumor cells. In conclusion, siRXFP1-CNNP 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-CNNP and this could explain the more subtle tumor
tissue response observed with these prostate cancer cells. One of the major differences
between two cell lines, PC3 and LNCaP, is the presence of active androgen receptor
signaling in LNCaP cells. It was shown that relaxin was acting through RXFP1 to stimulate
androgen independent growth of LNCaP cells by a mechanism that involved the activation of the androgen receptor signaling pathway. The specific mechanisms by which relaxin facilitates tumor growth may be different in different cancer cells. Previously it was shown that PI3K/Akt and components of the Wnt pathway can be responsible for relaxin-mediated activation of the AR pathway in androgen-responsive LNCaP prostate cancer cells (Liu et al. 2008). We have show that in AR negative PC3 cells the inactivation of relaxin receptor also caused a decrease in Ser308 Akt phosphorylation (Agoulnik, Feng, unpublished data). It is likely, therefore, that the relaxin 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 relaxin signaling in PC3 prostate cancer cells. Examples of these novel relaxin-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 non-polarized fashion 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 (Singh, et al. 2006; Wesseling, et al. 1996). 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; Wu, et al. 2001b). 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. 2005; Wu, et al. 2004; Wu et al. 2001a; Wu et al. 2001b).

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; Gesierich, et al. 2005; Kanetaka, et al. 2001; 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 (Glucose phosphate isomerase) is thought to facilitate tumor cell invasion and metastasis (Funasaka, et al. 2002). The downregulation of all these targets observed following silencing of relaxin signaling further supports the potential of siRXFP1-CNNP for prostate cancer therapy in men.

In summary, our data demonstrated that down-regulation of RXFP1 relaxin receptor by siRNA on chitosan nanoparticles effectively suppressed tumor growth in vivo through increased apoptosis and decreased proliferation of PC3 cancer cells. Furthermore, the suppression of relaxin signaling significantly reduced metastasis rates. We have identified several relaxin responsive genes in prostate cancer cells with defined effects on tumor progression implicating relaxin and its receptor RXFP1 as potential therapeutic targets for prostate cancer treatment.

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Figure legends:

Figure 1. Downregulation of RXFP1 expression by siRNAs *in vivo*. Intra-tumoral 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 hr after siRNA injection (n=3 in each group). Error bars denote ±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 µl of RPMI-1640:Matrigel B (1:1) were inoculated subcutaneously 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 (7 injections). Tumor volumes were measured by digital caliper. The difference in 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, ×400. Error bars denote ±S.E.M.

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 7 injections with siRXFP1-CNNP1 treatment (P=0.056). There was no downregulation of mouse Rxfp1 gene in host cells in xenografts. siNC-CNNP, n=10; siRXFP1-CNNP1, n=7. Error bars denote ±S.E.M. (B) Representative sections of siRNA-CNNP treated tumors
stained with anti-RXFP1 monoclonal antibody showing a significant reduction of RXFP1 expression in siRXFP1-CNNP treated xenografts. Magnification , ×400.

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 µl of DMEM/F12:Matrigel B (1:1) were inoculated subcutaneously 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 (6 injections). Tumor volumes were measured by digital caliper. The difference in tumor volume was significant (P<0.05) from days 6 to 23. (B) Reduction of tumor weight in the siRXFP1-treated group after resection at the end of experiment (P<0.05). (C) Decrease of metastasis rates in siRXFP1-CNNP treated animals. Metastases were exclusively detected in cervical lymph nodes shown above. The difference between siRXFP1-CNNP and siNC-CNNP group was statistically significant (P=0.0186). Yellow bars correspond to 200µm. (D) Small tumors in siRXFP1-CNNP treated group encapsulated in a thick collagen layer as shown with picrosirius red staining. Magnification , ×400. Error bars denote ±S.E.M.

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, x400) 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 200µm. Error bars
denote ±S.E.M.

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, 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 hr after siRNA transfection. Expression
differences between siRXFP1 and siNC were significant with P<0.05 for each gene. Error
bars denote ±S.E.M. Expression differences between siRXFP1 and siNC were significant
with P<0.05 for each gene.
References


Figure 1

- siNC
- siRXFP1-0 24hrs
- siRXFP1-0 48hrs

P<0.05

Expression of RXFP1/GAPDH
Figure 2

(A) Tumor volume (mm³) over time after first siRNA-CNNP injection. The black dots represent siRNA-CNNP and the red squares represent siRXFP1.

(B) Comparison of tumor weight (g) between siNC and siRXFP1. The graph shows a significant difference (P<0.05).

(C) Histological images of tumor sections stained with hematoxylin and eosin. The images are labeled siNC and siRXFP1.

(D) Immunohistochemical staining of the tumor sections with a marker of interest. The colors indicate the presence of staining.
Figure 3
Figure 4

A. Graph showing tumor growth rate over time for siNC and siRXFP1 treatments. The graph displays the tumor volume (mm³) over days after the first siRNA-CNNP injection. The x-axis represents the days after the first injection, ranging from 0 to 30. The y-axis shows the tumor volume from 0 to 2500 mm³. The graph includes error bars to indicate variability. A notable increase in tumor volume is observed for the siNC group compared to the siRXFP1 group, particularly after the last treatment.

B. Bar graph comparing tumor weight (g) between siNC and siRXFP1 treatments. The graph shows a significant difference in tumor weight, with the siRXFP1 group having a lower tumor weight compared to the siNC group (P<0.05).

C. Images of histological sections. The sections are stained with hematoxylin and eosin. The left image shows animals with lymph node metastases, while the right image shows animals without metastases.

D. Image of a histological section with immune staining. The section is stained with immune markers, showing cellular infiltration and tissue architecture.

C. Figure showing the total number of animals with lymph node metastases and without metastases for siNC and siRXFP1 treatments. The graph indicates that siRXFP1 treatment significantly reduces the number of animals with metastases compared to the siNC group (P=0.0186).
Figure 5

A

B

C

[Images of Ki67 positive cells and apoptosis stained cells for siNC and siRXFP1 conditions]

Graphs showing the percentage of Ki67 positive cells and apoptosis stained cells for siNC and siRXFP1 conditions.
Figure 6