Supporting Information

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SI Materials and Methods

In Vivo Studies. For the human xenograft model, 10^7 cells of the PC-3 human prostate cancer cell line were injected s.c. into the flanks of four donor animals. The tumors grown were removed aseptically and mechanically minced, and tumor pieces of ~3 mm^3 were transplanted s.c. using trocar needles into both flanks of animals. When tumors had grown to a mean volume of ~65 mm^3, the animals were assigned randomly to a control group (animals $n = 10$, tumors $n = 15$) and a group treated s.c. daily with 10 μg of the growth hormone-releasing hormone (GHRH) antagonist JMR-132 (animals $n = 10$, tumors $n = 16$). Tumor size was measured using microcalipers on days 0, 7, 14, 21, 28, and 49, and the tumor volume was calculated using the formula: length × width × height × 0.5236. Tumor doubling time was calculated at the end of the studies using the formula: study duration/(log final tumor volume − log initial tumor volume)/log2. Tumor inhibition was calculated using the formula: 100% ($\Delta volume_{control} - \Delta volume_{treatment}$)/$\Delta volume_{control}$, where Δvolume = final volume − initial volume. The experiment was terminated after 49 d of treatment.

At the end of the experiments, mice were anesthetized with phenobarbital and killed by severing the abdominal aorta. Tumors then were excised carefully, weighed, snap frozen, and stored at −80 °C for further investigations. Liver, heart, lungs, kidneys, and spleen were removed carefully and weighed.

Total DNA Isolation. To quantify the cellular content of PC-3 tumors, total DNA was prepared from 20 mg of tumor xenografts for each sample using the DNeasy Blood & Tissue kit (Qiagen). Five tumor samples from each group were analyzed. The yield and purity of DNA was determined according to manufacturer's instructions.

Immunohistochemical Staining. Serial 4-μm sections of xenografted PC-3 tumors were used for immunoperoxidase staining following standard protocols. Briefly, the paraffin was melted at 37 °C overnight and cleared in a bath of xylene for 10 min. The slides then were rehydrated in decreasing grades of ethanol (10 min each) and washed in PBS. Antigen retrieval was performed in a pressure cooker with Dako Target Retrieval Solution (S1968; Dako) at 90 °C for 15 min (pH 9.0). Antibody to Ki67 at 1:100 dilution (clone MIB-1; Dako) was added to the slides and incubated for 30 min at room temperature. Immunohistochemical analysis was performed using Dako Flex Detection System 1.0 (polymer-based). Diaminobenzidine was the chromogen in the presence of hydrogen peroxide. The reaction for Ki67 appeared as brown granules in the nuclei of positive cells.

Quantitative Evaluation of Cell Proliferation and Apoptosis in Xenografted PC-3 Tumors. Ki67-labeled cells were counted in 10 random fields at 40× objective magnification from three different individual PC-3 tumor sections. Slices of 5-μm thickness made from representative blocks of paraffin-embedded tissues were mounted on glass slides and stained with H&E for morphological analysis. The mitotic and apoptotic cells in the tumors from five animals in each group were counted in 10 random fields at 40× objective magnification from three different individual PC-3 tumor H&E sections. To eliminate the effects of small differences in cell counts per microscopic viewfield on the results, the ratio of apoptotic to mitotic indices also was calculated for each tumor section.

Total RNA Isolation and Reverse Transcription. Total RNA was isolated and DNase from cultured PC-3 human prostate cancer cells and from xenografted PC-3tumors was treated using the NucleoSpin kit according to the manufacturer's instructions (Macherey-Nagel). Three tumor samples from each group were analyzed. The yield and quality of RNA samples were determined spectrophotometrically using 260 nm and 260/280 and 260/230 nm ratios. Two micrograms of RNA from the sample was reverse-transcribed into cDNA by QuantiTect Reverse Transcription Kit (Qiagen) in a final volume of 40 μL. Reverse transcription was done in a Veriti 96-well thermal cycler (Applied Biosystems).

Real-Time RT-PCR. We evaluated the expression of mRNA for human phosphorylated GHRH receptor (pGHRH-R), splice variant 1 of GHRH-R (SV1), and β-actin. Probes and primers for pGHRH-R, and β-actin and sense- and antisense-specific primers for SV1 were described previously (1). All real-time PCR reactions were performed in the iQ5 real-time PCR detection system (Bio-Rad). All thermal cycling conditions were described in former studies (2). Briefly, samples were run in triplicate, and each well of PCR contained 25 μL as final volume including 2 μL of cDNA, 200 nM of gene-specific primers, and 400 nM of probes (3). iQ Supermix (Bio-Rad) was used in the PCR reactions for pGHRH-R and β-actin, and iQ SYBR-Green Supermix (Bio-Rad) was used for SV1. The efficiencies of all primers (Invitrogen Life Technologies) and probes (Integrated DNA Technologies) were tested before the experiments and found to be efficient in the range of 95–105%. Normal human pituitary was used as positive control, and human β-actin was used as a housekeeping gene. Negative samples were run in each reaction consisting of no-RNA in reverse transcriptase reaction and no-cDNA in PCR. Five microliters of each amplification reaction was separated electrophoretically and visualized using FlashGel DNA System (Lonza).

Human Cancer Pathway Finder PCR Array. The Human Cancer Pathway Finder Superarray (PAHS-033A; Qiagen) was used to analyze mRNA levels of 84 genes related to cell proliferation, apoptosis, cell cycle, angiogenesis, invasion, and metastasis. Quality control of RNA samples, synthesis of cDNA, and real-time RT-PCR arrays were performed as described (4). All genes represented by the array showed a single peak on the melting curve characteristic to the specific products. Data analysis of gene expression was performed using Excel-based PCR Array Data Analysis Software provided by manufacturer (Qiagen). Fold-changes in gene expression were calculated using the ΔΔCt method, and five stably expressed housekeeping genes (β2 microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, GAPDH, and β-actin) were used for normalization of the results.

Western Blot. Protein was isolated from cultured PC-3 cells and PC-3 tumor samples using the NucleoSpin Kit (Macherey-Nagel) and was sonicated. Equal amounts of protein were resuspended in 4× sample loading buffer [0.25 M Trizma Base, 8% SDS, 40% glycerol, 0.004% bromophenol blue, 4% β-mercaptoethanol (pH 6.8)], boiled for 3 min, and separated by 12% SDS-PAGE. Proteins from the gel were transferred onto nitrocellulose membrane, which was blocked with 50–50% Tris-buffered saline [20 mM Tris·HCl (pH 7.5), 150 mM NaCl]: Odyssey blocking buffer for 1 h at room temperature, followed by an overnight

incubation at 4 °C with primary antibodies for β-actin (A5441; Sigma), GHRH (ab-8899; Abcam Inc.), GHRH-R/SV1 (ab-28692; AbCam), p44/42 MAPK (Erk1/2) (#9102; Cell Signaling), phospho p44/42 MAPK (Erk1/2) (#9101; Cell Signaling), pan AKT (ab-8805; AbCam), phospho AKT (#9270; Cell Signaling), and proliferating cell nuclear antigen (PCNA) (sc25280; Santa Cruz Biotechnology). The signals were developed by incubating the membrane for 1 h at room temperature with the appropriate infrared IRDye-labeled secondary antibodies (LI-COR Biosciences) and then were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences). The protein bands were quantified using V3.0 software (LI-COR Biosciences). Relative intensity of the phosphorylated p44/42 MAPK (Erk1/2) and phosphorylated AKT was calculated by dividing their absolute signal intensity by that of p44/42 MAPK (Erk1/2), and pan AKT, respectively.

ELISA. To analyze phosphorylation of ERK, the cell-based ERK CASE ELISA kit (FE-002; Qiagen) was used. Assays were performed on the PC3 prostate carcinoma cell line according to the manufacturer's protocol, and cells were cultured as described before. Briefly, 20,000 cells per well were seeded in 96-well plates for 24 h in medium containing serum. Medium was changed to serum-free medium for an additional 24 h. Cells were treated for the appropriate time periods with 10 nM GHRH(1-29), 10 nM EGF, 10 nM insulin-like growth factor 1, and 1 μM of the GHRH antagonist JMR-132. Pretreatment of cells was performed with 1 μM JMR-132 for 30 min following 10 nM GHRH(1-29) for 15 min. Absorbance was measured with a Dynax plate reader at the wavelengths of 450 nm (first reading) and 595 nm (second reading).

Statistical Analysis. For statistical evaluation, SigmaStat 3.0 software (Sytat Software) was used. Results are expressed as means ± SEM. One-way ANOVA followed by Bonferroni *t* test or a two-tailed Student's *t* test was used where appropriate, and significance was accepted at $P < 0.05$.

1. Havt A, et al. (2005) The expression of the pituitary growth hormone-releasing hormone receptor and its splice variants in normal and neoplastic human tissues. *Proc Natl Acad Sci USA* 102:17424–17429.
2. Hohla F, et al. (2009) GHRH antagonist causes DNA damage leading to p21 mediated cell cycle arrest and apoptosis in human colon cancer cells. *Cell Cycle* 8: 3149–3156.
3. Rick FG, et al. (2011) LHRH antagonist Cetrorelix reduces prostate size and gene expression of proinflammatory cytokines and growth factors in a rat model of benign prostatic hyperplasia. *Prostate* 71:736–747.
4. Pozsgai E, et al. (2011) The effect of a novel antagonist of growth hormone releasing hormone on cell proliferation and on the key cell signaling pathways in nine different breast cancer cell lines. *Int J Oncol* 39:1025–1032.

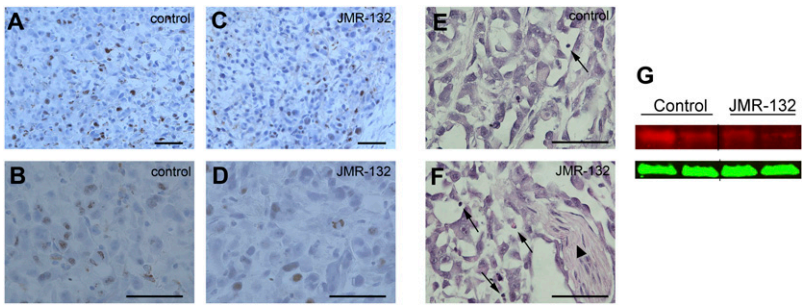

Fig. S1. The GHRH antagonist JMR-132 reduces cell division and induces apoptosis in PC-3 human androgen-independent tumor xenografts. (*A–D*) Ki67 staining of control (*A* and *B*) and JMR-132–treated (*C* and *D*) PC-3 tumors. The reaction for Ki67 appeared as brown-black granules in the nuclei of positive cells. (*E* and *F*) Representative apoptotic cells (arrows) are shown in H&E slides of control (*E*) and JMR-132–treated (*F*) PC-3 tumors. A part of nerve infiltrated by the tumor is shown (arrowhead). (Magnification: 20× in *A* and *C*, 40× in *B* and *D–F*.) (Scale bar: 50 μm.) (*G*) Western blot analysis of PCNA. Representative blots of three independent experiments are presented. Grouping of representative bands for each experimental group was performed digitally.

Table S1. Effect of GHRH antagonist JMR-132 on cell proliferation and apoptosis in PC-3 tumor xenografts

Group	Ki67-labeling indices	Mitotic indices (H&E staining)	Apoptotic indices (H&E staining)	Apoptotic to mitotic index ratios (H&E staining)
Control	68.0 ± 11.8	4.09 ± 0.31	2.23 ± 0.18	0.564 ± 0.308
JMR-132 (10 μg/d)	37.9 ± 2.4*	1.67 ± 0.22†	2.9 ± 0.22*	2.110 ± 0.224‡

Statistical analysis was performed by Student's *t* test.
*$P < 0.05$ compared with control.
†$P < 0.001$ compared with control.
‡$P < 0.01$ compared with control.

Table S2. Modulated genes with at least 1.5-fold change relative to the untreated control after treatment of PC-3 xenografts with the GHRH antagonist JMR-132

Name of gene	Gene symbol	Accession no.	Fold up- or down-regulation
V-akt murine thymoma viral oncogene homolog 1	*AKT1*	NM_005163	**−4.00**
Angiopoietin 1	*ANGPT1*	NM_001146	**−3.73**
Angiopoietin 2	*ANGPT2*	NM_001147	**−11.31**
Ataxia telangiectasia mutated	*ATM*	NM_000051	**−3.94**
BCL2-antagonist of cell death	*BAD*	NM_004322	**7.46**
BCL2-associated X protein	*BAX*	NM_004324	**1.87**
B-cell chronic lymphocytic leukemia/lymphoma 2	*BCL2*	NM_000633	**−2.30**
BCL2-like 1	*BCL2L1*	NM_138578	−1.52
Breast cancer 1, early onset	*BRCA1*	NM_007294	**−6.96**
Caspase 8, apoptosis-related cysteine peptidase	*CASP8*	NM_001228	**−3.97**
Cyclin E1	*CCNE1*	NM_001238	**−3.73**
Cyclin-dependent kinase 4	*CDK4*	NM_000075	−1.74
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*CDKN1A*	NM_000389	**−3.03**
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	*CDKN2A*	NM_000077	**−2.14**
CASP8 and FADD-like apoptosis regulator	*CFLAR*	NM_003879	−1.52
Collagen, type XVIII, alpha 1	*COL18A1*	NM_030582	**−4.00**
E2F transcription factor 1	*E2F1*	NM_005225	−1.62
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	*ERBB2*	NM_004448	**−.73**
Fas (TNF receptor superfamily, member 6)	*FAS*	NM_000043	**−2.14**
IFN, beta 1, fibroblast	*IFNB1*	NM_002176	**4.29**
Insulin-like growth factor 1	*IGF1*	NM_000618	**−2.16**
Integrin, alpha 1	*ITGA1*	NM_181501	**−6.50**
Integrin, alpha 2	*ITGA2*	NM_002203	−1.74
Integrin, alpha 3	*ITGA3*	NM_002204	**−2.83**
Integrin, alpha V	*ITGAV*	NM_002210	2.00
Integrin, beta 1	*ITGB1*	NM_002211	**−2.02**
Integrin, beta 3	*ITGB3*	NM_000212	**−2.46**
Integrin, beta 5	*ITGB5*	NM_002213	**−2.85**
Jun oncogene	*JUN*	NM_002228	**−4.29**
Mitogen-activated protein kinase kinase 1	*MAP2K1*	NM_002755	−1.74
Matrix metallopeptidase 1 (interstitial collagenase)	*MMP1*	NM_002421	**−1.82**
Matrix metallopeptidase 9 (type IV collagenase)	*MMP9*	NM_004994	**−3.03**
Metastasis associated 1 family, member 2	*MTA2*	NM_004739	−1.52
Metastasis suppressor 1	*MTSS1*	NM_014751	**−5.66**
Nuclear factor of kappa light polypeptide 1	*NFKB1*	NM_003998	**−2.92**
Nuclear factor of kappa light polypeptide inhibitor, alpha	*NFKBIA*	NM_020529	**2.46**
Nonmetastatic cells 4, protein expressed in	*NME4*	NM_005009	**−2.64**
Phosphoinositide-3-kinase, regulatory subunit 1	*PIK3R1*	NM_181504	−1.52
Plasminogen activator, urokinase receptor	*PLAUR*	NM_002659	**−2.30**
S100 calcium binding protein A4	*S100A4*	NM_002961	**2.64**
Serpin peptidase inhibitor, member 5	*SERPINB5*	NM_002639	**2.14**
Serpin peptidase inhibitor, member 1	*SERPINE1*	NM_000602	**−4.02**
Synuclein, gamma	*SNCG*	NM_003087	−1.74
TEK tyrosine kinase,	*TEK*	NM_000459	−1.62
Thrombospondin 1	*THBS1*	NM_003246	**−1.87**
TIMP metallopeptidase inhibitor 1	*TIMP1*	NM_003254	**−1.89**
Tumor necrosis factor receptor superfamily, member 10b	*TNFRSF10B*	NM_003842	**−2.83**
Tumor necrosis factor receptor superfamily, member 25	*TNFRSF25*	NM_003790	**−2.14**
Vascular endothelial growth factor A	*VEGFA*	NM_003376	**−2.07**

Multiple genes related to cell proliferation, apoptosis, cell cycle, angiogenesis, invasion, and metastasis were evaluated for expression using real-time PCR via the RT^2 Profiler PCR Array system. The table lists the genes of interest evaluated and their fold increase or decrease in PC-3 human prostate cancer tumor xenografts treated for 49 d with the GHRH antagonist JMR-132 (10 μg/d). Data represent fold differences in the expression of individual genes expression between the JMR-132–treated group and controls. Positive values indicate up-regulation of individual genes; negative values indicate down-regulation. Three experiments were run for each study group. The data were evaluated by two-tailed Student's *t* test. Boldface indicates significant changes ($P < 0.05$).