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Role of sirtuin histone deacetylase Sirt1 in prostate cancer: *A target for prostate cancer management via its inhibition?*

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Running Title: *Sirt1 in Prostate Cancer*

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Prostate cancer (PCa) is a major age-related malignancy and according to estimates from the American Cancer Society, a man's chance of developing this cancer significantly increases with increasing age; from 1 in 10,149 by age 39 to 1 in 38 by age 59 to 1 in 7 by age 70. Therefore, it is important to identify the causal connection between mechanisms of aging and PCa. Employing *in vitro* and *in vivo* approaches, in this study, we tested the hypothesis that Sirt1, which belongs to the Silent information regulator 2 (Sir2) family of sirtuin class III histone deacetylases (HDACs), is overexpressed in PCa and its inhibition will have anti-proliferative effects in human PCa cells. Our data demonstrated that Sirt1 was significantly overexpressed in human PCa cells (DU145, LNCaP, 22Rv1, and PC3) compared to normal prostate epithelial cells (PrEC) at protein, mRNA and enzymatic activity levels. Sirt1 was also found to be overexpressed in human PCa tissues compared to adjacent normal prostate tissue. Interestingly, our data demonstrated that Sirt1 inhibition via nicotinamide and sirtinol (at the activity level) as well as via short hairpin RNA (shRNA)-mediated RNA *interference* (at the genetic level) resulted in a significant inhibition in the growth and viability of human PCa cells while having no effect on normal prostate epithelial cells. Further, we found that inhibition of Sirt1 caused an increase in FoxO1 acetylation and transcriptional activation in PCa cells. Our data suggested that Sirt1, via inhibiting FoxO1 activation, could contribute to the development of PCa. We suggest that Sirt1 could serve as a target towards developing novel strategies for PCa management.

Prostate Cancer (PCa) is a major age-related malignancy and is rarely seen in men younger than 40 years; the incidence rises rapidly with each decade thereafter. Because the present life expectancy has significantly improved and Americans are living longer, it is believed that more cases of PCa will be diagnosed in the future. According to a prediction, by year 2010, the number of annual PCa cases will skyrocket to 330,000 (http://www.pcacoalition.org). Thus, it will be immensely useful to better understand the molecular mechanism and connection between aging and PCa.

Unraveling determinants such as genes and gene-products involved in aging and having a connection with PCa could be exploited in designing novel targets and approaches for the management of this age-related neoplasm. We hypothesized that sirtuins (Sirt proteins), which are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases, could serve as a connection between aging and cancer.

Originally discovered in yeast, sirtuins are a unique class of type III histone deacetylases that utilize NAD^+ as a cofactor for their functions (1-4). Seven homologs of yeast Sir2 have been identified in the human genome. Called Sirt1–7, they all contain a highly conserved catalytic domain and despite their enzymatic activity on histone substrates *in vitro*, Sirt proteins predominantly target nonhistone proteins for deacetylation, in both the nucleus and the cytoplasm (1-4). Each sirtuin is characterized by a conserved 275 amino acid catalytic core domain and a unique N-terminal and/or C-terminal sequences of variable length. The catayltic core domain may act preferentially as a mono-ADP-ribosyl transferase or NAD+ dependent deacetylase (3). Their functions and locations differ greatly and Sirt1 is the best characterized member among the mammalian sirtuins (1-4). Sirt1 has been reported to be a nuclear as well as cytoplasmic protein and demonstrated to be involved in a number of cellular processes including gene silencing at telomere and mating loci, DNA repair, recombination and aging (1-6).

Recent studies demonstrated that Sirt1 plays an important role in the regulation of cell death/survival and stress response in mammals. Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage and oxidative stress (1-6). An increasing number of proteins have been identified as substrates of Sirt1, including p53 (7-10), forkhead (FoxO) transcription factors (11-16), repair protein Ku70 (11,17,18), p300 (19), Rb (19,20) and p73 (19,21) just to name a few. Interestingly, Sirt1 has been shown to negatively regulate proliferative signaling via regulating i) p53 function (7-10,19,22) ii) FoxO pathway (1,11-13,23) and iii) MAPK signaling (24). Improper regulation of sirtuin proteins has been reported in a number of diseases including Bowen's disease (25), type I diabetic nephropathy (26), Alzheimer's disease and amyotrophic lateral sclerosis (27) and nonalchoholic fatty liver disease (28).

There are four reported human forkhead family members: FoxO1 (FKHR) (29), FoxO3a (FKHRL1) (30), FoxO4 (ARX) (31) and FoxO6 (32), which have been shown to regulate a variety of cellular processes including cell differentiation, transformation, and metabolism (11-13,15,33). The activities and localization of the FoxOs have been shown to be dependent on their phosphorylation and acetylation status. Phosphorylation of FoxO factors, with subsequent ubiquitination, has been shown to modulate a variety of downstream target genes (34). FoxO acetylation is believed to have multiple and opposing effects depending on the specific FoxO factor and model system (11,34). Regardless, the various FoxO factors have shown to be deacetylated by Sirt1 (11,13,15,35,36); however, the connection between Sirt1 and the FoxO factors in PCa is not well understood.

In this study, we have demonstrated that Sirt1 is significantly overexpressed in human PCa cell lines compared to normal human prostate epithelial cells. Further, we found that Sirt1 is significantly overexpressed in prostate cancer tissue samples versus adjacent normal prostate epithelium in patients with PCa. Furthermore, our data demonstrated that Sirt1 inhibition by nicotinamide, sirtinol or shRNA-mediated RNA interference causes an inhibition in growth and cell viability of human PCa cells while having no effect on normal prostate epithelial cells. We have also found that inhibition caused an increase in acetylated-FoxO1 protein with a concomitant increase in FoxO1 transcriptional activity. Our data suggests

that Sirt1 may be promoting PCa cell growth via inhibiting FoxO1.

EXPERIMENTAL PROCEDURES

Cell Culture. The human prostate carcinoma cell lines *viz*. LNCaP, 22Rν1, DU145 and PC3 (obtained from American Type Culture Collection, ATCC, VA) were maintained in RPMI-1640, MEM, and F12K media (ATCC, VA) supplemented with FBS and antibiotics (penicillin/streptomycin). Normal human prostate epithelial cells PrEC (Cambrex, NJ) and NPEC (Celprogen, CA) were maintained at standard cell culture conditions in PrEBM media or Human Prostate Culture Complete growth media respectively, with growth factors and supplements as recommended by the vendors (Cambrex, NJ and Celprogen, CA). Normal human keratinocytes, NHEK, (Invitrogen Corporation, CA) were maintained in Keratinocyte-SFM media (Invitrogen Corporation, CA). N/Tert-1 keratinocytes were obtained from the Brigham and Women's Hospital Cell Culture Core Facility (Boston, MA) and maintained in Keratinocyte-SFM media. Human mammary eptithelial cells, HMEC, (Cambrex, NJ) were maintained in Mammary Epithelial Cell Growth Medium with supplements (Cambrex, NJ). Normal human bronchial epithelial cells, NHBE**,** (Cambrex, NJ) were maintained in Bronchial Epithelial Cell Basal Media supplemented with BEGM Single Quots (Cambrex, NJ). All cells were maintained at standard cell culture conditions (37ºC, 5% CO_2 in a humidified incubator) as recommended by the vendors.

Primary Cell Culture. Prostate tissue was obtained under an approved Institutional Review Board protocol from men (ages 44–66) undergoing cystoprostatectomy for bladder cancer at the University of Wisconsin Hospital and Clinics. Histology confirmed the absence of cancer in the tissue. Prostate epithelial cultures were established as described by others (37,38). Briefly, prostate tissues were minced with a scalpel and digested in a solution containing collagenase (500 units/ml; Sigma, MO) and plated on collagen-coated plates. Cells were maintained in Ham's F-12 media (Invitrogen Corporation, CA) supplemented with 0.25 units/ml regular insulin, 1 μg/ml hydrocortisone, 5μg/ml human transferrin, 2.7 mg/ml dextrose, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml cholera toxin, 25 μg/ml bovine pituitary extract, and 1% FBS. Cells were passaged using trypsin-EDTA.

Preparation of Whole Cell Protein Lysates and Western Blot Analysis. PCa cells were washed with ice-cold PBS, trypsinized and collected by centrifugation. Cell lysates were prepared using 1X RIPA buffer, with freshly added PMSF and protease inhibitor cocktail (Cell Signaling, CA) and protein concentration was determined with BCA Protein Assay (Pierce, IL). Primary culture protein (designated P326 and P218) was extracted by freeze thawing three times in ECB buffer and protein concentration was determined with BCA Protein Assay. For immunoblot analysis, 30-40μg protein was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoblot analysis was performed using a variety of primary antibodies: anti-Sirt1, anti-TATA binding protein TBP (Abcam, MA), anti-FoxO1, anti-FoxO3a, anti-FoxO4 (Cell Signaling, MA), anti-actin and anti-Ac-FKHR (Santa Cruz, CA) and variety of secondary antibodies: goat anti-rabbit and goat anti-mouse HRP-conjugated antibodies (Upstate, MA), donkey anti-goat HRP-conjugated antibody (Santa Cruz, CA) followed by chemiluminescent detection. The quantification of protein was performed

by a digital analyses of protein bands (TIFF images) using UN-SCAN-IT software.

Preparation of Nuclear and Cytosolic Protein Lysates. Following treatments, the media was aspirated and the cells were washed twice with ice-cold 1X PBS. Cytoplasmic Lysis Buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L DTT, 1 mmol/L PMSF, 10µg/mL protease inhibitor cocktail] was added and cells were scraped off. The lysate was then incubated on ice for 15 minutes. 10% NP-40 was added to the suspension which was then centrifuged at 14,000 x *g*, 4°C for 2 minutes. Supernatant was collected for cytosolic protein lysate. The remaining cell pellet was resuspended in Nuclear Extraction Buffer [20mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1mmol/L DTT, 2 mmol/L PMSF and 10 µg/ml protease inhibitor cocktail]. Suspension was incubated on ice for 30 minutes and then centrifuged at 14,000 x *g* for 10 minutes at 4°C. Supernatant was collected for nuclear protein lystate. Nuclear and cytosolic protein concentrations were determined with BCA Protein Assay (Pierce, IL). Both nuclear and cytosolic lysates were used for subsequent Western blotting experiments (described above).

Immunoflourescene. For detection of Sirt1 by immunofluorescence, the cells were plated and grown on BD Falcon CultureSlides (BD Biosciences, CA) until a confluency of 80% was reached. The cells were fixed and then blocked for 1 hour at room temperature in 10% normal goat serum (Caltag Laboratories, CA) in PBS. Following blocking, rabbit anti-Sirt1 antibody (Santa Cruz, CA) was added and allowed to incubate for 2 hours at room temperature. Primary antibody was removed and Alexa Fluor 555 goat anti-rabbit secondary antibody (Molecular Probes, OR) was then added and incubated for 1 hour at room temperature in the dark. PBS diluted 4', 6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (Pierce, IL) counterstain was used for nuclear staining. Cells were mounted with ProLong anti-fade kit as per vendor's protocol (Molecular Probes, OR) and examined under Bio-Rad Radiance 2100 MP Rainbow Confocal/Multiphoton System.

Immunoprecipitation and Sirt1 Enzyme Activity Assay. For immunoprecipitation of Sirt1 protein, the lysates containing 500µg total protein were incubated with rabbit anti-Sirt1 antibody (Abcam, MA) overnight at 4°C with constant rotation. The specific antibody-antigen complex was collected by precipitation with Protein A-agarose beads (Pierce, IL) for 2.5 hours at 4°C with constant rotation. Sirt1 activity was determined in immunoprecipitates from cells using Sirt1 Fluorimetric Drug Discovery Kit (AK-555; Biomol, PA) as per vendor's protocol.

Quantitative Real Time Reverse Transcriptase-PCR. RNA was isolated using Trizol reagent (Invitrogen Corporation, CA) according to the vendor's protocol. RNA was treated with DNAse (Invitrogen Corporation, CA) and first strand cDNA was transcribed with 300 ng random primers, 10mM dNTPs and 200 units of M-MLV reverse transcriptase (Invitrogen Corporation, CA). Quantitative RT-PCR was performed in triplicate with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Corporation, CA) with 50ng first strand cDNA, 0.2µM each forward and reverse primers for Sirt1 (forward-5'TGCTGGCCTAATAGAGTGGCA3', reverse-5'CTCAGCGCCATGGAAAATGT3' with the product size of 102bp or GAPDH forward-

5’GAAGGTGAAGGTCGGAGTC3’, reverse-5'GAAGATGGTGATGGGATTTC 3' with the product size of 236bp). The samples were cycled once for 50ºC for 2 minutes for UDG incubation followed by 94ºC for 2 minutes then 40 cycles of 94ºC; 15 seconds, 55ºC; 30 seconds each. Relative Sirt1 mRNA was calculated using the ΔΔCT comparative method using GAPDH as an endogenous control. Purity of product was checked by dissociation curve analysis as well as running the samples on 3% agarose gel.

Human Tissues and Immunohistochemistry. Paraffin embedded tissue slides containing human prostate cancer tissue with adjacent normal prostate tissue and a custom tissue micro-array containing cancer and normal prostatic tissue from 41 patients (3-9 samples per patient depending on heterogenicity) with varying grades of PCa were obtained from the Department of Pathology and Laboratory Medicine, UW-Madison. The slides were deparaffinized and blocked for endogenous peroxidases with a 3% H_2O_2 in ddH_2O incubation. 1mM EDTA, pH 8.0 was heated to a boil and tissue slides were then boiled for 3 minutes for antigen retrieval. Slides were then blocked in 1.5% normal goat serum (Caltag Laboratories, CA) in PBS for 1 hour at room temperature in a humidified chamber followed by incubation with a rabbit anti-Sirt1 antibody (Santa Cruz, CA) overnight at 4°C in a humidified chamber. The slides were then incubated with goat anti-rabbit secondary antibody (Upstate, MA) for 1 hour at room temperature in a humidified chamber followed by cross reaction with freshly prepared liquid (3,3’-diaminobenzidine) DAB Substrate Chromogen System, 20µL DAB Chromogen per 1mL of Substrate Buffer (DakoCytomation, Denmark) . Hematoxylin (Vector Labs, CA) was diluted 1:5 in ddH_20 and used as a nuclear counterstain. Finally, slides were dehydrated and mounted with cover slips followed by microscopic analysis with digital image capture. Sirt1 staining was semiquantitatively graded as negative (-), weak (+), moderate (++) or strong (+++) staining in 50% of cells examined. Statistical analysis of the data was performed using Fisher’s exact test.

Treatment of Cells with Nicotinamide. Cells were grown to 60% confluency and then treated with 150µM, 300µM, 5mM or 20mM nicotinamide (Acros Organics, NJ) dissolved in the growth media. Cells were incubated with the nicotinamide treatment for 24 hours after which they were used for subsequent experiments.

Treatment of Cells with Sirtinol. Cells were grown to 60% confluency and then treated with 30µM or 120µM sirtinol (Sigma-Aldrich, MO; dissolved in DMSO). Cells were incubated with sirtinol for 24 or 48 hours after which they were used for subsequent experiments.

Transfection with Sirt1 shorthairpin RNA (shRNA). shSirt1 clone V2HS_20109 (sequenced with U6 5' TGT GGA AAG GAC GAA ACA CC sequencing primers) cloned into a pSHAG-MAGIC2 vector was purchased from Open Biosystems (Huntsville, AL). The transfections were done according to the protocol supplied with RNA*intro* shRNA Transfection Kit (Open Biosystems, Huntsville, AL). Plasmid DNA from cultures was prepared according to protocol given in QIAGEN Plasmid Maxi Kit (Qiagen, Valencia, CA). Appropriate restriction digests were performed to confirm correct shRNA plasmid DNA. Plasmid DNA was then diluted in serum free media and mixed with Arrest-In diluted in serum free media and incubated for 10 minutes at room temperature. The DNA/Arrest-In complex mixture was added

to 60-80% confluent cells and incubated for 6 hours at 37˚C, 5% CO_2 in a humidified incubator. After 6 hours, transfection media was removed and media with serum was added and incubated in a humidified chamber at 37°C, 5% CO_2 for 48 hours. The cells were then harvested and further studies were performed.

Trypan Blue Exclusion Assay. Following treatments, cells were trypsinized and collected in a 1.5mL Eppendorf tube. The cells were pelleted by centrifugation and re-suspended in PBS (120 µL). Trypan blue (0.4% in PBS; 10 µL) was added to a smaller aliquot (10 µL) of cell suspension, and the number of cells (viable-unstained and non-viable-blue) were counted.

Luciferase Reporter Assay. PCa cells were transfected with phRL-TK (Promega, WI) and 3xIRSLuc-FoxO1 (15) or FGHRE-Luc (33) (addgene plasmid 1789) (Addgene, MA) by Lipofectamine 2000 (Invitrogen, CA) as per the vendor's protocol. Transfected cells were then collected and re-plated into 12-well plates 48 hours post transfection so that they were 75% confluent the following day. 24 hours post replating, cells were treated in triplicate with media, DMSO (vehicle), or sirtinol (30µM or 120µM for 24 hours). Then, cell lysates were prepared and luciferase activity was determined using the dual luciferase assay system (Promega, WI). Luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis. Statistical analyses were performed with the Student's t test for independent samples and the data are expressed as means ± SD unless specified otherwise. Statistically significant p-values are provided for each individual experiment.

RESULTS AND DISCUSSION

Aging, an inevitable process in living organisms, has been linked to several unwanted disease conditions including several types of cancers. Studies suggest that certain genetic and epigenetic alterations are accumulated during aging and appear to possess a direct role in cell transformation. These events show a clear evolution during aging and are reversed in cancer. An interesting example of this is that telomere length (controlled by genetic and epigenetic modifications) decreases with age but rapidly increases after transformation (39). Similarly, the levels of sirtuins have been shown to be decreased during aging and elevated in cancer cells (39). It is being increasingly appreciated that sirtuins could be one of the lost links between aging and cancer (39). Sirt1 is the most well studied member of sirtuin family and known to modulate several cell signaling pathways which promote cell survival (3). Since prostate cancer is a major age-related malignancy, the involvement of Sirt1 in the development of PCa is an intriguing possibility. In this study, we tested the hypothesis that Sirt1 is overexpressed in PCa and its inhibition will have anti-proliferative effects in human PCa cells.

To test this hypothesis, we first determined the constitutive levels of Sirt1 in human prostate carcinoma cells versus normal prostate epithelial cells. We utilized a panel of PCa cells differing in androgen receptor (AR) and p53 status: LNCaP and 22Rv1 cells possess mutant but functional AR and wild type p53, DU145 cells are AR negative and mutant for p53 and PC3 cells are AR and p53 null. As shown by immunoblot analysis, we found that Sirt1 is overexpressed in the human PCa cell lines tested compared to normal prostate epithelial PrEC cells and normal prostate cells obtained from patients (P326 and P218) (Fig. 1A). A quantification of protein

bands showed a 8 to 13 fold overexpression of endogenous Sirt1 protein in PCa cells compared to normal prostate cells (Fig. 1B). Further, employed several different normal cell lines to compare the levels of Sirt1 protein. As compared to DU145 PCa cells, the normal cells (NHEK, N/Tert-1 keratinocytes, NHBE, and HMEC) were found to have markedly low levels of Sirt1 (Sup. Fig.1). We also assessed the levels of Sirt1 mRNA in PCa cells compared to the normal PrEC cells. As shown by the Quantitative Real Time PCR analysis, a significant overexpression of Sirt1 mRNA was observed in PCa cell lines, when compared to normal PrEC cells (Fig. 1C). The mRNA quantitation and mRNA dissociation curves for Sirt1 and GAPDH are provided as supplementary figures (Sup. Fig. 2A and B). Further, employing a Sirt1 activity assay kit that uses a fluorogenic peptide (encompassing residues 379 to 382 of p53, acetylated on lysine 382), we determined the deacetylation activity of Sirt1. As shown in Fig. 2, compared to normal PrEC cells, Sirt1 activity was found to be significantly elevated in human PCa cell lines tested. Purified Sirt1 enzyme was used as a positive control and nicotinamide was used as a negative control. Thus, our data suggested Sirt1 is overexpressed at the protein, mRNA and activity levels in PCa cells compared to normal PrEC cells.

Next, employing immunohistochemical analysis, we determined the levels of Sirt1 protein in the prostate tissue of patients with PCa. For this purpose, we utilized a custom tissue micro-array (made at the Department of Pathology and Laboratory Medicine) of human prostate cancer and normal or benign prostate epithelium tissues from 41 patients (3-9 samples per patient depending on heterogenicity) as well as paraffin embedded tissue slides containing human prostate cancer tissue with adjacent normal or benign prostate tissues. Interestingly, Sirt1 was found to be significantly overexpressed in human PCa tissues compared to adjacent normal or benign prostate tissues (Fig. 3). The staining was graded as negative (-), weak (+), moderate (++) or strong (+++) on the custom tissue micro-array. We found a significant overexpression of Sirt1 in PCa, especially in the tumor specimen of Gleason pattern 3 and 4, compared to normal or benign prostate epithelium tissues (Table 1). Only 2.6% of normal or benign prostate epithelium samples showed strong staining compared to 27.1% of Gleason pattern 3 tumors and 60% of Gleason pattern 4 tumors. Further, the immunohistochemical analyses also showed that Sirt1 was localized primarily to the nucleus, but did show some residual cytoplasmic localization (Fig. 3). These findings were confirmed by immunofluorescence analysis of endogenous Sirt1 levels where Sirt1 was found to be abundantly expressed in PCa cells and showed both nuclear and cytoplasmic localization (Sup. Fig. 3). This finding was further supported by the observed cytoplasmic and nuclear localization of Sirt1 by the western blot analyses (Fig. 7). Our findings are consistent with several published studies which have shown Sirt1 to be localized to both the cytoplasm and nucleus (40-42).

As our next aim, in order to ascertain the biological significance of Sirt1 in human PCa, we determined the consequence of Sirt1 inhibition on PCa cells. We treated cells with nicotinamide, which specifically inhibits sirtuins, with an IC_{50} of < 50μM (43). The proposed mechanism of nicotinamide's inhibition involves blocking Sirt1's deacteylation activity by binding to the conserved pocket adjacent to NAD^+ binding thus blocking Sirt1's hydrolysis (43). Nicotinamide (150μM; for 24 hours) treatment resulted in a significant decrease in Sirt1 enzyme activity in all PCa cell lines tested, albeit at different rates of inhibition,

probably dependent on endogenous levels of Sirt1 in the cells. However, Sirt1 activity was reduced to similar activity level in the different cell types, suggesting that probably a maximum Sirt1 inhibition was achieved (Fig. 4A). Although Sirt1 levels were very minimal in normal prostate epithelial cells compared to PCa cells, we determined the effect of nicotinamide treatment on cell growth and viability in these cells. Interestingly, as assessed by Trypan Blue Exclusion assay, treatment with 150μM and 300μM nicotinamide had no effect on the growth or viability of normal prostate cells (Fig. 4B). This is most likely due to the very low endogenous levels present in normal prostate epithelial cells. We next assessed the effect of the Sirt1 inhibition on the growth and viability of human PCa cells again using a Trypan Blue Exclusion assay to measure both cell growth and viability. Interestingly, 24 hour nicotinamide treatment was found to result in a significant dose dependent inhibition of growth and viability of PCa cells (DU145, LNCaP, 22Rv1, and PC3) with 150μM, 300μM, 5mM and 20mM nicotinamide treatment (Fig. 4C and D). Although cell growth and viability were found to decrease in all cell types with Sirt1 inhibition, the degree of the inhibition varied in different cell lines most likely because of the different genetic makeups of these cells. For example, these differences in cell growth and viability inhibition may be attributed to different endogenous Sirt1 levels, different androgen receptor and/or p53 status, or different overall growth patterns and rates. Overall, all PCa cell types in which Sirt1 was inhibited via nicotinamide resulted in a decrease in cell growth and viability.

Recently, another chemical Sirt1 inhibitor, sirtinol, has gained popularity for its greater specificity (24,24,44). First described in 2001, sirtinol (Sir two inhibitor napthol) was found to inhibit Sir2p transcriptional activity directly without affecting the other classes of HDACs (44). As shown in figure 5, We found that sirtinol (30μM and 120μM) treatment for 24 hours or 48 hours resulted in a significant decrease in the growth and viability of all the PCa cell lines tested; this response was much more pronounced at 48 hours post treatment. This data is in agreement with a recently published study (45) as well as with our data with nicotinamide (Fig. 4).

To concretely confirm our data and to firmly establish that the observed effect were due to solely Sirt1 inhibition, we employed an additional approach of RNA *interference* mediated knockdown of the Sirt1 gene. For this purpose, we employed three different shRNA constructs all of which were effective in knocking down Sirt1 protein, albeit at different levels, compared to the nonsense shRNA control (Fig. 6A). Of these three, we selected the sh-Sirt1-271 construct which was effective in inhibiting Sirt1 in all PCa cell lines *viz*. DU145, 22Rv1 and PC3 (Fig. 6A). The achieved knockdowns with sh-Sirt1-271 were found to be moderate and significant, but not complete as shown by the quantitation data (Fig. 6B). Further, we confirmed shRNA-mediated knockdown of Sirt1 at the enzyme activity level. As shown in Fig. 6C, we found sh-Sirt1-271 significantly decreased Sirt1 activity compared to control sh-non-sense in all PCa cells tested. sh-Sirt1-271 was specific for Sirt1 and did not affect other related proteins such as Sirt2 (data not shown). In our next experiment, we evaluated the effect of Sirt1 knockdown, using sh-Sirt1-271 construct on the growth and viability of human PCa cells. We found that sh-Sirt1-271-mediated knockdown of Sirt1 resulted in a marked decrease in the growth and viability of the PCa cells tested compared to the nonsense shRNA control (Fig. 6D and E). Although different levels of Sirt1 protein were present

to begin with as well as different levels of knockdown were achieved among the different cell types, the inhibition on cell growth and viability was very similar. This may suggest that more than one mechanism is responsible for the biological effects of Sirt1 in PCa cells or that the maximal inhibition of Sirt1 was achieved to produce a maximal inhibition on cell growth and viability in the PCa cells tested. These results coupled with the observed inhibition of Sirt1 by nicotinamide and sirtinol suggested that Sirt1 plays a functional role in PCa.

The exact mechanism by which Sirt1 controls the growth of PCa is not known at this time although several intriguing possibilities exist in this direction. First, it has been shown that when overexpressed, Sir2 extends the lifespan of both budding yeast and *C. elegans* via modulating daf-16, which is the only *C. elegans* homologue of the FoxO family of forkhead transcription factors (35). Several studies have shown that Sirt1 can regulate mammalian FoxO transcription factors through direct binding and/or deacetylation (1,11,13,15,35,36). Sirt1 deacetylation can either lead to activation or repression of FoxO-dependent transcription depending on the situation (1,12,13,23). Thus, it is believed that alterations of the acetylation status of FoxO factors by Sirt1 may lead to alterations in a set of stress-resistant factors, tipping the balance toward stress resistance and away from apoptosis (1,11,16,23,46).

To determine if Sirt1 modulates FoxO1 specifically in PCa, we assessed the effect of sirtinol-mediated inhibition of Sirt1 on FoxO1. Sirtinol (30μM and 120μM for 24 hours) was found to inhibit Sirt1 protein in the nucleus and cytoplasm (Fig. 7A), albeit at different levels in different cell types. Further, sirtinol treatment to all PCa cells tested (22Rv1, DU145 and PC3) caused an increase in acetylation of FoxO1 both the nuclear and cytosolic cellular fractions (Fig. 7A). Interestingly, the increase in acetylated FoxO1 was not accompanied by an increase in total FoxO1 protein, suggesting the increase in acetylation was not due to an increase in total FoxO1 protein (Fig. 7A). As a fractionation control, the nuclear and cytosolic lysates from each cell types were resolved on a 12% SDS-PAGE gel and subjected it western blot analysis with β-actin and TATA binding protein antibodies. TATA binding protein was found to be present only in the nuclear fraction, while β-actin was present in the cytosolic fraction (Sup. Fig.4). Previous reports have shown that the acetylated status of FoxO1 is indicative of its transcriptional activity in that acetylation causes an increase in FoxO1 transcriptional activity (15). Therefore, we measured the effect of sirtinol on the transcriptional activation of FoxO1 in PCa cells. As shown by the luciferase activity, we found that inhibition of Sirt1 resulted in an increase in FoxO1 transcription of all PCa cells tested (Fig. 7B). Thus our data suggested that Sirt1 may in part be promoting PCa growth via inhibiting FoxO1 acetylation and transcription.

Our data is in agreement with previous studies which observed Sirt1 binding to and deacetylating FoxO1 thereby inhibiting its transcriptional activity (15). Frescas *et al.* have shown that FoxO1 is mobile between the nucleus and cytoplasm when sirtuins are inhibited by nicotinamide and that deacetylation targets FoxO1 for nuclear retention, promoting FoxO1-dependent transcription of genes (12). Further, activation of forkhead transcription factors have been shown to induce apoptosis through the regulation of a number of target proteins such as Fas ligand, TRAIL, Bim, and $p27^{KIP1}$ (33,47-49). Birkenkamp *et al.* have suggested that FoxO transcription factors regulate transcription through both DNA-binding-dependent and -independent

mechanisms, thus questioning whether nuclear retention is essential for FoxO transcriptional activity (50). Importantly, another study has shown FKHR levels to be higher in normal prostate tissue than in PCa, suggesting that FKHR may be an important regulatory protein for homeostasis in normal tissue, whereas the apoptosis-inducing role of FKHR was inhibited in PCa (51). These studies support our findings on Sirt1 inhibition-mediated activation of FoxO1 transcription and subsequent decrease in PCa cell growth and viability.

However, we cannot rule out other mechanistic possibilities regarding the events downstream to Sirt1 in our system. One potential mechanism could involve the tumor suppressor gene, p53. Acetylation of p53 leads to enhanced transcriptional activation whereas by deacetylating p53, Sirt1 may turn off p53 to promote cellular proliferation (7-10,22). Some studies also suggest that although Sirt1 can deacetylate p53, this process may not have an effect on p53-mediated biological outcomes (41,52,53). Interestingly, an intricate interplay between FoxOs, Sirt1, and p53 is also believed to exist, with each being able to regulate the other two in at least some cellular contexts (1,23).

It is important to mention here that a few recent studies appear to contradict our findings and suggest that Sirt1 may act as a tumor suppressor in some cancer models. A study by Solomon *et al*. has shown that Sirt1 interacts and deacetylates Lys630 in the androgen receptor lysine motif, which represses its oncogenic signaling and inhibits PCa cells from growing in response to the dihydrotestosterone (53). Also, Dai and colleague have found that Sirt1 acts as a co-repressor of the androgen receptor and its down regulation increased the sensitivity of androgen-responsive genes to androgen stimulation, enhanced the sensitivity of PCa cells proliferative responses to androgens, and decreased their sensitivity to androgen antagonists (54). Firestin *et al*. reported that Sirt1 suppresses intestinal tumor formation *in vivo* via modulating β-catenin (55). However, contrary to these observations, a number of studies support our observations of Sirt1's tumor promoter role. Very recently, Kojima *et al.* demonstrated that sirtinol as well as Sirt1 siRNA inhibited cell growth and increased sensitivity to camptothecin and cisplatin in DU145 or PC3 cells (45). It has also been reported that Sirt1 in overexpressed in prostate tissues of transgenic adenocarcinoma of mouse prostate (TRAMP mice) which possesses many features of the human PCa (56). Further, in another study the four and a half LIM domain protein, FHL2, was shown to enhance the interaction and deacetylation of FoxO1 by Sirt1 in PCa cells to promote tumorigenesis in response to increased stress during aging (15). These studies clearly support our findings and hypothesis that Sirt1 acts as an oncogene in PCa. A definite reason for the observed differences between the two lines of thoughts and findings is not very clear at present. However, the observed differences could be i) due to the complexity of the androgen receptor, ii) different model systems and/or iii) other unknown mechanisms by which Sirt1 imparts its biological functions.

At present, only limited reports are available regarding the role of sirtuin proteins in human cancers and Sirt1-inhibition is being increasingly appreciated as a viable option for anti-cancer strategies (15,24,57,58). Wang and colleagues have demonstrated an involvement of Sirt1 in human melanoma A375-S2 cell death by an agent evodimine, which was isolated from *Evodia rutaecarpa* (59). Ota and colleagues have shown that the Sirt1 inhibitor sirtinol, induced a senescence-like growth arrest in human breast cancer MCF-7 cells and lung cancer H1299 cells (24), suggesting that

Sirt1 inhibitors may have anticancer potential (24). Ford and colleagues have demonstrated that Sirt1 silencing induced growth arrest and/or apoptosis in certain human epithelial cancer cells (57). Further, a study by Chu and colleagues has implicated Sirt1 in the control of multidrug resistance gene mdr1 and cancer resistance to chemotherapy (60). A very recent study by Liang *et al.* has shown that cisplatin-resistant (CP-r) cancer cells overexpressed Sirt1 and Sirt1 knockdown conferred sensitivity to cisplatin in these cells (61). Furthermore, Heltweg et al. identified cambinol as an inhibitor of Sirt1 and Sirt2 and demonstrated that it induced apoptosis of BCL6-expressing Burkitt lymphoma cells and inhibited growth of Burkitt lymphoma xenografts in mice (62).

In summary, our data, along with other published studies, suggested that Sirt1 functions as an oncogene in PCa via inhibiting FoxO1 acetylation and transcription, and could possibly be used as a potential target and a biomarker for the management of this age-related malignancy. However, future studies in appropriate *in vitro* and *in vivo* systems are needed to delineate detailed mechanism(s) by which Sirt1 imparts a growth advantage to PCa cells.

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FIGURE LEGENDS

Figure 1. Sirt1 protein and mRNA levels in PCa cell lines *versus* normal prostate epithelial cells. ***A) Western blot analysis of Sirt1:*** The cells were grown to 80% confluency and cell lysates were prepared. Sirt1 protein levels were determined by Western blot analysis. Equal loading was confirmed by reprobing the blot for β-actin. ***B) Quantitation of Sirt1 protein levels:*** Western blot analysis was quantitated by densitometric analysis of protein bands. The data (relative density normalized to β-actin) is expressed as mean ± SE of three experiments (*p<0.01); ***C) Sirt1 mRNA:*** The relative expression of Sirt1 transcript in PCa cell lines was determined by Quantitative Real Time Reverse Transcriptase-PCR using ABI-PRISM SDS software and comparative Ct methods analysis. The data is expressed as mean ± SE of three experiments (*p<0.01). Details of the experiments are given in "Materials and Methods".

Figure 2. Sirt1 activity in PCa cell lines *versus* normal prostate epithelial cells. Sirt1 protein was immunoprecipitated from protein lysate (500 μg protein) and Sirt1 enzyme activity was assessed using Sirt1 Activity assay kit (Biomol, PA) as per vendor's protocol. Pure Sirt1 enzyme (Sirt1) and nicotinamide (Nic) were used as a positive and negative control, respectively. Sirt1 activity is represented as arbitrary fluorescence units (AFU). The data is expressed as mean ± SE of three experiments (*p <0.01).

Figure 3. Immunohistochemical analysis of Sirt1 in prostate tissues from patients with PCa. Immunohistochemical analysis for Sirt1 was performed on a custom made tissue microarray containing normal or benign prostate epithelium, high-grade prostatic intraepithelial neoplasia (PIN) and prostate cancer samples with different Gleason patterns as well as on paraffin embedded tissue slides containing human prostate cancer tissue with adjacent normal prostate tissues. Details of the experiment are given in "Materials and Methods."

Figure 4. Effect of nicotinamide treatment on growth and viability of human PCa cells and normal prostate epithelial cells. ***A) Effect of nicotinamide on Sirt1 activity:*** PCa cells (60% confluent) were treated with 150μM nicotinamide or untreated control for 24 hours. Sirt1 protein was immunoprecipitated using a Sirt1 antibody. Sirt1 enzyme activity was assessed using Sirt1 activity assay kit and is represented as arbitrary fluorescence units (AFU) as previously stated. The data is expressed as mean ± SE of three experiments (*p<0.01); ***B) Effect of nicotinamide on cell growth and cell viability in normal prostate epithelial cells:*** The normal human prostate epithelial cells were treated with nicotinamide (150μM or 300μM nicotinamide) for 24 hours and analyzed by Trypan Blue assay to assess cell growth and viability. Cell growth is expressed as percent growth (from total number of cells) and cell viability is expressed as the percent viable cells out of the total number of cells. The data is expressed as the mean ± SE of three experiments; ***C) Effect of nicotinamide on cell growth in PCa cells:*** PCa cells were treated with nicotinamide (150μM, 300μM, 5mM or 20mM nicotinamide) for 24 hours and analyzed by Trypan Blue assay to assess cell growth and is expressed as percent cell growth (from total number of cells). The data is expressed as the mean ± SE of three experiments (*p<0.01); ***D) Effect of nicotinamide on cell viability in PCa cells:*** PCa cells were treated and analyzed using Trypan Blue assay as described above. Cell viability is expressed as the percent viable cells out of the total number of cells. The data represents mean ± SE of three experiments (*p<0.01). Details of the experiments are given in "Materials and Methods."

Figure 5. Effect of sirtinol treatment on growth and viability of human PCa cells. ***A) Effect of sirtinol on cell growth in PCa cells:*** PCa cells were treated with 30μM or 120μM sirtinol (dissolved in DMSO) for 24 or 48 hours and analyzed by Trypan Blue assay to assess cell growth and is expressed as percent cell growth (from total number of control cells). The data is expressed as the mean ± SE of three experiments (*p<0.01); ***B) Effect of sirtinol on cell viability in PCa cells:*** PCa cells were treated with 30μM or 120μM sirtinol for 24 or 48 hours and analyzed using Trypan Blue assay as described above. Cell viability is expressed as the percent viable cells out of the total number of control (DMSO treated) cells. The data represents mean ± SE of three experiments (*p<0.01).

Figure 6. Effect of shRNA-mediated knockdown of Sirt1 on growth and viability of human PCa cells. ***A) Effect of shRNAs on Sirt1 protein levels:*** Following transfection of PCa cells with Sirt1 shRNA or control nonsense shRNA (for 48 hours), Sirt1 protein levels were detected by Western blot analysis. Equal loading was confirmed by reprobing the blot for β-actin. Data represents three experiments with similar results; ***B) Quantitation of Sirt1 protein levels:*** Western blot data were quantitated by a densitometric analysis of protein bands. The data (relative density normalized to β-actin) are expressed as mean ± SE of three experiments (*p<0.01); ***C) Effect of Sirt1 knockdown on Sirt1 activity:*** PCa cells were transfected with Sirt1 shRNA or control shRNA (for 48 hours). Sirt1 protein was immunoprecipitated using a Sirt1 antibody. Sirt1 enzyme activity was assessed using Sirt1 activity assay kit (AK-555; Biomol, PA). The data (relative fold change in AFU normalized to nonsense control) are expressed as mean ± SE of three experiments (*p<0.01); ***D) Effect of Sirt1 knockdown on cell growth:*** The effect of Sirt1 knockdown on cell growth was analyzed by Trypan Blue assay and is expressed as percent growth (from total number of cells). The data are expressed as the mean ± SE of three experiments (*p<0.01); ***E) Effect of***

Sirt1 knockdown on cell viability: The effect of Sirt1 knockdown was measured using Trypan Blue assay. Cell viability is expressed as the percent viable cells out of the total number of cells. The data represents mean ± SE of three experiments (*$p<0.01$). Details of the experiments are given in "Materials and Methods".

Figure 7. Effect of sirtinol treatment on Sirt1 and FoxO1 in human PCa cells. ***A) Effect of sirtinol on Sirt1 and FoxO1and Ac-FoxO1 protein levels:*** PCa cells were treated with 30μM or 120μM sirtinol (in DMSO) for 24 hours and then nuclear and cytosolic protein lysates were separated and collected. Sirt1, FoxO1 and Ac-FoxO1 protein levels were detected by Western blot analysis. Equal loading was confirmed by reprobing the blots for β-actin (cytosolic) or TATA binding protein TBP (nuclear). Data represents three experiments with similar results; ***B) Effect of sirtinol treatment on the transcriptional activity of FoxO1:*** PCa cells were transfected with phRL-TK and 3xIRSLuc-FoxO1 using Lipofectamine 2000. After 48 hours, cells were replated at equal density and allowed to adhere overnight. 24 hours later, transfected cells were treated with 30μM or 120μM sirtinol (in DMSO) for 24 hours. Then, cell lysates were prepared and luciferase activity was determined using the dual luciferase assay system. Luciferase activity was normalized to Renilla luciferase activity. The data represents mean ± SE of two experiments (*$p<0.001$). The data represents mean ± SE of three experiments. Details of the experiments are given in "Materials and Methods".

Table 1. Quantitation of Sirt1 immunostaining*

Tissue Type	Number of Specimens	Staining Intensity			
		- (Negative)	+ (Weak)	++ (Moderate)	+++ (Strong)
Normal or Benign	78	29 (37.7%)	30 (38.5%)	17(21.8%)	2 (2.6%)
High Grade PIN	23	4 (17.4%)	8 (34.8%)	8 (34.8%)	3 (13.0%)
Gleason Pattern 2	11	2 (18.2%)	3 (27.3%)	6 (54.5%)	0
Gleason Pattern 3	118	11 (9.3%)	30 (25.4%)*	45 (38.1%)*	32 (27.1%)*
Gleason Pattern 4	20	1 (5%)	5 (25.0%)*	2 (10.0%)*	12 (60.0%)*

*Staining on the 41 patient (3-9 samples per patient depending on heterogenicity) tissue microarray was semiquantitatively graded as negative (-), weak (+), moderate (++) or strong (+++) staining in 50% of cells examined. Statistical analysis of the data was determined using Fisher's exact test (*p-value <0.005).

Fig.1

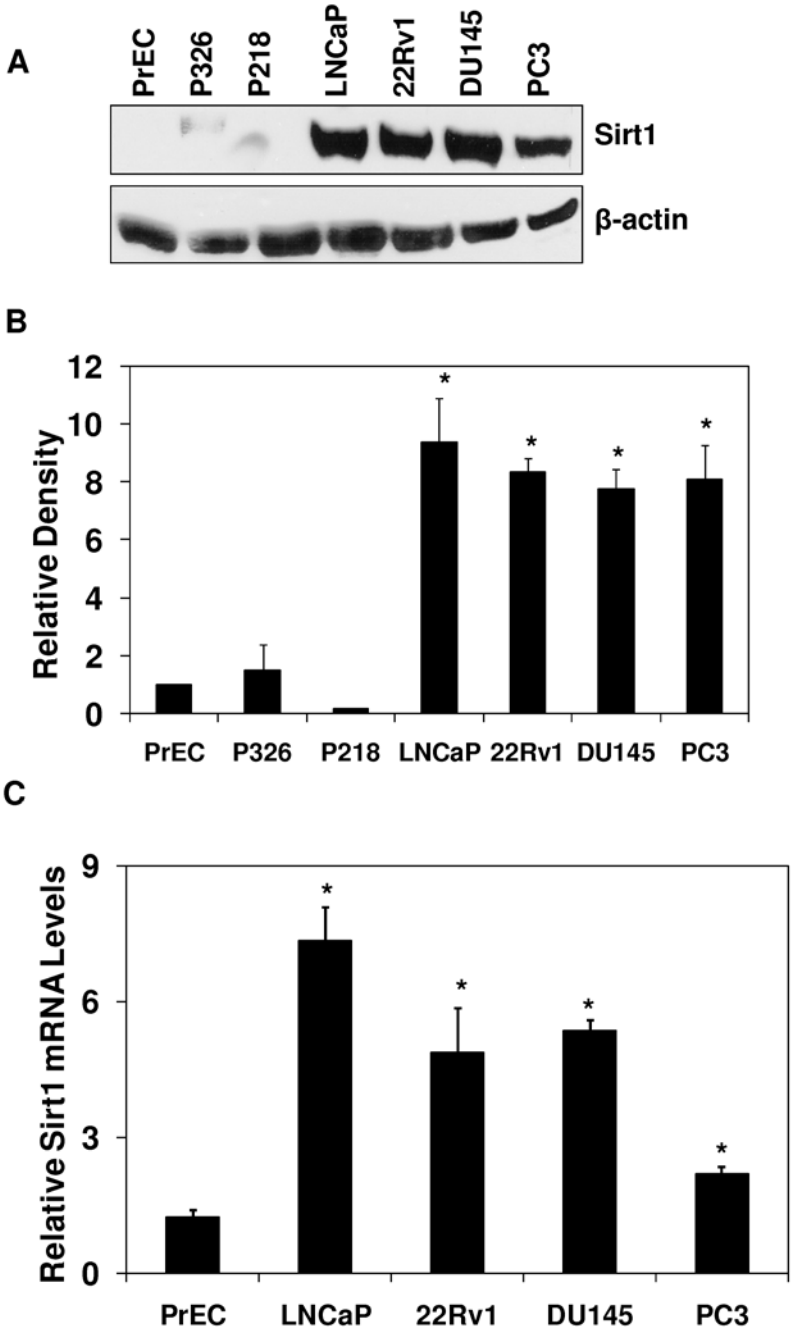

Fig. 2

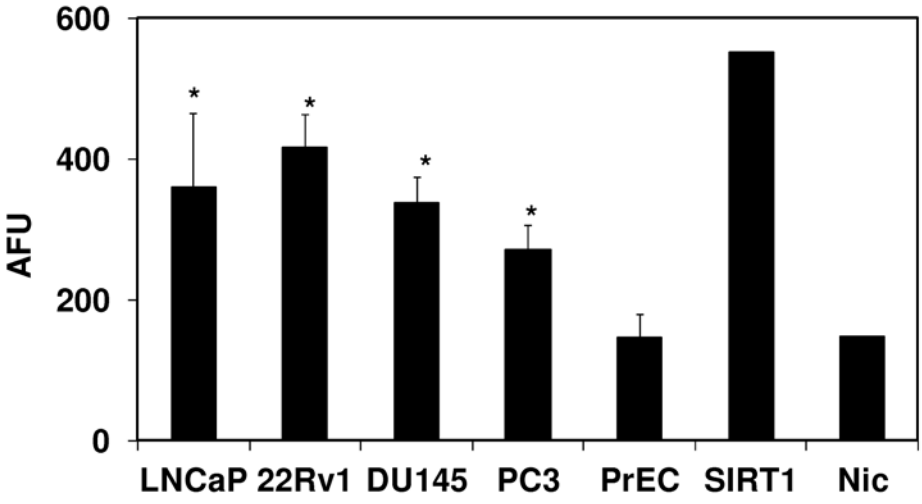

Fig. 3

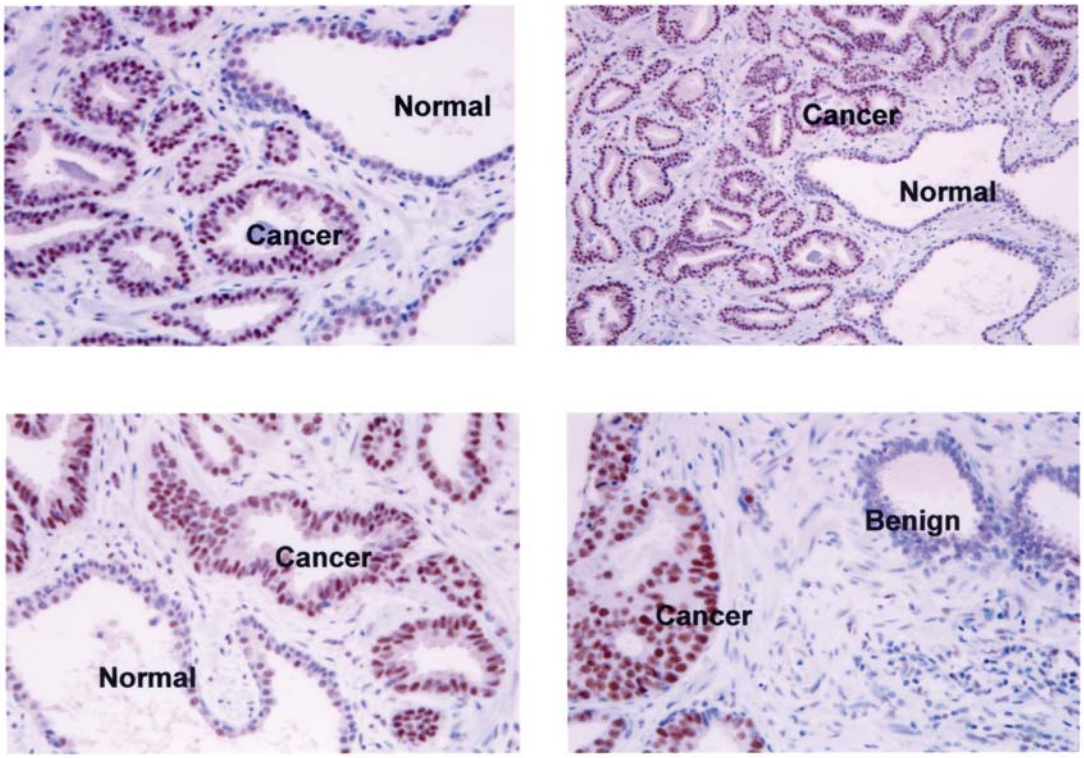

Fig. 4

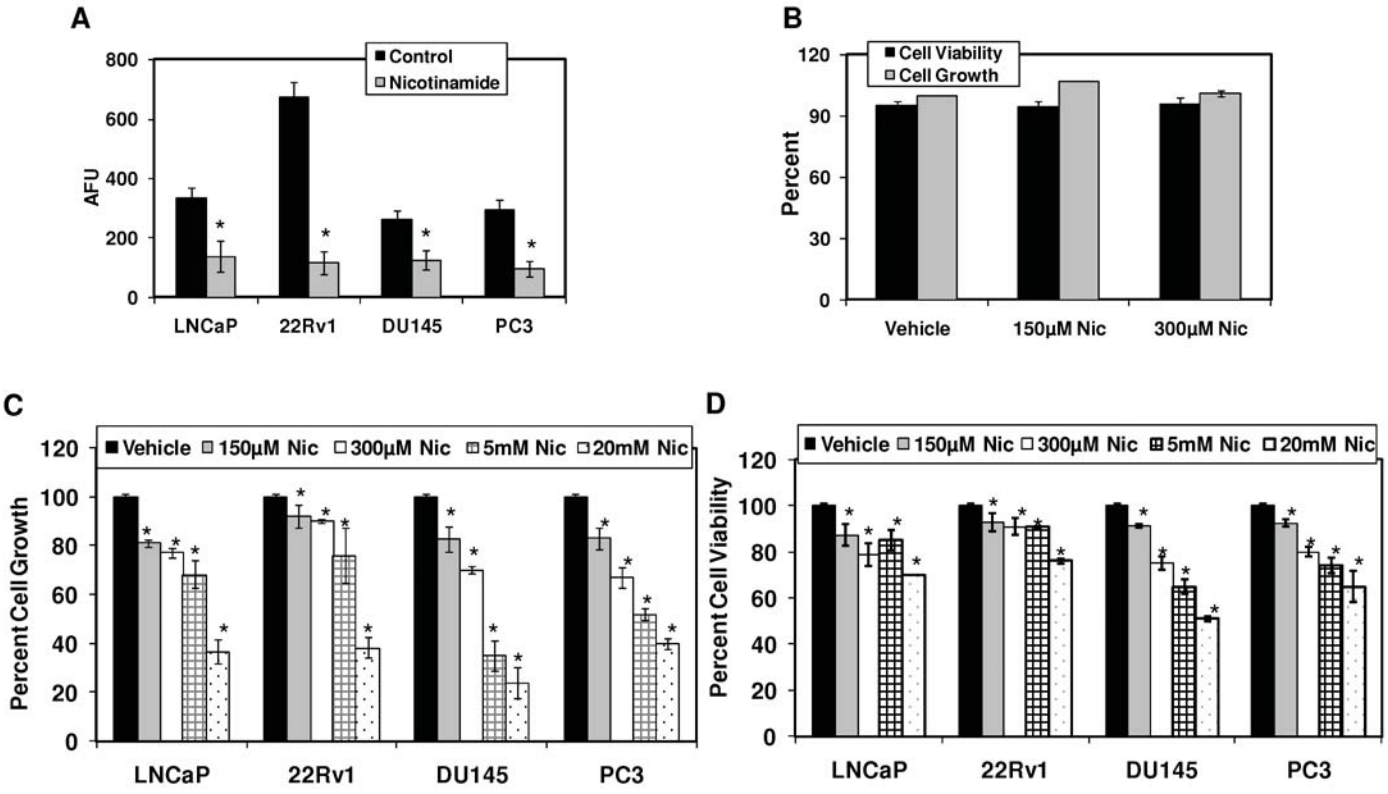

Fig. 5

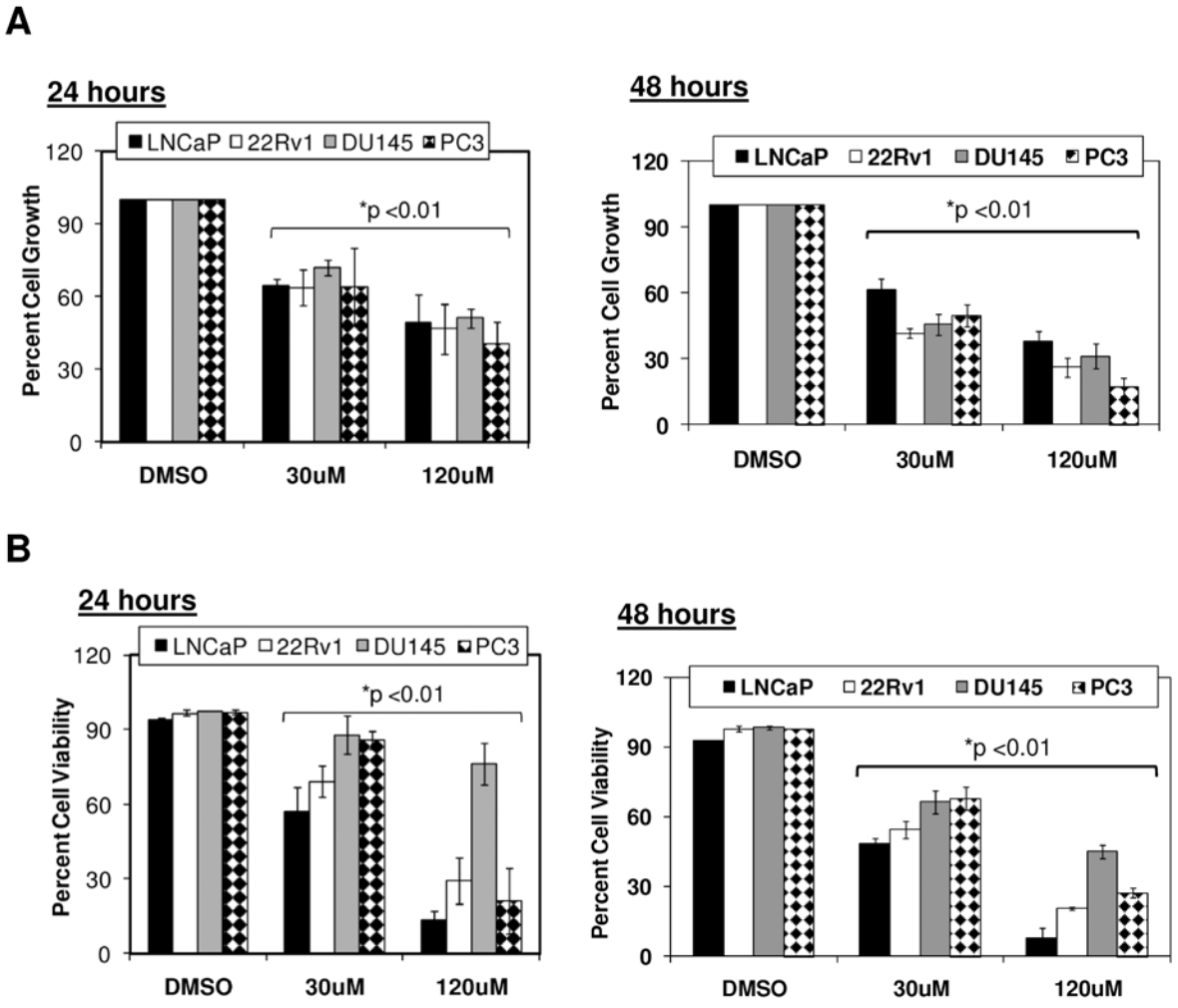

Fig. 6

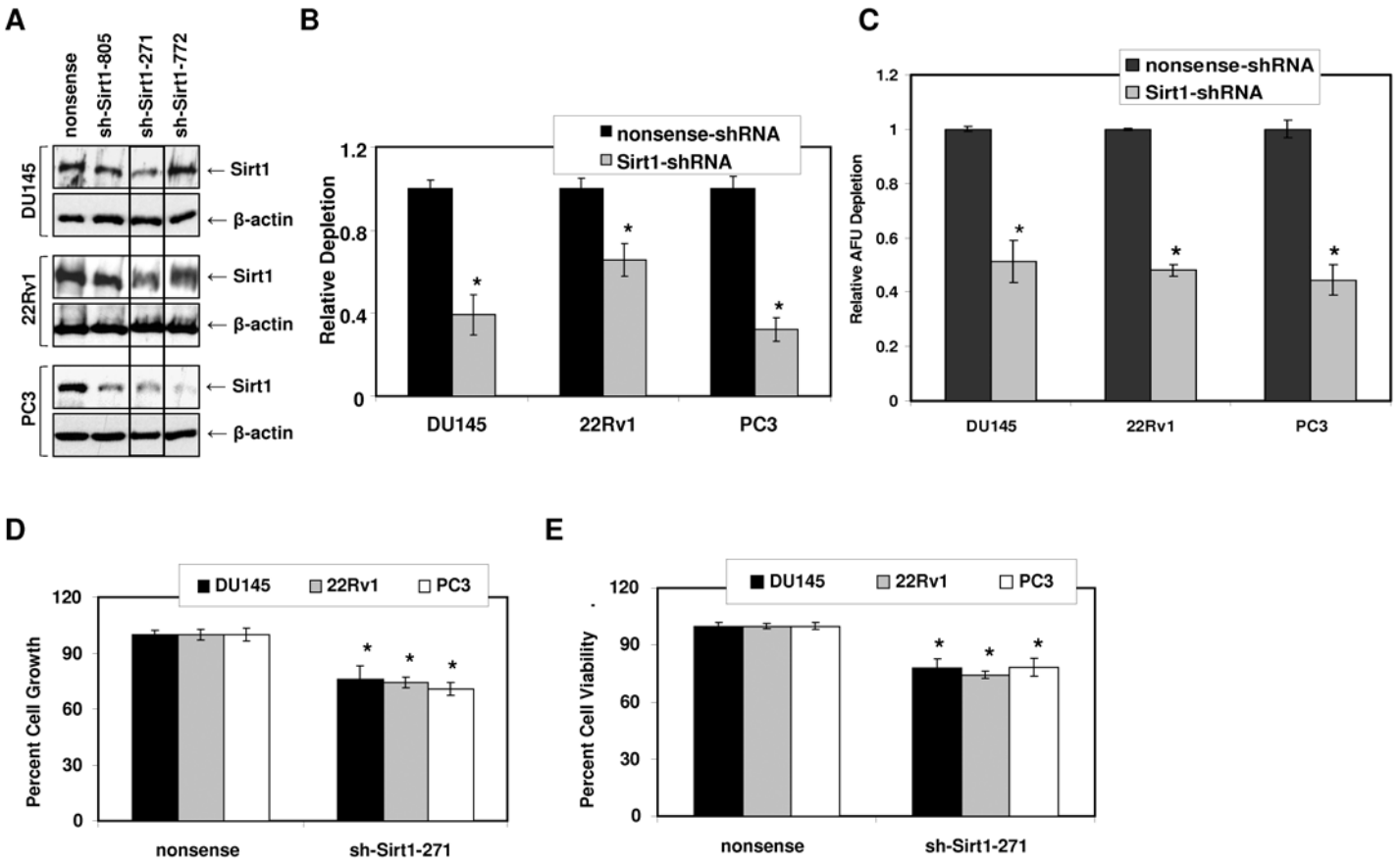

Fig. 7