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Original Research

FOXA1 knock-out via CRISPR/Cas9 altered Casp-9, Bax, CCND1, CDK4, and fibronectin expressions in LNCaP cells

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Impact statement

Knock-out studies offer a unique way of studying the function of genes especially for developmentally lethal genes. FOXA1 has prominent roles both in breast and prostate cancer pathogenesis due to its role in ER receptor signaling pathway. FOXA1 has also been identified as one of the seven molecular subtypes of primary prostate cancer. In the present study, we used an efficient gene knock-out method, CRISPR/Cas9, in order to investigate FOXA1 function on LNCaP prostate cancer cells *in vitro*. FOXA1 knock-out altered cell-cycle regulator CCND1 protein expression levels. Therefore, our results suggest that FOXA1 might be a plausible drug target for prostate cancer treatment.

Abstract

Prostate cancer is one of the most common types of cancer in men and the leading cause of death in developed countries. With the aid of molecular and genetic profiling of cancers, cancer molecular subtypes are paving the way for tailored cancer therapy. FOXA1 has been identified as one of the seven molecular subtypes of prostate cancer. FOXA1 is involved in a variety of metabolic process such as glucose homeostasis and deregulation of its expression is crucial in prostate cancer progression. In this study, we investigated the effects of FOXA1 gene knock-out on the expression levels of various cancer cell metabolism and cell cycle-related protein expressions. FOXA1 gene was knocked-out by using CRISPR/Cas9 technique. While FOXA1 gene knock-out significantly altered Casp-9, Bax, CCND1, CDK4, and fibronectin protein expressions ($P < 0.05$, fold change: $\sim$40, 4.5, 2.5, 4.5, and 4, respectively), it did not affect the protein expression levels of Casp-3, Bcl-2, survivin, β-catenin, c-Myc, and GSK-3B. Knocking-out FOXA1 gene in androgen-dependent LNCaP prostate cancer cells inhibited CCND1 protein expression. Our pre-clinical results demonstrate the importance of FOXA1 as a drug target in the treatment of prostate cancer.

Keywords: FOXA1, CRISPR/Cas9, prostate cancer, androgen-dependent, cell culture, western blotting

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Introduction

Tumor characterization holds great promise in patient stratification to enable precision medicine. The Cancer Genome Atlas project has recently analyzed 333 primary prostate carcinomas and defined seven molecular subtypes of primary prostate cancer. Most of the primary prostate tumors fell into one of the categories defined by specific gene fusions (ERG, ETV1/4, and FLI1) and mutations (SPOP, FOXA1, and IDH1).[1]

FOXA1 has prominent roles in prostate gland development and differentiation of prostatic cells and is required for prostate glandular morphogenesis.[2] FOXA1 is responsible for the expression of prostate specific genes by maintaining prostate epithelial phenotype.[3] FOXA1 mutant mice is developmentally lethal due to dehydration and hypoglycemia. These observations strongly suggest that FOXA1 is involved in glucose homeostasis.[4] Conditional FOXA1 knock-out mice exerts altered ductal development, immature epithelial cells surrounded by thick stromal layers.[5] FOXA1 also has a pivotal role in cell cycle by promoting G1-S transition and G2-M transition.[6]

Furthermore, FOXA1 is a pioneer transcription factor in chromatin remodeling that de-compacts chromatin structure to enable androgen receptor recruitment to the binding sites.[7,8] FOXA1 expression is overexpressed in different types of cancers such as acute myeloid leukemia, thyroid, lung, esophageal, breast, and prostate cancer.[4] Higher FOXA1 levels is associated with poorer prognosis.[9] FOXA1 is involved in regulating the transactivation of steroid hormone receptors and is strongly associated with

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tumor growth and hormone responsiveness in prostate cancer.[10,11] We, therefore, investigated the effects of FOXA1 knock-out on the protein expression profiles of some apoptotic, metastatic, and invasion-related genes in androgen-dependent LNCaP prostate cancer cell line. In this study, we investigated that some of the genes have a general overview of cancer cell hallmarks reflection in the form of protein expression. Therefore, we evaluated the effect of FOXA1 gene knock-out apoptosis-related effect via investigating Bax, Bcl-2, survivin, Casp-3, and Casp-9; epithelial-mesenchymal transition-related activity via integrin, FAK, fibronectin; anti-angiogenic activity via VEGFA, HIF1A; tumor cell metabolism and cell cycle progression via GSK3B, c-Myc, CCND1, CDK4, and B-catenin.

Materials and methods

Cell line and culture

Androgen-dependent prostate cancer cell line LNCaP was kindly gifted from Dr. Levent Turkeri. LNCaP cells were cultured in RPMI medium supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA). Cells were grown in 5% CO_2 incubator at 37°C.

CRISPR/Cas9 plasmid and design

FOXA1 genomic sequence around the first exon (ENSG00000129514) was submitted to Zhang Lab online gRNA design tool (http://crispr.mit.edu). Two target sites were selected according to a previous study.[12] The oligonucleotides used to construct gRNAs for the human FOXA1 gene were g-Foxa1 1s (5′-GTAGTAGCTGTTCCAG TCGC-3′) and g-Foxa1 1r (5′-GCGACTGGAACAGCTAC TAC-3′). A human codon-optimized SpCas9 and chimeric guide RNA expression plasmid (pX330-U6-Chimeric_BB-CBh-hSpCas9, Addgene, Cambridge, MA, USA) was digested by FastDigestBpiI FD1014 (Thermo Fisher Scientific, MA, USA) and then a pair of annealed oligonucleotides was cloned into the gRNA according to Ran *et al*.[13]

FOXA1 gene knock-out by CRISPR/Cas9

LNCaP cells were seeded in a six-well plate at a density of 5×10^4 cells/well and incubated for one day. Isolated gDNA from the transformed plasmid (see previous steps) were used to transfect cells using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, MA, USA) following the manufacturer's protocol. Three days after transfection, limiting dilution method was used at 0.5 cells/well of a 96-well plate in order to have efficiently modified colonies. When cells reached 70% confluency, they were lysed using 100 μl of RIPA buffer including 1 mM PMSF (Roche Diagnostics, Mannheim, Germany). Immunoblot screening of the clones were performed without an antibiotic selection according to a previously described method.[14] Western blot was used to examine FOXA1 protein expression of each clone. Subsequent experiments were performed using FOXA1 protein non-expressing clones. Later steps were performed using passage-matched parental cells in order to exclude passage-related cell division effects on FOXA1 expression.

Dot blotting

Protein samples were mixed with LDS sample buffer. PVDF membrane was pre-wetted using methanol then washed by distilled water and TBS-T (TBST, 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). Samples were loaded onto the PVDF membrane and were air-dried for 1.5 h at room temperature. PVDF membrane was blocked with 1% bovine serum albumin in TBS-T for 1 h at room temperature. Immunoblot was washed three times by TBS-T followed by 1 h incubation at room temperature with 1:1000 concentration of FOXA1 (PA5–27157) antibody (Thermo Fisher Scientific, MA, USA). The membrane was washed three times with TBS-T, blocked with anti-rabbit secondary antibody (Thermo Pierce, Rockford, IL, USA) and visualized by Luminata Forte Western HRP Substrate (Merck Millipore, Darmtadt, Germany). Blot was washed three times with TBS-T. Chemiluminescent signals of dot blot was documented using Gel Logic 2200 Pro (Carestream Health, Rochester, NY, USA).

Western blotting

Cells were washed with PBS and scraped into RIPA lysis buffer containing 1 mM PMSF followed by sonication. Samples were centrifuged for 15 min at 13,500 r/min at 4°C and supernatant was collected. Proteins were quantified using BCA Assay Kit (Thermo Pierce, Rockford, IL, USA). Protein lysates (20 μg) were heated for 5 min at 95°C in LDS non-reducing sample buffer (Pierce, Rockford, IL, USA) then loaded to 10% Tris-glycine gels, transferred to PVDF membrane (Pierce, Rockford, IL, USA) membranes at 250 mAmp for 2 h. Membranes were blocked with 5% bovine serum albumin in TBS-T for 1 h at room temperature and incubated overnight at 4°C with the antibodies for Casp-3 (PI-PA516335), Casp-9 (PA1–12506), Bcl-2 (PI-PA520068), Bax (PI-PA511378), c-Myc (PI-PA1981), CCND1 (PI-PA524173), CDK4 (PI-PA527827), HIF1A (PI-PA116601), GSK3B (PI-PA529251), VEGFA (PI-PA516754), FAK (PI-PA517591), and β-actin (PI-PA1183) at 1:1000 concentration (Thermo Pierce, Rockford, IL, USA). Antibodies for integrin (ENT5248) were used at 1:1000 dilution (Elabscience Biotechnology Co., Ltd). Fibronectin (sc-9068), survivin (sc-10811), β-catenin (sc-7199) was used at 1:1000 dilution (Santa Cruz Biotechnology Inc., USA) was used at 1:1000 dilution (Cell Signaling Technology, MA, USA). Blots were washed three times with TBS-T. Protein bands were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibody at 1:50,000 dilution (#31460) (Thermo Pierce, Rockford, IL, USA) and visualized by Luminata Forte Western HRP Substrate (Merck Millipore, Darmtadt, Germany). Chemiluminescent signals of immunoblots were documented using Gel Logic 2200 Pro (Carestream Health, Rochester, NY, USA). The band density of specific proteins was quantified using ImageJ (NIH, Bethesda, MD, USA).

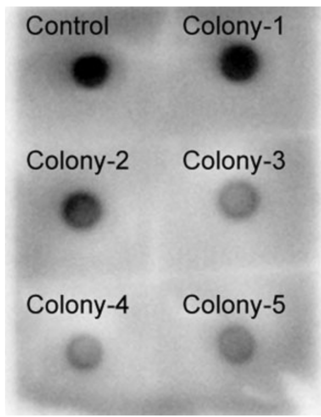

Figure 1. Immunoblot screening of the clones by dot blotting.

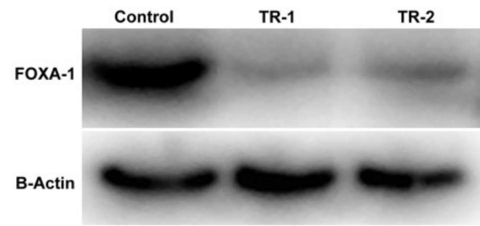

Figure 2. Knocking-out confirmation of FOXA1 by western blotting. β-actin was used as internal control. TR-1: transfected colony 1; TR-2: transfected colony 2.

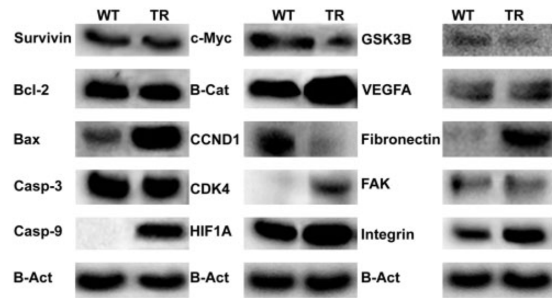

Figure 3. Effects of FOXA1 gene knock-out on several protein expression levels. TR: transfected cells; WT: wild-type cells.

Statistical analysis

Experiments were performed at least three times. Statistical analysis was performed using SPSS 21.0 software. Differences in protein expression were tested using nonparametric Mann–Whitney U test. $p < 0.05$ was considered statistically significant.

Results

Dot blot selection of FOXA1 knock-out clones

Figure 1 shows dot blot screening of FOXA1 non-expressing clones. It represents only a small subset of samples that were screened by dot blotting. Wild-type LNCaP cells were used as control while colonies 1–5 represent FOXA1 knock-out clones.

Confirmation of FOXA1 knock-out clones by western blotting

Colonies 4 and 5 (see above) were confirmed by western blotting to ensure the absence of FOXA1 protein expression therefore the successful knock-out of FOXA1 gene. β-actin was used as a control (Figure 2). These colonies were used for the other downstream applications of western blotting.

Effects of FOXA1 gene knock-out on apoptotic, cell-cycle regulator, epithelial to mesenchymal transition, and protein expression levels

In this study, we investigated the protein expression levels of Casp-3, Casp-9, Bcl-2, Bax, survivin, β-catenin, c-Myc, GSK-3B, HIF1A, VEGFA, CCND1, CDK4, fibronectin, integrin, and FAK. β-actin was used as a loading control. A minimum of two-fold protein expression change was considered to be significant in this study. FOXA1 gene knock-out significantly altered Casp-9, Bax, CCND1, CDK4, and fibronectin protein expressions ($p < 0.05$, fold change: ~40, 4.5, 2.5, 4.5, and 4, respectively) (Figure 3), whereas it did not affect the protein expression levels of Casp-3, Bcl-2, survivin, β-catenin, c-Myc, GSK-3B, integrin, VEGFA, HIF1A, and FAK. As two-fold change in protein expression profiles was considered statistically significant, the aforementioned protein expressions were identified as non-significant (Figure 4).

Anti-apoptotic protein Bcl-2 and survivin exerted similar patterns as Casp-3, which is known as the executioner caspase in apoptosis. In addition, pro-apoptotic proteins Bax and Casp-9 (initiator caspase) exhibited similar patterns. FOXA1 gene knock-out decreased CCND1 while it increased CDK4 protein expression level (Figure 3). Figure 4 demonstrates the differences in protein expression levels.

As an *in vitro* study design, FOXA1 gene expression did not affect the angiogenesis-related protein expressions: VEGFA and HIF1A. Anti-apoptotic survivin and Bcl-2 expression levels were correlated before and after FOXA1 gene knock-out. FOXA1 gene knock-out increased the Bax protein expression. As Bcl-2 protein expression was not affected significantly after gene knock-out, Bax/Bcl-2 ratio was increased after FOXA1 knock-out. This result is also correlated with the lower expression profile of CCND1. FOXA1 gene knock-out did not affect Casp-3 protein expression significantly, whereas it increased the protein expression levels of Casp-9. c-Myc protein expression level was decreased after FOXA1 knock-out. FOXA1 gene knock-out increased fibronectin protein expression, whereas it did not have a significant effect on other structural, epithelial-mesenchymal transition indicators integrin and FAK protein expression levels.

Protein–protein interaction analysis by STRING

As FOXA1 gene knock-out in LNCaP prostate cancer cells significantly altered Casp-9, Bax, CCND1, CDK4, and fibronectin protein expression levels, we further investigated the interaction of the aforementioned proteins using the STRING – the protein–protein interaction database (Figure 5). FOXA1 protein was found to interact via CCND1. Interactions were found between FOXA1,

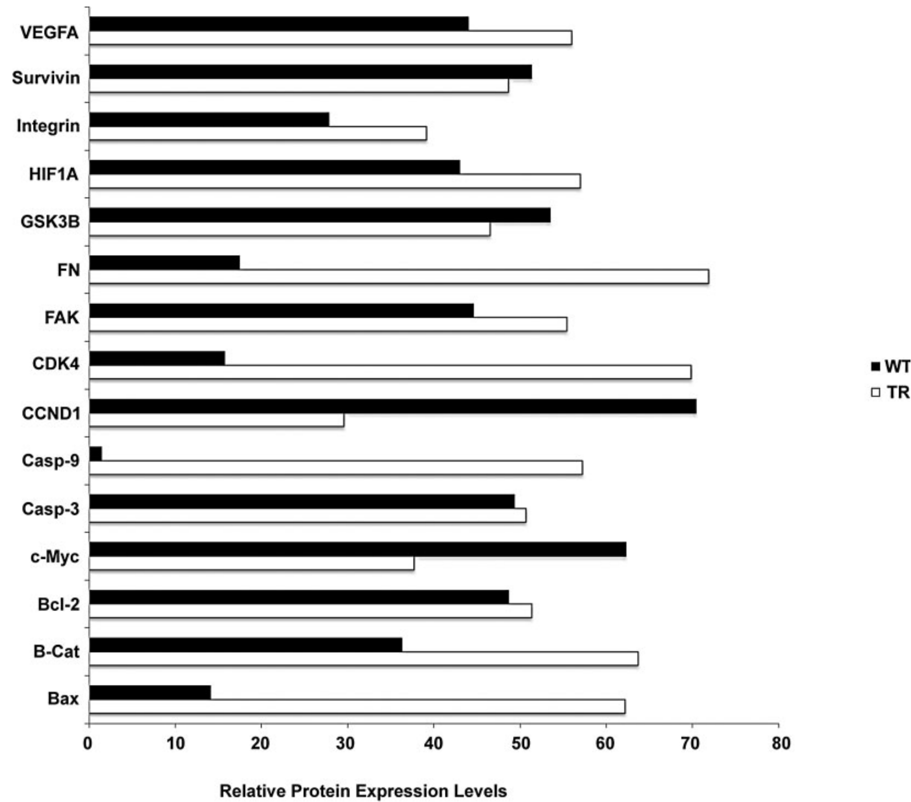

Figure 4. Quantifying the effects of FOXA1 gene knock-out on protein expression levels (Series 1: wild-type LNCaP cell line; Series 2: transfected LNCaP cell line).

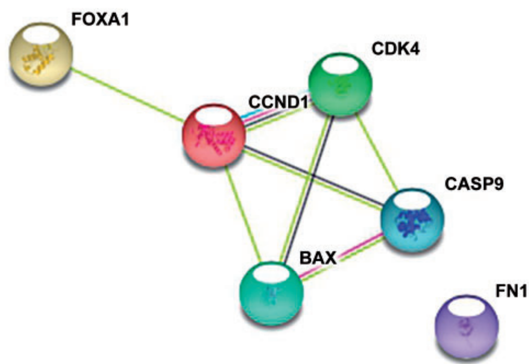

Figure 5. Protein–protein interactions of altered proteins during FOXA1 knock-out (STRING: protein–protein interactions database). (A color version of this figure is available in the online journal.)

CCND1, CDK4, Bax, and Casp-9, whereas FN1 protein was not found to interact with the proteins mentioned above.

Discussion

In this study, we examined the potential of FOXA1 as a drug target for primary prostate cancer using an *in vitro* model. The biological function of FOXA1 gene, therefore, should be examined prior to tailored therapy. In this study, we focused on FOXA1 gene as it was identified as being one of the molecular subtypes in prostate cancer progression. We identified that FOXA1 gene knock-out in androgen-dependent LNCaP prostate cancer cells resulted with lower CCND1 expression profile *in vitro*. We believe that it would be plausible to investigate the effects of cell cycle inhibitors in FOXA1 mutant patients. Our pre-clinical results demonstrate the importance of FOXA1 as a drug target in the treatment of prostate cancer. This study preliminarily indicates that it might be helpful to stratify prostate cancer patients for FOXA1 gene status for more precise treatment approaches.

Due to the known challenge to produce FOXA1 knock-out *in vivo*,[8] we believe such a patient-derived cell line should provide indications on the clinical response. Therefore, we used CRISPR/Cas9 method to knock-out FOXA1 gene. It is a simple and an efficient way of studying gene function even in the absence of a selection method. It is important to note that selection methods, such as puromycin selection or sorting of green fluorescent protein positive clones by flow cytometry, could also be used to improve transfection efficacy after transfection. Studying of gene knock-out using new techniques, therefore, opens opportunities for future conditional knock-out studies.

We demonstrated that knock-out of FOXA1 gene in androgen-dependent LNCaP prostate cancer cells lower CCND1 expression profile *in vitro*. Cancer cell-related proliferation indicator c-Myc protein expression level after gene knock-out is also consisted with the CCND1 protein expression. Increased Bax/Bcl-2 ratio after FOXA1 knock-out also contributes to the apoptotic process.

CCND1 plays an important and positive role during the key rate-limiting point of G1-S transition. Our results thus

suggest further investigation on the effects of cell cycle inhibitors in FOXA1 mutant patients, as FOXA1 has a pivotal role in cell cycle.[6] FOXA1 could be a drug target due to its inhibitory effects in cell cycle progression via down-regulating CCND1 protein expression level. Our pre-clinical results demonstrate the importance of FOXA1 and provide a foundation for future study on its potential as a drug target for prostate cancer treatment.

Although the existing literature contains studies that investigate the effects of FOXA1 gene knock-out, in other words the function of FOXA1 gene, there is no such research that investigates the FOXA1 gene knock-out effect on these diverse ranges of proteins. It was also interesting to find out the highly differential protein expression levels for fibronectin after FOXA1 knock-out. To the best of our knowledge, this is the first study that investigates the relationship between FOXA1 and fibronectin. We believe that this relationship needs deeper evaluation in order to better understand the therapeutical potential.

Conclusions

While FOXA1 gene knock-out significantly altered Casp-9, Bax, CCND1, CDK4, and fibronectin protein expressions ($p < 0.05$), it did not affect the protein expression levels of Casp-3, Bcl-2, survivin, β-catenin, c-Myc, GSK-3B, HIF1A, VEGFA, integrin, and FAK. Our results revealed that FOXA1 gene knock-out decreases CCND1 protein expression level *in vitro*. It is thus worth to investigate the response to cell cycle inhibitors that has FOXA1 mutant gene. *In vivo* confirmation of our results in this study should help in the stratification of prostate cancer patients to enable precision medicine.

Author contributions: All authors participated in the review of the manuscript; GA conducted the experiments; AUD participated in the analysis of the data; GA and EK designed the experiments, GA, EK, and CYB interpreted the data; GA and EK wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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