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TITLE: Functional Validation of H2 Relaxin, and its Downstream Effectors, as Mediators, Therapeutic Targets and Potential Biomarkers of Prostate Cancer Progression

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14. ABSTRACT The purpose of this study was to investigate the mechanism by which H2 relaxin mediates castrate resistant (CR) prostate cancer (CaP), and to determine whether H2 relaxin can be used as a biomarker to identify CaP progression. Elucidating the mechanisms by which prostate cancer disease progression occurs is critical to the development of new treatments for this disease, while the identification of a biomarker will facilitate the detection of CaP progression. We have demonstrated H2 relaxin (RLN2) facilitates castrate resistant (CR) growth of prostate cancer (CaP) cells through PI3K/Akt/ β -catenin-mediated activation of the androgen receptor (AR) pathway. As inhibition of this pathway caused only ~50% reduction in CR growth, we set out to identify additional RLN2-activated pathways that contribute to CR growth. Inhibition of PKA attenuated RLN2-mediated AR activity, inhibited proliferation and caused a small but significant increase in apoptosis. Combined inhibition of the PKA and NF κ B signaling pathways via inhibition of PKA and Akt induced significant apoptosis and dramatically reduced clonogenic potential, outperforming docetaxel, the standard of care treatment for CR CaP. Immunohistochemical (IHC) analysis of tissue microarrays (TMA) in combination with multispectral quantitative imaging comparing RLN2 levels in patients with BPH, PIN and CaP determined that RLN2 is significantly upregulated in CaP vs BPH (p=0.002). In summary, our data indicate that simultaneous inhibition of PKA and NF- κ B would prevent RLN2 mediated cell survival in CaP, and that in a setting of elevated RLN2 expression this combined inhibition is superior to docetaxel. The number of patients who would potentially benefit from this study is likely to be extensive since a significant portion of CaP patients overexpress RLN2.					
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INTRODUCTION

Currently, castrate resistant prostate cancer (CRPC) remains incurable. The identification of novel pathways that promote castrate resistant growth of prostate cancer (CaP) cells is critical for the development of successful new therapies to treat CaP. Our group has identified H2 relaxin as a facilitator of CRPC (1). We have demonstrated that blocking H2 relaxin expression, or expression of LGR7, the H2 relaxin receptor, can inhibit castrate resistant growth, and that the H2 relaxin signaling pathway in part mediates CRPC by promoting interaction between β -catenin and the androgen receptor (AR) (2). Our hypothesis is that H2 relaxin, and its downstream effectors, represent therapeutic targets for use in the prevention and treatment of CRPC, and that H2 relaxin itself can potentially be used as a biomarker to predict CaP progression in a subset of CaP patients. To test this hypothesis we proposed the following specific aims: (1) To further elucidate the mechanism(s) by which the H2 relaxin mediates CRPC, (2) To functionally validate H2 relaxin-mediated CaP progression to CRPC using *in vivo* models of CaP, and to use *in vivo* models of CRPC to determine whether blocking expression H2 relaxin is a viable treatment option for CRPC, (3) To establish the usefulness of H2 relaxin as a biomarker for progression to CRPC.

BODY

Task 1: To further delineate the pathway(s) by which H2 relaxin mediates AI CaP.

H2 relaxin induces activation of Akt and the phosphorylation of GSK-3B

To understand the mechanism whereby H2 relaxin activates AR, we first looked for GPCR signals elevated in LNCaP-R273H, as compared to wild type LNCaP. After preliminary screening with phospho-specific antibodies against different signal molecules, we found that the phosphorylation of Akt (Ser473) was consistently elevated in the LNCaP-R273H subline (Figure 1). The data are in agreement with the ability of the G_{sa} subunits of GPCR to activate PI3K/Akt (1, 2), and the recent report that H2 relaxin activates PI3K in THP-1 cells (3, 4). We noted that a basal level of phospho-Akt was detected in parental LNCaP, presumably due to the inactivating mutation of Pten in this cell line (5). The combined effect of H2 relaxin expression and Pten deficiency resulted in the hyperactivation of Akt in LNCaP-R273H. There are several down-stream targets of Akt. We found that the inhibitory-phosphorylation of GSK-3B at serine 9 is elevated in LNCaP R237H cell line (Figure 1).

H2 relaxin induces Akt and GSK-3B phosphorylation in LNCaP cells

LNCaP cells were treated with H2 relaxin (100ng/ml) for 0.5, 1, 3 and 6 hours, and the levels of phospho-Akt and phospho-GSK-3B were assessed to determine whether H2 relaxin could increase the phosphorylation of these molecules in a time dependent manner. Phosphorylation of Akt at Thr 308 peaked at 30 minutes after H2 relaxin treatment. Phosphorylation at Ser 473 occurred as soon as 30 minutes post-treatment and peaked at 180 minutes. Phosphorylation of GSK-3B Ser 9 followed a similar kinetics (Figure 2). These data provide strong evidence that H2 relaxin is able to activate the Akt pathway.

H2 relaxin increases B-catenin levels and the association of B-catenin with AR

GSK-3B is a kinase which causes destabilization of B-catenin by phosphorylating it at Ser33, 37 and Thr41 (6). The phosphorylated B-catenin is then degraded by the ubiquitin/proteasome pathway. Phosphorylation of GSK-3B at Serine 9 reduces the activity of GSK-3B resulting in decreased degradation of B-catenin and the subsequent accumulation of B-catenin in the cytoplasm and translocation into the nucleus (7, 8). Western blot analysis showed that treatment of LNCaP cells with H2 relaxin for 0.5 h resulted in decreased phosphorylation of B-catenin and a corresponding increase of the B-catenin protein level (Figure 3A). Increasing evidence suggests that stabilized B-catenin serves as a co-activator of AR (9-12). To determine whether H2 relaxin-stabilized B-catenin can interact with AR protein extracts from LNCaP treated with or without H2 relaxin were immunoprecipitated with an AR antibody, followed by SDS-polyacrylamide gel electrophoresis, and Western blot analysis with an antibody to B-catenin. Figure 3B showed that treatment of LNCaP with H2 relaxin increased the level of association between B-catenin and AR. Immunochemical analysis substantiated the above result and showed that H2 relaxin treatment of LNCaP cells increased not only B-catenin accumulation in the cytoplasm but also AR and B-catenin co-localization in the nucleus (Figure. 3C).

H2 relaxin induces the recruitment of B-catenin to PSA promoter.

We next proceeded to determine whether H2 relaxin induced B-catenin/AR complex was recruited to relevant promoters targeted by AR (e.g. PSA promoter). ChIP assay was employed for this analysis. We analyzed both the proximal (P) ARE I/II and the distal enhancer (E) ARE III sites for potential binding to the B-catenin/AR complex. The region in between, H, was used as a negative control. Interestingly, H2 relaxin induced B-catenin binding to the proximal (P) region of the PSA promoter, but not to the PSA enhancer (E) region or the PSA H region (Figure 4). By contrast, DHT induced the recruitment of B-catenin to both the P and E sites. This is consistent with reports by us and others that post-translationally activated AR, unlike androgen bound receptor, only binds the proximal ARE and not the distal ARE (13, 14).

LY294002 blocks both H2 relaxin -induced phosphorylation of Akt and GSK-3B and the co-localization of AR and B-catenin in the nucleus

To confirm that PI3K is involved in H2 relaxin-mediated AR activation, we used the PI3K inhibitor LY294002 to block PI3K/Akt activation. We hypothesized that inhibition of the PI3K/Akt pathway would prevent the interaction of AR and B-catenin in LNCaP R273H cells. Indeed, the association and co-localization of B-catenin and AR could be blocked by treatment with LY294002 (Figure 5A), even in the presence of 200ng/ml H2 relaxin (data not shown). To determine whether this observation was due to LY294002-induced changes in the intracellular level of B-catenin protein, we examined both AR and B-catenin protein levels using Western blotting. LY294002 treatment did not significantly affect AR protein level, but inhibited the accumulation of B-catenin induced by H2 relaxin (Figure 5B). These results together indicate that inhibition of PI3K can suppress H2 relaxin-mediated association of AR and B-catenin through decreasing B-catenin protein level. Consistent with the decrease in B-catenin protein levels, we found that H2 relaxin-mediated serine9 phosphorylation of GSK-3B proteins was also impaired by treatment with LY294002 in a dose-dependent manner (Figure 5C). Likewise, complete inhibition of H2 relaxin-induced Akt phosphorylation at sites thr 308 and ser 473 was observed in samples treated with LY294002 at a concentration above 12.5uM. The total amount of Akt protein remained the same in the presence or absence of LY294002 (data not shown).

B-catenin siRNA blocks H2 relaxin-mediated AR activation.

Our previous results suggest that H2 relaxin is able to influence the AR signaling pathway. Transient transfection assays were performed to further investigate the possible effect of B-catenin on AR-mediated transcription. Plasmids capable of expressing a PSA luciferase reporter and a B-catenin specific siRNA were transfected into LNCaP cells. H2 relaxin treatment induced a nearly 1.5-fold increase in transactivation in LNCaP cells. Co-transfection of the B-catenin siRNA decreased AR activity induced by H2 relaxin to base line (Figure 6). These data indicate that B-catenin is a critical component in H2 relaxin-mediated AR activation. Conversely, we were able to show that overexpression of B-catenin can directly augment the transcriptional activity of AR, using a B-catenin expression construct (15). As seen in Figure 6, elevated levels of B-catenin enhanced both basal as well as H2 relaxin-mediated AR transcription through the PSA promoter.

Generation of LNCaP sublines that stably overexpress H2 relaxin.

To understand the functional significance of increased RLN2 expression in CaP, we stably transfected LNCaP with plasmid expressing the RLN2 gene. LNCaP are an androgen dependent CaP cell line, and are the cell line that was used for all our previous RLN2-related studies (16, 17). Two clones (LNCaP-RLN2/C1 and LNCaP-RLN2/C2) were chosen for further investigation of the role played by RLN2 in CaP. These sublines expressed ~20-fold higher levels of RLN2 mRNA relative to LNCaP stably transfected with the vector control (LNCaP-vector) (Figure 7A), $p < 0.005$ for both LNCaP-RLN2/C1 and /C2 versus LNCaP-vector. LNCaP-R273H, an androgen independent LNCaP subline that stably expresses a p53^{R273H} mutant allele, expressed the highest level of RLN2 mRNA, ~60-fold higher than the androgen dependent LNCaP-vector subline ($p < 0.0005$), and was used as a positive control. Note that triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. We have previously demonstrated that the p53^{R273H} mutant, which is a hotspot mutation in CaP patients, can bind to the RLN2 promoter and drive RLN2 expression [44]. In the present study, the increase in RLN2 expression was observed at both the mRNA level (as determined by qRT-PCR) and at the protein level (as determined by immunocytochemistry in the same cell lines) (Figure 7B). It is noteworthy that the LNCaP-RLN2 sublines appear to be good models of CR CaP as they behave similarly to the CaP cells found in CR CaP patient tumors; they are able to grow in the absence of androgens and express normal levels of AR but high levels of PSA even in the absence of androgen, i.e. the AR pathway can be activated in a ligand independent manner.

Identification of downstream effectors of RLN2 using Next-Generation Sequencing (NGS) analysis.

To help identify downstream effectors of RLN2 and to further elucidate the role played by RLN2 in CaP, we conducted next-generation DNA sequencing (NGS) analysis (i.e. RNA-Seq) followed by GO analysis using LNCaP-RLN2/C1 and LNCaP-RLN2/C2 vs LNCaP-vector sublines. Comparison of gene sequence analysis (Figure 2C) revealed 12.7% of genes with >1.5-fold increased expression in the LNCaP-RLN2 sublines relative to the LNCaP-vector were related to proliferation. Other key processes associated with increased RLN2 expression were transcription (18.6%), metabolism (16.4%), signal transduction (11.7%) and proteolysis (6.2%) (Figure 7C). As expected, NGS analysis determined that the LNCaP-RLN2 sublines express high levels of RLN2 compared to the LNCaP-vector subline (350-fold and 582-fold increased expression in the LNCaP-RLN2/C1 and -RLN2/C2 sublines respectively). Table 1 lists the top 10 differentially expressed genes in the RLN2 versus vector control sublines. It is of note that H1 relaxin (RLN1) is also expressed at high levels in the

RLN2 sublines. While the RLN1 isoform has been shown to be expressed in the prostate at the mRNA level, only the RLN2 is translated and secreted (18-23) indicating that upregulation of RLN1 expression in the RLN2 sublines likely has no functional consequence. Expression of PSA (KLK3, 8-fold and 22-fold increased expression in LNCaP-RLN2/C1 and -RLN2/C2 respectively), a downstream target of the AR, and Cyclin D1 (CCND1, 1.44-fold and 1.2-fold increased expression), an important cell cycle regulator, was increased in the RLN2 sublines. Expression of TRAF1 and C-IAP1, which can be driven by NF κ B, were also overexpressed (TRAF1, 2.01-fold and 3.08-fold increased expression, C-IAP1, 1.47-fold and 1.5-fold increased expression) (Table 1). Both of these molecules have been shown to promote cell survival.

To verify the observations made with the NGS/GO analysis, we demonstrated that stable expression of RLN2 increased PSA levels, but not AR expression, in LNCaP cells, confirming that RLN2 affects AR transcriptional activity but not expression (Figure 7D). These results thereby validate the use of these clones as a model for determining RLN2 function in CaP. Increased expression of RLN2 has been observed during neuroendocrine differentiation (NED) of CaP cells (24), a process that is associated with the development of CR CaP in CaP patients (25). Our data also indicate that H2 relaxin is associated with NED, and thereby further support a role for H2 relaxin in progression to CR CaP. The LNCaP-RLN2/C1 and -RLN2/C2 sublines express increased levels of NSE and decreased levels of NEP compared to LNCaP-vector (Figure 7C). Neuron specific enolase (NSE) is a key marker of NED (review; (26)) and increased expression is associated with CaP progression [19]. Neural endopeptidase (NEP) is an enzyme that is expressed at high levels by normal CaP cells and is responsible for degrading neuropeptides such as bombesin, ET-1 and neurotensin that promote NED (27, 28). NGS analysis revealed that mRNA levels of NSE and NEP were also altered in the LNCaP-RLN2 sublines, 9.532-fold and 18.796-fold increase in NSE in LNCaP-RLN2/C1 and -RLN2/C2 respectively, and a 2.544-fold and 1.668-fold decrease in NEP expression. Since cyclin D1 and several survival-related genes appeared to be upregulated in H2-relaxin overexpressing cells (Table 1), we determined whether RLN2 overexpression stimulated cell numbers. MTT assay verified that RLN2 overexpressing sublines have a significantly increased rate of cell growth in culture medium containing charcoal stripped serum (containing castrate levels of androgens) compared to vector transfected LNCaP cells (Figure 7E, statistical analysis compared the day 5 data for each subline and revealed a significant difference between the LNCaP-vector compared to all LNCaP-RLN2/C1 and /C2 as well as LNCaP-R273H, $p < 0.005$). These observations are supported by our previous observations indicating ligand independent activation of the AR by RLN2 (17). It should be noted that increased AR activity and increase cell proliferation are not characteristics that associated with NED. Our data indicate that the LNCaP-RLN2 sublines have some NED-like characteristics they are clearly not NE cells. While this is somewhat unusual, other groups have reported similar findings. For example, Snail induces NSE and chromogranin A expression in LNCaP as well as mediating nuclear translocation of AR and increased PSA expression (29).

RLN2 promotes activation of an NF- κ B-dependent cell survival pathway in LNCaP prostate cancer cells.

Since the NGS/GO analysis demonstrated an increase in TRAF1 and C-IAP1, which can be driven by NF κ B, in LNCaP sublines overexpressing RLN2, we also investigated the activation of NF κ B in these cells. The NF- κ B subunits (p65, p50) remain bound to I κ B- α in the cytoplasm; upon stimulation, I κ B- α is degraded and p65/p50 released, which then translocates to the nucleus, and helps transcribe anti-apoptotic genes such as Bcl-xL (30). To determine whether the NF κ B pathway is active in the RLN2 LNCaP sublines we assessed I κ B- α expression

levels and phosphorylation state, NF κ B localization, and binding of NF κ B to its DNA consensus sequence. I κ B- α levels were significantly lower in the RLN2 LNCaP sublines and I κ B- α was phosphorylated indicating active degradation of I κ B- α occurs in these cells (Figure 8A). Increased expression of Bcl-xL, a downstream effector of NF κ B, was also observed in the LNCaP sublines overexpressing RLN2. Immunofluorescence analysis of the LNCaP-rlx sublines revealed a significant increase in levels of nuclear NF κ B compared to LNCaP-vector (Figure 8B). LNCaP treated with TNF- α were used as a positive control for nuclear staining. We also demonstrate that RLN2 is able to facilitate binding of the NF κ B p65 subunit to its DNA consensus sequence (Figure 8C). Significantly increased binding was observed in both LNCaP-vector treated with recombinant human (rh) RLN2 and in the RLN2 LNCaP sublines relative to the LNCaP-vector only control ($p < 0.05$ for all 3 LNCaP sublines compared to LNCaP-vector). These studies indicate that activation of NF- κ B is an important mediator of RLN2-mediated cell survival, and points to a mechanism by which RLN2 may induce CR CaP.

It is known that RLN2 signals via the G protein-coupled receptor (GPCR) RXFP1 in CaP cells. To determine whether the effects of RLN2 on NF- κ B are mediated by RXFP1, we investigated the effect of RXFP1 knockdown on NF- κ B activity. RXFP1-mediated activation of NF- κ B could be inhibited using siRNA specific to RXFP1, the RLN2 receptor (Figure 8D), indicating that the effects of RLN2 on NF- κ B are indeed mediated by RXFP1.

RLN2 stimulates cAMP production and PKA activation independent of NF- κ B.

Activation of the H2 relaxin receptor RXFP1 by RLN2 activates the G_s class of G-proteins (G_{sa}), resulting in cyclic AMP (cAMP) dependent PKA activation. Hence, we investigated whether RLN2s effects are mediated by the PKA pathway in CaP cells. Previous studies performed in other cell types have demonstrated that RLN2 causes activation of the adenylate cyclase/AMP/PKA pathway (31, 32). Our results validate these findings in CaP cells. Treatment of parental LNCaP with 10, 50 and 100 ng/ml recombinant human RLN2 (rhRLN2) induced a significant increase ($p < 0.005$), ~2.2 and 2.1-fold increase in cAMP activity respectively compared to a 3.2-fold forskolin-induced response (positive control, $p < 0.005$) (Figure 9A). However, this increase was transient as shown by the decrease in cAMP levels after 30 minutes of treatment. Similarly, treatment of parental LNCaP with 50 ng/ml RLN2 induced a ~3.3-fold increase in PKA activity ($p < 0.005$) compared to a ~4.9-fold forskolin-induced increase (positive control, $p < 0.005$) (Figure 9B). On the other hand, co-treatment with the PKA inhibitor H89 was able to completely inhibit this response to RLN2 indicating the assay is PKA specific (Figure 9B). Inhibition of PKA using H89 also caused a decrease in growth rate in both LNCaP-RLN2/C1 and -RLN2/C2 cultured in CSS media - in LNCaP-RLN2/C1 a ~1.8-fold decrease in growth rate was observed ($p < 0.05$), and in LNCaP-RLN2/C2 a ~2.1-fold decrease ensued (Figure 9C, $p < 0.05$). Inhibition of the PKA pathway did not directly affect the activation of NF- κ B (data not shown), indicating that the PKA and the NF- κ B pathways represent two different arms of the signaling mechanisms downstream of RLN2 (Figure 9F). Inhibition of PKA had a more dramatic effect on PSA expression (Figure 9D, E). Treatment with H89 caused a ~7-fold decrease in PSA levels in LNCaP-RLN2/C1 ($p < 0.005$) and a ~5.6-fold decrease in LNCaP-RLN2/C2 ($p < 0.05$). It is of note that the RLN2 LNCaP sublines express very high levels of PSA even when cultured in castrate conditions. These data indicate that H2 relaxin induces the activation of the AR signaling pathway and

cell growth in a ligand independent manner by a mechanism mediated by the activation of the cAMP/PKA pathway.

RLN2 overexpression confers resistance to treatment with therapeutic agents.

Activation of the NF- κ B pathway has been frequently associated with drug resistance. Since RLN2 induces an increase in NF- κ B activity, we investigated whether RLN2 expression is also associated with resistance to various therapeutic drugs. Annexin V/propidium iodide (PI) labeling followed by flow cytometry analysis to investigate the effects on apoptosis showed that LNCaP cells transfected with vector only are highly susceptible to induction of apoptosis by various inhibitors including LY294002 (PI3K inhibitor), perifosine (Akt inhibitor), rapamycin (mTOR inhibitor) and docetaxel (anti-mitotic), whereas the LNCaP-RLN2/C1 and -RLN2/C2 sublines are more resistant to treatment with the same drugs (Figure 10A). Perifosine, rapamycin and docetaxel are all clinical agents. Perifosine has been shown to reduce PSA levels in 20% CaP patients with recurrent disease (33). Several on-going clinical trials are testing the efficacy of rapamycin, and analogs of rapamycin alone and in combination with other agents (review; (34)). Docetaxel is the standard of care treatment for CaP patients with castrate resistant CaP (review; (35)). For LNCaP-vector, treatment with vehicle control, LY294002, perifosine, rapamycin and docetaxel induced ~10, 38, 22, 37, 29% apoptosis respectively (Figure 10A), but was reduced in LNCaP-RLN2/C1 (4, 20, 12, 13, 9%) and LNCaP-RLN2/C2 (1, 4, 10, 2, 1.5%). The levels of apoptosis in LNCaP-vector were statistically higher compared to those observed in the LNCaP RLN2 sublines regardless of the type of drug treatment ($p < 0.05$). These data indicate that there is a link between RLN2 expression and chemoresistance in LNCaP cells, and provide rationale for combining targeted inhibition of the RLN2 pathway with conventional chemotherapy.

Combined treatment with perifosine and a PKA inhibitor in CaP cells overexpressing RLN2 promotes apoptosis.

IKK causes phosphorylation of I κ B- α and subsequent proteasome-mediated degradation. This degradation allows NF κ B to translocate to the nucleus. Hence an IKK inhibitor would inhibit the activation of the NF- κ B pathway. Perifosine is known to be an Akt inhibitor; however, it inhibits NF- κ B activation to the same extent as the IKK inhibitor (Figure 10B). As perifosine has been FDA approved and is currently in clinical trials for the treatment of CaP, we investigated whether its effects on NF- κ B would be of significance in the treatment of patients who overexpress RLN2 and may have developed resistance to commonly used drugs as a result. Hence we compared the effects of perifosine to that of the IKK inhibitor (Figure 10B,C). Inhibition of IKK in the LNCaP-RLN2/C1 and -RLN2/C2 sublines did not cause a significant increase in apoptosis or decrease in clonogenic potential (Figure 10C), whereas perifosine alone caused only a moderate increase in apoptosis and decrease in clonogenic potential (~2-fold increase and ~40% decrease respectively, Figure 10C). Similarly, simultaneous blockade of IKK and Akt resulted in only a modest increase in apoptosis and decrease in clonogenic potential compared to treatment with perifosine alone (Figure 10C). We concluded that the lack of a clinically relevant increase with dual blockade is due to the fact that both the IKK inhibitor and perifosine are acting on the same target, either directly or indirectly (see scheme in Figure 9F) resulting in decreased binding of NF κ B to its DNA consensus sequence (Figure 10B).

We hypothesized that simultaneous blockade of pathways leading to PKA and NF- κ B would therefore be the only way to completely block signaling downstream of RLN2-induced cell proliferation and survival (based on scheme in Figure 4F). H-89 alone had little or no effect on the RLN2 overexpressing clones (Figure 10D); in addition, inhibition of either IKK or PKA caused only a moderate increase in the sensitivity of the RLN2 LNCaP sublines to treatment with docetaxel (Figure 9C,D). However, dual inhibition of both arms of the RLN2/RXFP1 pathway, with H-89 and perifosine, resulted in a larger and significant increase in apoptosis compared to blockade of either individual pathway (~2-3-fold increase in apoptosis compared to treatment with perifosine alone, ~15-18% apoptosis in the combination treatment, Figure 10D), and compared to treatment with docetaxel (docetaxel induced only ~2-3% apoptosis in the LNCaP-RLN2 sublines, Figure 10D). It is of note that this dual inhibition induced a similar level of apoptosis in the LNCaP-RLN2 sublines (15-18%) as docetaxel treatment in the LNCaP-vector subline (~25%). A significant decrease in clonogenic potential was also observed (~20-30% decrease compared to treatment with either the IKK inhibitor or perifosine alone (Figure 10D). Taken together, these results indicate that overexpression of RLN2, which is commonly seen in tumors from patients with CaP, provides a growth advantage to CaP cells by causing activation of both the NF- κ B and PKA pathways. A near complete inhibition of this growth advantage can be achieved only by simultaneous blockade of both pathways.

Task 2: To functionally validate H2 relaxin-mediated AI CaP growth using in vivo models of CaP, and to use in vivo models of AI CaP to determine whether blocking expression H2 relaxin and/or its downstream effectors is a viable treatment option for AI CaP.

Several attempts were made to generate mice bearing LNCaP-RLN2 xenograft tumors using the LNCaP sublines that were stably transfected with H2 relaxin. While some tumors were observed, the tumor take rate (~50%) was decided to be too low to justify the proposed experiments in which components of the H2 relaxin pathway would be blocked and the effect on tumor growth determined.

Task 3: To establish the usefulness of H2 relaxin as a biomarker for progression to AI CaP.

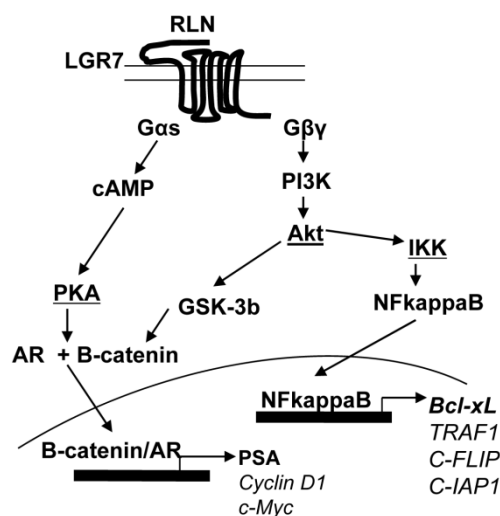
RLN2 is elevated in CaP patient specimens relative to benign prostatic hyperplasia (BPH).

To determine the significance of RLN2 in human prostate cancer (CaP) patients, we conducted IHC analysis of 49 CaP, 15 PIN and 24 BPH specimens taken from patients with primary CaP undergoing prostatectomy (for PIN and CaP specimens) or TURP (for BPH) as initial treatment for the disease (Figure 11A). Note that RLN2 stained the epithelial cells strongly while some staining could also be seen for the stromal cells. However, the nuclei did not stain for this peptide hormone at all, demonstrating the specificity of the staining. Specifically, the staining level increased from BPH<PIN=CaP indicating the increased accumulation of RLN2 during initiation of CaP. Quantification of staining using the Nuance multi-spectral imaging system demonstrated that RLN2 expression is significantly higher in CaP specimens relative to the BPH specimens (Figure 11B, 0.119 $\pm$ 0.032 versus 0.95 $\pm$ 0.021, $p=0.002$), a finding that has not previously been reported. The increase of RLN2 in CaP compared to BPH is of clinical relevance because in patients the serum levels of PSA increase in both cases. Since RLN2 can be detected in the serum (36), this means that the serum levels of this peptide could potentially be used as an independent marker of CaP as opposed to BPH. Further studies are required to test this

hypothesis. A significant difference in RLN2 expression was not observed between the PIN and CaP specimens ($p=0.475$) and RLN2 expression did not correlate with Gleason grade (Figure 11C). Specimens from CaP patients with CR disease were not assessed due to lack of availability, however, other studies have observed very high H2 relaxin expression levels in bone metastases specimens from CR CaP patients (37). Quantitative RT-PCR (qRT-PCR) analysis and RLN2 IHC was used to assess relative expression of RLN2 in “normal-like” RWPE-1 and multiple CaP cell lines (Figure 11D, E). Note that triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. Excluding PC3, the aggressive tumors C4-2 and CWR22Rv1 expressed significantly higher levels of RLN2 compared to the RWPE-1 cells ($p<0.05$) derived from a normal human prostate and compared to the PC-346C cells ($p<0.05$) derived from an androgen-dependent CaP thereby supporting our hypothesis that RLN2 plays a role in progression to CR CaP.

KEY RESEARCH ACCOMPLISHMENTS

1. Elucidation of H2 relaxin signaling pathway in prostate cancer



2. Generation of LNCaP that stably express H2 relaxin
3. Identification of treatment strategy that blocks H2 relaxin-mediated castrate-resistant growth

REPORTABLE OUTCOMES

Published abstracts:

1. Ruth Vinall, Ibitola Asaolu, Xu-Bao Shi and Ralph deVere White. H2 relaxin facilitates castrate resistant growth of prostate cancer cells by a mechanism that involves nuclear translocation of NFκappaB. American Association for Cancer Research Special Conference; Advances in Prostate Cancer Research. 2009 Jan 21-24th; San Diego, CA.
2. Ruth Vinall, Shangqin Liu, Hsing-Jien Kung and Ralph deVere White. H2 relaxin can influence the

expression of molecules associated with neuroendocrine differentiation. Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA.

Generation of cell lines:

LNCaP that stably express H2 relaxin (LNCaP-rlx#3 and LNCaP-rlx#5).

Published papers:

1. Liu S, Vinall RL, Tepper C, Shi XB, Xue LR, Ma AH, Wang LY, Fitzgerald LD, Wu Z, Gandour-Edwards R, deVere White RW, Kung HJ. Inappropriate activation of androgen receptor by relaxin via beta-catenin pathway. *Oncogene*. 2008 Jan 17;27(4):499-505.
2. Vinall RL, Mahaffey CM, Davis RR, Luo Z, Gandour-Edwards R, Ghosh PM, Tepper CG, de Vere White RW. Dual Blockade of PKA and NF-kappaB Inhibits H2 Relaxin-Mediated Castrate-Resistant Growth of Prostate Cancer Sublines and Induces Apoptosis. *Horm Cancer*. 2011 Jul 26:Epub ahead of print, PMID21789713

CONCLUSION

We previously showed that LNCaP-R273H, a LNCaP subline that expresses a high level of H2 relaxin, is able to grow in the absence of androgen *in vitro* (38), and that H2 relaxin treatment of wild type LNCaP resulted in its androgen independent growth via the activation of the AR (17). While virtually nothing is known about the signals engaged by H2 relaxin in prostate cancer cells, previous work in other systems has provided an outline of the pathways involved. Upon H2 relaxin treatment, cAMP, tyrosine kinases, PI3K/Akt and Erk pathways are stimulated in a cell-context dependent manner (2-4, 39-41). We have surveyed some of these pathways, and found that the Akt activation, as reflected by increased phosphorylation at the PDK1 site and the autokinase site, was most pronounced. In addition, the phosphorylation of GSK-3B, a downstream effector of Akt was increased and this phosphorylation (at Ser 9) inhibits the kinase activity of GSK-3B (42). GSK-3B is a component of the Wnt signaling pathway, which regulates the stability of B-catenin (43, 44). GSK-3B destabilizes B-catenin by phosphorylating it at Ser33, 37 and Thr41, with subsequent recognition by the ubiquitin/proteasome degradation system (6). The Akt-mediated phosphorylation and inactivation of GSK-3B should result in the stabilization of B-catenin. This was exactly what we observed, in both LNCaP-R273H and LNCaP treated with H2 relaxin. To our knowledge, this is the first time that B-catenin stabilization induced by relaxin has been described. The overall proposed pathway leading to B-catenin accumulation is summarized in Figure 7. We presume the initial activation of PI3K is caused by the free G_{sa} (45), coupled with the Pten deletion in this cell line, although at present we have no direct evidence for that. An alternative pathway is via G_{as} . The association of the G_{as} subunit with axin would release GSK-3B from the active axin-GSK-3B complex, resulting in the stabilization of B-catenin. This scenario is unlikely to operate in LNCaP cells since they express an undetectable level of Axin (data not shown). Previous studies showed that B-catenin can augment AR activity through a specific AR/B-catenin protein-protein interaction (10). In the current study we found H2 relaxin induced the association of b-catenin with AR through B-catenin stabilization and subsequent translocation of this complex into the nucleus (Figure 3C). This was echoed by the constitutive co-localization of AR and B-catenin in LNCaP-R273H cells which carry an autocrine loop of H2 relaxin. The involvement of the PI3K/Akt pathway in causing this co-localization was further confirmed by the application of LY294002, a

PI3K inhibitor, to H2 relaxin treated LNCaP or LNCaP-R273H; in both cases, the accumulation of B-catenin, the association of AR with B-catenin and the translocation of the AR/B-catenin complex into the nucleus of these cells were blocked. The consequence of increased association between B-catenin and AR is the increased activity of AR in the transcription of target genes (9). In the literature the B-catenin effect is mostly observed with liganded AR. Our data indicate that B-catenin can augment AR transcription in an androgen independent manner. Nonetheless, we anticipate that the effect of H2 relaxin may be even more pronounced in the presence of low levels of androgen. We found that H2 relaxin is able to activate AR transcriptional activity and induces the recruitment of B-catenin to the proximal region of the PSA promoter, coincident with AR. We also showed that over-expression of B-catenin enhanced the H2 relaxin-induced AR activity, whereas knockdown of B-catenin level by siRNA blocked such an activity. Our studies suggest that a modulation of AR activity by H2 relaxin released from surrounding cells including neuroendocrine cells may have significant impact on the growth prostate cancer cells, and that B-catenin provides a potential target for intervention.

Several groups have demonstrated a link between PKA activity and CaP progression (review; (46)). PKA can mediate ligand-independent activation of AR and can therefore play an important role in facilitating both androgen dependent and independent CaP. We have previously demonstrated that H2 relaxin mediates PI3K-dependent co-translocation of the androgen receptor (AR) and β -catenin to the nucleus and causes transactivation of the PSA promoter (16, 17). Our current data indicate that H2 relaxin can also activate AR via PKA. H2 relaxin has been demonstrated to cause activation of PKA in other cell types (47, 48). H2 relaxin signals via RXFP1 and 2, both of which are GPCRs that activate the G_s class of G-proteins (G_{sa}) resulting in cAMP dependent PKA activation. Only RXFP1 is expressed in CaP cells [44]. Our data demonstrate AR activity is very high in the LNCaP-RLN2 sublines relative to LNCaP-vector, and that inhibition of PKA in the LNCaP-RLN2 sublines causes a significant inhibition of this elevated AR activity. These data indicate that PKA-mediated activation of AR is very important in a setting of elevated H2 relaxin expression.

NF- κ B is a transcription factor which controls expression of genes associated with both cell proliferation and apoptosis (49). NF- κ B has been demonstrated to be constitutively active in several CaP cell lines and expressed at high levels in both PIN and CaP patient samples (50-54). Usually NF- κ B is sequestered in the cytoplasm through interaction of its p65 and p50 subunits with I κ B α . Growth and survival stimuli induce phosphorylation of I κ B α by IKK α resulting in I κ B α degradation, followed by p65 phosphorylation and NF- κ B translocation to the nucleus. We demonstrate that H2 relaxin is one of these growth stimuli; forced overexpression of H2 relaxin caused I κ B α degradation, nuclear translocation of NF- κ B and binding to the NF- κ B DNA binding consensus sequence. Relaxin has previously been shown to activate NF- κ B in other organs but this is the first time it has been shown to activate NF- κ B in CaP. While it has been shown that Akt phosphorylation such as that induced by H2 relaxin can phosphorylate the p65 subunit of NF- κ B (55), we show that H2 relaxin promotes NF- κ B activity by degrading I κ B α .

There is sound rationale to simultaneously block both the PKA and NF- κ B pathways in CaP cells that express elevated levels of H2 relaxin; both pathways are activated by H2 relaxin yet mediate proliferation by different mechanisms, and both pathways have been shown to be active in CaP patients. While inhibition of either pathway alone resulted in growth inhibition and/or a small increase in apoptosis, it was only when both pathways were inhibited simultaneously that a clinically relevant increase in apoptosis occurred. As inhibition

of both IKK and Akt caused similar levels of NF- κ B inhibition, we chose to use perifosine, an Akt inhibitor, for the drug combination studies as it has been FDA approved and tried in CaP clinical trials. In contrast, common IKK inhibitors such as Bay11-7082 have not and are therefore of limited relevance for translational studies. It is possible that our future studies may employ other inhibitors such as Bortezomib, a proteasome inhibitor that is currently in clinical use and has been found to inhibit NF- κ B (56). Currently, PKA inhibitors are not in clinical use in CaP patients. For future translational studies, we may employ drugs that lower cAMP levels such as beta-blockers, which are in clinical use, to determine if these have an effect on H2 relaxin signaling. It is of note that beta-blockers have been found to have a small effect on prevention of CaP (57). It is also of note that the simultaneous blockade of the PKA and NF- κ B pathways outperformed docetaxel, the standard of care treatment for advanced CR CaP.

In summary, we our research has resulted in a comprehensive understanding of how H2 relaxin contributes to the progression of castrate resistant prostate cancer, and has identified therapeutic strategies that can block signaling of the H2 relaxin signaling pathway. The number of patients who would potentially benefit from blockade of the H2 relaxin pathway is likely to be extensive since a significant portion of CaP patients overexpress RLN2.

SUPPORTING DATA

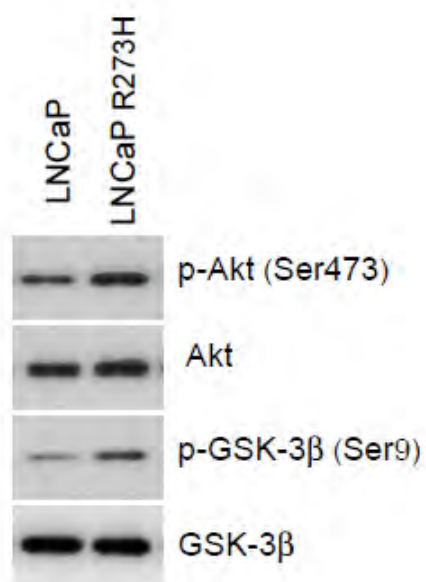


Figure 1

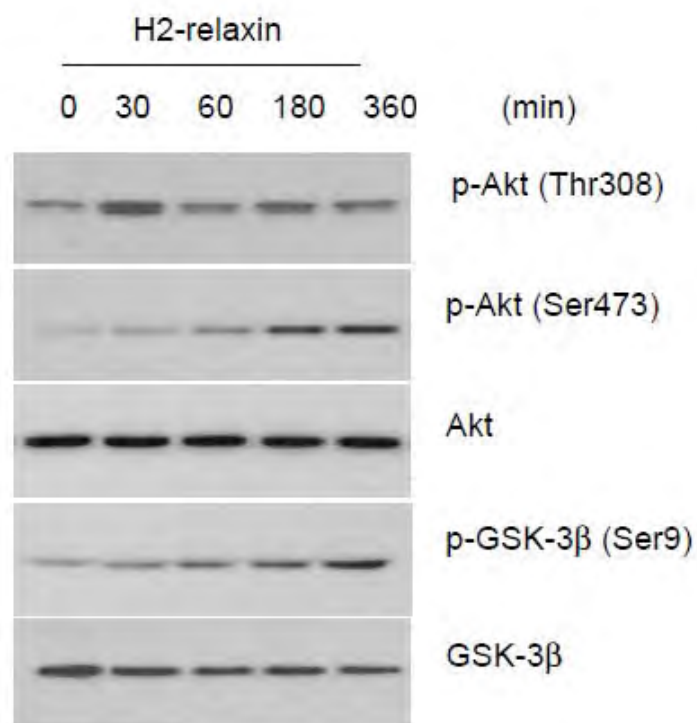


Figure 2

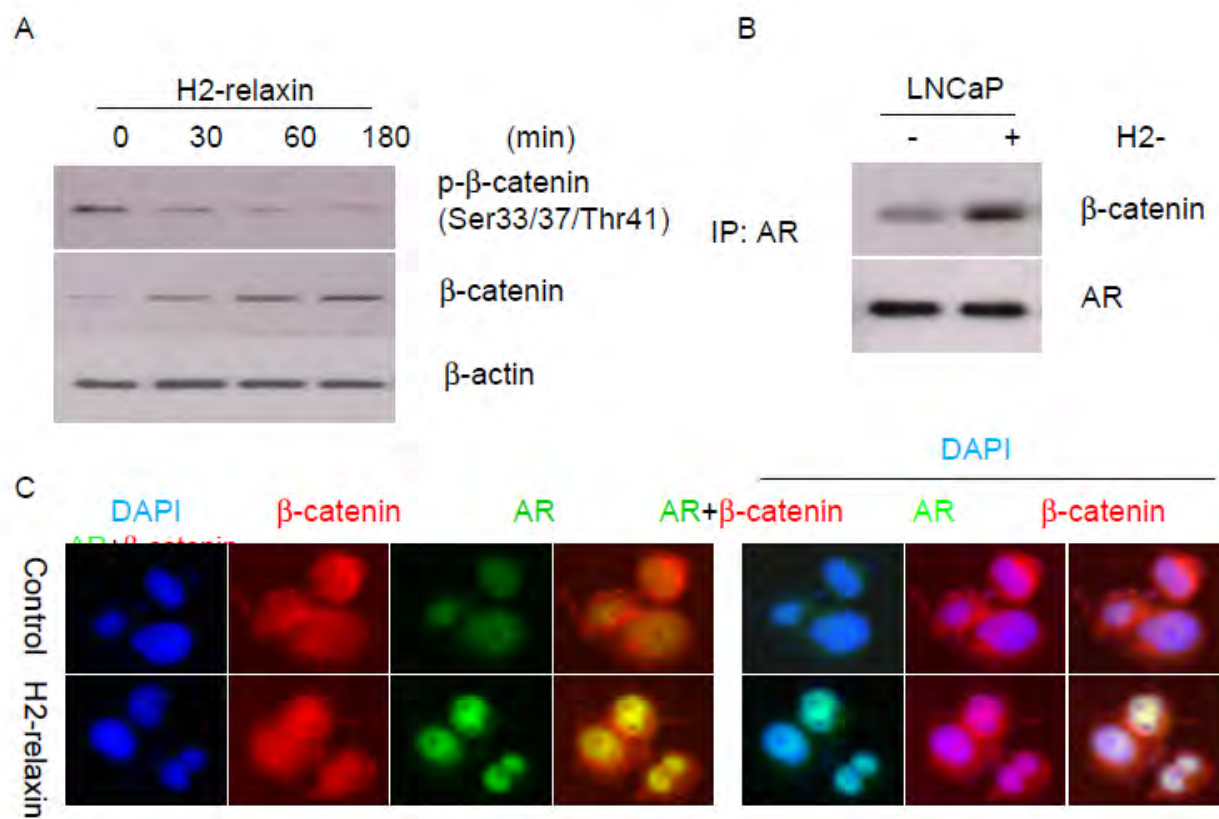


Figure 3

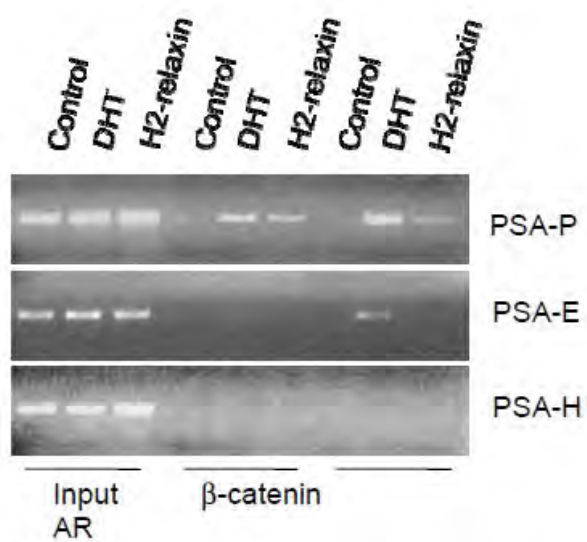


Figure 4

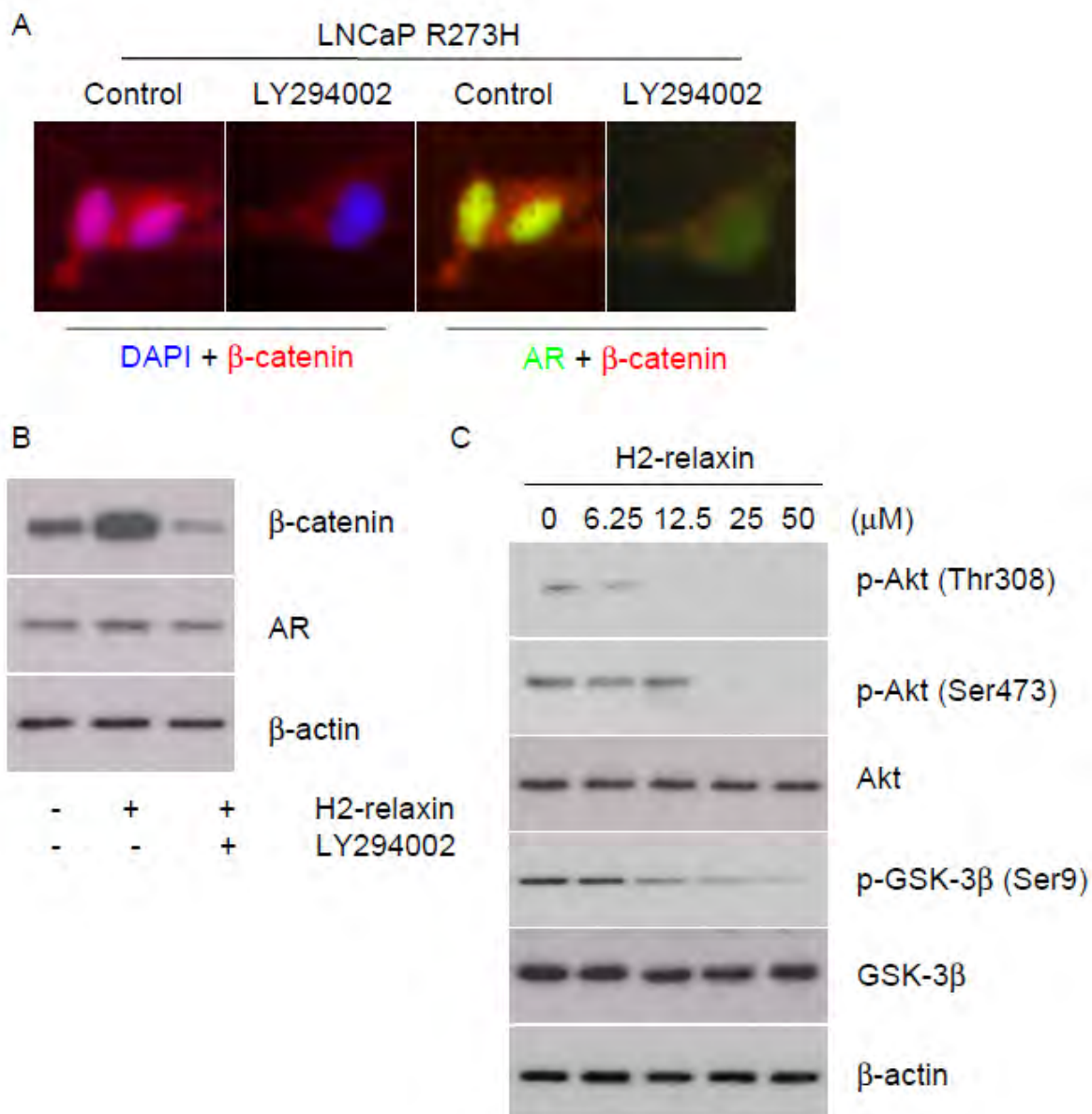


Figure 5

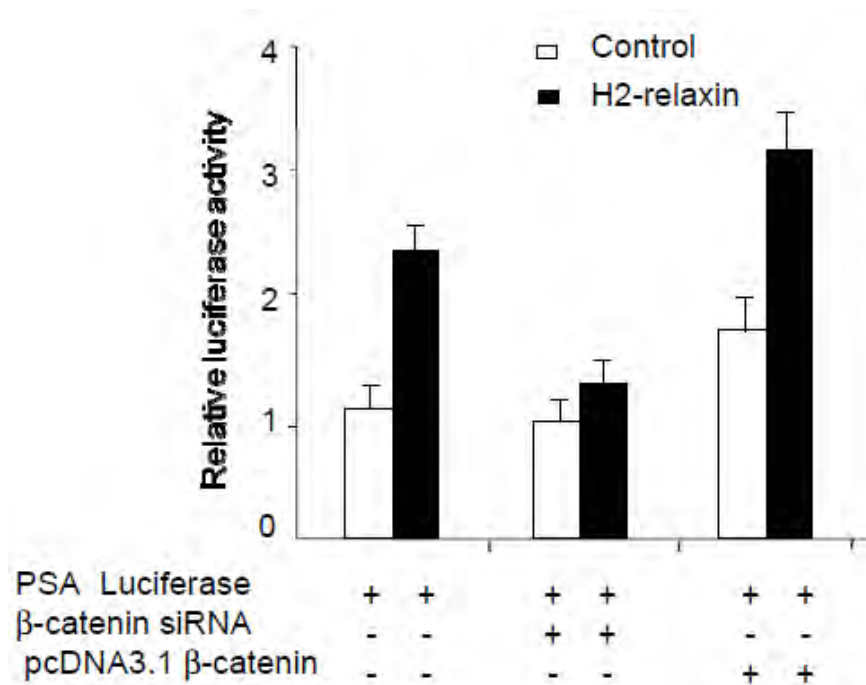


Figure 6

TABLE 1: Genes that are differentially expressed by LNCaP-RLN2 versus LNCaP-vector sublines.

NGS was performed on mRNA-Seq libraries prepared from total RNA isolated from LNCaP-RLN2 and vector control cell lines. Data analysis was performed as described in *Materials and Methods*. Normalized transcript expression (RPKM values) was used for calculation of fold expression changes in the RLN2 LNCaP sublines relative to the vector control. The numbers listed in the LNCaP-RLN2/C1 and –RLN2/C2 columns of the table are fold change in gene expression relative to LNCaP-vector. This table also lists the relative expression of genes that have been shown to be driven by either the beta-catenin/AR complex or by NFκB and those involved in neuroendocrine differentiation (in grey). Both PSA (KLK3) and cyclin D1 (CCND1) have increased expression in the LNCaP-RLN2 sublines, as does TRAF1 and C-IAP1 (CIAPIN1). C-Myc and Bcl-xL were not differentially expressed.

<i>Gene symbol</i>	<i>LNCaP-RLN2/C1</i>	<i>LNCaP-RLN2/C2</i>	<i>GO Function</i>
	<i>(fold-change relative to LNCaP- vector)</i>	<i>(fold-change relative to LNCaP-vector)</i>	
SALL2	932	1827	transcription
CCL20	701	166	chemotaxis
KLHL13	693	187	catabolism
TCEA3	498	705	transcription
RLN1	419	241	signal transduction
TUSC3	413	264	glycosylation
PEG3	401	231	transcription
RLN2	350	582	female pregnancy
S100A10	267	165	signal transduction
MEST	196	106	mesoderm development
KLK3	8	22	catalytic activity
MYC	1.18	0.94	survival
CCND1	1.44	1.20	cell cycle
BCL2L1	0.83	0.93	survival
TRAF1	2.01	3.08	survival
CIAPIN1	1.47	1.50	survival
NSE	9.532	18.796	NED
NEP	2.544	1.668	NED

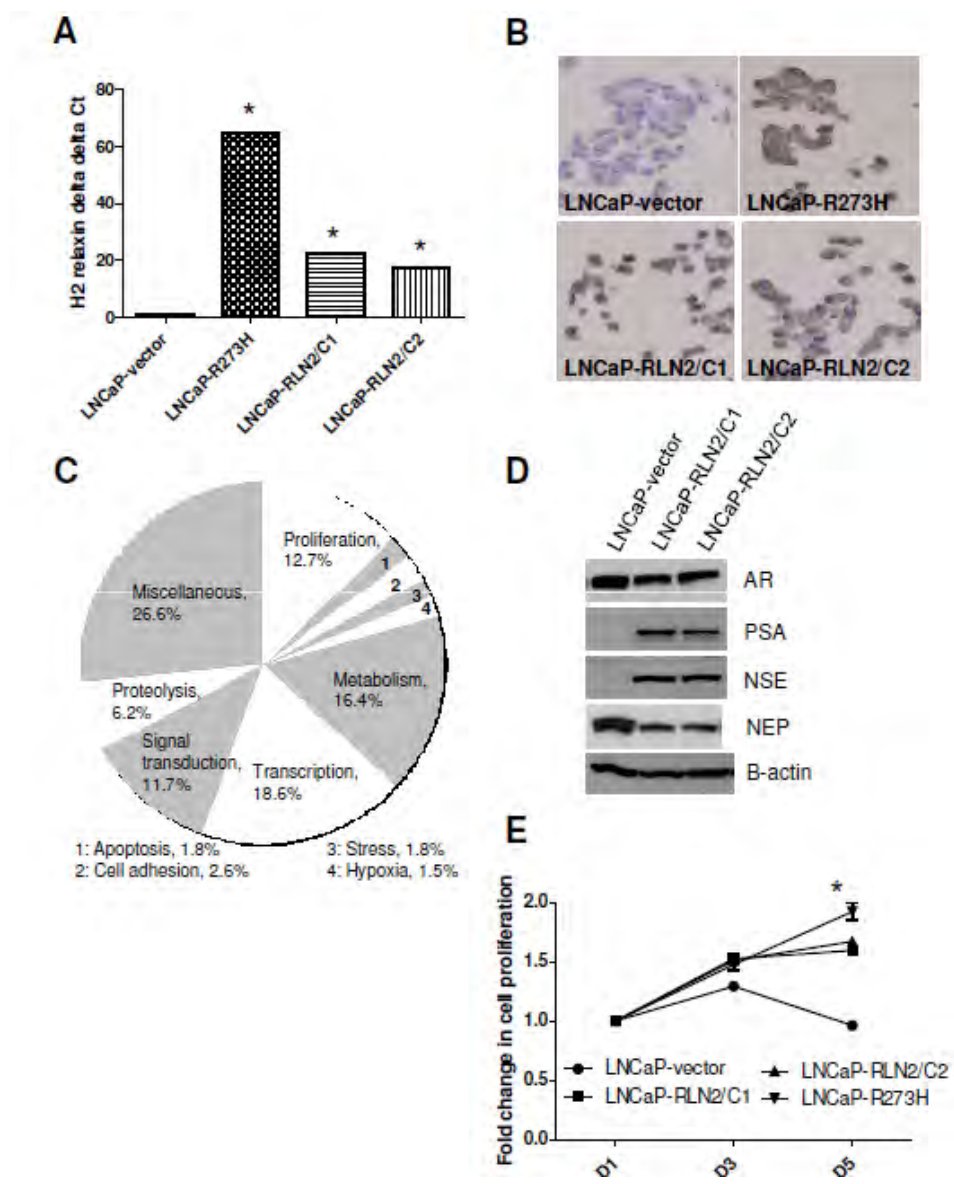


Figure 7

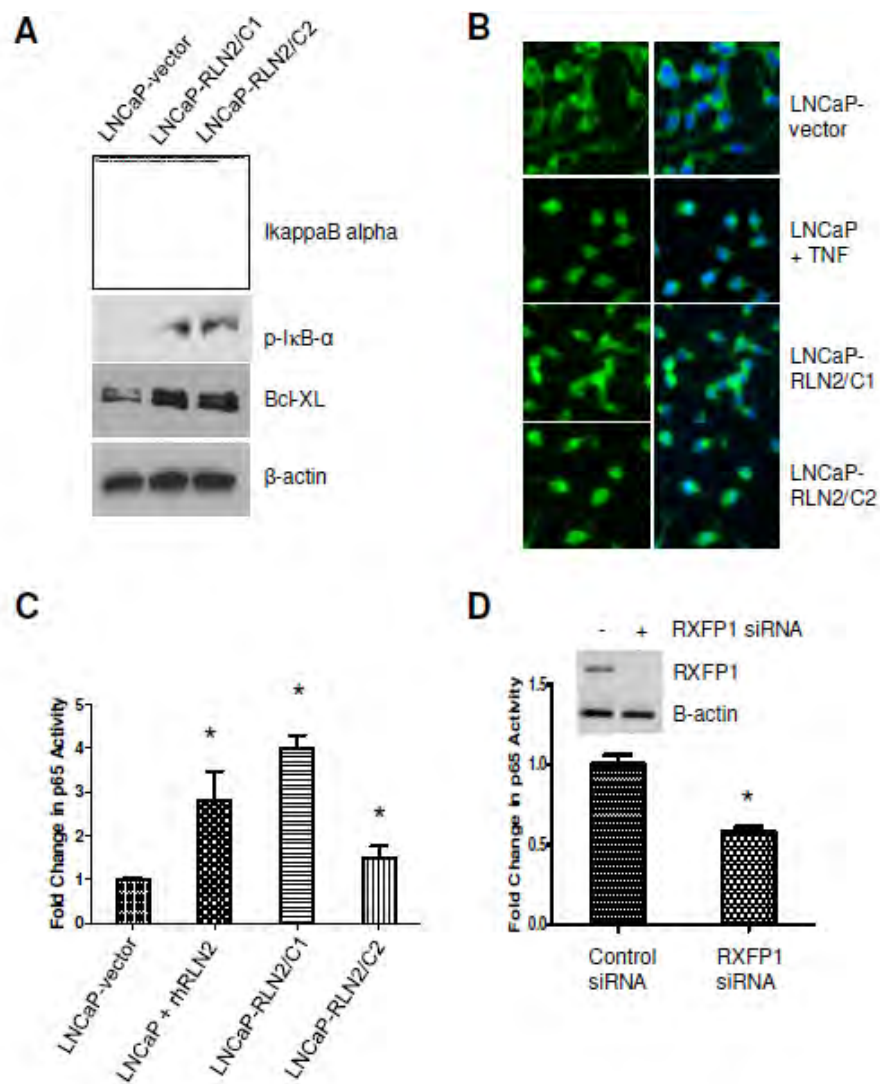


Figure 8

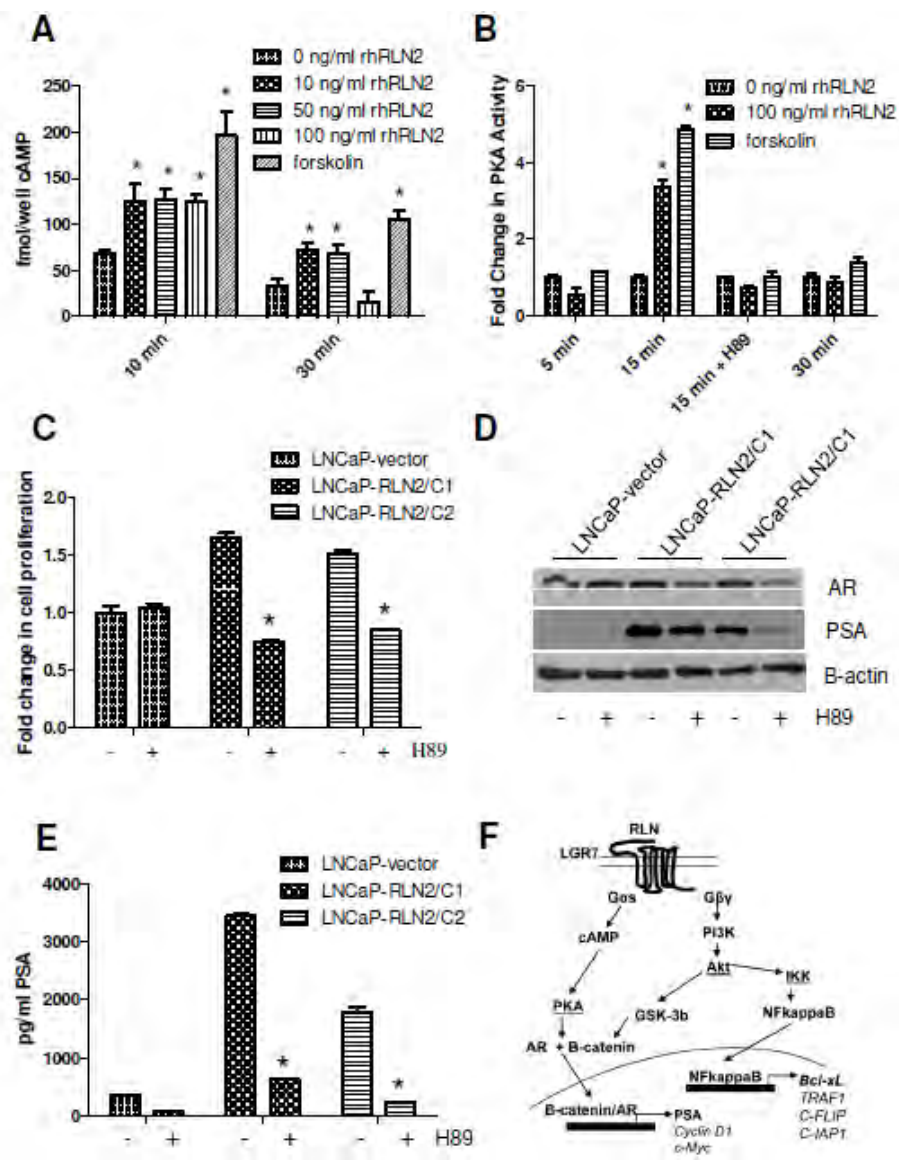


Figure 9

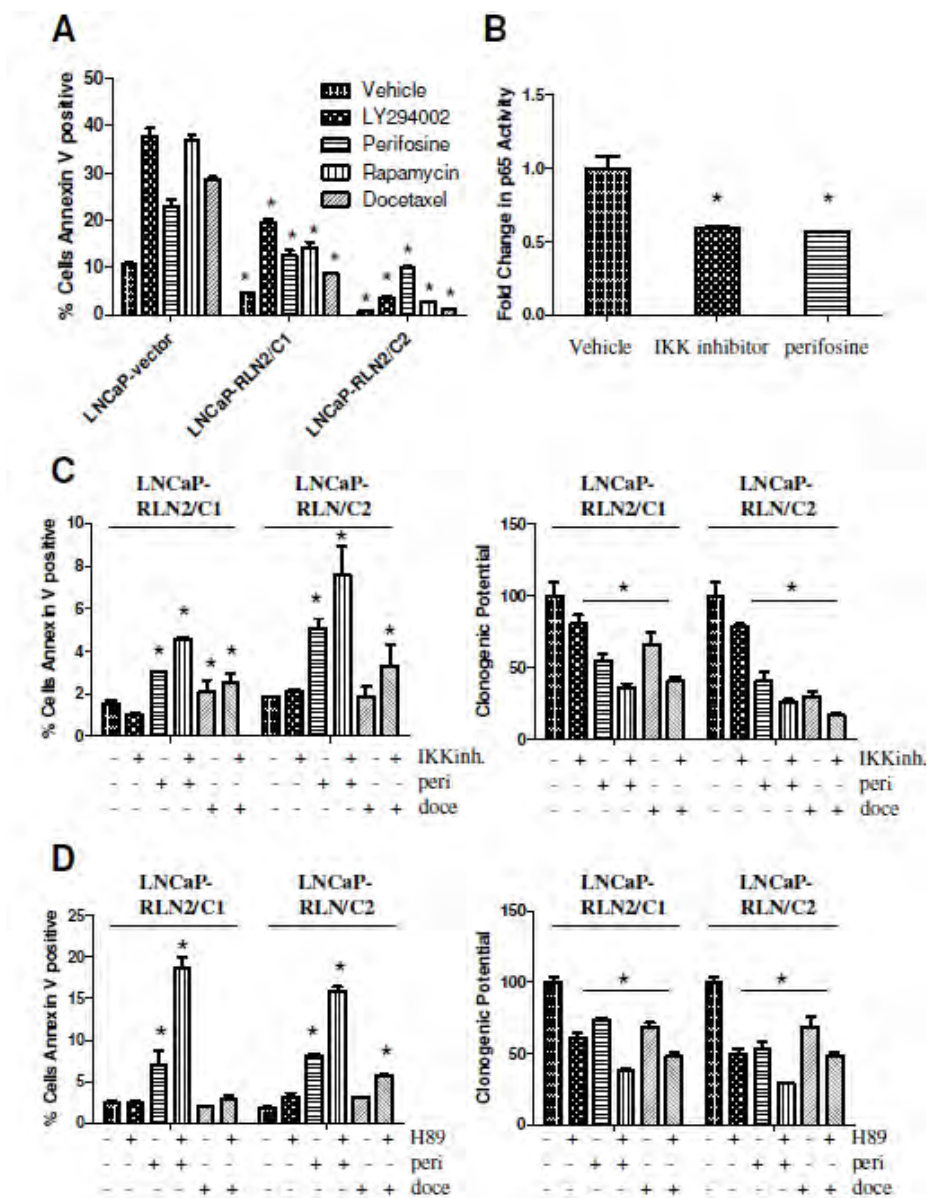


Figure 10

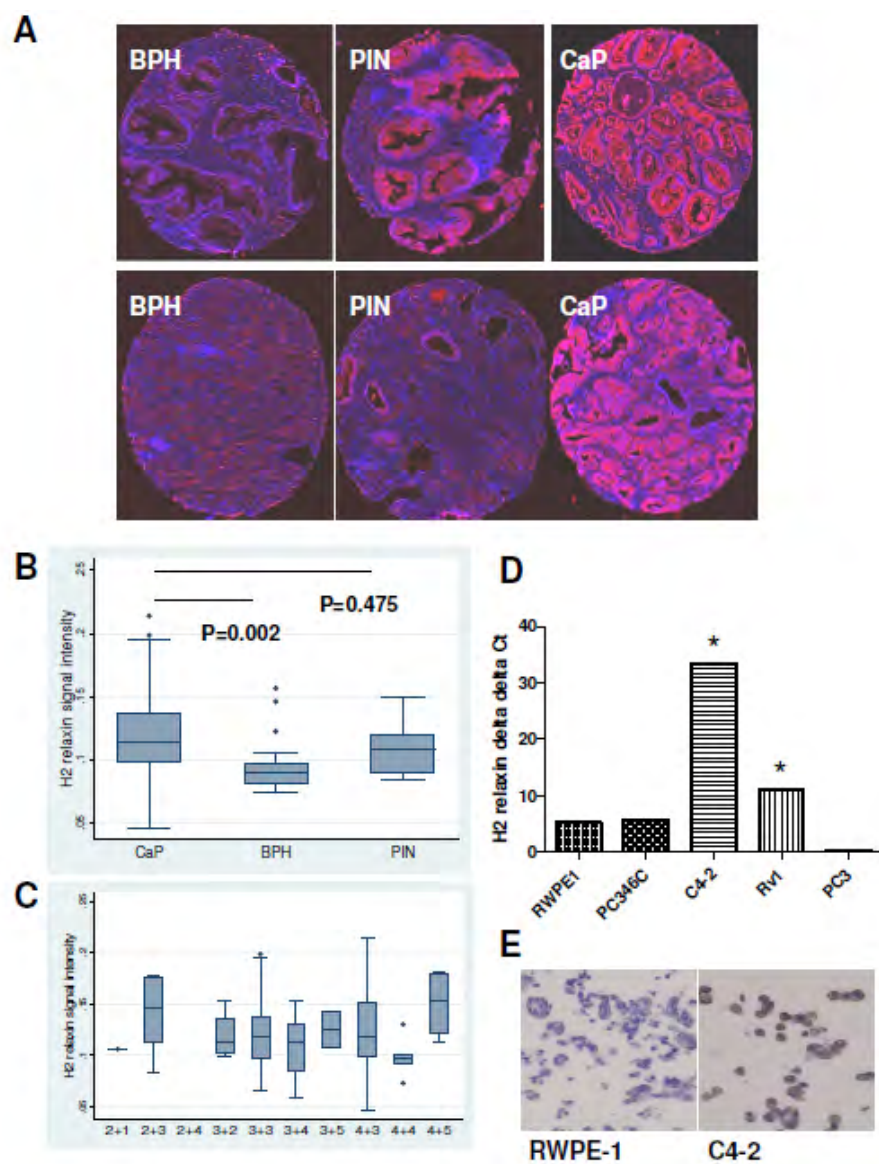


Figure 11

FIGURE LEGENDS

Figure 1. Akt and GSK-3B activation in LNCaP and LNCaP-R273H cells. Compared with LNCaP cells, Western blotting showed that phosphorylation of Akt and GSK-3 β was increased in LNCaP R273H cells. Total Akt and GSK-3B were used as internal loading controls.

Figure 2. H2 relaxin induced phosphorylation of Akt and its downstream effector GSK-3B in LNCaP cells. Treatment of serum starved LNCaP cells with H2 relaxin at a concentration of 100ng/ml induced phosphorylation of Akt at Thr308 and Ser473 at 60 and 30 minutes post-treatment respectively. H2 relaxin induced GSK-3B phosphorylation at serine9 in a time dependent manner. Total Akt and GSK-3B were used as internal loading controls.

Figure 3. H2 relaxin induced accumulation of B-catenin and promoted the association of B-catenin with AR. (A) LNCaP-R273H cells, serum-starved overnight, were treated with 100ng/ml rh H2 relaxin for 30, 60 and 180 minutes. Western blot showed that the phosphorylation of B-catenin was decreased and B-catenin was increased in a time-dependent manner. B-actin was used as an internal loading control. (B) Immunoprecipitation of B-catenin with AR showed that the association of B-catenin and AR could be induced by treatment of 100ng/ml H2 relaxin for 12 hr in LNCaP cells. (C) Immunocytochemistry showed that 100ng/ml H2 relaxin induced co-localization of B-catenin with AR in the nucleus of LNCaP cells.

Figure 4. ChIP analysis showed that the binding of B-catenin to the PSA promoter is induced by H2 relaxin. Serum-starved LNCaP R273H cells were treated with vehicle, 10nM DHT or 100ng/ml H2 relaxin overnight. ChIP analysis revealed that treatment of LNCaP-R273H with H2 relaxin causes B-catenin to bind to the P domain of the PSA promoter. DHT treatment caused B-catenin to bind to both the E and P domains of the PSA promoter.

Figure 5. LY294002, a PI3K inhibitor, blocked the effects of H2 relaxin on the association and nuclear co-localization of B-catenin/AR through inhibiting the phosphorylation of Akt and GSK-3B in LNCaP-R273H cells. (A) Overnight serum starved LNCaP R273H cells were treated with or without LY294002 at a concentration of 50 μ M for 3 hr. Immunocytochemical staining was performed using FITC-anti-mouse/anti-AR and CY3-anti-rabbit/anti-B-catenin. DAPI was used for nuclear stain. (B) Whole cell lysates were isolated from serum starved LNCaP-R273H cells treated without or with 200ng/ml H2 relaxin in the absence or presence of 50 μ M LY294002 for 3 h, and analyzed by Western blotting to detect the expression of AR and B-catenin. (C) Treatment of serum starved LNCaP R273H cells with 200ng/ml H2 relaxin in the presence of different concentrations of LY294002 for 3 h. Whole cell lysates were isolated and analyzed by Western blotting to detect the phosphorylation of Akt Thr308 and Ser473 and GSK-3B Ser9, B-actin was used as an internal loading control.

Figure 6. The inhibition of H2 relaxin-mediated AR transcription activity by B-catenin. SiRNA. LNCaP cells were transfected with PSA-luciferase reporter (100 ng) and B-catenin specific siRNA or control siRNA, and pcDNA3.1-B-catenin vector. After transfection for 24 h, cells were treated with or without 100ng/ml H2 relaxin for 24 h. Cell lysates were measured for luciferase and B-gal activities. The data represent the mean of three independent samples.

Figure 7: Effect of increased expression of RLN2 in LNCaP prostate cancer cells. **(A)** LNCaP sublines LNCaP-RLN2/C1 and LNCaP-RLN2/C2, which express elevated levels of RLN2, were generated by stable transfection of RLN2 in LNCaP cells. Quantitative RT-PCR (qRT-PCR) analysis for RLN2 mRNA levels in these sublines compared to vector-transfected LNCaP cells and LNCaP-R273H, a subline previously shown to express extremely high levels of this peptide hormone, demonstrated that the two RLN2 LNCaP sublines expressed RLN2 at significantly higher levels compared to LNCaP, but not as high as in p53^{R273H}-transfected cells. Triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. **(B)** Immunocytochemical analysis of the cell lines confirmed this trend. **(C)** Next generation sequencing (NGS) followed by Gene Ontology (GO) analysis revealed 12.7% of genes that are differentially expressed between the RLN2 LNCaP and LNCaP-vector sublines are linked to proliferation. Other key processes associated with increased RLN2 expression were transcription (18.6%), metabolism (16.4%), signal transduction (11.7%) and proteolysis (6.2%). **(D)** The RLN2 LNCaP sublines express high levels of NSE and low levels of NEP, suggesting a neuroendocrine-like phenotype. While lower levels of AR were observed in the RLN2 LNCaP sublines, assessment of PSA levels indicates that the AR pathway is much more active. **(E)** MTT proliferation assay determined that the RLN2 LNCaP sublines are able to grow in the absence of androgen, as was the LNCaP-R273H subline, and that the difference in proliferation at the day 5 time point was statistically significant when comparing the LNCaP-vector and all 3 LNCaP sublines. (* signifies $p < 0.05$).

Figure 8: RLN2 induces activation of the NF κ B pathway. **(A)** The RLN2 LNCaP sublines expressed decreased levels of I κ B- α and increased levels of P-I κ B- α , indicating RLN2 expression causes I κ B- α degradation. Increased levels of Bcl-xL, a downstream effector of NF κ B was also observed. **(B)** Increased nuclear translocation of NF κ B is observed in RLN2 LNCaP sublines. LNCaP cells or the RLN2 overexpressing sublines were immunostained with anti-p65 antibody (green) or with DAPI to detect the nuclei (blue). Merger of the two stains indicated NF- κ B nuclear localization. LNCaP cells treated with TNF- α (10 ng/ml) were used as positive control. Note that in LNCaP cells NF- κ B remained in the cytoplasm whereas in the RLN2-overexpressing sublines, the complex is localized to the nucleus. **(C)** NF- κ B transcriptional activity as determined by reporter assay was significantly elevated in RLN2 LNCaP sublines and in parental LNCaP treated with rhRLN2 (human recombinant). Nuclear localization of functional NF κ B was confirmed by assessment of the ability of NF κ B to bind to its DNA binding consensus sequence. **(D) (upper panels).** Knockdown of RXFP1, the RLN2 receptor, inhibited the ability of NF κ B to bind to its DNA consensus sequence in the RLN2 LNCaP sublines **(lower panels)**. (* signifies $p < 0.05$).

Figure 9: RLN2 stimulates cAMP production and PKA activation. **(A)** LNCaP cells were treated with 0, 10, 50, 100 ng/ml rhRLN2 (human recombinant) and cAMP levels measured by ELISA. 50 μ M Forskolin, which directly stimulates cAMP production, is used as a positive control. **(B).** 100 ng/ml rhRLN2 induced PKA activation comparable to the positive control Forskolin as measured by the phosphorylation of Kemptide, a phosphate group acceptor synthetic peptide. Activation of PKA was observed 15 minutes post-treatment upon treatment with 100 ng/ml rhRLN2. This activation could be inhibited using H89, a PKA inhibitor. **(C, D)** Inhibition of PKA activity in LNCaP-RLN2/C1 and LNCaP-RLN2/C2 cells resulted in inhibition of **(C)** cell growth as measured by MTT assay and **(D, E)** PSA levels, as measured by both Western blotting as well as PSA ELISA. Figure 4F shows a schematic representation of RLN2 signaling in CaP cells based on data

obtained from this and other studies of the RLN2 pathway in CaP cells. RLN2 is able to cause activation of both the cAMP/PKA and NF- κ B signaling pathways by two independent mechanisms. Note that LGR7 is an alternate name for RXFP1. (* signifies $p < 0.05$).

Figure 10: Combined blockade of the PKA and PI3K/Akt signaling pathways that are activated by RLN2 promotes apoptosis. **(A)** The RLN2 LNCaP sublines are resistant to apoptosis by several drugs, including LY294002, perifosine, rapamycin and docetaxel. The levels of apoptosis in LNCaP-vector were statistically higher compared to those observed in the LNCaP RLN2 sublines regardless of the type of drug treatment **(B)** Perifosine, similar to the IKK inhibitor decrease binding of NF κ B to its DNA consensus sequence. **(C)** Inhibition of IKK in the LNCaP-RLN2/C1 and -RLN2/C2 sublines did not cause an increase in apoptosis, while perifosine alone caused only a moderate increase in apoptosis (~2-fold increase). Simultaneous blockade of IKK and Akt resulted in only a minimal increase in apoptosis compared to treatment with perifosine alone **(left panel)**. A similar trend was observed by clonogenic assay **(right panel)**. **(D)** On the other hand, simultaneous blockade of PKA with H-89 together with perifosine resulted in a significant increase in apoptosis compared to blockade of either individual pathway (~2-3-fold increase in apoptosis compared to treatment with perifosine alone, ~15-18% apoptosis in the combination treatment) **(left panel)**, an increase that was far greater than that observed with docetaxel treatment (~2-3% apoptosis). Clonogenic assay showed this same trend **(right panel)**. (* signifies $p < 0.05$).

Figure 11: RLN2 expression is elevated in CaP patients. **(A)** Representative images from cores of BPH, PIN and CaP were obtained from patients with BPH (who underwent TURP) or CaP (who underwent prostatectomy) with IRB consent and assembled as a TMA. The cores were immunostained with an antibody to RLN2 and counterstained with hematoxylin. Multispectral imaging technologies were utilized to convert the brown staining (representing RLN2 localization) and the blue hematoxylin counterstain to false fluorescent imaging for better visualization and staining quantitation. Here shown are two representative cores from each group. **(B)** Box plot comparing median H2 relaxin expression values and interquartile ranges between the patient groups. Statistical analysis of RLN2 expression based on quantitation of multi-spectral imaging determined that RLN2 expression is higher in CaP patients compared to patients with BPH ($p=0.002$), while the difference in staining between BPH and PIN or PIN and CaP was not significant. All patient cores were assessed based on their DAB level to area score. **(C)**. RLN2 expression did not correlate with Gleason grade. **(D)** Quantitative RT-PCR (qRT-PCR) analysis of RWPE-1 cells (derived from a normal prostate) vs several CaP cell lines demonstrated that, excluding PC3 cells, RLN2 expression is elevated in CaP cell lines. Triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. **(E)** Immunocytochemical analysis in RWPE-1 vs C4-2 with RLN2 antibody (brown staining) cells confirmed this trend. The cells were counterstained with hematoxylin (blue) which allows visualization of unstained cells.

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H2 relaxin facilitates castrate resistant growth of prostate cancer cells by a mechanism that involves nuclear translocation of NFkappaB.

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Currently, castrate resistant (CR) prostate cancer (PC) remains incurable. The identification of novel pathways that promote androgen independent growth of prostate cancer cells is critical for the development of successful new therapies to treat CaP. Our group has identified H2 relaxin as a facilitator of castrate resistant prostate cancer (CRPC) (Vinall et al., 2006). The mechanism by which H2 relaxin causes CRPC remains to be fully elucidated. We have demonstrated that H2 relaxin promotes interaction between β -catenin and the androgen receptor (AR) (Liu et al., 2007). However, blocking this pathway causes only a modest decrease in H2 relaxin-mediated CR growth. In the current study we have determined that H2 relaxin can also mediate CR growth by causing translocation of NFkB to the nucleus. As part of these studies we generated LNCaP sublines that stably overexpress H2 relaxin. As expected, these LNCaP-relaxin sublines are able to grow in the absence of androgen and express high levels of PSA. In addition we found that the LNCaP-relaxin sublines are more resistant to treatment with either docetaxel or perfosine compared to the LNCaP-vector control sublines. Immunocytochemistry was used to demonstrate that the LNCaP-relaxin sublines express higher levels of nuclear NFkappaB compared to LNCaP-vector only control sublines. Western blot analysis demonstrated that levels of IkappaB-alpha are decreased in the LNCaP-relaxin sublines while levels of Bcl-XL are increased. Inhibition of LGR7, the H2 relaxin receptor, using an LGR7-specific siRNA caused increased expression of IkappaB-alpha indicating that H2 relaxin signaling plays a role in promoting nuclear translocation of NFkappaB. The combined data indicate that nuclear translocation of NFkappaB is another mechanism by which H2 relaxin facilitates CRPC.

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H2 relaxin can influence the expression of molecules associated with neuroendocrine differentiation.

Ruth Vinall, Shangqin Liu, Hsing-Jien Kung and Ralph deVere White.

It has been shown that neuroendocrine differentiation (NED) is present, at least focally, in all cases of prostate cancer (CaP). NE cells secrete neuropeptides such as bombesin and neurotensin that cause the inappropriate proliferation of surrounding cells. Abundant literature has demonstrated a link between NED in CaP and progression towards an androgen-independent state. Our group previously identified H2 relaxin as a facilitator of androgen independent prostate cancer (AI CaP) (Vinall et al., 2006). Our current data suggest that H2 relaxin may facilitate AI CaP by promoting NED. RT-PCR and Western blot analysis demonstrated that stable transfection of LNCaP with H2 relaxin or addition of H2 relaxin to LNCaP cultured in charcoal stripped serum (CSS) resulted in up-regulation of neuron specific enolase (NSE) and chromogranin A expression and down-regulation of neutral endopeptidase (NEP). NSE and chromogranin A are considered markers of NE cells. NEP is an enzyme that is able to degrade neuropeptides such as bombesin and neurotensin. Further investigation using enzyme assays revealed that H2 relaxin can cause activation of cAMP (peak activity 10 minutes post-treatment) and PKA (peak activity 15 minutes post-treatment) and that inhibition of PKA using H-89 can prevent an H2 relaxin-mediated decrease in NEP expression. In conclusion, these data indicate that H2 relaxin may promote AI CaP by a mechanism that involves components associated with NED.

ORIGINAL ARTICLE

Inappropriate activation of androgen receptor by relaxin via β -catenin pathway

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We have previously demonstrated that human H2-relaxin can mediate androgen-independent growth of LNCaP through a mechanism that involves the activation of the androgen receptor (AR) signaling pathway. The goal of the current study is to elucidate the mechanism(s) by which H2-relaxin causes activation of the AR pathway. Our data indicate that there is cross-talk between AR and components of the Wnt signaling pathway. Addition of H2-relaxin to LNCaP cells resulted in increased phosphorylation of protein kinase B (Akt) and inhibitory phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) with subsequent cytoplasmic accumulation of β -catenin. Immunoprecipitation and immunocytochemical studies demonstrated that the stabilized β -catenin formed a complex with AR, which was then translocated into the nucleus. Chromatin immunoprecipitation analysis determined that the AR/ β -catenin complex binds to the proximal region of the prostate-specific antigen promoter. Inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, using LY294002, prevented both H2-relaxin-mediated phosphorylation of Akt and GSK-3 β and translocation of β -catenin/AR into the nucleus. Knockdown of β -catenin levels using a β -catenin-specific small interfering RNA inhibited H2-relaxin-induced AR activity. The combined data demonstrate that PI3K/Akt and components of the Wnt pathway can facilitate H2-relaxin-mediated activation of the AR pathway.

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Keywords: H2-relaxin; GPCR; β -catenin; Wnt; androgen receptor; prostate cancer

Introduction

H2-relaxin is a small peptide hormone structurally related to the insulin family (Ivell, 2002). It is produced

by the ovary and/or placenta in pregnant women and the prostate in men and has shown to be essential for the proper development and function of these systems (Sherwood, 2004). LGR7, a G protein-coupled receptor (GPCR), mediates the action of H2-relaxin (Hsu *et al.*, 2002). Recent studies have demonstrated that overexpression of H2-relaxin can promote tumorigenesis in prostate by enhancing tumor cell growth, invasion and angiogenesis (Silvertown *et al.*, 2003). We previously reported that H2-relaxin induces growth of LNCaP in an androgen-independent (AI) manner through activation of the androgen receptor (AR) (Vinall *et al.*, 2006). H2-relaxin has been shown to be upregulated during neuroendocrine differentiation of LNCaP prostate cancer cells (Figueiredo *et al.*, 2005). Androgen depletion/ablation leads to increased level of neuroendocrine cells *in vitro* (Yuan *et al.*, 2006) and *in vivo* (Huss *et al.*, 2004), and abundant literature suggests that increased level of neuroendocrine differentiation in prostate cancer is associated with progression towards an AI state (di Sant'Agnese and Cockett, 1996). Mutation of p53 is another hallmark of prostate cancer progression (Shi *et al.*, 2004), the most common mutation found in prostate cancer is p53-R273H (Dinjen *et al.*, 1994). When stably transfected into androgen-sensitive cell lines, such as LNCaP and PC-346C, this mutant causes an upregulation of H2-relaxin and its receptor LGR7 (Vinall *et al.*, 2006). Transfection of other p53 mutants (G245S, R248W and R273C) into LNCaP also causes elevation of H2-relaxin expression (Vinall *et al.*, 2006). How H2-relaxin contributes to AR activation and AI growth is completely unknown. Studies in other systems revealed that H2-relaxin activates the cAMP pathway in a tyrosine kinase-dependent manner (Bartsch *et al.*, 2001). Using THP-1 cell line as a model, it was shown that H2-relaxin also stimulates protein kinase C ζ , as well as PI3 kinase, via both Gs and Gi proteins (Dessauer and Nguyen, 2005; Halls *et al.*, 2005b). These studies provide important basic information concerning the signal pathways emanating from H2-relaxin and set the stage for the present study in prostate cancer cells.

In prostate cancer, an increase in β -catenin expression level correlates with disease progression. β -Catenin was originally identified as a component of adherent

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junctions that facilitated the binding of cadherins and the actin cytoskeleton (Song *et al.*, 2004). Subsequent studies revealed that β -catenin is part of Wnt pathway and plays a role in signal transduction (Nusse, 2005). β -Catenin serves as a co-activator of T-cell factor (TCF)/lymphoid enhancing factor, a transcription factor, implicated in growth and early development (Eastman and Grosschedl, 1999). Inappropriate activation of Wnt signaling contributes to numerous human cancers (Polakis, 2000; Reya and Clevers, 2005). In prostate cancer, Wnt3a induces AR-mediated transcription and AI growth of LNCaP cell line, in a β -catenin-dependent manner (Verras *et al.*, 2004). Other studies showed that β -catenin is associated with AR and acts as an AR co-activator to enhance AR-mediated transcription (Truica *et al.*, 2000; Yang *et al.*, 2002). Indeed, in prostate cancer cell lines LNCaP and CWR22Rv1, the β -catenin-mediated AR response seems to dominate over the TCF response, and requires lower levels of β -catenin for activation (Cronauer *et al.*, 2005). In addition to being a co-activator of AR, β -catenin is able to modulate both the transcription and protein stability of AR in a complex fashion (Yang *et al.*, 2006). Thus, there is strong evidence linking β -catenin to AR activity and prostate cancer growth.

In this report, we show that H2-relaxin enhances AR activity via activation of β -catenin, which is accompanied by phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) activation and phosphorylation/degradation of glycogen synthase kinase-3 β (GSK-3 β). To our knowledge, this is the first report that connects the relaxin/GPCR pathway to the Wnt pathway.

Results

H2-relaxin induces activation of Akt and the phosphorylation of GSK-3 β

To understand the mechanism whereby H2-relaxin activates AR, we first looked for GPCR signals elevated in LNCaP-R273H, as compared to wild-type LNCaP. After preliminary screening with phospho-specific antibodies against different signal molecules, we found that the phosphorylation of Akt (Ser473) was consistently elevated in the LNCaP-R273H subline (Figure 1). The data are in agreement with the ability of the G $\beta\gamma$ subunits of GPCR to activate PI3K/Akt (Stoyanov *et al.*, 1995; Murga *et al.*, 1998). We noted that a basal level of phospho-Akt was detected in parental LNCaP, presumably due to the inactivating mutation of PTEN in this cell line (Vlietstra *et al.*, 1998). The combined effect of H2-relaxin expression and PTEN deficiency resulted in the hyperactivation of Akt in LNCaP-R273H. This is accompanied by the increase of the inhibitory-phosphorylation of GSK-3 β at Ser9 (Figure 1).

H2-relaxin induces Akt and GSK-3 β phosphorylation in LNCaP cells

LNCaP cells were treated with H2-relaxin (100 ng/ml) for 0.5, 1, 3 and 6 h, and the levels of phospho-Akt and

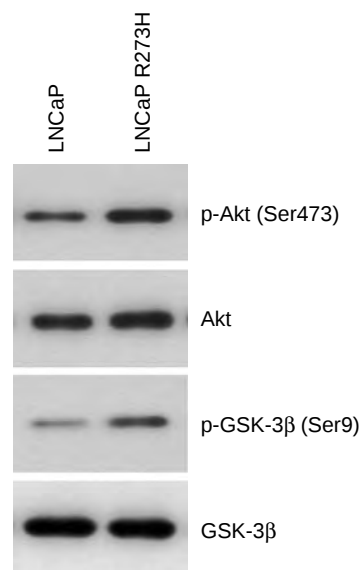


Figure 1 The phosphorylation of Akt and GSK-3 β in LNCaP and LNCaP-R273H cells. Cells were serum starved overnight. Lysates were analysed in immunoblotting with anti-p-Akt (Ser473), Akt, p-GSK-3 β (Ser9) and GSK-3 β . Total Akt and GSK-3 β were used as internal loading controls. Akt, protein kinase B; GSK-3 β , glycogen synthase kinase-3 β .

phospho-GSK-3 β were assessed to determine whether H2-relaxin could increase the phosphorylation of these molecules in a time-dependent manner. Phosphorylation of Akt at Thr308 peaked at 30 min after H2-relaxin treatment. Phosphorylation at Ser473 occurred as soon as 30 min post-treatment and peaked at 180 min. Phosphorylation of GSK-3 β Ser9 followed a similar kinetics (Figure 2). These data provide strong evidence that H2-relaxin is able to activate the Akt pathway.

H2-relaxin increases β -catenin levels and the association of β -catenin with AR

GSK-3 β is a kinase, which causes destabilization of β -catenin by phosphorylating it at Ser33, 37 and Thr41 (Yost *et al.*, 1996). The phosphorylated β -catenin is then degraded by the ubiquitin/proteasome pathway. Phosphorylation of GSK-3 β at Ser9 reduces the activity of GSK-3 β resulting in decreased degradation of β -catenin and the subsequent accumulation of β -catenin in the cytoplasm and translocation into the nucleus (Miller and Moon, 1996; Morin *et al.*, 1997). Western blot analysis showed that treatment of LNCaP cells with H2-relaxin for 0.5 h resulted in decreased phosphorylation of β -catenin and a corresponding increase of the β -catenin protein level (Figure 3a). Increasing evidence suggests that stabilized β -catenin serves as a co-activator of AR (Truica *et al.*, 2000; Yang *et al.*, 2002). To determine whether H2-relaxin-stabilized β -catenin can interact with AR, protein extracts from LNCaP treated with or without H2-relaxin were immunoprecipitated with an AR antibody followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blot analysis with an antibody to β -catenin. Figure 3b

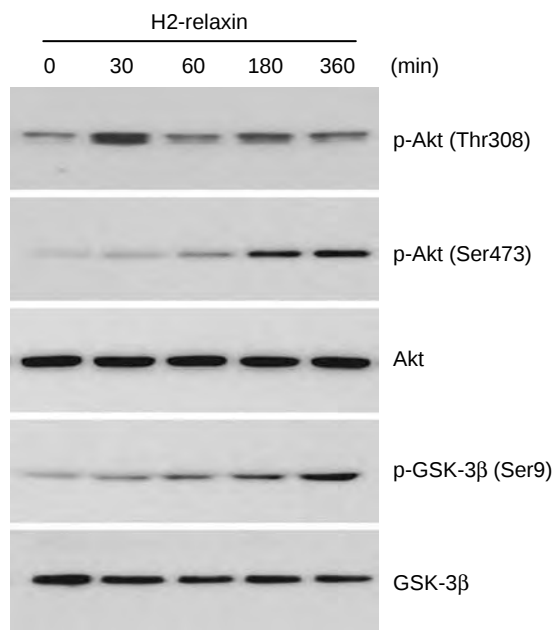


Figure 2 Phosphorylation of Akt and GSK-3 β induced by H2-relaxin in LNCaP cells. LNCaP cells were serum starved overnight and treated with 100 ng/ml H2-relaxin for different time points varying from 0.5 to 6 h. Lysates were analysed by immunoblotting with anti-p-Akt (Thr308), p-Akt (Ser473), Akt, p-GSK-3 β (Ser9) and GSK-3 β . Total Akt and GSK-3 β were used as internal loading controls. Akt, protein kinase B; GSK-3 β , glycogen synthase kinase-3 β .

showed that treatment of LNCaP with H2-relaxin increased the level of association between β -catenin and AR. Immunochemical analysis substantiated the above result and showed that H2-relaxin treatment of LNCaP cells increased not only β -catenin accumulation in the cytoplasm but also AR and β -catenin colocalization in the nucleus (Figure 3c).

H2-relaxin induces the recruitment of β -catenin to PSA promoter

We next proceeded to determine whether H2-relaxin-induced β -catenin/AR complex was recruited to relevant promoters targeted by AR (for example, prostate-specific antigen (PSA) promoter). Chromatin immunoprecipitation (ChIP) assay was employed for this analysis. We analysed both the proximal (P) androgen receptor response element (ARE) I/II and the distal enhancer (E) ARE III sites for potential binding to the β -catenin/AR complex. The region in between, H, was used as a negative control. Interestingly, H2-relaxin induced β -catenin binding to the proximal (P) region of the PSA promoter, but not to the PSA enhancer (E) region or the PSA H region (Figure 4). By contrast, dihydrotestosterone (DHT) induced the recruitment of β -catenin to both the P and E sites. This is consistent with reports by us and others that post-translationally activated AR, unlike androgen bound receptor, only binds the proximal ARE and not the distal ARE (Lee *et al.*, 2004; Desai *et al.*, 2006).

LY294002 blocks H2-relaxin-induced phosphorylation of Akt and GSK-3 β and the colocalization of AR and β -catenin in the nucleus

To confirm that PI3K is involved in H2-relaxin-mediated AR activation, we used the PI3K inhibitor LY294002 to block PI3K/Akt activation. We hypothesized that inhibition of the PI3K/Akt pathway would prevent the interaction of AR and β -catenin in LNCaP-R273H cells. Indeed, the association and colocalization of β -catenin and AR could be blocked by treatment with LY294002 (Figure 5a), even in the presence of 200 ng/ml H2-relaxin (data not shown). To determine whether this observation was due to LY294002-induced changes in the intracellular level of β -catenin protein, we examined both AR and β -catenin protein levels using western blotting. LY294002 treatment did not significantly affect AR protein level, but inhibited the accumulation of β -catenin induced by H2-relaxin (Figure 5b). These results together indicate that inhibition of PI3K can suppress H2-relaxin-mediated association of AR and β -catenin through decreasing β -catenin protein level. Consistent with the decrease in β -catenin protein levels, we found that H2-relaxin-mediated Ser9 phosphorylation of GSK-3 β proteins was also impaired by treatment with LY294002 in a dose-dependent manner (Figure 5c). Likewise, complete inhibition of H2-relaxin-induced Akt phosphorylation at sites Thr308 and Ser473 was observed in samples treated with LY294002 at a concentration above 12.5 μ M. The total amount of Akt and GSK-3 β proteins remained the same in the presence or absence of LY294002.

β -Catenin siRNA blocks H2-relaxin-mediated AR activation

Our previous results suggest that H2-relaxin is able to influence the AR signaling pathway. Transient transfection assays were performed to further investigate the possible effect of β -catenin on AR-mediated transcription. Plasmids capable of expressing a PSA luciferase reporter and a β -catenin-specific small interfering RNA (siRNA) were transfected into LNCaP cells. H2-relaxin treatment induced a nearly 1.5-fold increase in transactivation in LNCaP cells. Co-transfection of the β -catenin siRNA decreased AR activity induced by H2-relaxin to base line (Figure 6). These data indicate that β -catenin is a critical component in H2-relaxin-mediated AR activation. Conversely, we were able to show that overexpression of β -catenin can directly augment the transcriptional activity of AR, using a β -catenin expression construct (Pang *et al.*, 1997). As seen in Figure 6, elevated levels of β -catenin enhanced both basal as well as H2-relaxin-mediated AR transcription through the PSA promoter.

Discussion

We previously showed that LNCaP-R273H, a LNCaP subline that expresses a high level of H2-relaxin, is able

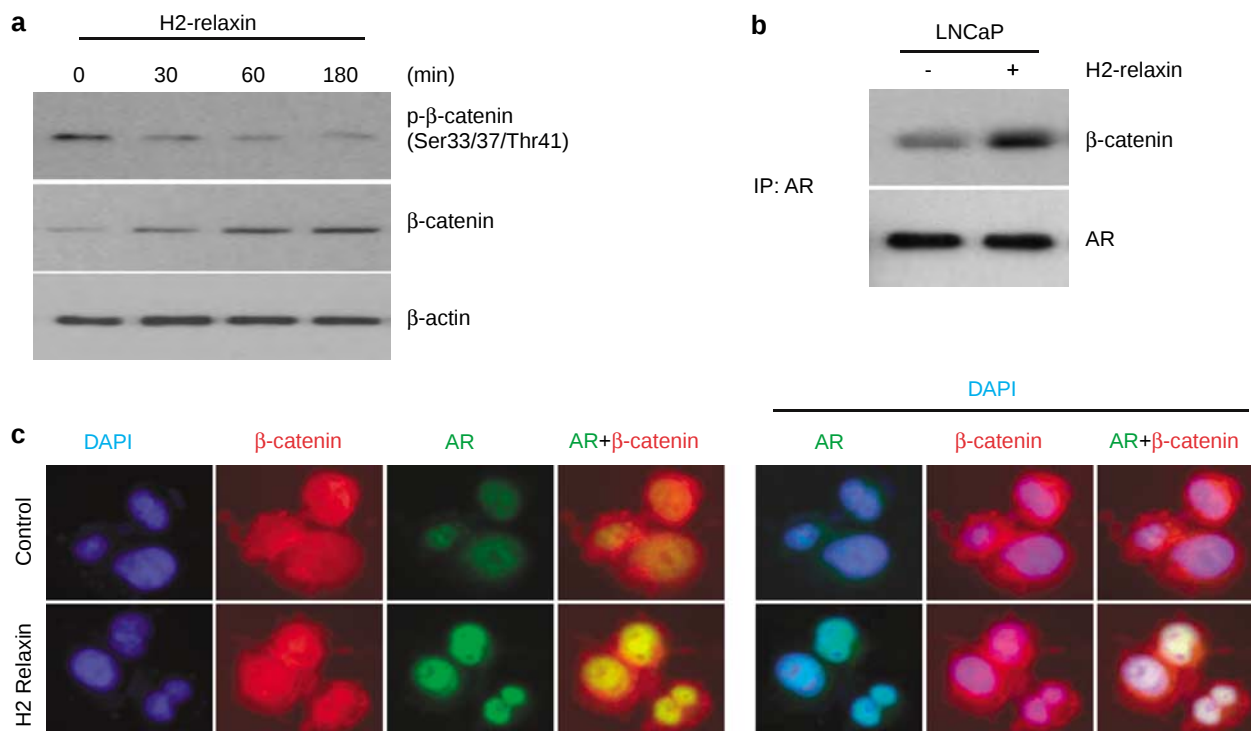


Figure 3 H2-relaxin induced accumulation of β -catenin and promoted the association of β -catenin with AR. (a) LNCaP cells were serum-starved overnight and treated with 100 ng/ml H2-relaxin for 30, 60 and 180 min. Lysates were analysed in immunoblotting with anti-p- β -catenin (Ser33/37/Thr41), β -catenin and β -actin. β -Actin was used as internal loading controls. (b) LNCaP cells were serum-starved overnight and treated with or without 100 ng/ml H2-relaxin and whole-cell extracts were prepared. Extracts were immunoprecipitated with anti-AR antibody and subjected to immunoblotting with anti- β -catenin and AR. (c) LNCaP cells were seeded on glass coverslips and incubated with or without H2-relaxin for 16 h in serum-free medium. After fixation, cells were incubated with anti-AR and anti- β -catenin antibodies. Anti-mouse conjugated with fluorescein (green) and anti-rabbit-conjugated Texas Red (red) were used for second antibodies. The coverslips were viewed by fluorescence microscopy. AR, androgen receptor.

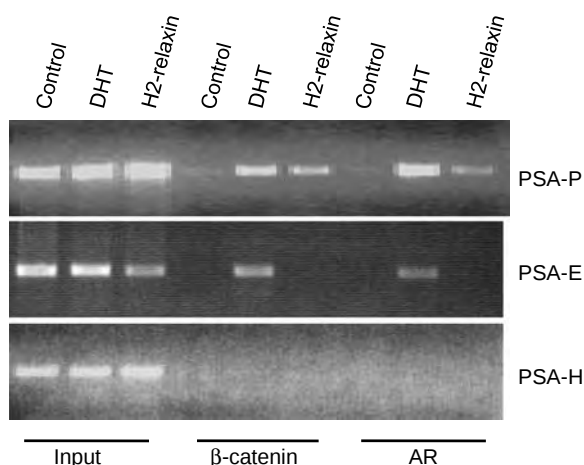


Figure 4 H2-relaxin induced β -catenin recruitment to PSA promoter. LNCaP cells were treated with vehicle, 10 nM DHT or 100 ng/ml H2-relaxin in serum-free medium overnight. ChIP assay was performed using anti-AR, anti- β -catenin antibodies to immunoprecipitate protein-DNA complex. PCR reactions products, including P domain, E domain and H domain of PSA promoter, were observed from input control DNA, anti- β -catenin and anti-AR immunoprecipitated chromatin from vehicle, DHT and H2-relaxin treated LNCaP cells in ethidium bromide-stained agarose gel. AR, androgen receptor; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; PSA, prostate-specific antigen.

to grow in the absence of androgen *in vitro* (Nesslinger *et al.*, 2003), and that H2-relaxin treatment of wild-type LNCaP resulted in its AI growth via AR activation (Vinall *et al.*, 2006). While virtually nothing is known about the signals engaged by H2-relaxin in prostate cancer cells, previous work in other systems has provided an outline of the pathways involved. Upon H2-relaxin treatment, cAMP, tyrosine kinases, PI3K/Akt and Erk pathways are stimulated in a cell-context-dependent manner (Palejwala *et al.*, 1998; Hsu *et al.*, 2002; Zhang *et al.*, 2002; Nguyen *et al.*, 2003; Dessauer and Nguyen, 2005). We have surveyed some of these pathways, and found that the Akt activation, as reflected by increased phosphorylation at the PDK1 site and the autokinase site, was most pronounced. In addition, the phosphorylation of GSK-3 β , a downstream effector of Akt was increased and this phosphorylation (at Ser9) inhibits the kinase activity of GSK-3 β (Cross *et al.*, 1995). GSK-3 β is a component of the Wnt signaling pathway, which regulates the stability of β -catenin (Nusse, 1997). GSK-3 β destabilizes β -catenin by phosphorylating it at Ser33, 37 and Thr41, with subsequent recognition by the ubiquitin/proteasome degradation system (Yost *et al.*, 1996). The Akt-mediated phosphorylation and inactivation of GSK-3 β should result in the stabilization of β -catenin. This was exactly what we observed in both LNCaP-R273H and

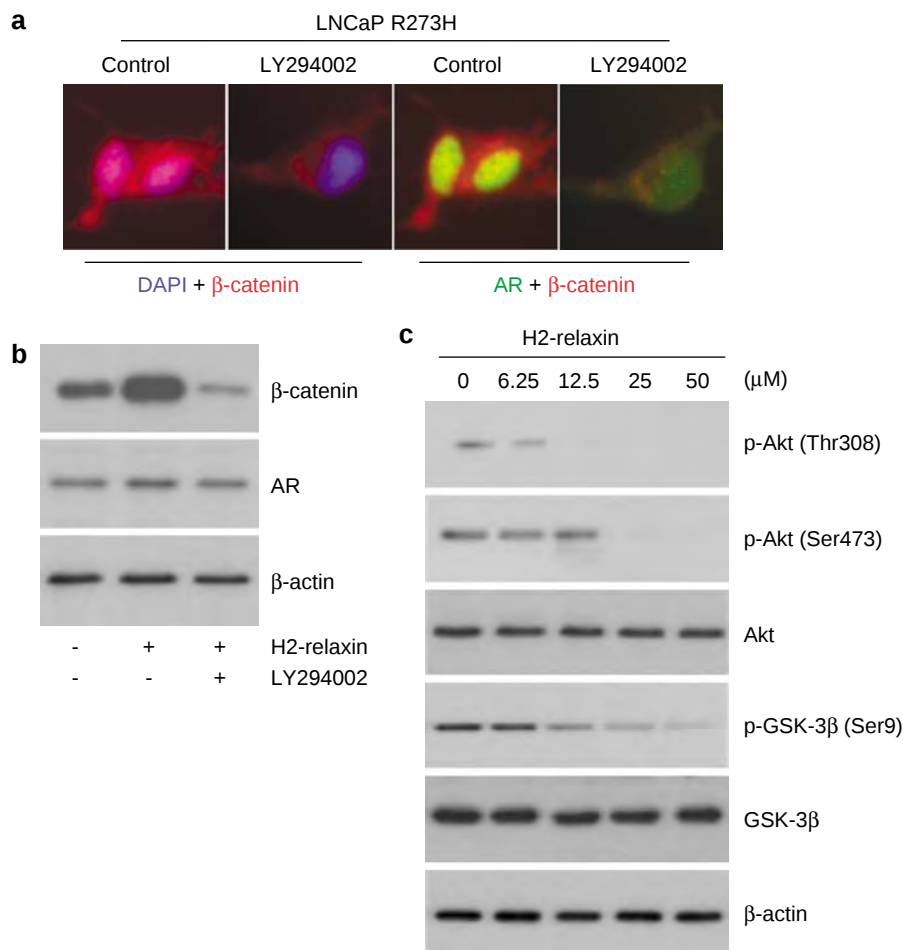


Figure 5 The effects of LY294002 on H2-relaxin-induced nuclear colocalization of β -catenin/AR, β -catenin levels, and the phosphorylation of Akt and GSK-3 β in LNCaP-R273H cells. **(a)** LNCaP-R273H cells were serum starved overnight and treated with or without LY294002 at a concentration of 50 μ M for 0.5 h. Immunofluorescence staining was performed using fluorescein-anti-mouse/anti-AR and Texas Red-anti-rabbit/anti- β -catenin. DAPI was used for nuclear staining. **(b)** LNCaP-R273H cells were serum starved overnight, pretreated with or without 50 μ M LY294002 for 0.5 h, and then treated with or without 200 ng/ml H2-relaxin for 3 h. Western blotting analysis was performed to detect the expression of AR and β -catenin. β -Actin was used as an internal loading control. **(c)** LNCaP-R273H cells were serum starved overnight, pretreated with or without LY294002 at varying concentration for 0.5 h, and then treated with 200 ng/ml H2-relaxin for 3 h. Lysates were analysed by immunoblotting with anti-p-Akt (Thr308), p-Akt (Ser473), Akt, p-GSK-3 β (Ser9) and GSK-3 β . β -Actin was used as an internal loading control. Akt, protein kinase B; AR, androgen receptor; DAPI, 4,6-diaminidino-2-phenylindole; GSK-3 β , glycogen synthase kinase-3 β .

LNCaP treated with H2-relaxin. To our knowledge, this is the first time that β -catenin stabilization induced by relaxin has been described. The overall proposed pathway leading to β -catenin accumulation is summarized in Figure 7. We presume the initial activation of PI3K is caused by the free G $\beta\gamma$ (Castellone *et al.*, 2005), coupled with the PTEN deletion in this cell line. An alternative pathway is via G α_s , where its association with axin would release GSK-3 β , resulting in the stabilization of β -catenin. This scenario is unlikely to operate in LNCaP cells, since they express an undetectable level of axin (data not shown). Previous studies showed that β -catenin can augment AR activity through a specific AR/ β -catenin protein-protein interaction (Yang *et al.*, 2002). In the current study, we found that H2-relaxin induced the association of β -catenin with AR through β -catenin stabilization and subsequent translocation of this complex into the nucleus (Figure 3c). This was

echoed by the constitutive colocalization of AR and β -catenin in LNCaP-R273H cells. The involvement of the PI3K/Akt pathway in causing this colocalization was further confirmed by the application of LY294002 to H2-relaxin-treated LNCaP or LNCaP-R273H; in both cases, the accumulation of β -catenin, the association of AR with β -catenin and their translocation into the nucleus of these cells were blocked. The consequence of increased association between β -catenin and AR is the increased activity of AR in the transcription of target genes (Truica *et al.*, 2000). In the literature, the β -catenin effect is mostly observed with liganded AR. Our data indicate that β -catenin can augment AR transcription in an AI manner. Nonetheless, we anticipate that the effect of H2-relaxin may be even more pronounced in the presence of androgen.

In summary, we provide evidence that H2-relaxin acts as an upstream signal to induce the transcriptional

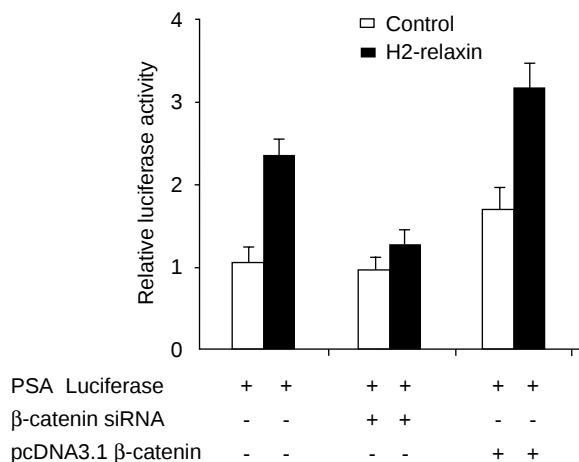


Figure 6 Inhibition of H2-relaxin-mediated AR transcription activity by β -catenin siRNA. LNCaP cells were transfected with PSA-luciferase reporter plasmid (100 ng), pRL-SV40 renilla luciferase plasmid (25 ng) and β -catenin-specific siRNA (100 nM) or pcDNA3.1- β -catenin (50 ng). After transfection for 24 h, cells were treated with or without 100 ng/ml H2-relaxin in serum-free medium for 24 h. Cell lysates were measured for luciferase activities. The data represent the mean of three independent samples. AR, androgen receptor; siRNA, small interfering RNA; PSA, prostate-specific antigen.

activity of AR and the growth of prostate cancer cells through increased expression of β -catenin. Our study further connects the GPCR and Wnt signaling pathways to AR activation and the development of hormone refractory prostate cancer.

Materials and methods

Cell lines and treatments

LNCaP and LNCaP-R273H cells were routinely maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Before treatment with H2-relaxin, cells were cultured in phenol red-free RPMI-1640 medium without serum for 12 h. In the experiments with the PI3 kinase inhibitor, cells were pre-treated with various concentrations of LY294002 (Cell Signaling Technology, Inc., Beverly, MA, USA) in dimethyl sulfoxide for 30 min and followed by 100 ng/ml H2-relaxin treatment for 3 h.

Antibodies

Mouse antibodies against AR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β -actin (Sigma, St Louis, MO, USA), rabbit antibodies against GSK-3 β , phospho-GSK-3 β (Ser 9), Akt, phospho-Akt (Thr 308 or Ser 473), β -catenin, phospho- β -catenin (Ser 33/37/Thr 41) (Cell Signaling), Gas and axin were used for immunoprecipitation, western blotting and immunofluorescence analysis.

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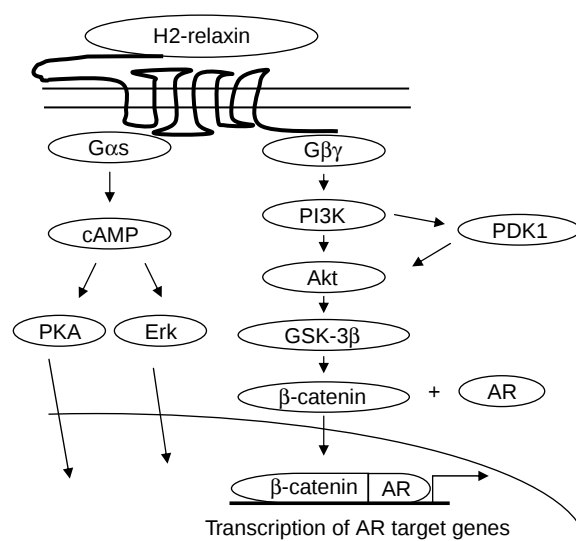


Figure 7 Schematic representation of β -catenin pathway activation in response to H2-relaxin. Overexpression of H2-relaxin in prostate cancer cells can activate LGR7 receptors that are coupled to heterotrimeric G proteins of the $G_{\alpha s}$ family. Upon exchange of GDP for GTP, free $\beta\gamma$ subunits stimulate the PI3K-PDK1-Akt signaling pathway (right), which causes the phosphorylation and inactivation of GSK-3 β . These events lead to β -catenin stability, AR association with β -catenin and expression of growth-promoting genes regulated by AR transcription factors. H2-relaxin can also increase cAMP level through $G_{\alpha s}$ signaling pathway (Hsu *et al.*, 2002), activate PKA (Halls *et al.*, 2005a) and Erk pathways (Zhang *et al.*, 2002; Dessauer and Nguyen, 2005) (left). AR, androgen receptor; GSK-3 β , glycogen synthase kinase-3 β .

ChIP assay

ChIP assay was performed as described previously (Vinall *et al.*, 2006), as well as, primers for the PSA promoter region (Louie *et al.*, 2003).

Western blot analysis and immunoprecipitation

The methods for isolation of cell lysate, western blot analysis and immunoprecipitation have been described previously (Desai *et al.*, 2006; Vinall *et al.*, 2006).

Supplementary methods

Detailed descriptions of immunofluorescence microscopy, siRNA and luciferase assays are provided in Supplementary methods.

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Dual Blockade Of PKA And NF κ B Inhibits H2 Relaxin-Mediated Castrate Resistant Growth Of Prostate Cancer Sublines And Induces Apoptosis.

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Running Title: H2 relaxin and castrate resistant prostate cancer.

Key Words: H2 relaxin, castrate resistant prostate cancer, androgen receptor, cyclic AMP, protein kinase A, NF kappaB

Abstract

We previously demonstrated H2 relaxin (RLN2) facilitates castrate resistant (CR) growth of prostate cancer (CaP) cells through PI3K/Akt/ β -catenin-mediated activation of the androgen receptor (AR) pathway. As inhibition of this pathway caused only ~50% reduction in CR growth, the goal of the current study was to identify additional RLN2-activated pathways that contribute to CR growth. Next-generation sequencing (NGS)-based transcriptome and gene ontology (GO) analyses comparing LNCaP stably transfected with RLN2 (LNCaP-RLN2) versus LNCaP-vector identified differential expression of genes associated with cell proliferation (12.7% of differentially expressed genes), including genes associated with the cAMP/PKA and NF κ B pathways. Subsequent molecular analyses confirmed that the cAMP/PKA and NF κ B pathways play a role in facilitating H2 relaxin-mediated CR growth of CaP cells. Inhibition of PKA attenuated RLN2-mediated AR activity, inhibited proliferation and caused a small but significant increase in apoptosis. Combined inhibition of the PKA and NF κ B signaling pathways via inhibition of PKA and Akt induced significant apoptosis and dramatically reduced clonogenic potential, outperforming docetaxel, the standard of care treatment for CR CaP. Immunohistochemical (IHC) analysis of tissue microarrays (TMA) in combination with multispectral quantitative imaging comparing RLN2 levels in patients with BPH, PIN and CaP determined that RLN2 is significantly upregulated in CaP vs BPH ($p=0.002$). The combined data indicate RLN2 overexpression is frequent in CaP patients and provides a growth advantage to CaP cells. A near complete inhibition of RLN2-induced CR growth can be achieved by simultaneous blockade of both pathways.

Introduction

Androgen ablation is the standard therapy for disseminated prostate cancer (CaP), however, patients develop resistance to this treatment regimen within 18-24 months [13]. Patients with castration resistant (CR) CaP are offered limited options, usually chemotherapy treatment with docetaxel, and more recently, the cancer vaccine “Provenge”, whose effect is to increase patient survival time by a median of 3 or 4 months, respectively [18, 42, 30]. The identification and elucidation of pathways that promote CR CaP is critical for the development of successful new therapies to treat this disease.

H2 relaxin (RLN2) is a peptide hormone that is a member of the insulin-like superfamily. Several groups, including ours, have demonstrated that H2 relaxin plays a role in prostate carcinogenesis. Overexpression of RLN2 can induce tumor growth in a mouse model of CaP [37], and stimulation with RLN2 increases cell proliferation, invasiveness, and adhesion of CaP cells *in vitro* [7, 8]. Inhibition of RLN2 using an inhibitory analog [38], or suppression of its receptor LGR7 (also called RXFP1) [9] blocks RLN2-mediated CaP growth. Studies in human CaP have demonstrated that RLN2 expression is increased in radical prostatectomy specimens after 6 months of androgen ablation and in CR CaP, and that expression is highest in bone metastases [44]. Our group has demonstrated RLN2 mediates CR growth of CaP cells by a mechanism that involves PI3K-dependent co-translocation of the androgen receptor (AR) and β -catenin to the nucleus and transactivation of the PSA promoter [21, 46]. Based on our studies, others have also shown that RLN2-induced β -catenin stabilization is mediated by ProtocadherinY [43]. While it is clear β -catenin plays an important role in mediating the effects of RLN2 in CaP cells, inhibition of β -catenin stabilization or of Akt activation only partially inhibits RLN2-mediated growth of LNCaP, while blocking the RLN2 receptor, RXFP1, causes near complete inhibition. Thus the goal of the current study was to further elucidate the mechanism(s) by which RLN2 contributes to AR activation and CaP progression.

In the current study, we confirm that H2 relaxin expression is elevated in CaP patient specimens and in addition demonstrate that RLN2 is expressed at low levels in BPH relative to CaP specimens ($p=0.002$). This finding has not previously been reported. We have used next-generation sequencing (NGS)-based transcriptome and gene ontology (GO) analyses to identify additional downstream effectors of RLN2 in CaP cells. These data, combined with molecular and inhibitor studies, have identified the NF- κ B and protein kinase A (PKA) signaling pathways as being activated by RLN2. Most importantly, when both pathways were simultaneously attenuated by the use of the PKA inhibitor H-89 as well as perifosine, which is an upstream inhibitor of NF- κ B, RLN2-induced cell growth and survival were effectively down-regulated.

Materials and Methods

Cell Lines and Culture. LNCaP, PC-3 and Rwpel cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). CWR22Rv1 and PC346C (an androgen dependent cell line [22]) were kindly provided to us by Drs. Hsing-Jien Kung and Van Weerden respectively. All cell lines were maintained as previously described [46].

Generation of Stable Cell Lines. The RLN2 LNCaP sublines (LNCaP-rlx3 and LNCaP-rlx5) were generated in house. Briefly, the RLN2 allele was cloned into the pCR3.1 vector (Invitrogen, Carlsbad, CA) downstream of the cytomegalovirus promoter. Plasmids containing the RLN2 allele or empty pCR3.1 plasmid (LNCaP-vector) were stably transfected into LNCaP cells using Effectene (Qiagen, Valencia, CA). After 48 h, cells were grown under G418 (500 µg/ml) selection for 2–3 weeks until isolated colonies appeared. Colonies were selected and expanded in 24-well plates before being transferred to culture flasks.

Reagents. Antibodies: Total and phospho IκB-α, and Bcl-xL (Cell Signaling Technology, Beverly, MA), B-actin (Sigma-Aldrich Corporation, St. Louis, MO), NFκB (Santa Cruz BioTechnologies, Santa Cruz, CA), H2 relaxin (ALPCO, Salem, NH). **Inhibitors:** IKK inhibitor (a 14-amino acid peptide corresponding to the active IκB phosphorylation recognition sequence fused to the hydrophobic region of the fibroblast growth factor signal peptide, Calbiochem, Gibbstown, NJ), PKA inhibitors, H89 and PKI, (Calbiochem, Gibbstown, NJ).

SiRNA. Pre-validated siRNA specific for beta-catenin was purchased from Dharmacon (Lafayette, CO). To achieve knockdown, cells were transfected with either 20nM or 50nM siRNA using Lipofectamine 2000 as previously described [21].

Clonogenic Assay. Single cells (12,000) were seeded into 60-mm culture dishes containing FBS media on day 0 and allowed to attach for 24 h at 37°C. After 24 hours, FBS media was replaced with charcoal stripped serum (CSS) media and cultured for 14 days. Colonies were fixed in 1.0% crystal violet and 0.5% glacial acetic acid in ethanol, and visible colonies containing approximately 50 or more cells were counted.

Flow Cytometry. Cell cycle analysis: Analyses were performed as previously described [45]. **Apoptosis analysis:** The TACS annexin V-FITC kit (R&D Systems) was used to quantitate both early and late apoptosis. The analysis was performed using a Coulter Epics XL flow cytometer (Beckman Coulter), compensation was performed using FlowJo software (FlowJo, Ashland, OR). The assay was performed in accordance with the protocol described by the manufacturer. All samples were run in triplicate, all experiments were performed at least 3 times.

Immunoblot Analysis. Analyses were performed as previously described [46].

Cell culture IHC. Cells (50,000 per chamber) were seeded in chamber slides (BD Falcon, Two Oak Park, MA) in FBS media and allowed to adhere for 24 hours. After 24 hours, FBS media was replaced with CSS media. After 3 days, cells were washed with PBS (pH7.4) then fixed in ice-cold methanol for 7 minutes. After further washing, cells were incubated with 0.5% BSA for 20 minutes prior to the NFκB antibody (see reagents section for details) being added at a dilution of 1:250. After 60 minutes, cells were washed and then incubated with a FITC labeled secondary antibody (Sigma-Aldrich), 1:500. Cells were again washed and then mounted using DAPI-containing mountant (Vector labs, Burlingame, CA). Staining was visualized by fluorescence microscopy using an Olympus BX61 microscope equipped with SlideBook digital imaging software.

cAMP and PKA kinase assays.

cAMP; The cAMP Biotrak competitive enzyme immunoassay (Amersham-Pharmacia, RPN225) was used to determine cAMP activity levels 10 minutes after treatment of parental LNCaP with recombinant human (rh) H2

relaxin. Forskolin (Calbiochem) was used as a positive control. LNCaP were serum starved for 24 hours prior to the addition of rh H2 relaxin or forskolin. The assay was performed in accordance with the Protocol 3 described by the manufacturer. *PKA*; The PKA kinase activity assay (Stressgen, EKS-390A) was used to determine PKA activity levels 5, 15 and 30 minutes after treatment of parental LNCaP with rh H2 relaxin or rh H2 relaxin and H89. LNCaP were serum starved for 24 hours prior to the addition of rh H2 relaxin or H89. The assay was performed in accordance with the protocol described by the manufacturer. All samples were run in triplicate, all experiments were performed at least 3 times.

NFκB DNA binding assay.

The NFκB p65 EZ-TFA Transcription Factor assay (Millipore) was used to determine whether H2 relaxin is able to mediate binding of NFκB to its DNA consensus sequence. This assay was performed using protein extracted from both LNCaP-vector treated with 100ng/ml rh H2 relaxin for 60 minutes and from the LNCaP-RLN2/C1 and LNCaP-RLN2/C2 sublines cultured in CSS media. The Whole Cell Extraction kit (Millipore) was used to prepare the protein extracts used in this assay. The assay was performed in accordance with the protocol described by the manufacturer. All samples were run in triplicate, all experiments were performed at least 3 times.

Tissue microarray (TMA) and TMA IHC. The tissue microarrays were constructed using a Semi-Automated Tissue Arrayer, TMAarrayer (Pathology Devices, Inc., Westminster, MD). Cores from 49 CaP (with known grade), 15 PIN and 24 BPH patients (4 core replicates of each) were analyzed. The protocol described by Thompson et al. was used to stain the CaP TMAs for H2 relaxin [44].

Multi-Spectral Imaging and Quantification of Staining.

Images were obtained using an Olympus BX51 microscope with an attached Nuance CRI multispectral imaging system version 2.10.0 (Cambridge Research and Instrumentation, Woburn, MA). This imaging system is able to separate signals generated by different chromagens and thereby improve quantitative accuracy (review; [20]). Isolation of background tissue and hematoxylin wavelengths was achieved by spectral analysis of hematoxylin only stained cores and slide mounted pure liquid hematoxylin. The 3,3'-Diaminobenzidine (DAB) wavelength was isolated by positive control core samples and slide mounted pure liquid DAB. Multispectral analysis was then used to assess the ratio of DAB level to TMA core area for each individual TMA core. Images were converted to a false fluorescence format.

RNA extraction and qRT-PCR. RNA extraction methods have previously been described [46]. RLN2 and GAPDH expression was assessed using pre-designed TaqMan primer/probes sets in combination with the TaqMan Reverse Transcription and Universal PCR Master Mix kits as per manufacturer's protocol (Applied Biosystems, Foster City, CA). Samples were run on an ABI 7900HT and data analyzed using the corresponding software. Triplicate samples were run for each experimental group. Care was taken to ensure that the resulting Ct values for each group were within 0.5 Ct of each other.

Cell proliferation assay. Described by Vinall et al. [46].

PSA ELISA. LNCaP were seeded into 24 well plates (50,000 cells/well) in FBS media and allowed to attach for 24 hours. After 24 hours the FBS media was replaced with CSS media and the relevant inhibitors added (see reagents section for details. The level of PSA present in culture supernatants was measured using a PSA ELISA kit (MEDICORP, Montre´al, Que´bec, Canada) according to the manufacturer's protocol. Absorbance was measured at OD 450 using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Labs, Hercules, CA).

Sequencing-based transcriptome analysis. Next-generation sequencing (NGS) was utilized for whole transcriptome analysis of LNCaP-vector and the LNCaP-RLN2 and LNCaP-p53/R273H sublines. For this, RNA-Sequencing (RNA-Seq) libraries were prepared from 1 ug total RNA using the mRNA-Seq Sample Prep

Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. The libraries were then loaded on paired-end flow cells for cluster generation (cBot) followed by sequencing (2 x 40-bp) with the Genome Analyzer Iix (Illumina, Inc.) using kitted reagents (Illumina) and according to the manufacturer's protocols [4]. Data and bioinformatics analysis: Image processing, base calling, and quality scoring (Phred) were executed by SCS 2.6/RTA 1.6 software (Illumina, Inc.). CASAVA 1.7 (Illumina) was used for read alignment to the reference human genome sequence (GRCh37/hg19) using ELANDv2 and allowing for a maximum of two mismatches. Normalized transcript levels were quantified by calculation for RPKM (reads per kilobase of exon model per million mapped reads) [25]. Comparison analysis was performed in order to identify genes differentially expressed in the LNCaP-RLN2 clones as compared to the LNCaP-vector control. For this, genes up- or down-regulated in LNCaP-RLN2 were filtered based upon having RPKM values ≥ 25 (*i.e.*, moderate expression) in either the LNCaP-RLN2 or LNCaP-vector cell lines, respectively, exhibiting the same trend in regulation in both LNCaP-RLN2 clones, and having ≥ 1.5 -fold change in expression (RLN2/Vector). Subsequently, Gene Ontology (GO) analysis [2] was performed in order to classify the genes according to biological process and molecular function.

Statistical analysis. At least three independent experiments were completed for each analysis described in this article. Data are shown as mean $\pm$ SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for comparison of means using STATA software (College Station, TX). $p < 0.05$ was considered statistically significant (* signifies $p < 0.05$).

Results

RLN2 is elevated in CaP patient specimens relative to benign prostatic hyperplasia (BPH).

To determine the significance of RLN2 in human prostate cancer (CaP) patients, we conducted IHC analysis of 49 CaP, 15 PIN and 24 BPH specimens taken from patients with primary CaP undergoing prostatectomy (for PIN and CaP specimens) or TURP (for BPH) as initial treatment for the disease (Figure 1A). Note that RLN2 stained the epithelial cells strongly while some staining could also be seen for the stromal cells. However, the nuclei did not stain for this peptide hormone at all, demonstrating the specificity of the staining. Specifically, the staining level increased from BPH<PIN=CaP indicating the increased accumulation of RLN2 during initiation of CaP. Quantification of staining using the Nuance multi-spectral imaging system demonstrated that RLN2 expression is significantly higher in CaP specimens relative to the BPH specimens (Figure 1B, 0.119 $\pm$ 0.032 versus 0.95 $\pm$ 0.021, $p=0.002$), a finding that has not previously been reported. The increase of RLN2 in CaP compared to BPH is of clinical relevance because in patients the serum levels of PSA increase in both cases. Since RLN2 can be detected in the serum [37], this means that the serum levels of this peptide could potentially be used as an independent marker of CaP as opposed to BPH. Further studies are required to test this hypothesis. A significant difference in RLN2 expression was not observed between the PIN and CaP specimens ($p=0.475$) and RLN2 expression did not correlate with Gleason grade (Figure 1C). Specimens from CaP patients with CR disease were not assessed due to lack of availability, however, other studies have observed very high H2 relaxin expression levels in bone metastases specimens from CR CaP patients [44]. Quantitative RT-PCR (qRT-PCR) analysis and RLN2 IHC was used to assess relative expression of RLN2 in “normal-like” RWPE-1 and multiple CaP cell lines (Figure 1D, E). Note that triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. Excluding PC3, the aggressive tumors C4-2 and CWR22Rv1 expressed significantly higher levels of RLN2 compared to the RWPE-1 cells ($p<0.05$) derived from a normal human prostate and compared to the PC-346C cells ($p<0.05$) derived from an androgen-dependent CaP thereby supporting our hypothesis that RLN2 plays a role in progression to CR CaP.

Generation of LNCaP sublines that stably overexpress H2 relaxin.

To understand the functional significance of increased RLN2 expression in CaP, we stably transfected LNCaP with plasmid expressing the RLN2 gene. LNCaP are an androgen dependent CaP cell line, and are the cell line that was used for all our previous RLN2-related studies [21, 46]. Two clones (LNCaP-RLN2/C1 and LNCaP-RLN2/C2) were chosen for further investigation of the role played by RLN2 in CaP. These sublines expressed ~20-fold higher levels of RLN2 mRNA relative to LNCaP stably transfected with the vector control (LNCaP-vector) (Figure 2A), $p<0.005$ for both LNCaP-RLN2/C1 and /C2 versus LNCaP-vector. LNCaP-R273H, an androgen independent LNCaP subline that stably expresses a p53^{R273H} mutant allele, expressed the highest level of RLN2 mRNA, ~60-fold higher than the androgen dependent LNCaP-vector subline ($p<0.0005$), and was used as a positive control. Note that triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. We have previously demonstrated that the p53^{R273H} mutant, which is a hotspot mutation in CaP patients, can bind to the RLN2 promoter and drive RLN2 expression [44]. In the present study, the increase in RLN2 expression was observed at both the mRNA level (as determined by qRT-PCR) and at the protein level (as determined by immunocytochemistry in the same cell lines) (Figure 2B). It is noteworthy that the LNCaP-RLN2 sublines appear to be good models of CR CaP as they behave similarly to the CaP cells found in CR CaP patient tumors; they are able to grow in the absence of androgens and express normal levels of AR but high levels of PSA even in the absence of androgen, i.e. the AR pathway can be activated in a ligand independent manner.

Identification of downstream effectors of RLN2 using Next-Generation Sequencing (NGS) analysis.

To help identify downstream effectors of RLN2 and to further elucidate the role played by RLN2 in CaP, we conducted next-generation DNA sequencing (NGS) analysis (i.e. RNA-Seq) followed by GO analysis using LNCaP-RLN2/C1 and LNCaP-RLN2/C2 vs LNCaP-vector sublines. Comparison of gene sequence analysis (Figure 2C) revealed 12.7% of genes with >1.5-fold increased expression in the LNCaP-RLN2 sublines relative

to the LNCaP-vector were related to proliferation. Other key processes associated with increased RLN2 expression were transcription (18.6%), metabolism (16.4%), signal transduction (11.7%) and proteolysis (6.2%) (Figure 2C). As expected, NGS analysis determined that the LNCaP-RLN2 sublines express high levels of RLN2 compared to the LNCaP-vector subline (350-fold and 582-fold increased expression in the LNCaP-RLN2/C1 and -RLN2/C2 sublines respectively). Table 1 lists the top 10 differentially expressed genes in the RLN2 versus vector control sublines. It is of note that H1 relaxin (RLN1) is also expressed at high levels in the RLN2 sublines. While the RLN1 isoform has been shown to be expressed in the prostate at the mRNA level, only the RLN2 is translated and secreted [16, 35, 31, 12, 17, 47] indicating that upregulation of RLN1 expression in the RLN2 sublines likely has no functional consequence. Expression of PSA (KLK3, 8-fold and 22-fold increased expression in LNCaP-RLN2/C1 and -RLN2/C2 respectively), a downstream target of the AR, and Cyclin D1 (CCND1, 1.44-fold and 1.2-fold increased expression), an important cell cycle regulator, was increased in the RLN2 sublines. Expression of TRAF1 and C-IAP1, which can be driven by NF κ B, were also overexpressed (TRAF1, 2.01-fold and 3.08-fold increased expression, C-IAP1, 1.47-fold and 1.5-fold increased expression) (Table 1). Both of these molecules have been shown to promote cell survival.

To verify the observations made with the NGS/GO analysis, we demonstrated that stable expression of RLN2 increased PSA levels, but not AR expression, in LNCaP cells, confirming that RLN2 affects AR transcriptional activity but not expression (Figure 2D). These results thereby validate the use of these clones as a model for determining RLN2 function in CaP. Increased expression of RLN2 has been observed during neuroendocrine differentiation (NED) of CaP cells [10], a process that is associated with the development of CR CaP in CaP patients [48]. Our data also indicate that H2 relaxin is associated with NED, and thereby further support a role for H2 relaxin in progression to CR CaP. The LNCaP-RLN2/C1 and -RLN2/C2 sublines express increased levels of NSE and decreased levels of NEP compared to LNCaP-vector (Figure 2C). Neuron specific enolase (NSE) is a key marker of NED (review; [33]) and increased expression is associated with CaP progression [19]. Neural endopeptidase (NEP) is an enzyme that is expressed at high levels by normal CaP cells and is responsible for degrading neuropeptides such as bombesin, ET-1 and neurotensin that promote NED [26, 27]. NGS analysis revealed that mRNA levels of NSE and NEP were also altered in the LNCaP-RLN2 sublines, 9.532-fold and 18.796-fold increase in NSE in LNCaP-RLN2/C1 and -RLN2/C2 respectively, and a 2.544-fold and 1.668-fold decrease in NEP expression. Since cyclin D1 and several survival-related genes appeared to be upregulated in H2-relaxin overexpressing cells (Table 1), we determined whether RLN2 overexpression stimulated cell numbers. MTT assay verified that RLN2 overexpressing sublines have a significantly increased rate of cell growth in culture medium containing charcoal stripped serum (containing castrate levels of androgens) compared to vector transfected LNCaP cells (Figure 2E, statistical analysis compared the day 5 data for each subline and revealed a significant difference between the LNCaP-vector compared to all LNCaP-RLN2/C1 and /C2 as well as LNCaP-R273H, $p < 0.005$). These observations are supported by our previous observations indicating ligand independent activation of the AR by RLN2 [46]. It should be noted that increased AR activity and increase cell proliferation are not characteristics that associated with NED. Our data indicate that the LNCaP-RLN2 sublines have some NED-like characteristics they are clearly not NE cells. While this is somewhat unusual, other groups have reported similar findings. For example, Snail induces NSE and chromogranin A expression in LNCaP as well as mediating nuclear translocation of AR and increased PSA expression [23].

RLN2 promotes activation of an NF- κ B-dependent cell survival pathway in LNCaP prostate cancer cells.

Since the NGS/GO analysis demonstrated an increase in TRAF1 and C-IAP1, which can be driven by NF κ B, in LNCaP sublines overexpressing RLN2, we also investigated the activation of NF κ B in these cells. The NF- κ B subunits (p65, p50) remain bound to I κ B- α in the cytoplasm; upon stimulation, I κ B- α is degraded and p65/p50 released, which then translocates to the nucleus, and helps transcribe anti-apoptotic genes such as Bcl-xL [49]. To determine whether the NF κ B pathway is active in the RLN2 LNCaP sublines we assessed I κ B- α expression levels and phosphorylation state, NF κ B localization, and binding of NF κ B to its DNA consensus sequence. I κ B- α levels were significantly lower in the RLN2 LNCaP sublines and I κ B- α was phosphorylated indicating

active degradation of I κ B- α occurs in these cells (Figure 3A). Increased expression of Bcl-xL, a downstream effector of NF κ B, was also observed in the LNCaP sublines overexpressing RLN2. Immunofluorescence analysis of the LNCaP-rlx sublines revealed a significant increase in levels of nuclear NF κ B compared to LNCaP-vector (Figure 3B). LNCaP treated with TNF- α were used as a positive control for nuclear staining. We also demonstrate that RLN2 is able to facilitate binding of the NF κ B p65 subunit to its DNA consensus sequence (Figure 3C). Significantly increased binding was observed in both LNCaP-vector treated with recombinant human (rh) RLN2 and in the RLN2 LNCaP sublines relative to the LNCaP-vector only control ($p < 0.05$ for all 3 LNCaP sublines compared to LNCaP-vector). These studies indicate that activation of NF- κ B is an important mediator of RLN2-mediated cell survival, and points to a mechanism by which RLN2 may induce CR CaP.

It is known that RLN2 signals via the G protein-coupled receptor (GPCR) RXFP1 in CaP cells. To determine whether the effects of RLN2 on NF- κ B are mediated by RXFP1, we investigated the effect of RXFP1 knockdown on NF- κ B activity. RXFP1-mediated activation of NF- κ B could be inhibited using siRNA specific to RXFP1, the RLN2 receptor (Figure 3D), indicating that the effects of RLN2 on NF- κ B are indeed mediated by RXFP1.

RLN2 stimulates cAMP production and PKA activation independent of NF- κ B.

Activation of the H2 relaxin receptor RXFP1 by RLN2 activates the G_s class of G-proteins (G_{sa}), resulting in cyclic AMP (cAMP) dependent PKA activation. Hence, we investigated whether RLN2s effects are mediated by the PKA pathway in CaP cells. Previous studies performed in other cell types have demonstrated that RLN2 causes activation of the adenylate cyclase/AMP/PKA pathway [3, 15]. Our results validate these findings in CaP cells. Treatment of parental LNCaP with 10, 50 and 100 ng/ml recombinant human RLN2 (rhRLN2) induced a significant increase ($p < 0.005$), ~2.2 and 2.1-fold increase in cAMP activity respectively compared to a 3.2-fold forskolin-induced response (positive control, $p < 0.005$) (Figure 4A). However, this increase was transient as shown by the decrease in cAMP levels after 30 minutes of treatment. Similarly, treatment of parental LNCaP with 50 ng/ml RLN2 induced a ~3.3-fold increase in PKA activity ($p < 0.005$) compared to a ~4.9-fold forskolin-induced increase (positive control, $p < 0.005$) (Figure 4B). On the other hand, co-treatment with the PKA inhibitor H89 was able to completely inhibit this response to RLN2 indicating the assay is PKA specific (Figure 4B). Inhibition of PKA using H89 also caused a decrease in growth rate in both LNCaP-RLN2/C1 and -RLN2/C2 cultured in CSS media - in LNCaP-RLN2/C1 a ~1.8-fold decrease in growth rate was observed ($p < 0.05$), and in LNCaP-RLN2/C2 a ~2.1-fold decrease ensued (Figure 4C, $p < 0.05$). Inhibition of the PKA pathway did not directly affect the activation of NF- κ B (data not shown), indicating that the PKA and the NF- κ B pathways represent two different arms of the signaling mechanisms downstream of RLN2 (Figure 4F). Inhibition of PKA had a more dramatic effect on PSA expression (Figure 4D, E). Treatment with H89 caused a ~7-fold decrease in PSA levels in LNCaP-RLN2/C1 ($p < 0.005$) and a ~5.6-fold decrease in LNCaP-RLN2/C2 ($p < 0.05$). It is of note that the RLN2 LNCaP sublines express very high levels of PSA even when cultured in castrate conditions. These data indicate that H2 relaxin induces the activation of the AR signaling pathway and cell growth in a ligand independent manner by a mechanism mediated by the activation of the cAMP/PKA pathway.

RLN2 overexpression confers resistance to treatment with therapeutic agents.

Activation of the NF- κ B pathway has been frequently associated with drug resistance. Since RLN2 induces an increase in NF- κ B activity, we investigated whether RLN2 expression is also associated with resistance to various therapeutic drugs. Annexin V/propidium iodide (PI) labeling followed by flow cytometry analysis to investigate the effects on apoptosis showed that LNCaP cells transfected with vector only are highly susceptible to induction of apoptosis by various inhibitors including LY294002 (PI3K inhibitor), perifosine (Akt inhibitor), rapamycin (mTOR inhibitor) and docetaxel (anti-mitotic), whereas the LNCaP-RLN2/C1 and -RLN2/C2 sublines are more resistant to treatment with the same drugs (Figure 5A). Perifosine, rapamycin and docetaxel are all clinical agents. Perifosine has been shown to reduce PSA levels in 20% CaP patients with recurrent

disease [5]. Several on-going clinical trials are testing the efficacy of rapamycin, and analogs of rapamycin alone and in combination with other agents (review; [11]). Docetaxel is the standard of care treatment for CaP patients with castrate resistant CaP (review; [39]). For LNCaP-vector, treatment with vehicle control, LY294002, perifosine, rapamycin and docetaxel induced ~10, 38, 22, 37, 29% apoptosis respectively (Figure 5A), but was reduced in LNCaP-RLN2/C1 (4, 20, 12, 13, 9%) and LNCaP-RLN2/C2 (1, 4, 10, 2, 1.5%). The levels of apoptosis in LNCaP-vector were statistically higher compared to those observed in the LNCaP RLN2 sublines regardless of the type of drug treatment ($p < 0.05$). These data indicate that there is a link between RLN2 expression and chemoresistance in LNCaP cells, and provide rationale for combining targeted inhibition of the RLN2 pathway with conventional chemotherapy.

Combined treatment with perifosine and a PKA inhibitor in CaP cells overexpressing RLN2 promotes apoptosis.

IKK causes phosphorylation of $\text{I}\kappa\text{B}-\alpha$ and subsequent proteasome-mediated degradation. This degradation allows NF κ B to translocate to the nucleus. Hence an IKK inhibitor would inhibit the activation of the NF- κ B pathway. Perifosine is known to be an Akt inhibitor; however, it inhibits NF- κ B activation to the same extent as the IKK inhibitor (Figure 5B). As perifosine has been FDA approved and is currently in clinical trials for the treatment of CaP, we investigated whether its effects on NF- κ B would be of significance in the treatment of patients who overexpress RLN2 and may have developed resistance to commonly used drugs as a result. Hence we compared the effects of perifosine to that of the IKK inhibitor (Figure 5B,C). Inhibition of IKK in the LNCaP-RLN2/C1 and -RLN2/C2 sublines did not cause a significant increase in apoptosis or decrease in clonogenic potential (Figure 5C), whereas perifosine alone caused only a moderate increase in apoptosis and decrease in clonogenic potential (~2-fold increase and ~40% decrease respectively, Figure 5C). Similarly, simultaneous blockade of IKK and Akt resulted in only a modest increase in apoptosis and decrease in clonogenic potential compared to treatment with perifosine alone (Figure 5C). We concluded that the lack of a clinically relevant increase with dual blockade is due to the fact that both the IKK inhibitor and perifosine are acting on the same target, either directly or indirectly (see scheme in Figure 4F) resulting in decreased binding of NF κ B to its DNA consensus sequence (Figure 5B).

We hypothesized that simultaneous blockade of pathways leading to PKA and NF- κ B would therefore be the only way to completely block signaling downstream of RLN2-induced cell proliferation and survival (based on scheme in Figure 4F). H-89 alone had little or no effect on the RLN2 overexpressing clones (Figure 5D); in addition, inhibition of either IKK or PKA caused only a moderate increase in the sensitivity of the RLN2 LNCaP sublines to treatment with docetaxel (Figure 5C,D). However, dual inhibition of both arms of the RLN2/RXFP1 pathway, with H-89 and perifosine, resulted in a larger and significant increase in apoptosis compared to blockade of either individual pathway (~2-3-fold increase in apoptosis compared to treatment with perifosine alone, ~15-18% apoptosis in the combination treatment, Figure 5D), and compared to treatment with docetaxel (docetaxel induced only ~2-3% apoptosis in the LNCaP-RLN2 sublines, Figure 5D). It is of note that this dual inhibition induced a similar level of apoptosis in the LNCaP-RLN2 sublines (15-18%) as docetaxel treatment in the LNCaP-vector subline (~25%). A significant decrease in clonogenic potential was also observed (~20-30% decrease compared to treatment with either the IKK inhibitor or perifosine alone (Figure 5D). Taken together, these results indicate that overexpression of RLN2, which is commonly seen in tumors from patients with CaP (Figure 1A), provides a growth advantage to CaP cells by causing activation of both the NF- κ B and PKA pathways. A near complete inhibition of this growth advantage can be achieved only by simultaneous blockade of both pathways.

Discussion

The key finding of this study is that dual blockade of the PKA and NF- κ B signaling pathways inhibits H2 relaxin-mediated castrate resistant growth of prostate cancer cells. This finding is of clinical relevance as our group and others have determined H2 relaxin is expressed at increased levels in CaP patients and increases in CaP patients following androgen ablation [44]. In addition, H2 relaxin has been demonstrated to play an important role in mediating CR CaP growth [37, 7, 8, 21, 46]. Focus was placed on determining the importance of the NF- κ B and PKA pathways in facilitating H2 relaxin-mediated CR CaP growth because 1. NGS analyses identified the differential expression of several PKA and NF- κ B-related genes in the LNCaP-RLN2 sublines, 2. These pathways are known to be dysregulated in CaP and are linked to CaP progression, 3. Both pathways have been demonstrated to be activated by H2 relaxin in other cell types.

Several groups have demonstrated a link between PKA activity and CaP progression (review; [24]). PKA can mediate ligand-independent activation of AR and can therefore play an important role in facilitating both androgen dependent and independent CaP. We have previously demonstrated that H2 relaxin mediates PI3K-dependent co-translocation of the androgen receptor (AR) and β -catenin to the nucleus and causes transactivation of the PSA promoter [21, 46]. Our current data indicate that H2 relaxin can also activate AR via PKA. H2 relaxin has been demonstrated to cause activation of PKA in other cell types [34, 14]. H2 relaxin signals via RXFP1 and 2, both of which are GPCRs that activate the G_s class of G-proteins (G_{sa}) resulting in cAMP dependent PKA activation. Only RXFP1 is expressed in CaP cells [44]. Our data demonstrate AR activity is very high in the LNCaP-RLN2 sublines relative to LNCaP-vector, and that inhibition of PKA in the LNCaP-RLN2 sublines causes a significant inhibition of this elevated AR activity. These data indicate that PKA-mediated activation of AR is very important in a setting of elevated H2 relaxin expression.

NF- κ B is a transcription factor which controls expression of genes associated with both cell proliferation and apoptosis [36]. NF- κ B has been demonstrated to be constitutively active in several CaP cell lines and expressed at high levels in both PIN and CaP patient samples [1, 6, 19, 28, 40]. Usually NF- κ B is sequestered in the cytoplasm through interaction of its p65 and p50 subunits with I κ B α . Growth and survival stimuli induce phosphorylation of I κ B α by IKK α resulting in I κ B α degradation, followed by p65 phosphorylation and NF- κ B translocation to the nucleus. We demonstrate that H2 relaxin is one of these growth stimuli; forced overexpression of H2 relaxin caused I κ B α degradation, nuclear translocation of NF- κ B and binding to the NF- κ B DNA binding consensus sequence. Relaxin has previously been shown to activate NF- κ B in other organs but this is the first time it has been shown to activate NF- κ B in CaP. While it has been shown that Akt phosphorylation such as that induced by H2 relaxin can phosphorylate the p65 subunit of NF- κ B [41], we show that H2 relaxin promotes NF- κ B activity by degrading I κ B α .

There is sound rationale to simultaneously block both the PKA and NF- κ B pathways in CaP cells that express elevated levels of H2 relaxin; both pathways are activated by H2 relaxin yet mediate proliferation by different mechanisms, and both pathways have been shown to be active in CaP patients. While inhibition of either pathway alone resulted in growth inhibition and/or a small increase in apoptosis, it was only when both pathways were inhibited simultaneously that a clinically relevant increase in apoptosis occurred. As inhibition of both IKK and Akt caused similar levels of NF- κ B inhibition, we chose to use perifosine, an Akt inhibitor, for the drug combination studies as it has been FDA approved and tried in CaP clinical trials. In contrast, common IKK inhibitors such as Bay11-7082 have not and are therefore of limited relevance for translational studies. It is possible that our future studies may employ other inhibitors such as Bortezomib, a proteasome inhibitor that is currently in clinical use and has been found to inhibit NF- κ B [32]. Currently, PKA inhibitors are not in clinical use in CaP patients. For future translational studies, we may employ drugs that lower cAMP levels such as beta-blockers, which are in clinical use, to determine if these have an effect on H2 relaxin signaling. It is of note that beta-blockers have been found to have a small effect on prevention of CaP [29]. It is also of note that the

simultaneous blockade of the PKA and NF- κ B pathways outperformed docetaxel, the standard of care treatment for advanced CR CaP.

In summary, our data indicate that simultaneous inhibition of PKA and NF- κ B would prevent RLN2 mediated cell survival in CaP, and that in a setting of elevated RLN2 expression this combined inhibition is superior to docetaxel. The number of patients who would potentially benefit from this study is likely to be extensive since a significant portion of CaP patients overexpress RLN2.

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Conflicts of Interest

The authors declare no conflicts of interest.

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TABLE 1.

Genes that are differentially expressed by LNCaP-RLN2 versus LNCaP-vector sublines.

NGS was performed on mRNA-Seq libraries prepared from total RNA isolated from LNCaP-RLN2 and vector control cell lines. Data analysis was performed as described in *Materials and Methods*. Normalized transcript expression (RPKM values) was used for calculation of fold expression changes in the RLN2 LNCaP sublines relative to the vector control. The numbers listed in the LNCaP-RLN2/C1 and –RLN2/C2 columns of the table are fold change in gene expression relative to LNCaP-vector. This table also lists the relative expression of genes that have been shown to be driven by either the beta-catenin/AR complex or by NFκB and those involved in neuroendocrine differentiation (in grey). Both PSA (KLK3) and cyclin D1 (CCND1) have increased expression in the LNCaP-RLN2 sublines, as does TRAF1 and C-IAP1 (CIAPIN1). C-Myc and Bcl-xL were not differentially expressed.

<i>Gene symbol</i>	<i>LNCaP-RLN2/C1</i> <i>(fold-change relative to LNCaP- vector)</i>	<i>LNCaP-RLN2/C2</i> <i>(fold-change relative to LNCaP-vector)</i>	<i>GO Function</i>
SALL2	932	1827	transcription
CCL20	701	166	chemotaxis
KLHL13	693	187	catabolism
TCEA3	498	705	transcription
RLN1	419	241	signal transduction
TUSC3	413	264	glycosylation
PEG3	401	231	transcription
RLN2	350	582	female pregnancy
S100A10	267	165	signal transduction
MEST	196	106	mesoderm development
KLK3	8	22	catalytic activity
MYC	1.18	0.94	survival
CCND1	1.44	1.20	cell cycle
BCL2L1	0.83	0.93	survival
TRAF1	2.01	3.08	survival
CIAPIN1	1.47	1.50	survival
NSE	9.532	18.796	NED
NEP	2.544	1.668	NED

FIGURE LEGENDS

Figure 1: RLN2 expression is elevated in CaP patients. (A) Representative images from cores of BPH, PIN and CaP were obtained from patients with BPH (who underwent TURP) or CaP (who underwent prostatectomy) with IRB consent and assembled as a TMA. The cores were immunostained with an antibody to RLN2 and counterstained with hematoxylin. Multispectral imaging technologies were utilized to convert the brown staining (representing RLN2 localization) and the blue hematoxylin counterstain to false fluorescent imaging for better visualization and staining quantitation. Here shown are two representative cores from each group. (B) Box plot comparing median H2 relaxin expression values and interquartile ranges between the patient groups. Statistical analysis of RLN2 expression based on quantitation of multi-spectral imaging determined that RLN2 expression is higher in CaP patients compared to patients with BPH ($p=0.002$), while the difference in staining between BPH and PIN or PIN and CaP was not significant. All patient cores were assessed based on their DAB level to area score. (C). RLN2 expression did not correlate with Gleason grade. (D) Quantitative RT-PCR (qRT-PCR) analysis of RWPE-1 cells (derived from a normal prostate) vs several CaP cell lines demonstrated that, excluding PC3 cells, RLN2 expression is elevated in CaP cell lines. Triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. (E) Immunocytochemical analysis in RWPE-1 vs C4-2 with RLN2 antibody (brown stain) cells confirmed this trend. The cells were counterstained with hematoxylin (blue) which allows visualization of unstained cells.

Figure 2: Effect of increased expression of RLN2 in LNCaP prostate cancer cells. (A) LNCaP sublines LNCaP-RLN2/C1 and LNCaP-RLN2/C2, which express elevated levels of RLN2, were generated by stable transfection of RLN2 in LNCaP cells. Quantitative RT-PCR (qRT-PCR) analysis for RLN2 mRNA levels in these sublines compared to vector-transfected LNCaP cells and LNCaP-R273H, a subline previously shown to express extremely high levels of this peptide hormone, demonstrated that the two RLN2 LNCaP sublines expressed RLN2 at significantly higher levels compared to LNCaP, but not as high as in p53^{R273H}-transfected cells. Triplicate samples were run for each experimental group and the resulting Ct values for each group were within 0.5 Ct of each other. (B) Immunocytochemical analysis of the cell lines confirmed this trend. (C) Next generation sequencing (NGS) followed by Gene Ontology (GO) analysis revealed 12.7% of genes that are differentially expressed between the RLN2 LNCaP and LNCaP-vector sublines are linked to proliferation. Other key processes associated with increased RLN2 expression were transcription (18.6%), metabolism (16.4%), signal transduction (11.7%) and proteolysis (6.2%). (D) The RLN2 LNCaP sublines express high levels of NSE and low levels of NEP, suggesting a neuroendocrine-like phenotype. While lower levels of AR were observed in the RLN2 LNCaP sublines, assessment of PSA levels indicates that the AR pathway is much more active. (E) MTT proliferation assay determined that the RLN2 LNCaP sublines are able to grow in the absence of androgen, as was the LNCaP-R273H subline, and that the difference in proliferation at the day 5 time point was statistically significant when comparing the LNCaP-vector and all 3 LNCaP sublines. (* signifies $p < 0.05$).

Figure 3: RLN2 induces activation of the NF κ B pathway. (A) The RLN2 LNCaP sublines expressed decreased levels of I κ B- α and increased levels of P-I κ B- α , indicating RLN2 expression causes I κ B- α degradation. Increased levels of Bcl-xL, a downstream effector of NF κ B was also observed. (B) Increased nuclear translocation of NF κ B is observed in RLN2 LNCaP sublines. LNCaP cells or the RLN2 overexpressing sublines were immunostained with anti-p65 antibody (green) or with DAPI to detect the nuclei (blue). Merger of the two stains indicated NF- κ B nuclear localization. LNCaP cells treated with TNF- α (10 ng/ml) were used as positive control. Note that in LNCaP cells NF- κ B remained in the cytoplasm whereas in the RLN2-overexpressing sublines, the complex is localized to the nucleus. (C) NF- κ B transcriptional activity as determined by reporter assay was significantly elevated in RLN2 LNCaP sublines and in parental LNCaP treated with rhRLN2 (human recombinant). Nuclear localization of functional NF κ B was confirmed by assessment of the ability of NF κ B to bind to its DNA binding consensus sequence. (D) (upper panels).

Knockdown of RXFP1, the RLN2 receptor, inhibited the ability of NF κ B to bind to its DNA consensus sequence in the RLN2 LNCaP sublines (**lower panels**). (* signifies $p < 0.05$).

Figure 4: RLN2 stimulates cAMP production and PKA activation. (A) LNCaP cells were treated with 0, 10, 50, 100 ng/ml rhRLN2 (human recombinant) and cAMP levels measured by ELISA. 50 μ M Forskolin, which directly stimulates cAMP production, is used as a positive control. (B). 100 ng/ml rhRLN2 induced PKA activation comparable to the positive control Forskolin as measured by the phosphorylation of Kemptide, a phosphate group acceptor synthetic peptide. Activation of PKA was observed 15 minutes post-treatment upon treatment with 100 ng/ml rhRLN2. This activation could be inhibited using H89, a PKA inhibitor. (C, D) Inhibition of PKA activity in LNCaP-RLN2/C1 and LNCaP-RLN2/C2 cells resulted in inhibition of (C) cell growth as measured by MTT assay and (D, E) PSA levels, as measured by both Western blotting as well as PSA ELISA. Figure 4F shows a schematic representation of RLN2 signaling in CaP cells based on data obtained from this and other studies of the RLN2 pathway in CaP cells. RLN2 is able to cause activation of both the cAMP/PKA and NF- κ B signaling pathways by two independent mechanisms. Note that LGR7 is an alternate name for RXFP1. (* signifies $p < 0.05$).

Figure 5: Combined blockade of the PKA and PI3K/Akt signaling pathways that are activated by RLN2 promotes apoptosis. (A) The RLN2 LNCaP sublines are resistant to apoptosis by several drugs, including LY294002, perifosine, rapamycin and docetaxel. The levels of apoptosis in LNCaP-vector were statistically higher compared to those observed in the LNCaP RLN2 sublines regardless of the type of drug treatment (B) Perifosine, similar to the IKK inhibitor decrease binding of NF κ B to its DNA consensus sequence. (C) Inhibition of IKK in the LNCaP-RLN2/C1 and -RLN2/C2 sublines did not cause an increase in apoptosis, while perifosine alone caused only a moderate increase in apoptosis (~2-fold increase). Simultaneous blockade of IKK and Akt resulted in only a minimal increase in apoptosis compared to treatment with perifosine alone (**left panel**). A similar trend was observed by clonogenic assay (**right panel**). (D) On the other hand, simultaneous blockade of PKA with H-89 together with perifosine resulted in a significant increase in apoptosis compared to blockade of either individual pathway (~2-3-fold increase in apoptosis compared to treatment with perifosine alone, ~15-18% apoptosis in the combination treatment) (**left panel**), an increase that was far greater than that observed with docetaxel treatment (~2-3% apoptosis). Clonogenic assay showed this same trend (**right panel**). (* signifies $p < 0.05$).