Asian Journal of Andrology (2013) 15, 753–758
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www.nature.com/aja

ORIGINAL ARTICLE

The miRNA *let-7a*1 inhibits the expression of insulin-like growth factor 1 receptor (*IGF1R*) in prostate cancer PC-3 cells

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Reduced microRNA (miRNA) *let-7a* expression and the activation of insulin-like growth factor-1 receptor (IGF1R) signalling are both involved in prostate cancer and progression. In the present study, we demonstrated that the growth inhibitory effect of *let-7a*1 is directly related to targeting *IGF1R* gene expression in PC-3 cells. TargetScan predicted three potential target sites (T1, T2 and T3) of *let-7a* in the 3′ untranslational region (3′ UTR) of *IGF1R* mRNA. Real-time PCR, Western blot and luciferase reporter assays were used to detect the effects of *let-7a*1 overexpression or *let-7a*1 inhibitor on the *IGF1R* gene expression in PC-3 cells. The results indicated that *let-7a*1 could inhibit *IGF1R* expression by directly targeting the T1 and T2 sites in the 3′ UTR of the *IGF1R* mRNA. We then used RT-PCR, luciferase reporter assays, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2*H*-tetrazolium bromide (MTT) assay, flow cytometry and Hoechst 33342 staining to examine whether *let-7a*1-mediated inhibition of *IGF1R* expression also affects the IGF1R-mediated signalling events, including Elk1 activity and *c-fos* gene expression, proliferation, apoptosis and cell cycle. We demonstrated that *let-7a*1-mediated IGF1R downregulation was accompanied by attenuation of Elk1 activity and *c-fos* expression, inhibition of cell proliferation, enhanced apoptosis and cell cycle arrest, and that loss function of *let-7a*1 *via* inhibition can upregulate IGF1R accompanied by an increase of Elk1 activity and *c-fos* expression, thereby enhancing cell proliferation. Altogether, these findings suggest that *let-7a* may be novel therapeutic candidate for prostate cancer.
Asian Journal of Andrology (2013) **15,** 753–758; doi:10.1038/aja.2013.84; published online 26 August 2013

Keywords: IGF1R; microRNA (miRNA); prostate cancer

INTRODUCTION

Prostate cancer is one of the most common cancers[1] and responds well to androgen ablation initially, before invariably progressing to treatment resistance characterized by a high proliferation rate and strong metastasis propensity.[2,3] The progression of these tumours is reportedly facilitated by growth factors activating critical signalling cascades, thereby promoting prostate cancer cell growth, survival and migration.[4] Among these growth and survival signalling pathways, the insulin-like growth factor-1/its receptor (IGF-1/IGF1R) system has been suggested to play a key role in the malignant transformation of prostate cancer cells.[5–8] IGF-1 binding to IGF1R results in tyrosine kinase activation and the stimulation of downstream signalling pathways that regulate proliferation, cell cycle and apoptosis. Several studies have reported that IGF1R expression is elevated in metastatic prostate cancer and hormone resistance progression.[9–11] Therefore, inhibition of IGF1R signalling may provide an alternative approach to prostate cancer treatment.

MicroRNAs (miRNAs) are ~22 nucleotides long, non-coding and endogenous RNA molecules with important functions in development, cell differentiation, regulation of cell cycle and apoptosis. miRNAs exert their functions by base pairing with target mRNAs to regulate protein-coding gene expression *via* mRNA degradation or translation inhibition. More than 60% of human protein coding genes have been under selective pressure to maintain pairing to miRNAs, which suggests that most mammalian mRNAs are conserved targets of miRNAs.[12] Much evidence demonstrates that miRNAs play important roles in the establishment and progression of human tumours. The miRNAs act as either oncogenes[13–15] or tumour suppressors.[16–18] The miRNA *let-7a* has been characterised as a tumour suppressor in several human cancers.[19–24] Dong *et al.*[25] reported that *let-7a* is downregulated in resected prostate cancer samples and in prostate cancer cells. PC-3 and DU145 cells (androgen-independent) express less *let-7a* than LNCaP cells (androgen-dependent). Target prediction algorithms indicate that *let-7a*1 can target a number of oncogenes, including *IGF1R*. Several studies have reported that the activation of IGF1R signalling is critical for prostate cancer cell growth and progression. In this study, we examine whether *let-7a*1 targets *IGF1R* expression to inhibit the proliferation of prostate cancer cells.

MATERIALS AND METHODS

Plasmid construction

Three miRNA *let-7a*1 targeting sequences were identified in the 3′ untranslational region (3′ UTR) of *IGF1R* mRNA. They are located at position 99–105, 2619–2626, 6661–6667 of the *IGF1R* 3′ UTR. The targeting sequences and their mutants (shown in the following list)

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Received: 16 March 2013; Revised: 12 May 2013; Accepted: 1 June 2013; Published online: 26 August 2013

were synthesised and cloned into a pMIR-Report vector (Ambion, Grand Island, NY, USA) to form pMIR-T1, pMIR-T2 pMIR-T3, pMIR-T1m, pMIR-T2m and pMIR-T3m.

IGF1R-3′ UTR-T1F: 5′-cCCATTCACAAGCCTCCTGTACCTC-AGTGa-3′

IGF1R-3′ UTR-T1R: 5′-agcttCACTGAGGTACAGGAGGCTTGT-GAATGGgagct-3′

IGF1R-3′ UTR-T2F: 5′-cTTCCCCCAAACATTTATCTACCTCA-CTCa-3′

IGF1R-3′ UTR-T2R: 5′-agcttGAGTGAGGTAGATAAATGTTTG-GGGGAAgagct-3′

IGF1R-3′ UTR-T3F: 5′-cAGGTTTGCCAGAGTTTGTCTACCTC-TGGa-3′

IGF1R-3′ UTR-T3R: 5′-agcttCCAGAGGTAGACAAACTCTGGC-AAACCTgagct-3′

IGF1R-3′ UTR-T1mF: 5′-cCCATTCACAAGCCTCCTGATGGA-GTGTGa-3′

IGF1R-3′ UTR-T1mR:5′-agcttCACTCTCCATCAGGAGGCTTG-TGAATGGgagct-3′

IGF1R-3′ UTR-T2mF: 5′-cTTCCCCCAAACATTTATGATGGAG-ACTCa-3′

IGF1R-3′ UTR-T2mR: 5′-agcttGAGTCTCCATCATAAATGTTT-GGGGGAAgagct-3′

IGF1R-3′ UTR-T3mF: 5′-cAGGTTTGCCAGAGTTTGTGATGG-AGTGGa-3′

IGF1R-3′ UTR-T3mR: 5′-agcttCCACTCCATCACAAACTCTGG-CAAACCTgagct-3′

For construction of the miRNA *let-7a*1 expression plasmid, the *let-7a*1 precursor was obtained by RT-PCR amplification using primers 7a1F (5′-cccggatccCCTGGATGTTCTCTTCACTG-3′) and 7a1R (5′-cccaagcttGCCTGGATGCAGACTTTTCT-3′) and cloned into a pSilencer 4.1-CMV neo vector (Ambion) to form pSilencer4.1-*let7a*1.

For construction of the Elk1-reporter plasmid, the Elk1 element DNA sequences (Elk1-S: cTTCCCCCAAACATTTATCTACCTCACTCa, Elk1-AS: agcttGAGTGAGGTAGATAAATGTTTGGGGGAAgagct) was synthesised and inserted upstream of the TATA box of the pGL4-LUC2[minp] vector (Premega, Madison, WI, USA) to form pGL4-Elk1.

Cell culture and transfection

The prostate cancer cell line PC-3 cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), which was supplemented with 10% foetal bovine serum, 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37 °C with 5% CO_2. Cell transfections were performed using FuGENE® HD (Roche, Mannheim, Germany) or siPORT *NeoFX* Transfection Agent (Ambion) according to the manufacturer's instructions. For RT-PCR and Western blot analysis, the cells were grown in 6-well plates and transfected for 48 h with either pSilencer4.1-*let7a*1 or parental vector, with either *let-7a*1 inhibitor or normal control (NC) inhibitor (Ambion). For the luciferase reporter assay, the cells were grown in 24-well plates to 80% confluence and cotransfected for 48 h with pSilencer4.1-*let7a*1 (or parental vector) and pMIR-T (pMIR-T1, pMIR-T2, pMIR-T3, pMIR-T1m, pMIR-T2m, pMIR-T3m) plasmids (or parental vector) and pRL-TK Renilla luciferase vector (Promega) as an internal control. For the proliferation and apoptosis assays, the cells were grown in 96-well plates and transfected with either pSilencer4.1-*let7a*1 or parental vector, with either *let-7a*1 inhibitor or NC inhibitor.

Western blot analysis

Western blotting was performed to detect IGF1R protein expression. Whole-cell lysates were prepared using lysis buffer (containing 50 mmol l^{-1} Tris-Cl, pH 8.0, 150 mmol l^{-1} NaCl, 0.1% SDS, 1% NP-40 and 100 μg ml^{-1} PMSF). Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Extracted protein (50 μg) was processed in a 10% SDS–PAGE and electrophoretic transferred to a polyvinylidene fluoride membrane. IGFIR protein expression was determined using an IGF1R β-subunit polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA). β-Actin (Sigma, St Louis, MO, USA) was used as an internal control. Immunoblots were detected using an ECL kit (Santa Cruz Biotechnology) and were visualised after exposure to X-ray film.

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from PC-3 cells using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Pittsburgh, PA, USA). The real-time PCR primers for amplification of miRNA *let-7a*1 used were 7a1F (5′-CCTGGATGTTCTCTTCACTG-3′) and 7a1R (5′-GCCTGGATGCAGACTTTTCT-3′). Quantitative PCR was conducted using SYBR Premix EX Taq (Takara Biotechnology, Dalian, China). The samples were analysed in triplicate, and their relative expression levels were normalised to the expression of *5S rRNA*.

Dual-luciferase reporter gene assay

The luciferase reporter assay was performed at 48 h post-transfection. The cells were lysed using 1× reporter lysis buffer and were harvested by manual scraping. Luminescence was detected using a Mithras LB 940 (Berthold Technologies, Oak Ridge, TN, USA). The firefly luciferase activity of the pMIR-Report plasmid was measure 1 (M1), and the Renilla luciferase activity (internal control) of pRL-TK plasmid (Promega) was measure 2 (M2). The relative luciferase activity was calculated as the ratio of M1/M2.

Proliferation assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2*H*-tetrazolium bromide (MTT) method. At 48 h post-transfection, the transfection medium in each well was replaced by 100 μl of fresh serum-free medium with 0.5 g l^{-1} MTT. After incubation at 37 °C for 4 h, the MTT medium was removed, and 50 μl of DMSO was added to each well. After incubation at 37 °C for another 10 min, the $A_{570\ nm}$ of each sample was measured using a plate reader.

Flow cytometry-based apoptosis and cell-cycle analysis

At 48 h post-transfection, the PC-3 cells were harvested and resuspended in phosphate-buffered saline (PBS) and then fixed in ethanol at −20 °C overnight. The cells were washed with PBS and resuspended in staining solution (50 μg ml^{-1} propidium iodide, 1 mg ml^{-1} RNase A and 0.1% Triton X-100 in PBS). The stained cells (1×10^5) were then analysed for apoptosis and cell cycle with an FACScalibur; Becton Dickinson Flow Cytometer (PT. Madagasi Brosa Inc., Batang Hari, Propinsi Sumatera Utara, Indonesia).

Nuclear staining with Hoechst 33342

At 48 h post-transfection, apoptotic cells were detected using Hoechst 33258 staining (Beyotime, Shanghai, China) according to the manufacturer's protocols. Stained cells were imaged under a fluorescent microscope using 350-nm excitation and 460-nm emission.

Statistical analysis

Statistical analysis was performed using *t*-test. Differences with $P<0.05$ were considered to be statistically significant.

RESULTS

The miRNA *let-7a*1 reduces *IGF1R* expression in PC-3 cells

With performing analysis using TargetScan-Human 6.2 (http://www.targetscan.org/), *IGF1R* is one miRNA *let-7a*1 candidate target. PC-3 cells were transfected either with pSilencer4.1-*let7a*1 or parental vector, with either *let-7a*1 inhibitor or NC inhibitor, for 48 h. The total RNA was extracted, and the whole-cell lysate was prepared. Western blot (**Figure 1a**) and real-time RT-PCR (**Figure 1b**) analyses were performed to detect the effects of miRNA *let-7a*1 or *let-7a*1 inhibitor on *IGF1R* expression. The results indicated that *let-7a*1 could reduce the protein level, while *let-7a*1 inhibition enhanced the IGF1R protein level; however, no apparent change in the *IGF1R* mRNA level was observed.

The miRNA *let-7a*1 directly interacts with its targeting sequences in the 3′ UTR of *IGF1R* mRNA

Three miRNA *let-7a*1 targeting sequences were found in the 3′ UTR region of *IGF1R* mRNA (**Figure 2a**) using TargetScan-Human 6.2. To confirm that miRNA *let-7a*1 directly targets these sequences, dual-luciferase reporter assays were performed using cell transfection with the constructs in which these targeting sites or their mutants were cloned into the 3′ UTR of the luciferase reporter gene. The results revealed that the luciferase activities in the cells transfected with

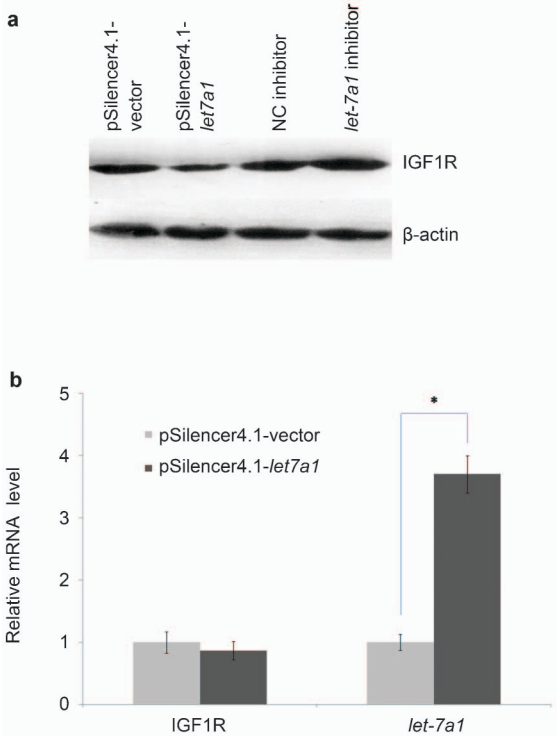

Figure 1 miRNA *let-7a*1 effects on *IGF1R* mRNA and protein expression. PC-3 cells were transfected with either pSilencer4.1-*let7a*1 or parental vector, with either *let-7a*1 inhibitor or NC inhibitor, for 48 h. Western blot (**a**) and real-time PCR (**b**) were conducted to detect the *let-7a*1 level and the effects of *let-7a*1 or *let-7a*1 inhibitor on the *IGF1R* expression. β-actin or *5S rRNA* expression was used as the internal control in the Western blots or in real-time PCRs, respectively. The results shown in **b** are the means±s.d. *$P<0.05$. IGF1R, insulin-like growth factor-1 receptor; miRNA, microRNA; NC, normal control.

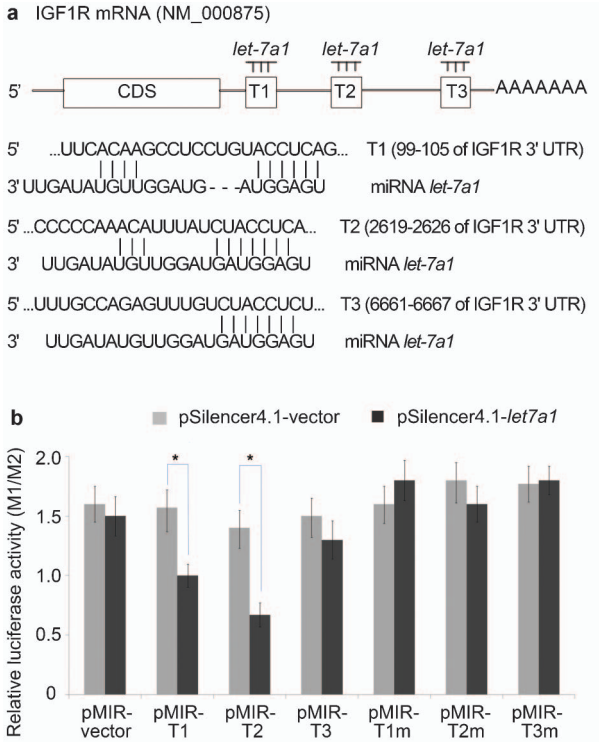

Figure 2 miRNA *let-7a*1 directly targets the 3′ UTR of *IGF1R* mRNA. (**a**) Schematic representation of *IGF1R* mRNA showing the positions and sequences of the three predicted miRNA *let-7a*1 binding sites located in its 3′ UTR. (**b**) PC-3 cells were co-transfected with pSilencer4.1-*let7a*1 (or parental vector), pMIR-T1 (or pMIR-T2 or pMIR-T3) or pMIR-T1m (or pMIR-T2m or pMIR-T3m) and pRL-TK for 48 h. Luciferase activity was detected using a Dual-Luciferase Assay System and was plotted as ratio of firefly to Renilla luciferase activity (M1/M2). The data are the means of three individual values ± s.d. *$P<0.05$. IGF1R, insulin-like growth factor-1 receptor; miRNA, microRNA; 3′ UTR, 3′ untranslational region.

pMIR-T1 or pMIR-T2 were reduced as compared with the cells treated with the parental vectors, whereas transfection with pMIR-T3 or pMIR-T1m, pMIR-T2m and pMIR-T3m had no obvious effect on the luciferase activity (**Figure 2b**). These results combined with the results in **Figure 1** demonstrate that *let-7a*1 regulates *IGF1R* expression by directly targeting the T1 and T2 sites in 3′ UTR of the *IGF1R* mRNA to inhibit its protein expression.

The miRNA *let-7a*1 reduces Elk1 activity and *c-fos* expression

Elk1 is a transcriptional factor activated by IGF1R signalling. Activated Elk1 binds with the Elk1 element to regulate the target gene expression. C-fos is one of the Elk1-regulating genes involved in cancer cell phenotypes. To confirm that miRNA *let-7a*1 or *let-7a*1 inhibitor can influence Elk1 activity *via* changing the expression level of IGF1R, dual-luciferase reporter assays were performed using cell transfection with the construct in which the Elk1 element was cloned upstream of the TATA box of the pGL4-LUC2[minp] vector to form pGL4-Elk1. RT-PCR and Western blot were performed to detect the effects of *let-7a*1 or *let-7a*1 inhibitor on *c-fos* expression. The results revealed that *let-7a*1 could reduce, whereas the *let-7a*1 inhibitor increased, luciferase activity (**Figure 3a**), *c-fos* mRNA (**Figure 3b**) and protein (**Figure 3c**) expression.

The effects of *let-7a*1 on PC-3 proliferation, cell cycle and cell apoptosis

IGF1R is a tyrosine kinase receptor and mediates IGF1-induced signalling events in cell survival and proliferation. Our present results

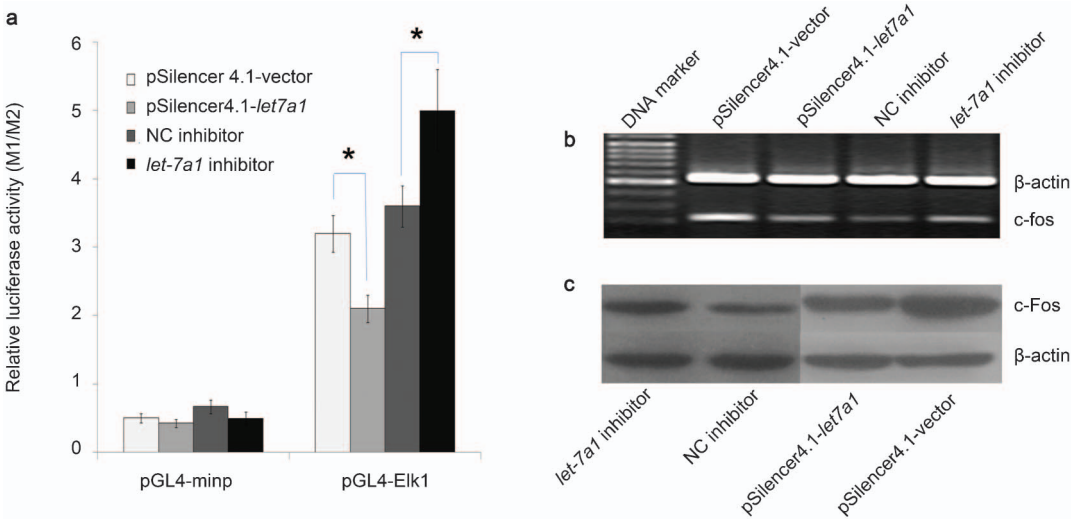

Figure 3 The effects of the miRNA *let-7a*1 on Elk1 activity and *c-fos* expression. Dual-luciferase reporter assays (**a**) were performed using cell transfection either with pSilencer4.1-*let7a*1 (or parental vector), or with *let-7a*1 inhibitor (or NC inhibitor), pGL4-Elk1 (or parental vector) and pRL-TK for 48 h. The results are expressed as the relative luciferase activity (M1/M2). The data are represented as the mean of three individual values ± s.d. *$P<0.05$. RT-PCR (**b**) and Western blot (**c**) were performed to detect the effects of miRNA *let-7a*1 or *let-7a*1 inhibitor on *c-fos* mRNA and protein expression. β-actin expression was used as the internal control in Western blot and RT-PCR. miRNA, microRNA; NC, normal control.

clearly demonstrate that *let-7a*1 reduces IGF1R expression in PC-3 cells by directly targeting the 3′ UTR of *IGF1R* mRNA. We also detect the effects of *let-7a*1 or *let-7a*1 inhibitor on PC-3 proliferation, the cell cycle and cell apoptosis. As illustrated in **Figure 4**, the *let-7a*1-mediated downregulation of *IGF1R* expression in PC-3 cells was accompanied by inhibition of cell proliferation (**Figure 4a**), G_0/G_1 cell-cycle arrest (**Figure 4b**) and enhanced cell apoptosis (**Figure 4c** and **d**), whereas *let-7a*1 inhibitor resulted in G_0/G_1-cell decrease, S-cell increase and cell proliferation.

DISCUSSION

miRNAs are increasingly being discovered to be involved in regulating the malignant progression of cancer by directly targeting oncogenes and tumour suppressor genes. Aberrant expression of microRNAs has now been associated with many types of cancer. Some miRNAs exert oncogenic function by targeting tumour suppressor genes, and some exert anti-tumour function by targeting oncogenes. *Let-7a* has been reported to act as a tumour suppressor in some cancer types, such as lung and colon cancer,[26–28] and the reduced expression levels of *let-7* is correlated with poor clinical prognosis.[29]

Each miRNA can potentially interact with several mRNA targets *via* perfect or imperfect base pairing, primarily in the 3′ UTR portion. A number of target prediction algorithms relying on seed sequence pairing rules and conservational analysis have been developed to score possible recognition sites and identify putative gene targets. In the present study, we found that *IGF1R* is one of the *let-7a*1 target genes, and there are three potential target sites (T1, T2 and T3) of the miRNA *let-7a*1 in the *IGF1R* 3′ UTR as predicted by TargetScan. To confirm that miRNA *let-7a*1 directly targets these sequences, dual-luciferase reporter assays were performed using cell transfection with constructs in which these targeting sites were cloned into the 3′ UTR of the reporter gene. Luciferase reporter assays, as well as real-time RT-PCR and Western blot, demonstrated that *let-7a*1 regulates IGF1R expression by directly targeting the T1 and T2 sites in the 3′ UTR of the *IGF1R* mRNA to inhibit its expression.

The inability of a cell to regulate its growth and proliferation is a distinctive feature of cancer. Activation of insulin-like growth factor-1 receptor (IGF1R) signalling is reportedly critical for prostate cancer cell growth and progression. IGF1R is a receptor tyrosine kinase that mediates IGF1-induced signalling events, including cell survival and proliferation. Our present results clearly demonstrate that *let-7a*1 downregulates IGF1R by directly targeting the 3′ UTR of *IGF1R* mRNA. This *let-7a*1-mediated IGF1R downregulation is accompanied by the attenuation of Elk1 activity and *c-fos* expression, inhibition of cell proliferation, enhanced apoptosis and cell-cycle arrest. This action is consistent with the well-established IGF1R/RAS/MAPK/ELK1 signalling pathway, which plays major roles in cell survival and proliferation.

Both *let-7a* and *IGF1R* are involved in prostate cancer and progression. Our finding that *let-7a*1 directly targets *IGF1R* in prostate cancer cells could further reveal the mechanism of prostate carcinogenesis and progression. *let-7a* might partly contribute to IGF1R over-expression in prostate cancer cells, leading to the cell survival and proliferation. *let-7a* may be a novel therapeutic candidate for prostate cancer given its ability to induce cell-cycle arrest, inhibit cell growth and induce apoptosis, especially in hormone-refractory prostate cancer.

AUTHOR CONTRIBUTIONS

LNW assisted in the design of the study, conducted the cloning of reporter plasmid constructs, transfection studies, RT-PCR, MTT assays and Western blotting and helped draft the manuscript. WWC assisted in the design of the study, participated in the sequence alignment and use of computer database, assisted in the construction of recombinant plasmids and helped draft the manuscript. JZ performed flow cytometry, assisted with the transfection experiments, participated in the maintenance of cell lines and performed luciferase reporter assays. CYL conducted the isolation of RNA and purification of plasmids and assisted with the RT-PCR and Western blotting. CYL conducted the Western blotting and assisted with the purification of plasmids and luciferase reporter assays. JX assisted with the maintenance of the cell lines and the transfection experiments. PJZ participated in the construction of recombinant plasmids and the design of the study and

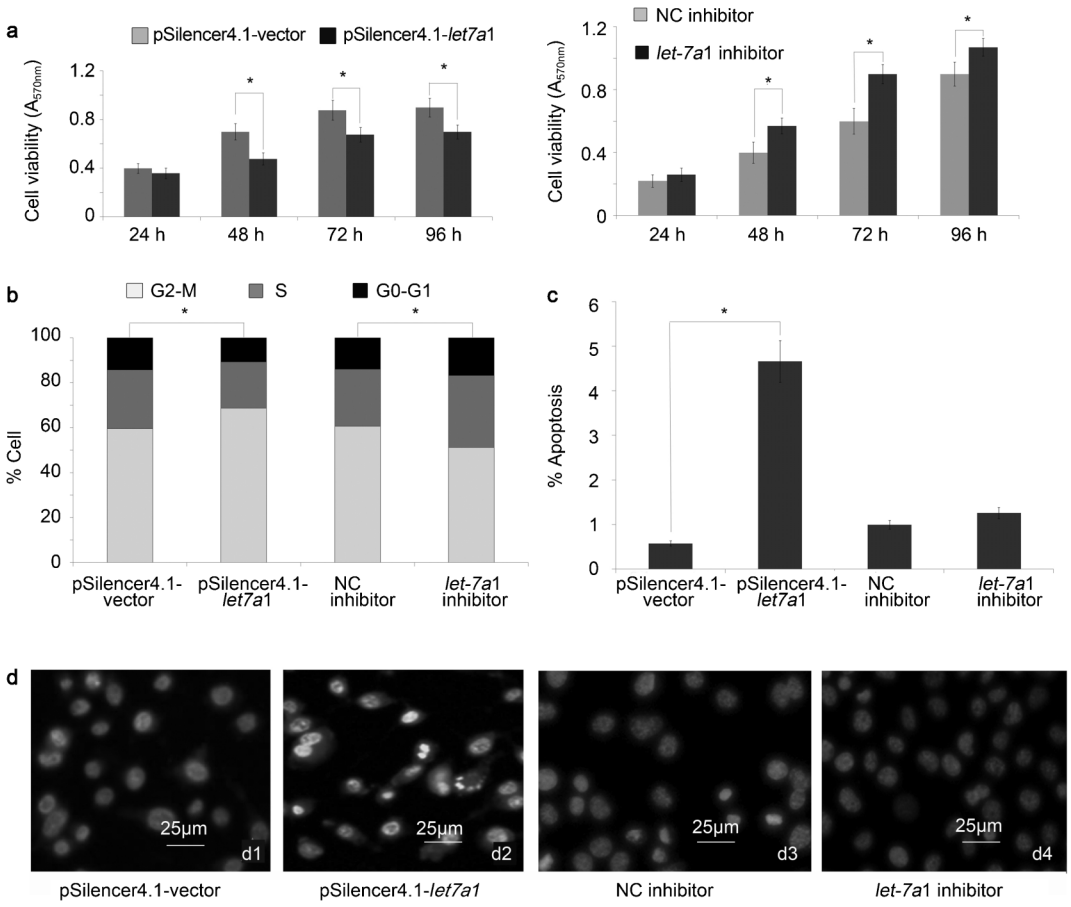

Figure 4 The effects of the miRNA *let-7a*1 on PC-3 proliferation, cell cycle and apoptosis. The changes in cell proliferation (**a**), cell-cycle distribution (**b**) and cell apoptosis (**c**, **d**) were examined as described in the experimental section in PC-3 cells that were transfected either with pSilencer4.1-*let7a*1 or parental vector with *let-7a*1 inhibitor or NC inhibitor for 48 h. All results are representative of three independent experiments. **P*<0.05. miRNA, microRNA; NC, normal control.

assisted with the revising of the manuscript. ALJ participated in the design of the study and revision of the manuscript, helped draft the manuscript and assisted with the construction of recombinant vectors. All authors have read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (Nos. 81071720 and 81172045), Shandong Provincial Programs for Science and Technology Development (No. 2012GSF11820) and Foundation for Outstanding Young Scientist in Shandong Province (No. 2006BS03066).

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