

## Relaxin Promotes Prostate Cancer Progression

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**Abstract Purpose:** To understand the role of relaxin peptide in prostate cancer, we analyzed the expression of relaxin and its receptor in human prostate cancer samples, the effects of relaxin signaling on cancer cell phenotype *in vitro*, and the effects of increased serum relaxin concentrations on cancer progression *in vivo*.

**Experimental Design:** The relaxin and its receptor leucine-rich repeat containing G protein-coupled receptor 7 (LGR7) expression were studied by quantitative reverse transcription-PCR (11 benign and 44 cancer tissue samples) and by relaxin immunohistochemistry using tissue microarrays containing 10 normal and 69 cancer samples. The effects of relaxin treatment and endogenous relaxin/LGR7 suppression via short interfering RNA in PC-3 and LNCaP cells were analyzed *in vitro*. The effect of transgenic relaxin overexpression [Tg(*RLN1*)] on cancer growth and survival was evaluated in autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP).

**Results:** The relaxin mRNA expression was significantly higher in recurrent prostate cancer samples. In tissue microarrays of the 10 normal tissues, 8 had low staining in epithelial cells, whereas only 1 of 9 high-grade prostatic intraepithelial neoplasia lesions had low expression ( $P = 0.005$ ) and only 29 of 65 cancers had low expression ( $P = 0.047$ ). Stimulation with relaxin increased cell proliferation, invasiveness, and adhesion *in vitro*. The suppression of relaxin/LGR7 via short interfering RNAs decreased cell invasiveness by 90% to 95% and growth by 10% to 25% and increased cell apoptosis 0.6 to 2.2 times. The Tg(*RLN1*) TRAMP males had shorter median survival time, associated with the decreased apoptosis of tumor cells, compared with non-Tg(*RLN1*) TRAMP animals.

**Conclusions:** Relaxin signaling plays a role in prostate cancer progression.

Prostate cancer is the second most common malignancy in men in Western countries. After advancing to the androgen-independent stage, prostate cancer is unresponsive to androgen ablation therapy and is refractory (1). Identification of novel endogenous factors responsible for proliferation, survival, and migration of the prostate cancer cells may generate new therapeutic targets for treatment.

Relaxin is a short circulating peptide hormone (2, 3). Two highly homologous genes on human chromosome 9 encode RLN1 and RLN2 peptides with predicted 82% identity at amino acid level. In the corpus luteum, the main source of circulating relaxin in females, only RLN2 is expressed (2-4). Prostate is

the main site of relaxin expression in men (5-7). Relaxin, secreted from the prostate gland into seminal fluid, is the product of the RLN2 gene; however, the expression of both RLN1 and RLN2 genes has been detected in prostate at mRNA level (8). In nonprimates, there is only one orthologous RLN1 gene. Related peptides, such as RLN3 or INSL3, have a more restricted expression pattern (3). It has been shown that relaxin plays a role in connective tissue remodeling, suppression of fibrosis, dilation of blood vessels, and angiogenesis (2, 3). The relaxin receptor called leucine-rich repeat containing G protein-coupled receptor 7 (LGR7) or relaxin family peptide receptor 1 belongs to the same subfamily of G protein-coupled receptors as the glycoprotein hormone receptors (3, 9) and is expressed in the majority of tissues studied (10, 11). Recombinant RLN1 and RLN2 peptides activate LGR7 with the same efficiency. Additionally, it was shown that the human receptor for INSL3, LGR8, also binds RLN2 in 293 cells transfected with the receptor albeit with much lower affinity (6).

The ability of the RLN2 peptide to stimulate cancer cell growth has been shown *in vitro*. At low concentrations over a short period, relaxin promotes growth of breast adenocarcinoma cells (12, 13). Elevated relaxin serum concentrations were found in breast cancer patients, especially in patients with metastatic disease (14). Stimulation with RLN2 increases the invasiveness and migration of breast, endometrial, and thyroid adenocarcinoma cells *in vitro* accompanied by the up-regulation of matrix metalloproteinase activity and vascular

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endothelial growth factor expression, which are directly related to cancer progression (15–17). Similarly, adenoviral-mediated delivery of *prorelaxin 2* gene increases the invasiveness of canine breast cancer cells (18). The lentiviral delivery of RLN2 into PC-3 prostate cancer cells increases xenograft tumor growth (19). It was shown that RLN2 is a direct downstream target of R273H p53 mutation in prostate carcinoma cells (20), and relaxin expression is up-regulated by androgen withdrawal *in vitro* and *in vivo* (21).

In this study, we examined the expression of relaxin and *LGR7* genes in human samples of normal prostate and prostate cancer. The effects of relaxin treatment and endogenous relaxin signaling suppression on prostate adenocarcinoma cells were analyzed *in vitro*. We also conducted an *in vivo* study of tumor progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice with transgenic overexpression of *Rln1*.

## Materials and Methods

**Cell lines and reagents.** Human prostate carcinoma cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 and in F-12 nutrient mixture (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Porcine relaxin was kindly provided by Dr. O.D. Sherwood (University of Illinois, Urbana, IL). It was shown previously that the porcine relaxin stimulates human *LGR7* (9). Matrix metalloproteinase inhibitor FN439 was purchased from Calbiochem (San Diego, CA).

**Tissue acquisition.** The human investigations were done after approval by an institutional review board and in accordance with an assurance filed with and approved by the Department of Health and Human Services. Normal peripheral zone and cancer tissues for RNA analysis were collected from men undergoing radical prostatectomy for clinically localized prostate cancer by the Baylor Prostate Cancer SPORE Tissue Core and snap frozen. Benign tissues (11 samples) were confirmed to be free of cancer and cancer tissues contained at least 70% carcinoma. We analyzed 14 cancers with no evidence of prostate-specific antigen (PSA) recurrence after 5 years of follow-up and 30 cancers with PSA recurrence within 5 years of surgery. PSA recurrence was defined as serum PSA >0.2 ng/mL more than 30 days following the surgery. The differences in the clinical and pathologic characteristics of these cancers were consistent with known prognostic variables influencing biochemical recurrence in prostate cancer. In particular, recurrent cancers had higher preoperative PSA (16.5 ng/mL in recurrent versus 6 ng/mL in nonrecurrent), higher Gleason scores (7.3 versus 6.6), and more pathologically detected extracapsular extension (80% versus 44%), seminal vesicle invasion (48% versus 0%), and pelvic lymph node metastasis (38% versus 6%).

**Tissue microarrays and immunohistochemistry.** Two tissue microarrays with 2-mm tissue cores were used to study relaxin expression. The first array consisted of tissues from 10 radical prostatectomy cases with triplicate cores each representing normal peripheral zone, high-grade prostatic intraepithelial neoplasia, and cancer, respectively. The second array consisted of 69 prostate cancers from radical prostatectomies done for clinically localized cancer. In this array, only a single tissue core was punched from each case. Of these, 29 patients had a PSA recurrence within between 3 and 73 months following surgery (mean time to recurrence, 28.5 months). Thirty-six patients were recurrence-free with at least 48 months of follow-up (mean, 87.3 months) and 4 were lost to follow-up. Tissue microarray slides were cut at 4  $\mu$ m and subjected to immunohistochemistry. Briefly, antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) at 90°C for 25 min in a vegetable steamer. Anti-relaxin antibody (22), kindly provided by Dr. G.D. Bryant-Greenwood (University of Hawaii, Honolulu, HI), was incubated with each tissue array section at 1:50 dilution at room

temperature for 60 min followed by the antibody detection with a rabbit EnVision detection protocol (DAKO, Carpinteria, CA) and counterstaining with hematoxylin. Staining was evaluated in the normal glandular epithelium and prostate cancer as described previously (23, 24) taking into account the staining intensity and the extent of staining, yielding a 10-point tumor staining index ranging from 0 (no staining) to 9 (extensive, strong staining) for each case.

**Cell proliferation assay.** To assess the effect of relaxin or short interfering RNAs (siRNA) on cell proliferation, cells were seeded in 100  $\mu$ L of serum-free medium at a density of  $3.5 \times 10^3$  per well in 96-well plates. At 48 h, cell proliferation was measured with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) and the absorbance was read at 490 nm on a Bio-Rad microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA). All measurements were done in quadruplicate, and the experiments were repeated thrice.

**Cell adhesion assay.** Cells grown in 24-well plates to 80% confluence were washed once with PBS and twice with serum-free RPMI 1640 and then incubated with a varying concentrations of relaxin in serum-free medium at 37°C for 24 h. Cells were then detached with 0.25% trypsin-EDTA, washed with PBS, resuspended in serum-free medium with 0.5% bovine serum albumin, and seeded with a density of  $2 \times 10^4$  per well into 96-well plates precoated with type I collagen (Sigma, St. Louis, MO) and blocked with 1% bovine serum albumin. After incubation at 37°C for 1 h, unbound cells were washed away with PBS and the attachment of cells was analyzed with CellTiter 96 Aqueous One Solution Cell Proliferation Assay.

**Apoptosis.** Apoptosis in LNCaP and PC-3 cells was analyzed by sub-G<sub>0</sub>-G<sub>1</sub> phase cell fluorescence-activated cell sorting analysis. Seventy-two hours after siRNA transfection, cells were harvested, fixed in 70% ethanol, incubated for 30 min in 1 mL of PBS containing 50  $\mu$ g/mL propidium iodide and 20  $\mu$ g/mL DNase-free RNaseA (Sigma), and analyzed on an EPICS XL fluorescence-activated cell sorter (Beckman Coulter, Fullerton, CA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used for detection of apoptosis in mouse tumors using ApopTag In Situ Apoptosis Detection kit (Serologicals Corp., Norcross, GA). A total of 500 cells per field in eight random fields per section was scored at  $\times 400$  magnification. All analyses were done blindly [13 TRAMP/non-Tg and 18 TRAMP/Tg(*Rln1*) mice].

**Matrigel invasion assay.** BD BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA) were used for cell invasion assay as described before (16). The charcoal-treated media were used for LNCaP cells. After incubation for 48 h (LNCaP) or 36 h (PC-3), cells on the lower surface of the membrane were stained with Diff-Quik Stain Set (Dade Behring, Inc., Newark, DE). Cells were counted using a Nikon (Melville, NY) TMS inverted microscope equipped with Olympus (Center Valley, PA) DP70 digital imaging system. Five fields of view ( $\times 20$ ) taken at random were photographed, and cells were counted. All experiments were done thrice; each point was repeated in triplicate.

**Real-time quantitative reverse transcription-PCR.** Total RNA was extracted from cell cultures and human prostate and prostate cancer specimens using the RNeasy kit (Qiagen, Valencia, CA). Real-time quantitative reverse transcription-PCR (RT-PCR) for relaxin and *LGR7* gene expression was done using Taqman One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems, Foster City, CA) and the following primers and Taqman dual-labeled probe set: relaxin, 5'-CACCTGGAG-CAAAAGGTCTC-3' (forward); 5'-TGGTTCTGTATCTTTGTTGATGA-3' (reverse); and 5'-FAM-CTCCTCAGACACCTAGACCAGGTGGCA-GAAATT-TAMRA-3' (probe); *LGR7*, 5'-TGTCATTTGCATGCCACCTT-3' (forward); 5'-AGTCGGCACAGCAGAGAGAAA-3' (reverse); and 5'-FAM-AGGTCTGAGAACAAAGCTGTATGCCATGTCA-TAMRA-3' (probe). Relaxin amplicon detects both *RLN1* and *RLN2*. As a template, 100 to 400 ng of RNA solution were used for real-time quantitative RT-PCR using the iCycler instrument (Bio-Rad Laboratories). The amplification of the serial dilutions of relaxin cDNA RT-PCR fragment or *LGR7* plasmid DNA (*LGR7* expression construct) was used to provide the reference. All

data were normalized to  $\beta$ -actin RNA expression. Each point was done in quadruplicate and the means and SDs were calculated.

**LGR7 and RLN2 siRNAs.** The siRNAs (sense strand) used for *LGR7* gene silencing were GGAUGUCAAGUCUCCUUtt (exon 2, LGR7-1 siRNA) and GGCAGAAACACCUGAAUGUtt (exon 3, LGR7-2 siRNA), and the siRNAs (sense strand) used for *RLN2* gene were GAAUUU-GUUGCUAAUUUGCtt (RLN2-1 siRNA, second exon) and GGUCC-CAUCAAUUCUAGAtt (RLN2-2 siRNA, second exon; Ambion, Austin, TX). Silencer-negative control siRNA (Ambion) with no significant sequence similarity to human gene sequences was used as control. LNCaP and PC-3 cells were seeded in 12-well plate 24 h before transfection. siLGR7-1, siLGR7-2, and negative control siRNA (100 nmol/L each) were transfected into the cells with Silent siRNA Transfection kit (Ambion) following the manufacturer's instruction. After 24-h incubation, cells were used in invasion and proliferation assays. In parallel experiments, transfected cells were harvested after 48 h, the total RNA was isolated, and the level of *LGR7* mRNA was examined by quantitative RT-PCR. RLN2-1/2 siRNAs and negative control siRNA (9  $\mu$ mol/L each) were transfected into LNCaP cells using the Cell Line Nucleofector Kit R with Nucleofector device (Amaxa Biosystem, Gaithersburg, MD) according to the manufacturer's instruction. The experiments were done twice in quadruplicate.

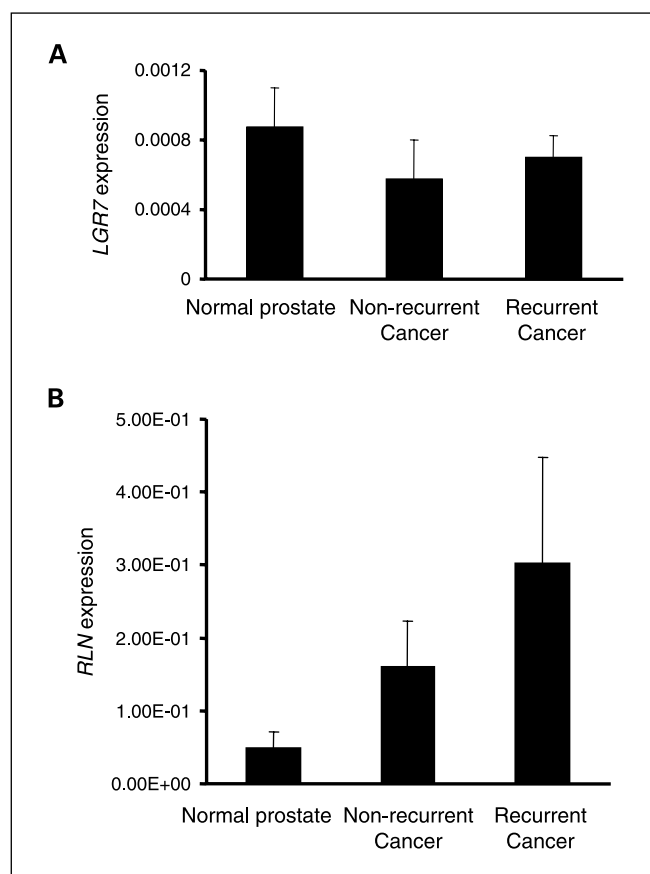
**ELISA assay for relaxin.** The concentration of the secreted relaxin produced by prostate cancer cells was determined in 100  $\mu$ L of conditioned medium using the Relaxin ELISA kit from ALPCO (Windham, NH) according to the manufacturer's protocol. The amount of relaxin in conditioned medium was normalized by cell number. The sensitivity of the assay is 2 pg/mL.

**Generation of TRAMP/Tg(*Rln1*) mice, necropsy, and survival analysis.** Production and characterization of mice with transgenic relaxin under rat insulin 2 promoter [Tg(*Rln1*)] was described previously (25). These mice have at least 20 times higher relaxin concentration in peripheral blood comparing with the nontransgenic controls. Tg(*Rln1*) hemizygous females (FVB background) were crossed to the TRAMP homozygous males (C57BL/6 background; ref. 26). All male progeny were kept until they met the euthanasia criteria as described in earlier studies (26). Some animals died unexpectedly without displaying obvious moribund signs. The experiment was terminated at 29 weeks, as it was shown previously that the majority of older TRAMP males had an endophytic growth of the tumors with an extensive invasion of the tumor into the seminal vesicles with a possible contribution of nonprostate tumorigenesis (27). The presence of Tg(*Rln1*) in mouse genomic DNA was determined by PCR (25). A full necropsy was done on all animals, which included a macroscopic and microscopic examination of all pelvic, abdominal, and thoracic organs and cervical lymph nodes. The classification of the differentiation of the primary tumor was based on previously described criteria (28). At the time of euthanasia, no specific lobes of the prostate can be separated in tumor. 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 Institutional Committee.

**Statistical analysis.** Student's *t* test and one-way ANOVA were used to compare the results between the different treatments. Kaplan-Meier survival plots were generated and compared with log-rank statistic using GraphPad Prism software (GraphPad Software, San Diego, CA).  $P < 0.05$  was considered statistically significant.

## Results

**Expression of relaxin and LGR7 in prostate cancer.** We first examined the combined expression of *RLN1* and *RLN2* and relaxin receptor *LGR7* in clinical samples of the normal prostate, nonrecurrent cancers, and recurrent cancers using quantitative RT-PCR (Fig. 1). The analysis showed that the relaxin receptor maintained the same level of expression both in normal tissues and in cancer samples (Fig. 1A). The expression



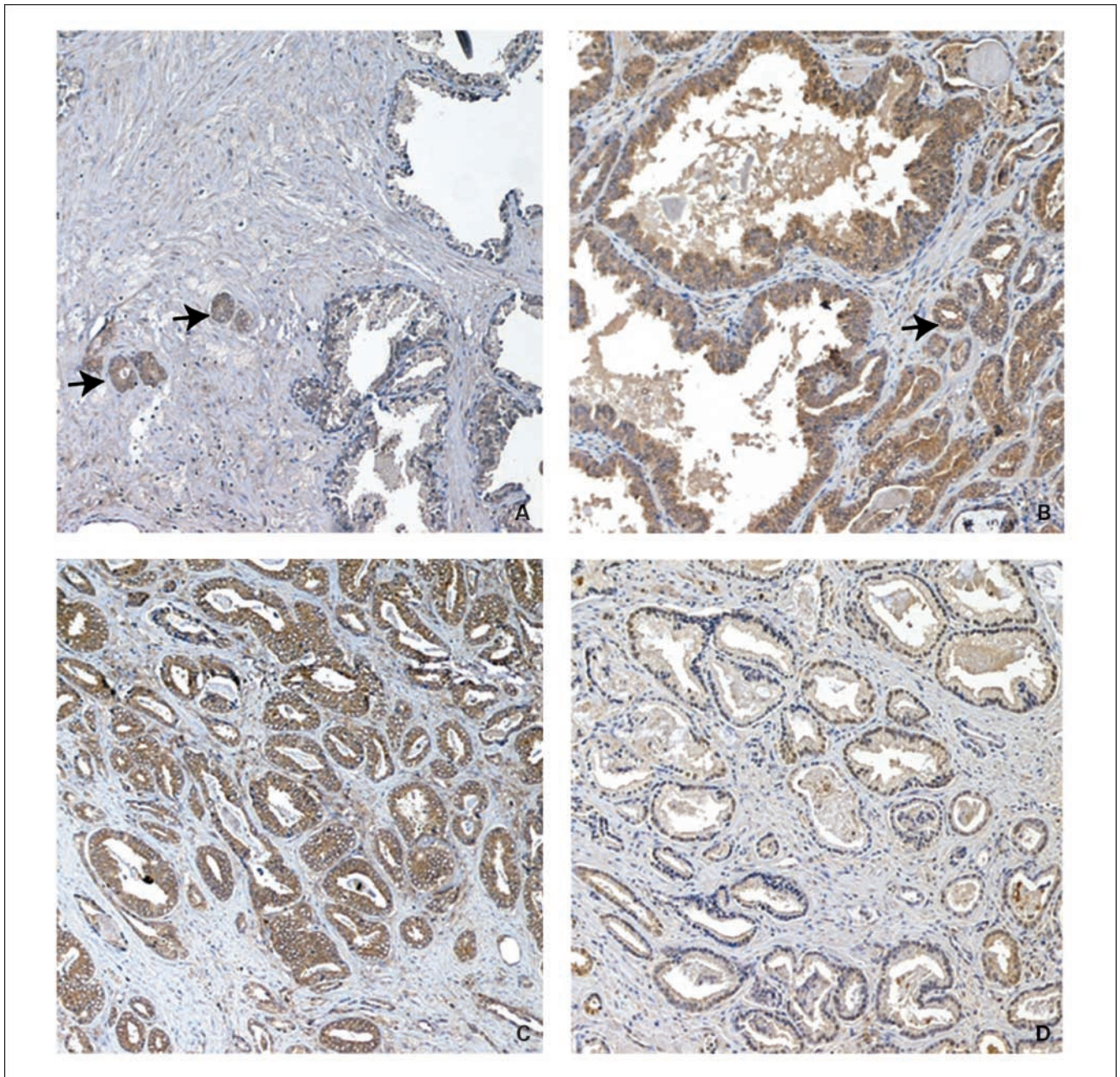
**Fig. 1.** Expression of the *LGR7* and *RLN* genes in normal prostate, nonrecurrent, and recurrent cancer samples. **A**, the *LGR7* expression is maintained at the same level in normal and cancer samples. **B**, there is statistically significant increase in the level of relaxin expression in recurrent prostate cancers compared with normal prostate. PCR primers for *RLN* were designed to amplify both *RLN1* and *RLN2* genes. The gene expression was normalized to  $\beta$ -actin expression. Columns, mean; bars, SE. \*,  $P < 0.05$ .

of the relaxin was significantly ( $P < 0.05$ ) higher in recurrent cancer samples compared with the normal tissues (Fig. 1B). We also analyzed the *RLN1* + *RLN2* and *LGR7* expression in the normal prostate (PNTIA) and prostate cancer (DU145, LAPC4, LNCaP, and PC-3) cell lines. All tested cell lines expressed similar levels of *LGR7*; however, there was a distinct difference in the expression of the relaxin genes. The highest level of relaxin gene expression was detected by quantitative RT-PCR in LNCaP cells, and the lowest level was detected in PC-3 cells (0.04% of LNCaP level; data not shown). These two cell lines were used in subsequent experiments. Relaxin was readily detectable in the conditioned medium from the LNCaP cells (Fig. 4B, bottom) by specific anti-relaxin ELISA assay. However, no relaxin was detected in the PC-3 medium by this method, consistent with the results obtained by quantitative RT-PCR.

The increase in relaxin expression detected by quantitative RT-PCR might be in part attributed to the increased proportion of cancer versus host cells in the tissue samples. To confirm an increased expression of relaxin at the protein level in cancer cells, we analyzed the expression of relaxin protein in tissue microarrays by immunohistochemistry using a specific anti-human RLN2 antibody (22). Relaxin expression was present in both normal glandular epithelial cells and cancer cells as reported previously (5, 6). However, there was significant

variability in expression between samples (Fig. 2). No staining was detected in samples treated with control rabbit IgG used at the same concentration as anti-RLN2 primary antibody (data not shown). Overall, 10 normal peripheral zone tissues, 9 high-grade prostatic intraepithelial neoplasia lesions, and 65 cancers gave assessable results, with some cases lost due to tissue depletion or technical artifact. Of the 10 normal tissues, 8 had low RLN2 staining in epithelial cells (index  $\leq 3$ ; Fig. 2A). In contrast, only 1 of 9 high-grade prostatic intraepithelial neoplasia lesions and 29 of 65 cancers had low RLN2 expression. This difference was statistically significant for both

high-grade prostatic intraepithelial neoplasia ( $P = 0.005$ ) and cancer ( $P = 0.047$ ). We then examined the association of high RLN2 expression (staining index of 9 versus  $\leq 6$ ) with clinical and pathologic variables of aggressive disease in all cases for which such data were available. There was a significant association between high RLN2 staining index and extracapsular extension (6 of 6 positive for index 9 cases versus 23 of 47 for index  $< 9$ ;  $P = 0.027$ ) and seminal vesicle invasion (4 of 6 index 9 cases positive versus 8 of 47;  $P = 0.019$ ). These two pathologic variables, particularly seminal vesicle invasion, are associated with more aggressive disease (29). There was no

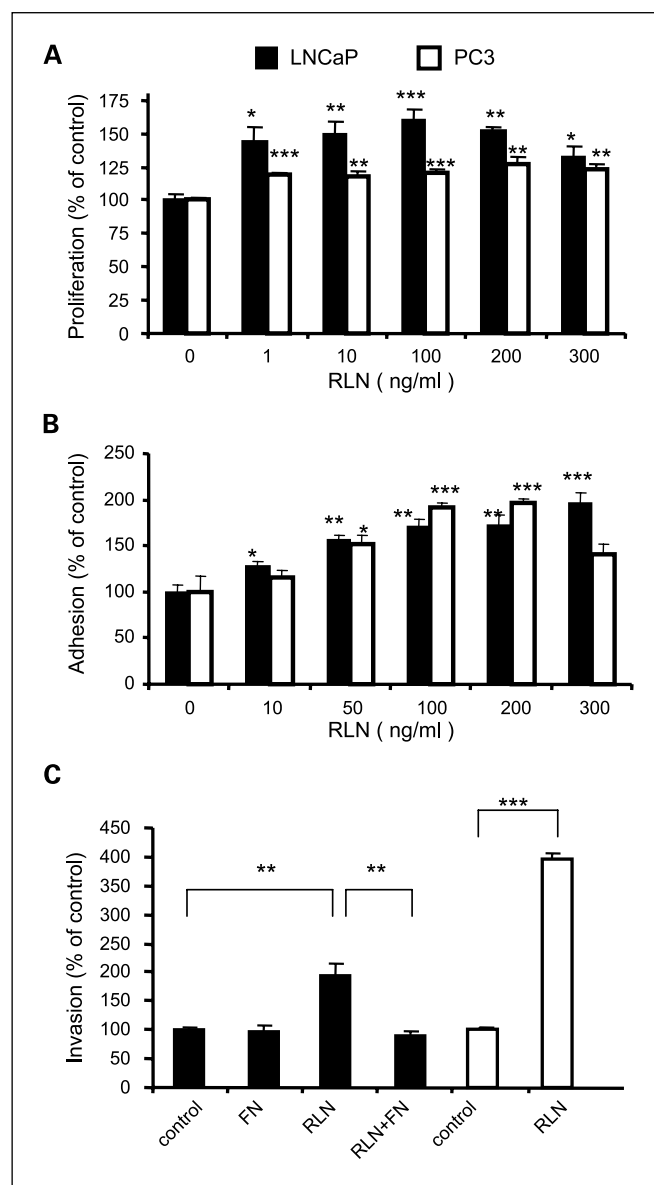


**Fig. 2.** Immunohistochemistry with anti-relaxin antibody. *A*, benign epithelium with low relaxin expression. Arrows, note strongly staining, infiltrating cancer cells. *B*, high-grade prostatic intraepithelial neoplasia with high relaxin expression (*left*) and adjacent infiltrating cancer glands with similar expression (*arrow*). *C*, cancer with high relaxin expression. *D*, prostate cancer with low relaxin expression. Original magnification,  $\times 100$ .

statistically significant association with Gleason score (Gleason 5/6 versus  $\geq 7$ ;  $P = 0.16$ ) or PSA recurrence ( $P = 0.19$ ). It should be noted that the recurrent cancers analyzed by quantitative RT-PCR described above included a much higher proportion of early PSA recurrences (<1 year) and thus were more highly aggressive cancer overall than the cancers analyzed by immunohistochemistry. Thus, RLN2 was more often expressed at higher levels in prostate cancer, particularly in aggressive prostate cancers.

**Effects of relaxin on prostate cancer cells.** To investigate the relaxin effects on prostate cancer cells *in vitro*, we analyzed cell proliferation, adhesion, and invasiveness after addition of relaxin to the culture medium. In all experiments, we used native porcine relaxin, shown to activate human receptor (9), and both androgen-responsive LNCaP and androgen receptor-negative PC-3 human prostate adenocarcinoma cells. We first analyzed the effect of relaxin on cell proliferation after 48 h of incubation in serum-free medium. Relaxin at concentrations as low as 1 ng/mL had a significant effect on net cancer cell proliferation compared with untreated controls (Fig. 3A). We evaluated the effect of relaxin on cell adhesion to type I collagen after preincubation with different concentrations of relaxin and detected a clear dose-dependent response (Fig. 3B). Previously, it was reported that relaxin increased the invasive potential of different cell types, including breast, thyroid, and endometrial cancer cells *in vitro*, mediated by matrix metalloproteinases (15–17). The data shown in Fig. 3C indicate a significant increase in invasiveness of relaxin-treated LNCaP and PC-3 cells in the Matrigel invasion assay. The addition of matrix metalloproteinase inhibitor FN439 completely negated the relaxin-induced increase in LNCaP cell invasiveness.

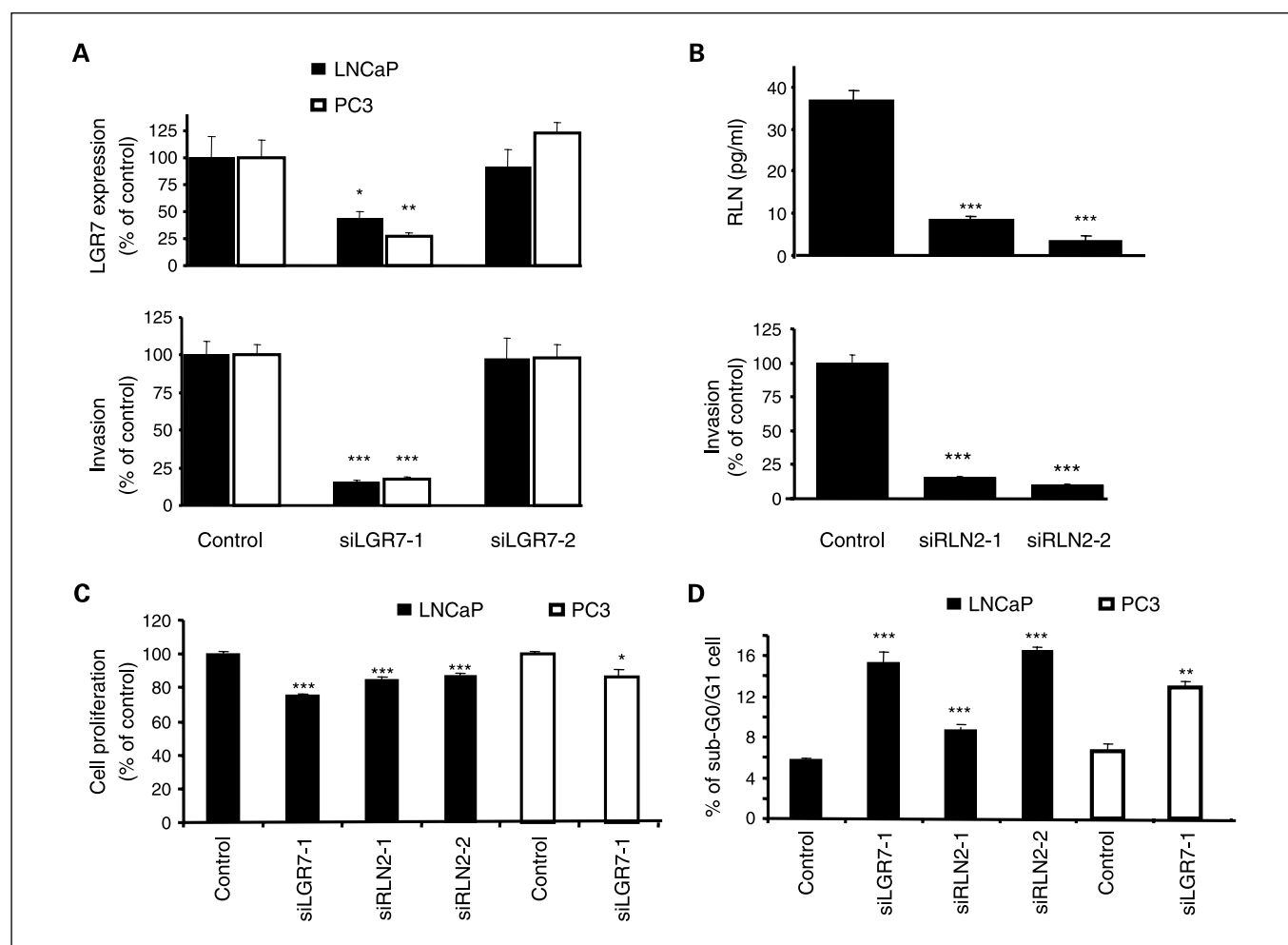
**Suppression of RLN2 or LGR7 reduces invasion and proliferation and increases apoptosis of prostate cancer cells.** We analyzed the effect of endogenous relaxin signaling suppression using siRNA against RLN2 and LGR7. Two different LGR7 siRNA constructs were tested (Fig. 4A). One of the constructs (siRNA-LGR7-1) was highly effective in reducing the RNA expression in both LNCaP and PC-3 cells. The invasive potential of the cells transfected with this siRNA decreased as much as 90% (Fig. 4A). The noncoding control or inactive siLGR7-2 did not have any effect on cell invasion or proliferation. It was established previously that the prostate gland secretes only RLN2 peptide into the seminal fluid (4). Two anti-RLN2 siRNA were used in LNCaP cells. As shown in Fig. 4B, both constructs were efficient in reducing total relaxin protein expression as evaluated in the conditioned medium using relaxin ELISA. This assay can detect both RLN1 and RLN2. Using gene-specific quantitative RT-PCR, we showed that the level of both RLN1 and RLN2 transcripts was decreased after siRNA treatment (data not shown). Suppression of relaxin expression reduced cell invasion by as much as 95% after 40 h. There was a modest but significant inhibitory effect of RLN2 and LGR7 reduction on cell proliferation after 48 h (Fig. 4C). Importantly, even after normalization to the 10% to 15% reduction in cell proliferation, the decrease in cell invasion after siRNA treatment was highly significant (in all cases,  $P < 0.001$ ). Finally, we evaluated cell apoptosis after depletion of LGR7 or relaxin using flow cytometry. The sub-G<sub>0</sub>-G<sub>1</sub> population of cells was measured by fluorescence-activated cell sorting analysis. The suppression of LGR7 or relaxin was highly effective in



**Fig. 3.** Relaxin effects on prostate cancer cell characteristics. **A**, dose-dependent effects of relaxin on proliferation of LNCaP and PC-3 cells. Cell proliferation was evaluated after 48 h of incubation with indicated amounts of relaxin (RLN; ng/mL). Proliferation as the percentage of control (no relaxin). The difference is significant for 1 to 300 ng/mL relaxin. **B**, relaxin causes dose-dependent increase of LNCaP and PC-3 cell adhesion to type I collagen. Cells treated with various amount of relaxin for 24 h were seeded into plates precoated with type I collagen. After incubation at 37°C for 1 h, the attachment of cells was assayed with CellTiter 96 Aqueous One Solution Cell Proliferation Assay. **C**, relaxin enhances the invasive potential of prostate cancer cells. An invasion of LNCaP and PC-3 cells through a Matrigel membrane increased following relaxin (100 ng/mL). Addition of matrix metalloproteinase inhibitor FN439 (FN; 0.3 mmol/L) abrogated relaxin effects on LNCaP cell invasiveness. Cell invasion under different conditions as percentage of control. Combined results of two to three independent experiments. Columns, mean; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

increasing cell apoptosis (Fig. 4D), which shows the anti-apoptotic role of relaxin signaling in cancer cells.

**Overexpression of relaxin decreases the survival of TRAMP mice.** Recently, we have produced transgenic mice with Rln1 overexpression (25). The expression of transgenic Rln1 was directed by the insulin 2 promoter in pancreatic cells, with subsequent secretion into the blood (25). To evaluate the effect



**Fig. 4.** Suppression of *LGR7* and relaxin gene expression by siRNA decreases prostate cancer cell invasiveness and proliferation and increases cell apoptosis. **A**, effect of two *LGR7* siRNAs on invasiveness of LNCaP and PC-3 cells. Transfection with siLGR7-1 construct caused significant reduction of *LGR7* mRNA expression (*top*), which correlated with a decrease of cell invasiveness (*bottom*). The expression of *LGR7* was normalized to  $\beta$ -actin expression. **B**, the suppression of relaxin expression in LNCaP cells by two siRNA constructs caused significant reduction of relaxin protein expression (*top*) associated with the decrease of cell invasiveness (*bottom*). Relaxin content in cell conditioned medium was measured with relaxin ELISA and normalized to the cell number. **C**, the suppression of *RLN2* and *LGR7* expression caused decrease of prostate cancer cell proliferation. LNCaP cells were transfected with *LGR7* and *RLN2* siRNAs, and PC-3 cells were transfected with *LGR7* siRNA. Cell number was determined 48 h after transfection as a percentage of the control. Experiment was repeated twice in triplicates. **D**, the suppression of *RLN2* and *LGR7* expression induced apoptosis in prostate cancer cells. LNCaP cells were transfected with *LGR7* and *RLN2* siRNAs, and PC-3 cells were transfected with *LGR7* siRNA. Seventy-two hours after transfection, the sub-G<sub>0</sub>-G<sub>1</sub> population of cells was detected by fluorescence-activated cell sorting analysis. Columns, mean; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  versus control.

of relaxin on prostate tumors, we generated TRAMP males with and without *Rln1* transgene. A total of 40 males with clearly identifiable tumors died or met the criteria for euthanasia within 29 weeks. Six males were found dead without exhibiting these criteria. The presence of the *Rln1* transgene was determined only after euthanasia to prevent bias in selection for euthanasia. Given that euthanasia was required by our institutional criteria for mice with palpable tumors  $\geq 10\%$  of body weight, most of the mice were sacrificed based on tumor size, and indeed, the mean tumor weight (3.44 g) was almost exactly 10% of body weight (34.3 g). Thus, differences between the transgenic and nontransgenic mice were more reflective of primary tumor size. The Tg(*Rln1*) TRAMP mice ( $n = 21$ ) with a lethal tumor had a median survival time of 153 days, whereas non-Tg TRAMP animals ( $n = 19$ ) had median survival time of 182 days. Kaplan-Meier analysis of the survival of the TRAMP animals showed that the presence of Tg(*Rln1*) significantly ( $P = 0.02$ ) decreased the survival time compared with control

(Fig. 5A). There was no significant difference in the total body weight, weight of the primary tumor, number of metastases, or histologic grade of the tumors in both Tg(*Rln1*) and non-Tg animals in either euthanasia group (data not shown).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used for detection of apoptosis in 13 non-Tg and 18 Tg(*Rln1*) early TRAMP tumors (Fig. 5B). The results indicate that an increased level of serum relaxin caused a decreased rate of apoptosis in prostate carcinomas. The cell proliferative index in the tumor measured by Ki-67 staining did not reveal significant differences between the two groups (data not shown). Thus, the relaxin overexpression caused the decrease of survival in TRAMP mice with prostate tumors.

## Discussion

The combined results of increased relaxin expression in human prostate cancer samples; the ability of relaxin to

stimulate effect of cancer cell invasion, proliferation, and adhesion; the sharp reduction of cancer cell invasiveness and the increase of apoptosis of prostate cancer cells after relaxin and *LGR7* gene knockdown; and, finally, the antiapoptotic and tumor growth-promoting role of relaxin *in vivo* clearly indicate the importance of relaxin signaling in progression of prostate cancer.

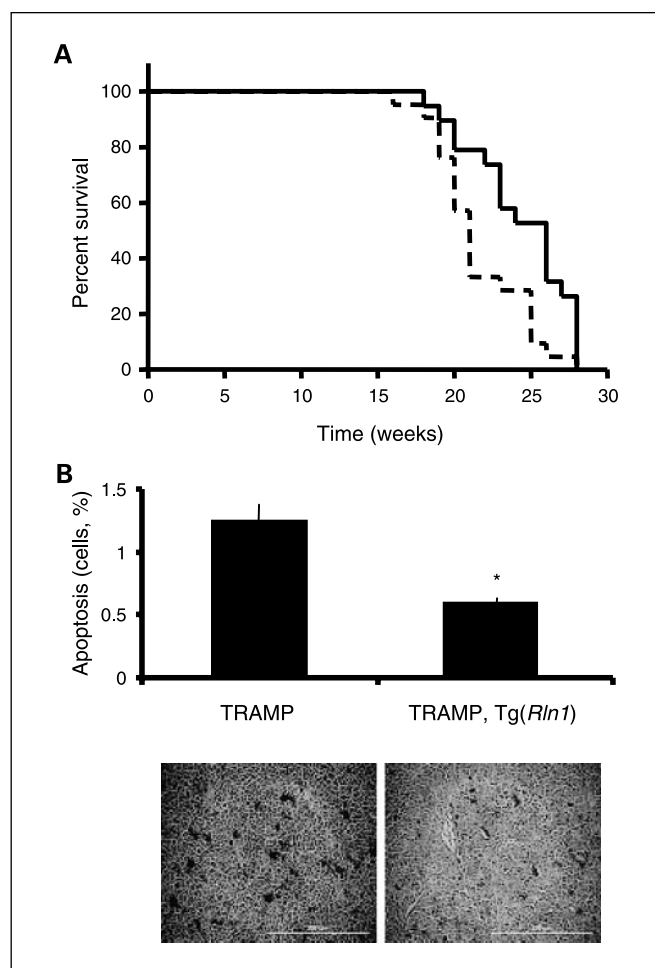
The expression of relaxin was previously detected specifically in normal glandular epithelium of adult prostate gland (4, 5), commonly believed to be a primary source of the cells giving rise to the majority of prostate tumors (30). In addition to relaxin, normal prostate cells express its receptor *LGR7*, suggesting a possible autocrine signaling of this hormone. The analysis of gene expression in clinical cancer samples showed that, whereas the expression of *LGR7* was maintained at the same level as in normal prostate, the expression of relaxin often increased in cancer, particularly aggressive prostate cancer. These data correspond to our previous findings of

increasing relaxin expression in endometrial cancer (16), increased serum levels of relaxin in patients with metastatic breast cancer (14), and recently reported increase of relaxin expression in prostate cancer after androgen ablation (21). Thus, both an increase in the number of relaxin-producing epithelial cells and an increase of the relaxin expression per cell might be responsible for the overall increase of the hormone production. Further analysis of the patient samples and the underlying mechanisms of relaxin expression regulation is required.

What are the biological consequences of the relaxin action on prostate cancer cells? The stimulation of human prostate adenocarcinoma cell lines with relaxin promoted the proliferation and survival of the cancer cells, supporting previously noted role of this peptide as a growth factor (2, 18, 19). We have shown that stimulation of LNCaP and PC-3 cells with relaxin at concentrations close to hormone levels detected in human seminal fluid (7) increased cell growth. Similar to other peptides of the insulin/relaxin family (31, 32), relaxin caused an increase in cell adhesion of the prostate cancer cells as reported in thyroid cancer cells (17).

As prostate cancer cells express both relaxin and its receptor, the autocrine/paracrine action of this peptide might be more essential than its endocrine functions. To verify this hypothesis, we targeted endogenous relaxin signaling in prostate cancer cells via siRNA. The efficiency of the targeting was confirmed at the RNA level for *LGR7* and relaxin and at the protein level for relaxin. Whereas inactivation of relaxin signaling only slightly affected the proliferation of the cells, the effect on cell migration through the Matrigel was very strong. Additionally, we showed that the suppression of relaxin signaling led to a significant increase in cancer cell apoptosis. Notably, both androgen-sensitive LNCaP cells and androgen receptor-negative PC-3 cells responded similarly to modulation in relaxin signaling. In summary, these experiments indicate that relaxin signaling may play a major role in survival and dissemination of prostate cancer cells.

The conclusions about the significance of relaxin in prostate cancer progression received further support in transgenic mouse studies. We have previously produced mice with transgenic pancreatic overexpression of mouse relaxin that had significantly elevated levels of hormone in peripheral blood (25). We showed here that an increased level of relaxin promoted tumor progression in the TRAMP mouse model of prostate cancer. The elevated level of relaxin caused a significant reduction of the median survival time by 29 days. The primary criterion for euthanasia was the tumor size, and thus, the differences seen in this group reflected primary tumor growth rates. The level of cancer cell apoptosis in the tumors of *Tg(Rln1)* transgenic mice was lower than that in control TRAMP cancers, confirming the previously described antiapoptotic effect of this hormone on prostate cancer cells *in vitro* and consistent with the promotion of net tumor growth. It should be noted that the level of apoptosis in analyzed advanced cancers was rather small, and thus, the possible antiapoptotic relaxin effects might have a greater role at earlier stages of tumorigenesis. Additional functions of relaxin, such as stimulation of angiogenesis (19), might have also contributed to cancer growth in this group. Despite the clear evidence of the induction of metastasis-related characteristics in cancer cells *in vitro*, we did not find an increased number of distant metastases in the relaxin transgenic



**Fig. 5.** *A*, Kaplan-Meier analysis of survival for TRAMP mice with or without relaxin transgene, *Tg(Rln1)*. Male with *Tg(Rln1)* transgene had an increased level of serum Rln1. Solid line, non-*Tg* mice; hatched line, *Tg(Rln1)*. The difference between the two groups is statistically significant ( $P = 0.0213$ ). *B*, apoptosis analysis in prostate TRAMP cancers in mice with and without *Tg(Rln1)*. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used to evaluate the number of apoptotic cells on cancer tissue sections. The results show the means of apoptosis cell percentage for each group of mice. Bottom, black staining, representative microscopic views with apoptotic cells. Columns, mean; bars, SE. \*\*\*,  $P < 0.001$ .

group. One can speculate that, perhaps, the aggressive nature of the TRAMP cancer can mask some of the effects of relaxin on tumor dissemination especially in mice with fast-growing exophytic primary tumors. In summary, we have shown that the increased level of serum relaxin was associated with more rapid progression of prostate cancer *in vivo*. However, it should be noted that, because relaxin was expressed at a distant site (the pancreas) in Tg(*Rln1*) transgenics, the relaxin reached the prostate via the bloodstream and the actual *Rln1* levels within the prostate may not have been as high as if the relaxin were expressed within the prostate itself. Indeed, recently published data indicate that the *RLN2* overexpression in prostate cancer cells may significantly increase *in vivo* xenograft tumor growth (19).

Our findings about relaxin in prostate cancer raise two important clinical issues. First, can serum relaxin be used as

additional prognostic marker in men with prostate cancer? This would be expected based on our studies but needs to be directly examined in retrospective and prospective studies. Second, our studies suggest that the suppression of relaxin signaling may be used as a potential treatment modality in managing this disease. Further studies using these and other approaches should help to evaluate the role of relaxin signaling in prostate cancer progression and its potential as a therapeutic target.

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