Long Noncoding RNA IGF2AS is Acting as an Epigenetic Tumor Suppressor in Human Prostate Cancer

Qiang Chen, Ting Sun, Fang Wang, Binbin Gong, Wenjie Xie, Ming Ma, and Xiaorong Yang

OBJECTIVE To assess the expression profile and functional mechanism of long noncoding RNA (lncRNA) insulin growth factor 2 antisense (IGF2AS) in human prostate cancer (PCa).

METHODS Quantitative reverse transcriptase-polymerase chain reaction was applied to assess IGF2AS expression in immortal PCa cell lines and in situ human PCa tumors. IGF2AS was overexpressed in VCaP and PC3 cells to assess its effect on PCa cell proliferation and invasion in vitro, and xenograft in vivo. The effect of IGF2AS overexpression on IGF2 was also assessed in PCa cells. Then, IGF2 was upregulated in IGF2AS-overexpressed PCa cells to assess the functional involvement of IGF2 in IGF2AS-mediated PCa cell development.

RESULTS IGF2AS was downregulated in both PCa cell lines and human PCa tumors. In VCaP and PC3 cells, lentivirus-induced IGF2AS overexpression suppressed cancer cell proliferation and invasion in vitro, and xenograft development in vivo. IGF2 was downregulated by IGF2AS overexpression. Conversely, IGF2 upregulation revered the suppressing function of IGF2AS on PCa proliferation and invasion.

CONCLUSION LncRNA IGF2AS is acting as an epigenetic tumor suppressor in human PCa, likely through inverse regulation on IGF2. IGF2AS/IGF2 axis may be a future therapeutic target for PCa treatment. UROLOGY 124: 310.e1–310.e8, 2019. © 2018 Elsevier Inc.

Prostate cancer (PCa) is one of the most commonly diagnosed malignant diseases in both young and old men around the world.[1-3] In the United States, PCa alone accounts for nearly 20% of all newly diagnosed cancer in men, with more than 160,000 cases every year.[4] In China, based on NCCR annual report in 2015, the overall incidence of PCa were 7.1/105 populations in 2011, which ranked ninth in the highest cancer incidences for all sexes and seventh in male.[5] Specifically, between 2000 and 2011, the incidence rate of PCa had increased dramatically.[6] Although mortality rate had steadily fallen for PCa patients for the past decade, the pathological mechanisms underlying PCa ontogenesis, maturation, and metastasis are still largely unknown.

Long noncoding RNAs (lncRNAs) are families of long (>200 n.t), nonprotein-coding RNAs, which were recently discovered to have critical roles in regulating human epigenome.[7-10] Recently, lncRNAs have been identified as dynamic prognostic biomarkers or active tumor oncogenes/suppressors in various types of human cancers.[11-13] Specifically, in human PCa, several lncRNAs were discovered to play functional roles in modulating cancer development. For example, Chakravarty and colleagues demonstrated that lncRNA nuclear enriched abundant transcript 1 (NEAT1) was overexpressed in PCa and its expression was closely correlated with PCa progression.[14] Also, Malik and colleagues demonstrated that downregulation of lncRNA prostate cancer-associated transcript 29 (PCAT29) was correlated with poor prognosis among PCa patients and overexpressing PCAT29 had suppressive effect on PCa cell growth and metastasis.[15] In addition, it was suggested that discriminative potential of exosomal lncRNA-p21 levels might predict the malignant state for PCa patients.[16]

Among many of the human cancer-associated lncRNAs, insulin growth factor 2 antisense (IGF2AS) was initially identified as the antisense transcript of IGF2 locus among gene-array study in Wilms' tumor.[17] Later on, IGF2AS was also identified as a functional modulator in neurons in response to anesthesia-induced neurotoxicity.[18,19] Most recently, Zhang and colleagues demonstrated that IGF2AS

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might act a prognostic biomarker, or therapeutic target in nonsmall cell lung cancer.[20]

In this study, we suspected that IGF2AS might also have functional implication in PCa. We first used quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantitatively assess IGF2AS expression in both immortal PCa cell lines and clinical human PCa tumor samples. Second, lentivirus-induced IGF2AS overexpression was performed in PCa cell lines to assess the functional effects of IGF2AS on PCa cell in vitro and in vivo development. Third, the possible involvement of IGF2 was investigated in the process of IGF2AS-induced cancer regulation in PCa cells. Overall, the goal of this study is to provide an insight on the epigenetic function of IGF2AS in PCa.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Clinical Research and Ethic Committee at The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi Province, China. In addition, consent forms were signed by all participating patients and all protocols were performed in full accordance with the World Medical Association Declaration of Helsinki.[21]

Prostate Cancer Cell Lines and Clinical Tissues

Six immortal human PCa cell lines, VCaP, PC3, MDA/Pca/2b, DU145, NCI-H660, and LNCaP, and 3 normal human prostate epithelial cell lines, RWPE1, RWPE2 and HPrEC, were commercially bought from the American Type Culture Collection (ATCC, www.atcc.rog USA). All PCa cell lines were maintained in 6-well tissue-culture plates (BD Biosciences, USA) in RPMI-640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin (10,000 U/mL, Gibco, USA) in a humidified environment of 5% CO_2 /air at 37°C.

From September 2010 to June 2018, clinical tissues were collected from 89 PCa patients who underwent prostatectomy in the Department of Urology at the First Affiliated Hospital of Nanchang University in Nanchang, Jiangxi Province, China. Both PCa tissues and paired adjacent normal (non-tumor) prostate epithelial tissues were collected. Upon collection, all samples were immediately snap-frozen in liquid nitrogen and stored at an −80°C freezer until further processing.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Quantitative assessment on gene expression was conducted using method of qRT-PCR. Briefly, RNA was extracted and purified from PCa cell lines or clinical tissues using a RiboPure RNA Purification Kit (Invitrogen, USA) according to the manufacturer's protocol. Then, purified RNA was reversely transcribed into cDNA using a PrimeScript RT reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. QRT-PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, USA) using a SYBR Green PCR Master Mix (Applied Biosystems, USA). For IGF2AS assessment, U6 small nuclear RNA was applied as housekeeping gene. For IGF2 assessment, GAPDH was applied as housekeeping gene. Relative expression level of each probed mRNA was then normalized to the expression level of housekeeping gene, using the $2^{-\Delta\Delta CT}$ method.

IGF2AS Overexpression Assay

A LV5 lentiviral plasmid with the insertion of human lncRNA IGF2AS (L/IGF2AS) was synthesized, and corresponding lentiviruses was generated and tittered by GenePharma (GenePharma, Shanghai, China). An empty LV5 plasmid (GenePharma, Shanghai, China) was used as nonspecific control plasmid (L/NS) to generate corresponding lentiviruses. In two PCa cell lines of VCaP and PC3, cells were infected with L/NS or L/IGF2AS lentiviruses (10^{13} U/ml) in the presence of polybrene (8 μg/ml, Gibco, USA) for 48 hours. Then, cells were selected with puromycin (10 μg/ml, Gibco, USA) for 10 days. After that, healthy multi-cell colonies were collected and reseeded in 48-well plates in culture medium supplemented with puromycin (5 μg/ml) to proliferate. After 3 passages, qRT-PCR was performed to verify the overexpression efficiency.

PCa Cell Proliferation In Vitro

VCaP and PC3 cells were lifted from 6-well plate and equally reseeded in new 96-well tissue-culture plates (BD Biosciences, USA) (5×10^3 cells / well). Cells were allowed to proliferate for 5 consecutive days. Every 24 hours, a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was performed for 4 hours according to the manufacturer's protocol. Then, 96-well plates were mounted onto a Varioskan Flash Microplate reader (Thermo Fisher Scientific, USA). In each well, PCa cell proliferation was characterized as optical density measured at 570 nm.

PCa Cell Invasion In Vitro

In a 24-well transwell invasion assay, VCaP and PC3 cells were equally plated inside the Matrigel precoated transwell inserts with a pore size of 8.0 μM (Becton Dickinson, USA) (2.5×10^5 cells / well) in serum-free culture medium. Underneath the transwell inserts, the wells were filled with regular culture medium supplemented with 10% FBS as chemo-attractant. Twenty-four hours later, all medium and transwell inserts were removed. VCaP or PC3 cells invaded onto the bottoms of 24-well plates were quickly by 70% ethanol (Gibco, USA) and stained with 1% crystal violet (Gibco, USA) for 20 minutes at room temperature. Then, 24-well plates were mounted onto an IX71 inverted microscope (Olympus, Japan). For each well, invaded cells were counted in five randomly selected fields using ImageJ software (ImageJ, NIH, USA). Relative invasion was then quantified as the percentage of invaded cells in experimental wells against the invaded cells in controlled wells.

PCa Xenograft In Vivo

Nude female BALB/c mice (6-week-old) were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China). VCaP cells were subcutaneously grafted into the dorsal compartments of mice (1 million cells/graft, total of 13 mice). The left sides were grafted with VCaP cells infected with L/NS, and right sides were grafted with VCaP cells infected with L/IGF2AS. The PCa xenograft assay was carried out for 5 weeks. Each week, surface dimension of cancer xenograft, including length (L, mm) and width (W, mm) were measured and the total volume (V, mm^3) of xenograft was estimated using the equation $V = L \times W \times W/2$. After 5 weeks, mice were killed and pairs of left-side and right-side xenografts were retrieved and directly compared under light scope.

IGF2 Overexpression Assay

A mammalian pcDNA3.1(+) overexpression plasmid containing the full-sequence of human IGF2 gene, pc/IGF2, was commercially synthesized by GenePharma (GenePharma, Shanghai, China). An empty pcDNA3.1(+) was also purchased from GenePharma (GenePharma, Shanghai, China) to be used as a nonspecific control plasmid, pc/NS. In L/IGF2AS-infected VCaP and PC3 cells, they were transfected with either pc/NS or pc/IGF2 using Lipofectamine 3000 (Gibco, USA) for 48 hours. Then, qRT-PCR was performed to verify the overexpression efficiency.

Statistical Analysis

In our study, all experiments were independently conducted for biological triplicates or more repeats. Data were presented as means ± standard errors. A windows-based SPSS software (version 13.0, SPSS, USA) was used for all statistical analysis. Comparison among clinical samples, namingly between normal prostate epithelial tissues and PCa tissues, was conducted using a Mann-Whitney U test. All other statistical analysis was conducted using a one-way ANOVA test. In all comparisons, P values <.05 were determined as significant differences.

RESULTS

IGF2AS is Downregulated in Both PCa Cell Lines and PCa Tumors

First in our study, we investigated the gene expression pattern of lncRNA IGF2AS in in vitro PCa cell lines. As compared to a normal human prostate epithelial cell line, RWPE1, expressions of IGF2AS were found to be significantly downregulated in tumor cell lines of VCaP, PC3, MDA/Pca/2b, DU145,

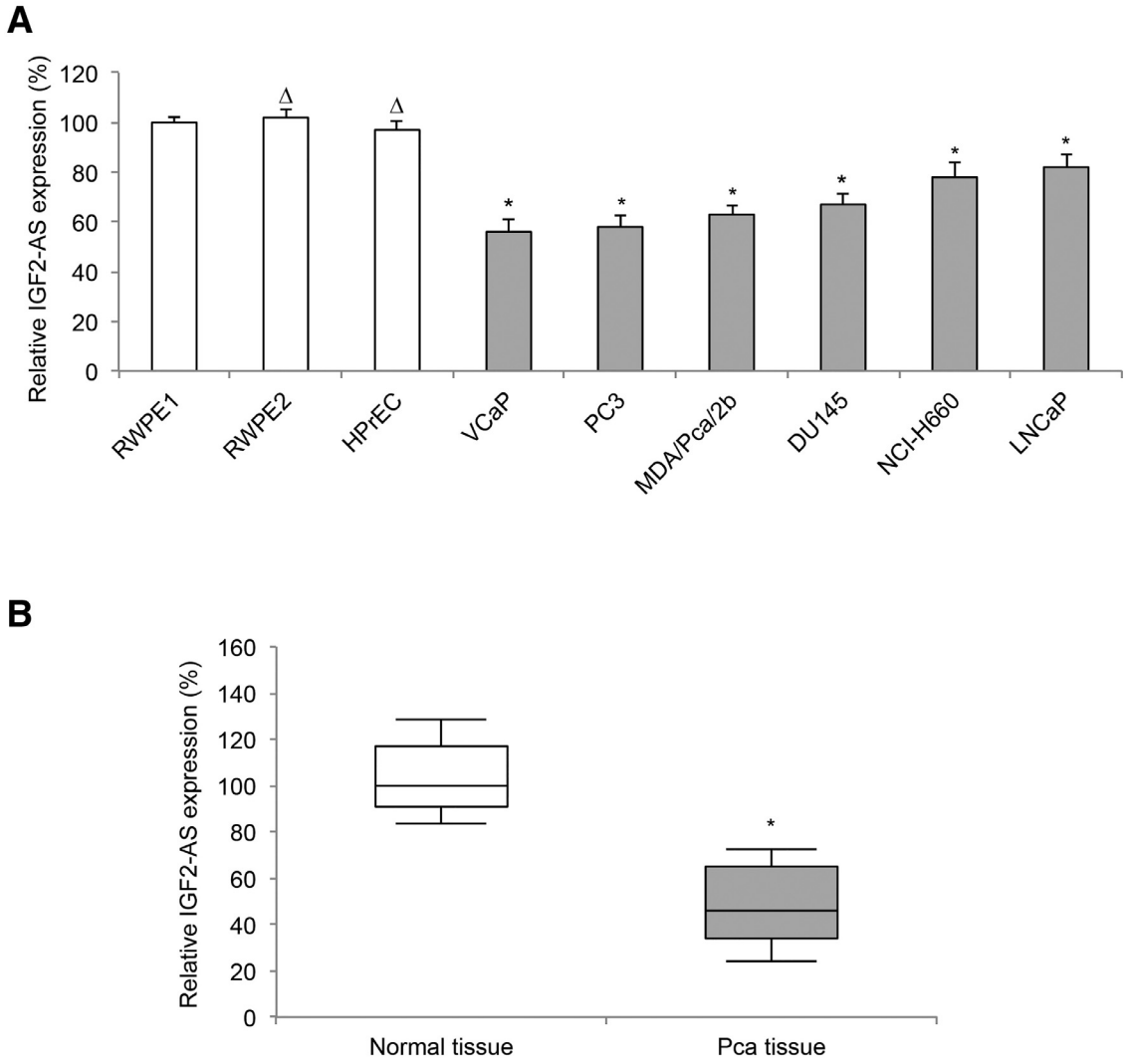

Figure 1. Expressing pattern of IGF2AS in human prostate cancer. (**A**) IGF2AS expression was assessed by qRT-PCR in 6 PCa cell lines, including VCaP, PC3, MDA/Pca/2b, DU145, NCI-H660 and LNCaP cells, and 2 normal human prostate epithelial cell lines, RWPE2 and HPEC cells. Their expressions were compared to the IGF2AS expression in a normal human prostate epithelial cell line, RWPE1 (*P <.05, ΔP <.05). (**B**) IGF2AS expression was also assessed in PCa tissues obtained from cancer patients, and compared to their adjacent normal (nontumor) prostate epithelial tissues (* P <.05, Mann-Whitney U test). IGF2AS, insulin growth factor 2 antisense; PCa, prostate cancer; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction.

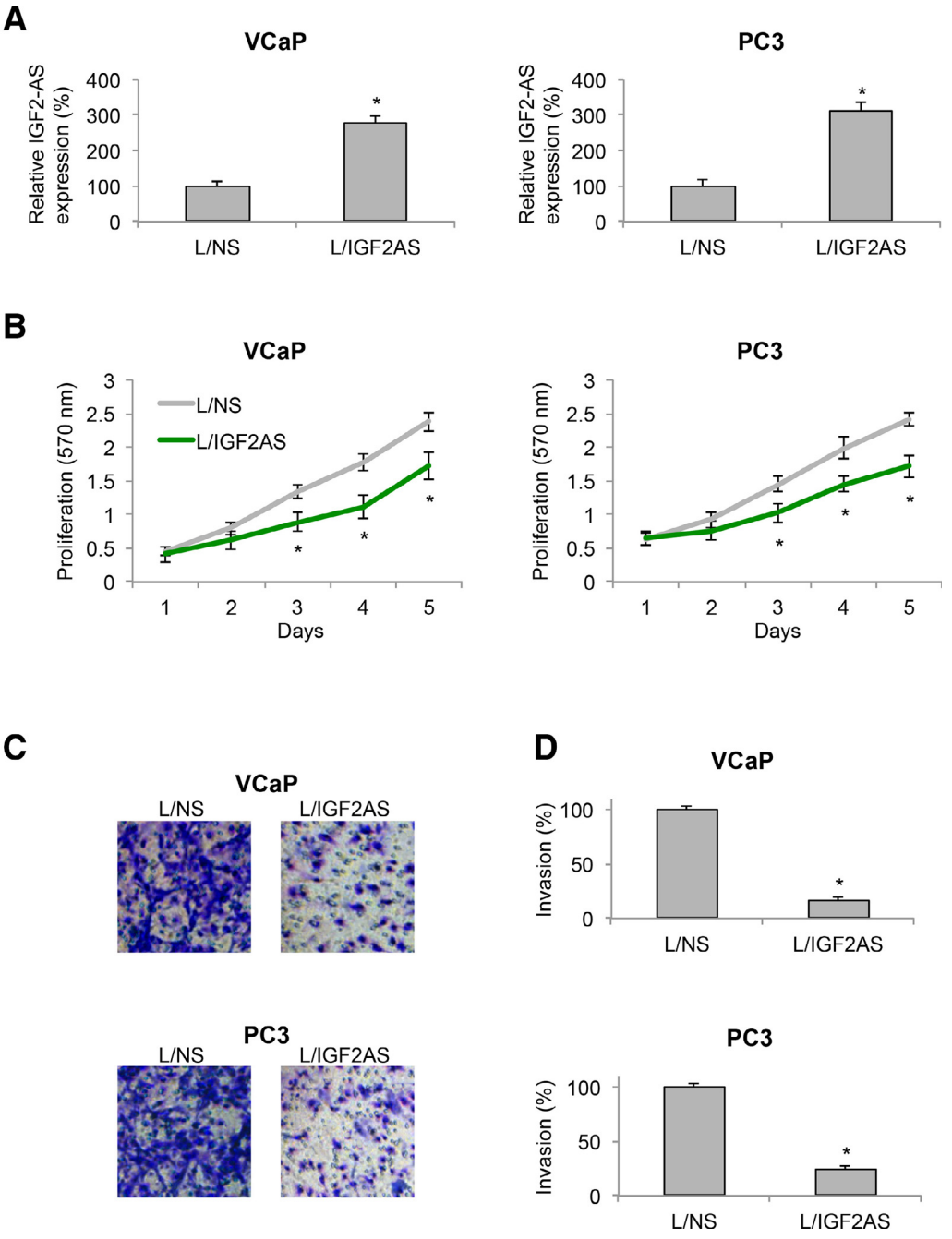

Figure 2. Inhibitory function of IGF2AS on PCa cell proliferation and invasion. (**A**) VCaP and PC3 cells were infected with lentivirus containing a nonspecific vector, L/NS, or lentivirus containing an IGF2AS overexpressing vector, L/IGF2AS. Post infection, cells were passaged 3 times, followed by qRT-PCR to assess their IGF2AS expressions (* P <.05). (**B**) Infected VCaP and PC3 cells were assessed by a 5-day CCK-8 assay, to compare *in vitro* proliferation between L/NS- and L/IGF2AS-infected PCa cells (*P <.05). (**D**) Infected VCaP and PC3 cells were assessed by a 24-well transwell assay. After 24 hours, PCa cells invaded onto the bottoms of wells were stained, and representative images were compared between L/NS- and L/IGF2AS-infected PCa cells. (**D**) For invaded PCa cells shown in (C), relative invasive capability was quantitatively measured and compared between L/NS- and L/IGF2AS-infected PCa cells (*P <.05). IGF2AS, insulin growth factor 2 antisense; PCa, prostate cancer. (Color version available online.)

NCI-H660, and LNCaP (Fig. 1**A**, * P <.05). On the other hand, IGF2AS was not found to be downregulated in the other two normal human prostate epithelial cell lines, RWPE1 or HPrEC (Fig. 1**A**, ΔP >.05).

Second, we measured IGF2AS expression in human PCa tumors. Analysis of qRT-PCR demonstrated that IGF2AS was significantly downregulated in PCa tissues, than in adjacent normal (nontumor) prostate epithelial tissues (Fig. 1**B**, * P <.05).

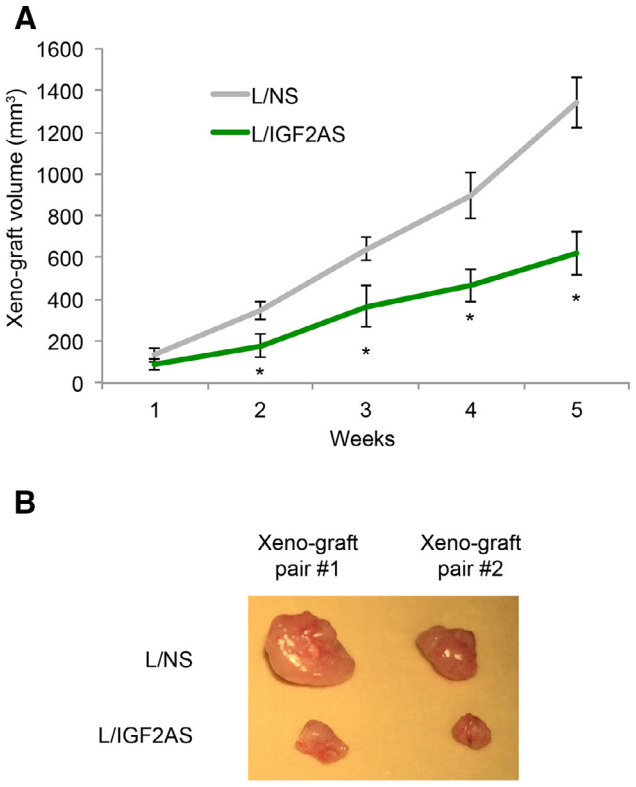

Figure 3. Suppressive effect of IGF2AS on PCa in vivo xenograft. (**A**) L/NS- and L/IGF2AS-infected VCaP cells were subcutaneously grafted into the dorsal compartments of mice (1 million cells/inoculation, total of 13 mice). The left sides were grafted with L/NS-infected VCaP cells, whereas right sides were grafted with L/IGF2AS-infected VCaP cells. The volumes (mm^3) of subcutaneous xenograft were measured every week for 5 consecutive weeks (*P <.05). (**B**) Five weeks later, VCaP xenografts were taken out and examined under light microscope. IGF2AS, insulin growth factor 2 antisense; PCa, prostate cancer. (Color version available online.)

IGF2AS Overexpression Suppressed PCa Cell In Vitro Proliferation and Invasion

As we discovered IGF2AS was downregulated in PCa, we tried to assess its functional implication in regulating PCa development. In 2 in vitro PCa cell lines, VCaP and PC3 cells, they were infected by lentiviruses to overexpress IGF2AS. After infection was stabilized, qRT-PCR demonstrated that L/IGF2AS-infected VCaP and PC3 cells had significantly higher IGF2AS expressions than L/NS-infected cells (Fig. 2**A**, * P <.05).

Infected VCaP and PC3 cells were then equally plated in 96-well plates, and their in vitro proliferation was assessed for 5 consecutive days. Using a CCK-8 assay, it showed that L/IGF2AS-infected VCaP and PC3 cells had significantly slower proliferating rates then L/NS-infected VCaP and PC3 cells (Fig. 2**B**, * P <.05).

In addition, cancer cell invasive capability was assessed in infected VCaP and PC3 cells. Using a 24-hour transwell assay, it showed that significantly fewer VCaP or PC3 cells were discovered at the bottoms of wells while they were infected with L/IGF2AS instead of L/NS (Fig. 2**C**). Then, quantitative assessment confirmed that, invasive capability of L/IGF2AS-infected PCa cells was significantly reduced, as compared L/NS-infected PCa cells (Fig. 2**D**, * P <.05).

IGF2AS Overexpression Suppressed PCa In Vivo Xenograft

Infected VCaP cells were subcutaneously grafted into nude BALB/c mice to assess the effect of IGF2AS overexpression on PCa in vivo development. During the course of 5 weeks, the tumor volumes were estimated weekly, and compared between L/NS- and L/IGF2AS-infected VCaP xenografts. It demonstrated that, the in vivo growth of VCaP xenograft was dramatically suppressed by IGF2AS overexpression (Fig. 3**A**, * P <.05). In addition, while mice were sacrificed and 5-week-old VCaP xenografts were taken out. Imaging results confirmed our in vivo estimation on tumor volumes, demonstrating that L/IGF2AS-infected xenografts were significantly smaller than L-/NS-infected xenografts (Fig. 3**B**).

IGF2 Reversed the Tumor-suppressing Function of IGF2AS in PCa Cells

We suspected that IGF2 might be directly involved in the regulation of IGF2AS-induced tumor suppression in PCa. To examine this hypothesis, we first assessed the gene expression of IGF2 in IGF2AS-overexpressed VCaP and PC3 cells. QRT-PCR demonstrated that, in responding to IGF2AS overexpression, IGF2 was reversely downregulated in PCa cells (Fig. 4**A**, * P <.05).

Second, we overexpressed IGF2 in IGF2AS-infected VCaP and PC3 cells. QRT-PCR showed that, in PCa cells

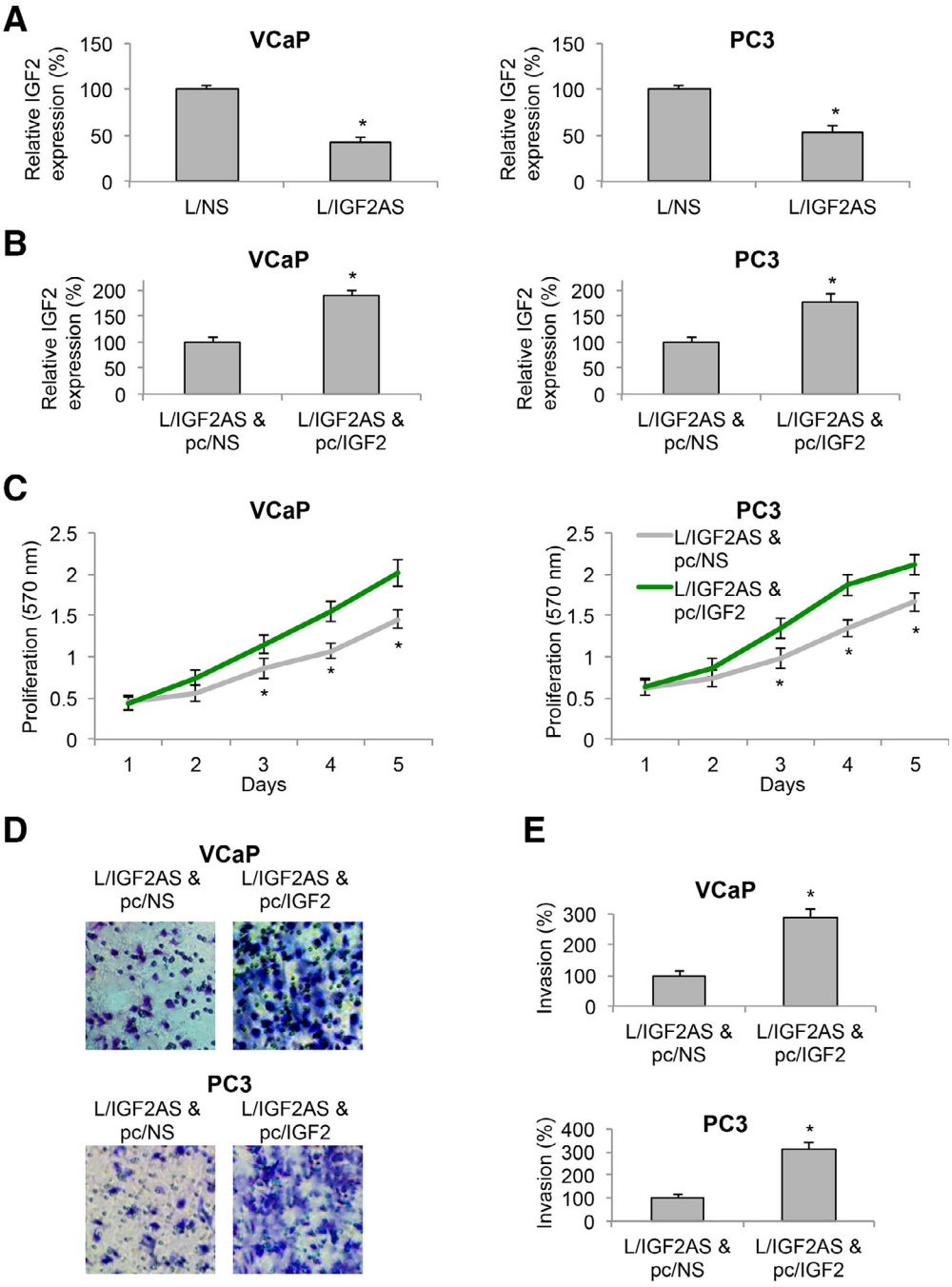

Figure 4. Reverse effect of IGF2 on IGF2AS-mediated inhibition on PCa cell proliferation and invasion. (**A**) In lentivirus infected VCaP and PC3 cells, qRT-PCR was used to assess their IGF2 gene expressions (*P <.05). (**B**) L/IGF2AS-infected VCaP and PC3 cells were transfected with an IGF2 overexpression plasmid, pc/IGF2, or an empty nonspecific plasmid, pc/NS. Then, qRT-PCR was used to assess their IGF2 gene expressions (*P <.05). (**C**) Double-infected VCaP and PC3 cells were assessed by a 5-day CCK-8 assay, to compare in vitro proliferation between pc/NS- and pc/IGF2-transfected PCa cells, which were already infected with L/IGF2AS (*P <.05). (**D**) Double-infected VCaP and PC3 cells were assessed by a 24-well transwell assay. After 24 hours, PCa cells invaded onto the bottoms of wells were stained, and representative images were compared between pc/NS- and pc/IGF2-transfected PCa cells, which were already infected with L/IGF2AS. (**E**) For invaded PCa cells shown in (C), relative invasive capability was quantitatively measured and compared between pc/NS- and pc/IGF2-transfected PCa cells, which were already infected with L/IGF2AS (*P <.05). IGF2AS, insulin growth factor 2 antisense; PCa, prostate cancer; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction. (Color version available online.)

transfected with pc/IGF2, IGF2 gene expressions were significantly upregulated, as compared to PCa cells transfected with pc/NS (Fig. 4**B**, * P <.05).

Third, double-infected cells were seeded in 96-well plates, and their in vitro proliferation was assessed by a CCK-8 assay for 5 consecutive days. The result of proliferation assay

showed that, in IGF2AS-infected VCaP and PC3 cells, IGF2 overexpression reversely accelerated cancer cell proliferating rates (Fig. 4**C**, * P <.05).

Finally, cancer cell invasive capability was assessed in double-infected VCaP and PC3 cells. Using a 24-hour transwell assay, it showed that considerably more L/IGF2AS-infected VCaP or PC3 cells were discovered at the bottoms of wells if they were further transfected with pc/IGF2 instead of pc/NS (Fig. 4**C**). Then, quantitative assessment confirmed that, in L/IGF2AS-infected VCaP and PC3 cells, invasive capability of PCa cells was reversely enhanced by IGF2 overexpression (Fig. 4**D**, * P <.05).

DISCUSSIONS

In recent decade, strong evidence has demonstrated that lncRNAs may by aberrantly deregulated in human PCa tumors, as well as acting as prognostic biomarkers or functional modulators for PCa development and progression.[14-16] In the present study, we suspected that human-cancer-associated lncRNA, IGF2AS, might be mechanistically involved in PCa development. Therefore, we adapted a systemic approach to assess the expression profile and possible functional roles of IGF2AS in human PCa.

First in the present study, qRT-PCR was applied and we noticed that IGF2AS was significantly downregulated in both immortal PCa cell lines and clinical human PCa tumors. This result is consistent with a very recent study, showing IGF2AS was also aberrantly downregulated in nonsmall cell lung cancer (NSCLC).[20] Therefore, it is likely that IGF2AS may be predominantly downregulated in various types of human cancers.

Second in the present study, as we discovered the deregulated expression pattern of IGF2AS in PCa, we then sought the possible functional role of IGF2AS. To do so, we used lentivirus to overexpress IGF2AS in VCaP and PC3 cells. Using several in vitro and in vivo cancer assays, we found that IGF2AS was acting as a tumor suppressor as its overexpression inhibited PCa cell proliferation and invasion in vitro and xenograft development in vivo. Similar anticancer function of IGF2AS was also reported in a very recent study, showing IGF2AS also inhibited NSCLC in vitro and in vivo progressions, as well as cancer-drug chemoresistance.[20] However, the present study was the first publication to demonstrate the mechanistic role of IGF2AS in human PCa. Specifically, we demonstrated that IGF2AS had tumor suppressing functions in both VCaP cells, a prostate-specific antigen (PSA)-positive cell line, and PC3 cells, a PSA-negative cell line. These results suggest that IGF2AS might be a universal tumor suppressor across various PCa sub-types.

Since IGF2AS was originally identified as a natural antisense transcript of IGF2 gene,[17] we then sought the possible involvement of IGF2 in IGF2AS' anti-cancer regulation in PCa. First, qRT-PCR assessment showed that IGF2 was inversely downregulated in IGF2AS-overexperssed VCaP and PC3 cells. Second, after we successfully upregulated IGF2 in IGF2AS-overexperssed VCaP and PC3 cells, we noticed that the tumor suppressing function of IGF2AS on PCa cell proliferation and invasion was significantly reversed. Thus, these data strongly suggested that IGF2 was the associated or downstream target gene of IGF2AS in cancer regulation in PCa. It is worth noting that, in the very recent publication revealing the functional role of IGF2AS in human NSCLC, the authors did not elaborate the associating signaling pathways of IGF2AS.[20] Therefore, the present study was the first report to demonstrate the inverse correlation of IGF2AS and IGF2 in human cancers.

Taken together, this study presented strong evidence showing IGF2AS acting as an epigenetic modulator to suppress cancer development in PCa. And the underling molecular mechanism may very well involve IGF2. Future studies, especially those focusing on the clinical relevance of IGF2AS in correlating with PCa patients' clinicopathological features or prognosis would further advance our understanding on the epigenetic regulation of lncRNA in PCa.

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