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ID4 promotes AR expression and blocks tumorigenicity of PC3 prostate cancer cells

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ABSTRACT

Deregulation of tumor suppressor genes is associated with tumorigenesis and the development of cancer. In prostate cancer, ID4 is epigenetically silenced and acts as a tumor suppressor. In normal prostate epithelial cells, ID4 collaborates with androgen receptor (AR) and p53 to exert its tumor suppressor activity. Previous studies have shown that ID4 promotes tumor suppressive function of AR whereas loss of ID4 results in tumor promoter activity of AR. Previous study from our lab showed that ectopic ID4 expression in DU145 attenuates proliferation and promotes AR expression suggesting that ID4 dependent AR activity is tumor suppressive. In this study, we examined the effect of ectopic expression of ID4 on highly malignant prostate cancer cell, PC3. Here we show that stable overexpression of ID4 in PC3 cells leads to increased apoptosis and decreased cell proliferation and migration. In addition, in vivo studies showed a decrease in tumor size and volume of ID4 overexpressing PC3 cells, in nude mice. At the molecular level, these changes were associated with increased androgen receptor (AR), p21, and AR dependent FKBP51 expression. At the mechanistic level, ID4 may regulate the expression or function of AR through specific but yet unknown AR co-regulators that may determine the final outcome of AR function.

1. Introduction

ID4, a dominant negative helix-loop-helix transcriptional regulator is highly expressed in normal epithelial cells of the prostate [1,2]. In PCa, ID4 expression is progressively lost with increasing stage of the disease [1] due to promoter hypermethylation [3]. We have previously reported that knockdown of Inhibitor of differentiation-4 (ID4) in prostate cancer LNCaP cells, promoted tumorigenicity with a gene expression signature that resembles that of constitutively activated AR in castrated mice [4]. Conversely, ectopic ID4 expression induced re-expression of AR that led to decreased proliferation and increased apoptosis in otherwise androgen receptor negative prostate cancer cell line DU145 [5].

Androgen receptor (AR) is a critical survival pathway for prostate cancer cells. AR may act both as a tumor suppressor and a proliferator in the prostate [6]. Over-expression of AR in PC3 cells results in decreased invasion in vivo mouse models whereas mice lacking the prostate epithelial AR (PEARKO) have increased apoptosis in epithelial luminal cells. The PERKO mice developed larger and more invasive metastatic tumors in lymph nodes and died earlier than wild-type littermates [7].

AR activity and function is regulated by many co-factors and chaperones [8]. In this context, ID4 appears to be one of the key regulators of AR function. Results suggest that in the presence of ID4, AR functions as a tumor suppressor whereas loss of ID4 promotes AR to act as a tumor promoter. Identifying new or complex interactions between AR co-/regulators could provide some insight into possible mechanisms by which AR undergoes transition between a tumor suppressor vs. tumor promoter. Here, we report that ID4 acts as a regulator of AR by not only inducing AR expression but promoting its tumor suppressor activity, leading to induction of apoptosis and inhibition of cell migration and growth, in more metastatic PC3 cells.

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2. Materials and methods

2.1. Cell lines

PC3 prostate cancer cell line was purchased from ATCC and cultured as per ATCC recommendations.

2.2. ID4 overexpression in prostate cancer cell line

Overexpression of pCMV + ID4 vector and pCMV vector alone in PC3 and subsequent selection of transfectants was performed in as described previously [5].

2.3. Real-time quantitative RT-PCR (qRT-PCR)

Total RNA (2 μg) from cell lines was reverse transcribed in a final volume of 25 μL as per standard protocols [9]. Reverse-transcribed RNA was used for qRT-PCR with gene-specific primers [9] (Supplemental Table 1).

2.4. Western blot analysis

Western blot analysis with 30 μg of total protein, extracted from cultured prostate cancer cell lines using respective protein specific antibodies (Supplemental Table 2) was performed as described earlier [10].

2.5. Proliferation assay

Cell proliferation was determined using a Cell Titer 96 nonradioactive cell proliferation assay (Promega) according to the manufacturer's protocol.

2.6. Apoptosis assay

Apoptosis was quantified using Propidium Iodide and Alexa Fluor 488 conjugated Annexin V (Molecular Probes) as described previously [11].

2.7. Migration assay

In vitro cell migration assay was performed using 24-well transwell inserts (8 mm, BD Biosciences, Palo Alto, California). Cells were harvested and centrifuged at 1500 rpm for 5 min at room temperature. The pellets were suspended into F12 supplemented with 0.2% BSA. Aliquots of 100 μl cell suspension (3×10^4 cells/inserts) were added to each insert. Chemoattractant solutions were made by diluting EGF (10 ng/ml) into F12 supplemented with 0.2% BSA and the cells were allowed to migrate through a porous membrane coated with rat tail collagen (50 mg/ml) at 37 °C for 5 h. F-12 containing 0.2% BSA served as the control medium. The cleaned inserts were fixed in 4% paraformaldehyde (pH 7.5). Cells on the outside of the transwell insert membranes were stained using DAPI (3 μg/ml). The images were captured in five areas using Axiovert 200 M, Carl Zeiss microscope. Stained nuclei were counted using image analysis software (ZEN 2012; Carl Zeiss). Results were expressed as migration index defined as: the average number of cells per field for test substance/the average number of cells per field for the medium control.

2.8. Immunocytochemistry (ICC)

ICC studies on cells grown in glass chamber slides were performed as described previously [12] using protein specific antibodies (Supplemental Table 2).

2.9. Tunel assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was used to detect fragmented DNA as marker for apoptosis in FFPE tissue sections using TACS 2 TdT-DAB apoptosis detection kit (Trevigen). The slides were counterstained in hematoxylin and mounted with Immuno-mount (Thermo Scientific).

2.10. Luciferase reporter assays

Cells were plated in a 96-well plate at a density of 2.5×10^4 cells/well and experiment was further conducted according to our previous study [10].

2.11. Animal studies

PC3 − CMV and PC3 + ID4 cells (2×10^6) suspended in 100 μL of serum-free F12 medium containing Matrigel (1:1 [v/v]; BD Biosciences) were injected subcutaneously into both flanks of 3-week-old noncastrated (NC) male *nu/nu* athymic nude mice (Taconic) using a 27-gauge syringe, then followed the procedure previously performed from our lab for harvesting the tumors [13]. The *nu/nu* mice were maintained at the Mercer University Vivarium. All studies were approved by the Clark Atlanta and Mercer University committee for the use and care of animals.

2.12. ChIP assay

Chromatin immuno-precipitation in cell lines was performed using the ChIP assay kit (Millipore, Billerica, MD) as per manufacturer's instructions. The chromatin (total DNA) extracted from cells was sheared (Covaris S220), subjected to immuno-precipitation with respective antibodies (Supplemental Table 1), reverse cross linked and subjected to quantitative ChIP- PCR in Bio-Rad CFX.

2.13. Statistical analysis

qRT-PCR data were analyzed using the $\Delta\Delta C_t$ method [1]. The within-group Student *t*-test was used for evaluating the statistical differences between groups.

3. Results

Previous study from our lab showed that in DU145 cells, ID4 functions as a tumor suppressor [5]. To further elucidate the role of ID4 in prostate cancer, we utilized more metastatic and tumorigenic PC3 cells, which do not express AR and have very low levels of ID4, due to promoter methylation [3].

3.1. Generation of ID4 expressing prostate cancer cell line

Stably transfected PC3 with pCMV+ID4 vector expressed nearly 2.5 fold higher ID4 expression (PC3+ID4) cells as compared to the control vector (PC3+CMV) transfected cells. The ID4 expression in control vector transfected cells was negligible and was comparable to that in parental PC3 cells. Expression of ID4 was measured by quantitative PCR (Fig. 1A) and western blotting (Fig. 1B).

3.2. Effect of ID4 on morphology, cell proliferation and migration

A change in morphology in PC3+ID4 cells was observed (Fig. 1C, Left panel). PC3+ID4 cells had an “epithelial like” morphology that was associated with increased cell-cell adhesion as compared to a mesenchymal morphology of the PC3-CMV cells (Fig. 1C, Right