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Combinatorial Screenings in Patients: The Interleukin-11 Receptor α as a Candidate Target in the Progression of Human Prostate Cancer

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Abstract

Direct screening of combinatorial peptide libraries in patients may allow the identification of ligands that target biochemical differences in the endothelium of blood vessels. In a screening performed in a patient, we selected and isolated a mimic motif of interleukin 11 (IL-11) from prostate biopsies after an i.v. administration of a phage display peptide library. We also demonstrated that the IL-11 phage mimic (displaying the cyclic nonapeptide CGRRAGGSC) bound specifically to a corresponding IL-11 receptor (IL-11Rα). Here we show that IL-11Rα is a potential target for intervention in human prostate cancer through morphological and functional analyses. First, a comprehensive serial immunohistochemical analysis of primary and metastatic prostate cancer samples showed increased stage-specific expression of IL-11Rα during disease progression. Second, a proapoptotic peptide was specifically targeted and internalized through this functional IL-11Rα-based ligand-receptor pair: treatment of prostate cancer cells *in vitro* with a proapoptotic peptide guided by the CGRRAGGSC peptide to the IL-11Rα resulted in dose-dependent apoptosis. Together, these data indicate that the IL-11Rα is a candidate target for translational clinical trials against advanced and metastatic prostate cancer. Moreover, our results illustrate the ability of direct combinatorial screening systems in cancer patients for identification of relevant targets in the context of human disease.

Introduction

In vivo phage display explores the surface of endothelial cells in their anatomical microenvironment (1). By taking advantage of the structural and functional differences existing among blood vessels in normal and diseased tissues, this technology enables the identification of molecular signatures on blood vessels that could allow targeted systemic delivery of therapeutic agents (1, 2). Our group has reported recently a phage display-based random peptide library screening in a human subject. In that work, we described that motifs localized nonrandomly to different organs (3, 4). To gain insight into candidate native ligands, a panel of proteins that contained the selected peptides was identified by homology blast against established protein databases. Among the selected peptide sequences, the motif Arg-Arg-Ala-Gly-Gly-Ser had homology to interleukin (IL) 11 (3). We showed that an IL-11 mimic phage displaying the cyclic peptide CGRRAGGSC (single letter amino acid code) specifically bound to immobilized IL-11 receptor (R) α and also to tissue sections of human prostate in overlay assays; additional studies with archival human tissues revealed that the expression of the IL-11Rα appeared to be increased in prostate cancer (3).

On the basis of these observations, we reasoned it might be possible to develop a system for selective targeting to the prostate and its tumors. Here we evaluate the cell surface receptor IL-11Rα as a potential target in primary and metastatic prostate cancer by using morphological and functional analyses.

Materials and Methods

Tissue Specimens and Immunohistochemistry. We studied 99 formalin-fixed, paraffin-embedded human primary and metastatic prostate cancer samples, derived from 90 patients (1 sample in 81 patients and 2 samples in 9 patients; median age: 61; range, 40–81). Samples consisted of 81 primary adenocarcinomas, obtained either from radical prostatectomy ($n = 71$ androgen-dependent and $n = 3$ androgen-independent), cystoprostatectomy ($n = 6$ androgen-independent), or pelvic exenteration ($n = 1$ androgen-independent); and 18 lymph node and bone metastases (Table 1). Human samples were selected to reflect: (*a*) stages in prostate cancer progression; (*b*) differing Gleason scores; and (*c*) zonal origin (peripheral zone and transition zone). Additional blocks from the same specimens, including benign prostatic tissues from peripheral ($n = 62$), transition ($n = 51$), and central zone ($n = 40$), were included.

Tissue samples were stained within 2 weeks of sectioning. Four-μm sections were antigen-retrieved by heat with EDTA (pH 8.0; Zymed, San Francisco, CA), and biotin and protein blocked (both from DAKO Corp., Carpinteria, CA). Incubation with the antihuman IL-11Rα antibody K20 (1:15 for 45 min; Santa Cruz Biotechnology, Santa Cruz, CA) and the LSAB+ kit (DAKO) followed. Endothelial cells were immunostained by JC/70A monoclonal antibody (anti-CD31; DAKO). Positive cases were defined by the presence of cytoplasmic staining, as seen in the positive controls (paraffin sections from a pellet of HeLa cells; American Type Culture Collection, Manassas, VA; Ref. 5). Categories 1+ to 3+ (intensity of staining in the luminal cells) were used for evaluation of benign prostatic tissues and comparison to prostatic intraepithelial neoplasia and primary prostate cancer; a scoring system based on combined intensity and percentage of immunostained tumor cells (from 0 to 300) was used to evaluate differences among specimens (6). All of the statistical analyses were done with S-PLUS 2000 (Math Soft Inc., Seattle, WA).

Phage Overlay Assays. Representative cases from the previous panel were selected, including primary androgen-dependent tumors of various Gleason scores and pathological stages ($n = 10$), primary androgen-independent ($n = 5$), and prostate cancer lymph node ($n = 5$) and bone metastases ($n = 6$). Phage was immunolocalized as described (3). To confirm specificity for the CGRRAGGSC sequence, phage-staining inhibition was tested by coincubation with the soluble CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide.

Phage Internalization Assays and Immunocytochemistry. Five × 10^4 LNCaP or MDA-PCa-2b cells (American Type Culture Collection) were incubated with 5 × 10^9 transducing units, IL-11-mimic phage in a chamber slide (Lab-Tek II; Nalge Nunc International, Naperville, IL). Rabbit anti-fd bacteriophage antibody (Sigma, St. Louis, MO) and Cy3-conjugated antirabbit antibody (Jackson, West Grove, PA) were used for phage immunodetection. Insertless fd phage was used as negative control for internalization. Cell expression of IL-11Rα was evaluated with a rabbit antibody (N20; Santa Cruz Biotechnology) that cross-reacts with both human and mouse receptors.

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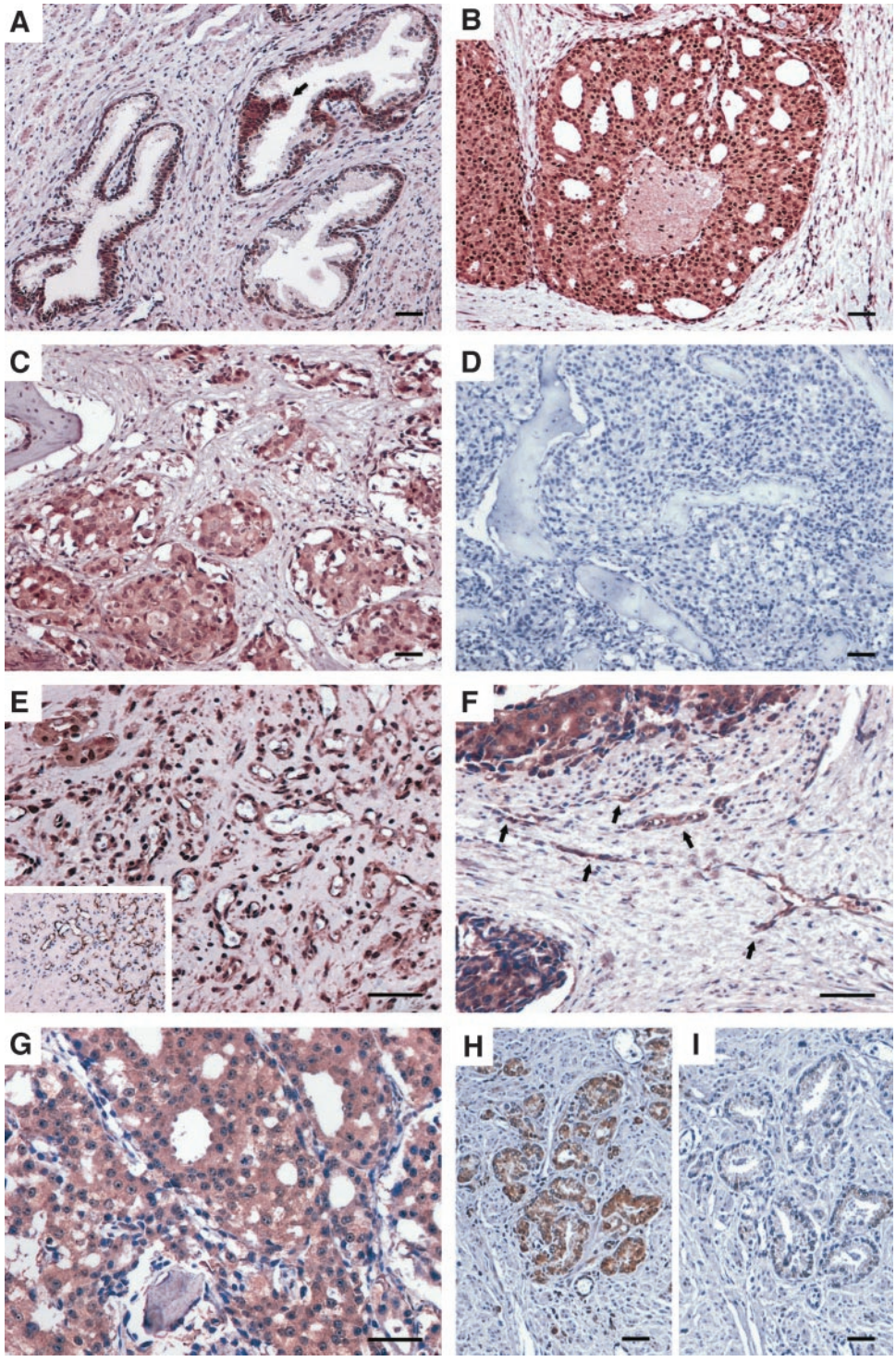

Fig. 1. Interleukin (IL)-11 receptor (R) α expression in normal prostate, and primary and metastatic prostate cancer. *A*, normal glands from the peripheral zone showing predominant staining in the basal cell compartment and area of transitional metaplasia (*arrow*), and no staining in the luminal cell layers. *B*, strong (3+) positive staining in high-grade primary androgen-dependent prostatic adenocarcinoma. *C*, homogeneous (3+) expression in prostate cancer metastatic to bone. *D*, negative control (normal immunoglobulin). *E*, positive staining in small blood vessels around malignant tumor tissue in bone matrix, confirmed by CD31 immunostaining on serial tissue sections (see *inset* for a representative section). *F* and *G*, IL-11-mimic phage overlays. *F*, high-grade, androgen-independent primary tumor showing strong (3+) and homogeneous staining in malignant epithelium and associated blood vessels (*arrows*). *G*, strong homogeneous expression in prostate cancer metastatic to bone. *H* and *I*, IL-11-mimic phage-staining inhibition. Phage localization to primary prostate cancer glands (*H*) was abolished (serial tissue sections) by coincubation with soluble CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide (*I*). *Bar*, 50 μm in all panels.

***In Vitro* Protein Binding Assays.** CGRRAGGSC-displaying phage (IL-11-mimic) binding to recombinant mouse IL-11Rα (R&D Systems, Minneapolis, MN) was assessed as described (3). Scramble phage clones displaying the peptides CRGSGAGRC or CSGGGRARC, phage clones displaying the unrelated peptides CKGGRAKDC or CGSPGWVRC, and insertless phage (fd-tet) were used as controls.

Induction and Quantification of Apoptosis with CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ Synthetic Peptide. Soluble CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$, CGRRAGGSC, and $_D$(KLAKLAK)$_2$ peptides, and the unrelated control peptide CKGGRAKDC-GG-$_D$(KLAKLAK)$_2$, were synthesized to our specifications by AnaSpec (San Jose, CA). The unrelated control peptide CGSPGWVRC-GG-$_D$(KLAKLAK)$_2$ was synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). LNCaP, MDA-PCa-2b cells (each at 3×10^4/well), and EF43.*fgf*-4 cells (7) at 2×10^4/well were seeded in triplicates and incubated in 96-well plates (Becton Dickinson, Franklin Lakes, NJ) for 24–72 h at 37°C, with serially increasing concentrations (10–100 μM) of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide, CGRRAGGSC peptide alone, $_D$(KLAKLAK)$_2$ peptide alone, or an equimolar mixture of the unconjugated peptides CGRRAGGSC and $_D$(KLAKLAK)$_2$. LNCaP cells were also exposed in parallel to increasing concentrations (20–100 μM) of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ and unrelated control peptides CKGGRAKDC-GG-$_D$(KLAKLAK)$_2$ or CGSPGWVRC-GG-$_D$(KLAKLAK)$_2$, under the same conditions. Specificity of binding to IL-11Rα was additionally tested by incubating LNCaP cells with either IL-11Rα antibody (50 μg/ml; Santa Cruz Biotech-

Table 1 *Interleukin-11 receptor α expression in prostate cancer*

Specimen	*n*	Median score (range)	*P*
Normal prostate			
Peripheral zone	62	1+ (1–2)	NS[a]
Transition zone	51	1+ (1–2)	
Central zone	40	1+ (1–2)	
Benign conditions			
Benign prostatic hyperplasia	15	1+ (1–2)	—
Stromal nodule	2	1+ (1–2)	—
Atrophy	10	2+ (1–2)	—
Transitional metaplasia	18	2+ (1–2)	—
Prostatic intraepithelial neoplasia, high grade	23	2+ (1–3)	—
Primary prostate cancer			
Androgen-dependent	71	2+ (1–3)/180 (50–290)	—
Zonal origin			
Peripheral zone	55	190 (50–290)	0.0003[b]
Transition zone	16	135 (50–250)	
Gleason score			
≤7 (3 + 4)	26	150 (50–260)	0.004[b]
≥7 (4 + 3)	38	200 (100–290)	
Pathological stage			
pT_2–pT_{3a}	42	175 (50–290)	0.046[b]
pT_{3b}–$pT_{any}pN_1$	22	210 (100–280)	
Serum PSA (ng/mL)[c]			
<10	48	180 (50–280)	NS
≥10	14	200 (100–290)	
Androgen-independent	10	250 (80–300)	—
Metastatic prostate cancer			
Lymph nodes			
Androgen-dependent	4	235 (200–290)	NS
Androgen-independent	8	235 (190–300)	
Bone marrow	6	270 (140–290)	—

[a] NS, nonsignificant.
[b] Mann-Whitney rank sum test.
[c] Serum prostate-specific antigen not available in 2 of 64 samples.

nology) or rabbit IgG (Zymed Labs.) for 1 h and then by adding 40 μM CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide for 3 h. Response was evaluated by a cell viability assay (WST-1; Roche, Mannheim, Germany).

Results and Discussion

To begin to evaluate IL-11Rα in the context of human prostate cancer, we used morphological (immunohistochemistry) and functional (targeting and internalization) assays. First, we analyzed the expression of IL-11Rα in a large panel of androgen-dependent and androgen-independent prostate cancer specimens ($n = 99$) by using both a specific antibody and an IL-11-mimic ligand phage clone (displaying the peptide CGRRAGGSC). Moreover, we tested the targeting of the IL-11-mimic peptide in human prostate cancer-derived cells. Finally, we assessed the internalization capability of the IL-11Rα by measuring uptake of IL-11-mimic phage and programmed cell death induction *in vitro* mediated by a targeted pro-apoptotic synthetic peptide.

We studied the immunohistochemical expression of IL-11Rα in formalin-fixed, paraffin-embedded tissue samples including the entire spectrum of prostate cancer from premalignant prostatic intraepithelial neoplasia to androgen-independent metastatic tumors, and normal prostate from the peripheral, transition, and central zones (Table 1). As examined with an anti-IL-11Rα antibody (Fig. 1, *A–D*), expression in normal prostatic glands from the different zones was low, typically localized in the basal cell compartment with or without staining of the luminal cells. Expression of the receptor in prostatic intraepithelial neoplasia and primary androgen-dependent prostate cancer samples was significantly higher than in their benign counterparts from the

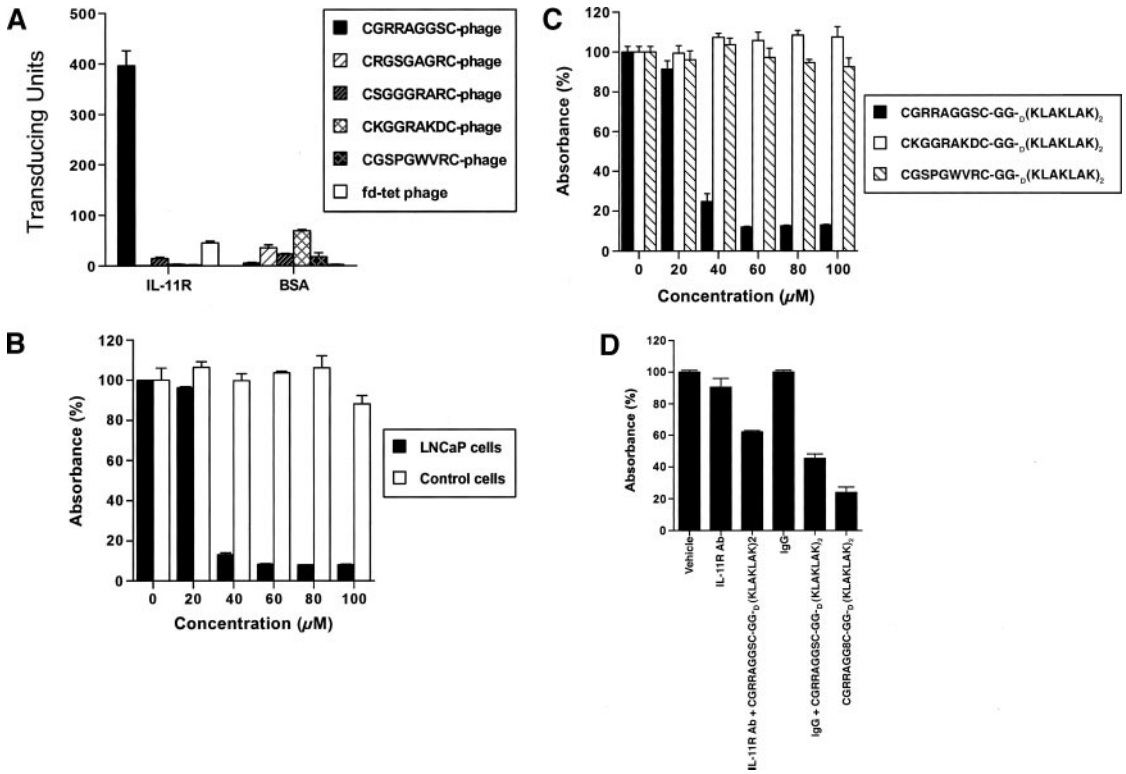

Fig. 2. CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ binds specifically to interleukin (IL)-11 receptor (R) α and induces apoptosis in IL-11Rα-positive prostate cancer cell lines. *A, in vitro* binding to immobilized IL-11Rα of CGRRAGGSC-displaying or control phage, including: scrambled peptides (CRGSGAGRC or CSGGGRARC), unrelated peptide sequences (CKGGRAKDC or CGSPGWVRC), and insertless phage (fd-tet). *B,* dose-response effect of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ on IL-11Rα-expressing LNCaP cells and lack of effect on IL-11Rα-deficient EF43.*fgf*-4 cells. Both cell lines were treated with increasing concentrations of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ for 24 h. *C,* cell killing selectivity of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ *versus* control peptides. LNCaP cells were independently incubated for 72 h with increasing concentrations of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide or the unrelated peptides CKGGRAKDC-GG-$_D$(KLAKLAK)$_2$ or CGSPGWVRC-GG-$_D$(KLAKLAK)$_2$. *D,* IL-11Rα antibody-mediated inhibition of proapoptotic effect of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$. LNCaP cells were incubated for 4 h with anti-IL-11Rα antibody (*IL-11R Ab*), anti-IL-11Rα antibody plus CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide, nonspecific IgG, nonspecific IgG plus CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide, or CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide alone. Drug response was assessed by the WST-1 cell viability assay. Absorbance obtained for cells incubated with vehicle alone was set to 100% in graphs *B–D*. *Bars*, mean ± SE in all graphs.

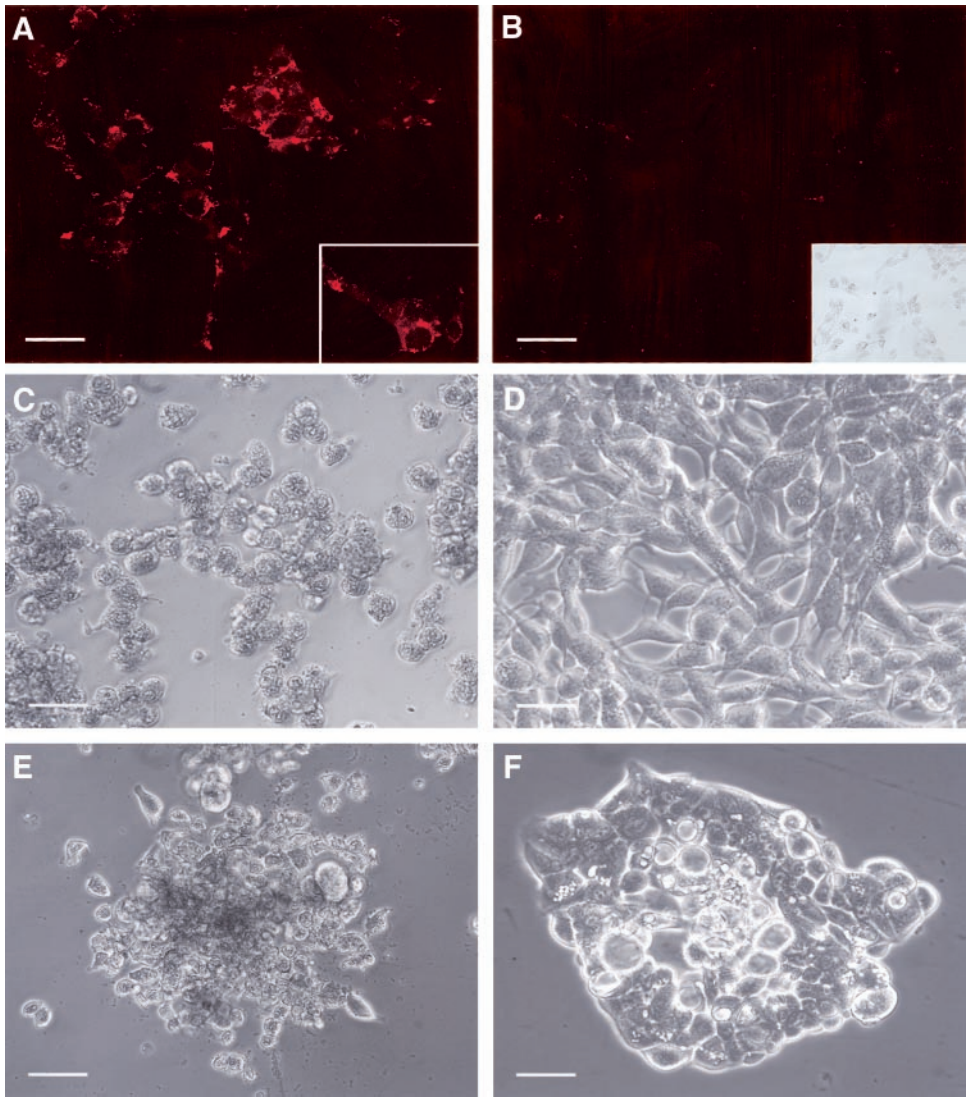

Fig. 3. Interleukin (IL) -11-mimic phage internalization and induction of programmed cell death with CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ synthetic peptide. *A*, IL-11-mimic phage internalization on LNCaP cells. Note distribution in cell projections and around the nucleus (*inset*). *B*, insertless fd phage was used as negative control for internalization (phase-contrast in *inset*). *C–F*, induction of programmed cell death with CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ synthetic peptide. LNCaP (*C* and *D*) or MDA-PCa-2b (*E* and *F*) cells were incubated with 50 μM CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ (*C* and *E*) or an equimolar mixture of unconjugated CGRRAGGSC and $_D$(KLAKLAK)$_2$ (*D* and *F*). Morphological evidence of programmed cell death is observed after treatment with the targeted proapoptotic peptide. *Bar*, 50 μm in all panels.

same areas ($P < 0.0001$ for both comparisons, Wilcoxon signed rank test). The extent and intensity of staining were heterogeneous among and within androgen-dependent tumor samples, but clearly increased in association with rising Gleason score and tumor stage (Table 1). In contrast, primary androgen-independent cancer showed a more homogeneous pattern of staining, with >80% of cells displaying moderate/strong intensity in 8 of 10 (80%) samples. Expression in lymph node metastases ($n = 12$) was also intense in most of the tumor cells regardless of their androgen-sensitivity status or anatomical origin. Similarly, prostate cancer cells metastatic to the bone marrow displayed a homogeneous moderate to strong intensity of staining in 5 of 6 (83%) specimens (all androgen-independent). Moreover, some small-caliber blood vessels in androgen-independent primary and metastatic tumors showed striking IL-11Rα immunoreactivity in 17 of 24 (71%) samples, confirmed by CD31 (PECAM-1) staining on serial sections, as opposed to a less consistent pattern in benign tissues and androgen-dependent tumors analyzed (Fig. 1*E*).

To establish whether similar differences in expression were also apparent and detectable for the epitope recognized by the IL-11-mimic phage, we performed phage overlay assays (3) on representative cases from the previous panel ($n = 26$) including primary androgen-dependent and independent tumors, and prostate cancer metastases (Fig. 1, *F* and *G*). The pattern of phage-bound staining matched that of the antibody, confirming that the IL-11 mimic phage colocalizes with the IL-11Rα receptor in tissue sections. Specificity was additionally confirmed when the staining was inhibited by coincubation with the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide (Fig. 1, *H* and *I*). Indeed, differential expression of normal *versus* tumor tissues appeared more evident than in cases with previous anti-IL-11Rα antibody low to moderate expression. In general agreement with our previous findings, most endothelia in these samples were recognized by the IL-11-mimic phage (3).

To model the functionality of our targeting system *in vitro*, we chose the human prostate cancer-derived cell lines MDA-PCa-2b and LNCaP because of their androgen-sensitive and prostate-specific antigen-expressing features, and also because such cells express IL-11Rα; as a negative control, the mouse mammary tumor-derived cells EF43.*fgf*-4 (7) were selected because expression of IL-11Rα was not detectable (data not shown). By using this panel of cells, we evaluated the targeting of the IL-11Rα and internalization of a synthetic peptide consisting of an IL-11-mimic domain linked to a well-established proapoptotic domain, $_D$(KLAKLAK)$_2$ (8–10). $_D$(KLAKLAK)$_2$ is an amphipathic, α-helix-forming antimicrobial peptide that preferentially disrupts eukaryotic mitochondrial membranes rather than plasma membranes when internalized by a ligand-receptor system (8).

We evaluated the *in vitro* binding of CGRRAGGSC-displaying

phage (3) and several control phage for IL-11Rα (Fig. 2*A*). Binding of CGRRAGGSC-displaying phage was significantly higher than that of control phage, including phage displaying scrambled IL-11-mimic peptides (CRGSGAGRC or CSGGGRARC), unrelated peptide sequences (CKGGRAKDC or CGSPGWVRC), and insertless phage (fd-tet; $P < 0.0001$ for each case, *t* test). We demonstrate by immunofluorescence peptide-mediated IL-11-mimic phage internalization in LNCaP (Fig. 3, *A* and *B*) and MDA-PCa-2b cells (data not shown). The chimeric synthetic peptide CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ induced dose-dependent programmed cell death in the prostate cancer cells tested. In contrast, no significant effect was observed on the IL-11Rα-deficient EF43.*fgf*-4 cells within the same dose range (Fig. 2*B*). In experiments performed under similar conditions, incubation of LNCaP and MDA-PCa-2b cells with control peptides CGRRAGGSC, $_D$(KLAKLAK)$_2$, an equimolar mixture of uncoupled CGRRAGGSC and $_D$(KLAKLAK)$_2$ (Fig. 3, *C–F*), or unrelated peptides CKGGRAKDC-GG-$_D$(KLAKLAK)$_2$ or CGSPGWVRC-GG-$_D$(KLAKLAK)$_2$ (Fig. 2*C*), showed no measurable toxic effects. The proapoptotic effect of CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ on LNCaP cells was also significantly inhibited by coincubation with an anti-IL-11Rα antibody, both when compared with CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ alone ($P = 0.008$, *t* test) or nonspecific IgG ($P = 0.02$, *t* test; Fig. 2*D*).

Together, our histological and functional findings establish the presence of a high and homogeneous IL-11Rα expression in primary androgen-independent and metastatic prostate cancer, and blood vessels in the majority of these specimens. On an expanded set of clinically annotated samples, we show up-regulation of IL-11Rα expression in primary androgen-dependent prostate cancer. These data indicate a gradual increase in epithelial expression of IL-11Rα that correlates directly with the clinical and pathological progression of prostate cancer. We also demonstrate a potential function for the ligand-receptor system IL-11:IL-11Rα. Consistently, by using unrelated technology, a role for the IL-11 molecular pathway in the progression of malignant human tumors metastatic to bone has been proposed recently (11), possibly related to the activation of STAT3 downstream from the IL-11Rα (12). Prospective studies on the pathogenic or prognostic value for this receptor in prostate cancer are ongoing.

In summary, the high expression of the IL-11Rα in androgen-independent disease and its associated blood vessels offers an opportunity for therapeutic targeting of a tumor with no curative treatment when metastatic. The tools provided in this report may enable therapeutic targeting of the IL-11Rα in prostate cancer. Finally, this study provides additional support for the use of direct combinatorial screenings in patients (3, 4) for the development of anticancer targeted therapies in the context of human disease.

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