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The focus of the project for this report period was the establishment of prostate cancer cell lines with inducible expression of prostasin, for which a role as a tumor suppressor has been implicated. Meanwhile, mapping of prostasin signal transduction pathways was underway using a gene expression knock-down approach in the LNCaP human prostate cancer cell line. A tetracycline-inducible, prostasin expressing DU-145 cell line has been established, this cell line will allow for proper assessment of prostasin's role as an invasion suppressor and a tumor suppressor. Prostasin serine protease and its serine active-site mutant were able to reduce the motility of DU-145 human prostate cancer cells. Prostasin gene silencing by siRNA in the LNCaP human prostate cancer cells resulted in a down-regulation of the cyclin-dependent kinase inhibitor, p21CIP1 at the protein level but not the mRNA level, further suggesting a tumor suppressor role for prostasin serine protease. The protein level change of p21CIP1 is a lead for pathway mapping of prostasin signal transduction.

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INTRODUCTION

The purpose of this project is to reveal the mechanism by which prostatic serine protease suppresses *in vitro* invasion of prostate cancer cells. The working hypothesis in the original proposal was that reduced tyrosine phosphorylation of either FAK or p130Cas (or both); and reduced protein expression of PKC α , were the signaling steps involved in prostatic's anti-invasion action. Four specific aims were proposed: 1. Identification of the 120-130-kDa tyrosine-phosphoprotein. 2. Prostatic's role in FAK-integrin signaling. 3. Prostatic's interaction with signaling proteins in caveolae. 4. Investigation of prostatic anti-invasion signal transduction by introducing active site-inactivated prostatic in prostate cancer cell lines DU-145 and PC-3.

BODY

Specific Aim 1: We will show that the 120-130-kDa tyrosine-phosphorylated protein being FAK or p130Cas, or both, using reciprocal immunoprecipitation/western blot analysis. We will confirm a role for prostatic in regulating cell movement by performing a motility assay.

Approved Statement of Work:

a). Reciprocal immunoprecipitation for the identification of FAK and/or p130Cas.

This task will be initiated at the onset of the project and is expected to be completed within months 1-6.

b). Cell migration assay

This task will be initiated at the onset of the project and is expected to be completed within months 1-6, however, the assay will continue to be employed in months 7-36 to serve the need of pathway investigation.

Overview:

In the previous two reports, we have indicated a potential tumor suppressor role for the prostatic serine protease in human prostate cancer cells, as well as the human embryonic kidney cell line HEK-293. Along with this finding, we have realized that the previously used episomal expression system for recombinant prostatic expression in the prostate cancer cell lines, namely DU-145 and PC-3, was unsuitable for the proposed studies. The transfected cells would gradually lose prostatic expression despite the presence of drug selection, presumably due to a viral promoter DNA methylation mechanism (1). The revised plan was to establish an inducible expression of prostatic in the prostate cancer cell lines for the proposed studies, the primary advantage of an inducible expression of prostatic is that the expression, when desired for the experiments, is uniformly in all the cells.

We have chosen the newly developed Flp-In T-REx system (Invitrogen, Carlsbad, CA) for the purpose of establishing tetracycline-inducible prostatic-expressing DU-145 and PC-3 cell lines. This system allows specific integration of any gene-of-interest at the same single chromosomal location once a parental Flp-In T-REx cell line is established. The biggest advantage of using this system is that the controls for experiments represent the true controls because they are genetically identical with the experimental groups.

Results:

1. Establishment of inducible human prostatic expression in DU-145 subline FT-145:

The Flp-In T-REx System is designed for the generation of stable mammalian cell lines with tetracycline-inducible expression of any gene-of-interest from one single chromosomal location.

The steps required for generating a cell line variant are somewhat complex and difficult but the end-product has many advantages, the most relevant in relation to cancer research is that the control (non-induced) cells are isogenic to the experimental (induced) cells. The major technical steps are as follows:

Phase 1. Independent integration of the following two plasmids into the genome of the parental cell line (*i.e.*, DU-145)

- a) A plasmid containing a Flp Recombination Target (FRT) site (pFRT//lacZeo)
- b) A plasmid expressing the Tet repressor (pcDNA6/TR)

Phase 2. Integration of an expression vector containing the gene-of-interest (*e.g.*, prostasin cDNA) under the control of a tetracycline-inducible promoter into the genome *via* Flp recombinase-mediated DNA recombination at the FRT site

Phase 3. Induction of the gene-of-interest by the addition of tetracycline

A). *Transfection and selection of colonies with a single chromosomal integration of the FRT*

Confluent cultures of the DU-145 cells (Passage number 67) were harvested *via* trypsinization and re-suspended in OPTI-MEM I medium (Invitrogen) at a density of 1×10^7 cells/ml. Four million cells in a volume of 400 μ l were mixed with 10 μ g (20 μ l) of *Apa* I-linearized pFRT//lacZeo plasmid for electroporation in a BTX-640 cuvette with the following parameters: 1600 μ F, 500V/CAP, 72 ohms, and 200 volts (2 x pulses) using an ECM-600 electroporator (Harvard Apparatus, Holliston, MA). Following the electroporation cells were rested at room temperature for 15 minutes before being plated on 2 x 100-mm Petri dishes in regular culture medium.

Cells were then placed under zeocin selection (with a predetermined kill-curve at 10 μ g/ml media) for 2 weeks until colonies were visible to the naked eye. Colonies were then picked into 24-well plates containing the zeocin selection media to propagate. These colonies were named DU-145-FI (numbers 1-4). Cells were further expanded into 60-mm Petri dishes for genomic DNA isolation and Southern blot analysis using a lacZ fragment (part of the pFRT//lacZeo plasmid released by restriction enzymes *Hind* III and *Eco*R V). Single chromosomal integration of the FRT in the cells was identified by a single hybridizing band in the genomic Southern blot, *per* Invitrogen Flp-In T-REx technical manual. As a result, all four DU-145-FI clones were shown to have single chromosomal integration of the pFRT//lacZeo plasmid (data not shown).

B). *Identification of a DU-145-FI clone with the highest transcription activity at the FRT integration site*

Next, we evaluated the transcriptional activities of the FRT chromosomal integration site of these DU-145-FI variants by a β -galactosidase assay (Promega, Madison, WI) to determine which clones to use for the ensuing steps. Cells were grown to confluence in 24-well plates before being lysed for the assay. The results are shown in Table 1 below.

Table 1. Transcriptional Activities of the FRT Chromosomal Integration Site

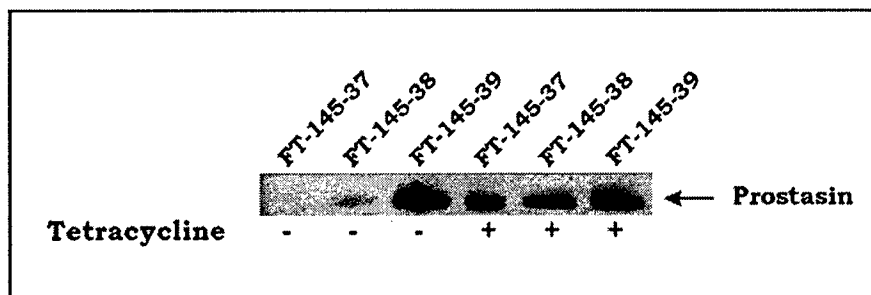
Clones	Relative β -Galactosidase Activity (Units/mg)
DU-145-FI-1	0.117
DU-145-FI-2	0.105
DU-145-FI-3	0.423* \rightarrow Selected for further use.
DU-145-FI-4	0.098
DU-145 Parent Control	0.000

C). *Transfection and selection of a DU-145-FI-3 clone with the best tet-repression*

Clone DU-145-FI-3 was grown to confluence for use in electroporation with the pcDNA6/TR plasmid (linearized with *Fsp I*) following the electroporation protocol described above, except that 2 million cells (in 200 μ l) and 15 μ g plasmid DNA (in 8 μ l) were used. Cells were selected in regular culture medium containing 15 μ g/ml blasticidin (selecting for pcDNA6/TR plasmid integration) for 2 weeks until colonies were visible. Colonies, named FT-145-3-numbers (a total of 12), were picked into 24-well plates, expanded, and selected in a transient expression experiment.

A transient transfection of the colonies with a full-length human prostaticin cDNA cloned in the pcDNA5/FRT/TO plasmid was used to establish the best repression (*i.e.*, without tetracycline) and induction (*i.e.*, with tetracycline). In Figure 1, the results of a western blot analysis for the FT-145-3 series cells (a sample of 3 clones) transiently expressing prostaticin under tetracycline induction are shown. Clone FT-145-37 gave the best repression (*i.e.*, no prostaticin protein expression without the addition of tetracycline in the medium) while expressing the prostaticin protein under induction/de-repression (*i.e.*, with tetracycline in the medium). The FT-145-37 clone/cell line was chosen for establishing inducible prostaticin expression.

Figure 1 (right):
Western blot analysis of prostaticin expression in FT-145-3-series. Cells were transfected with a full-length human prostaticin cDNA cloned in the pcDNA5/FRT/TO plasmid and were divided in two equal



portions in 6-well plates. One set of the cells were treated with 1 μ g/ml tetracycline in the culture medium for 24 hours for induction of prostaticin expression, the other set were cultured without tetracycline. A western blot analysis using a prostaticin-specific antibody was performed on both sets of cells to determine the best repression/induction.

D). *Transfection and selection of a stable DU-145 cell line with inducible expression of wild-type human prostaticin or the serine active-site mutant*

A full-length human prostaticin cDNA, or one that codes for a Ser195Ala active-site mutant form of prostaticin, cloned in the pcDNA5/FRT/TO plasmid (20 μ g), and the pOG44 plasmid (20 μ g, plasmid for transient expression of the Fip recombinase) were mixed with 2 million FT-145-37 cells for electroporation. Cells were then cultured in T-25 tissue culture flasks under the selection of blasticidin (15 μ g/ml) and hygromycin (5 μ g/ml) for 1 week. Colonies were dispersed, mixed, and kept as an isogenic polyclonal culture, and these cells are named FT-145-Pro or FT-145-ProM.

2. Prostaticin and its serine active-site mutant inhibit wound healing/motility of DU-145 cells:

A wound-healing assay was performed to assess prostaticin's effect on cell motility. FT-145-37, FT-145-Pro, or FT-145-ProM cells were cultured in 60-mm dishes with 1 μ g/ml tetracycline until confluence. A linear wound was made with a 1-ml pipette tip and the cells were cultured for another 24 hours and photographed. Wound healing was expressed as the extent of closure. The experiment was repeated and the results are shown in Figure 2 and Figure 3.

Figure 2 (right). Effect of prostaticin and its serine active-site mutant on DU-145 cell motility in a wound healing assay: The percent wound healed was determined by the following formula: Percent wound healed = $100 \times (\text{width of wound at 0 hr} - \text{width of wound at 24 hr}) \div \text{width of wound at 0 hr}$. The experiment has been repeated and the differences between the experimental groups were evaluated by ANOVA, $p < 0.05$.

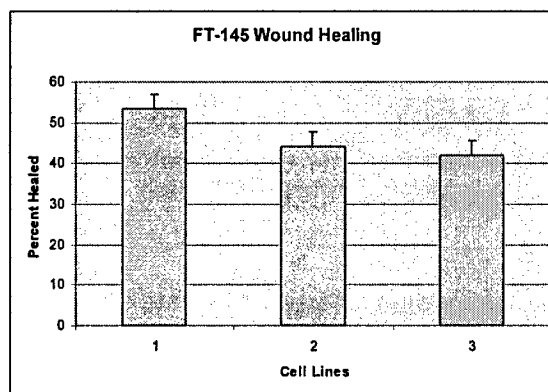
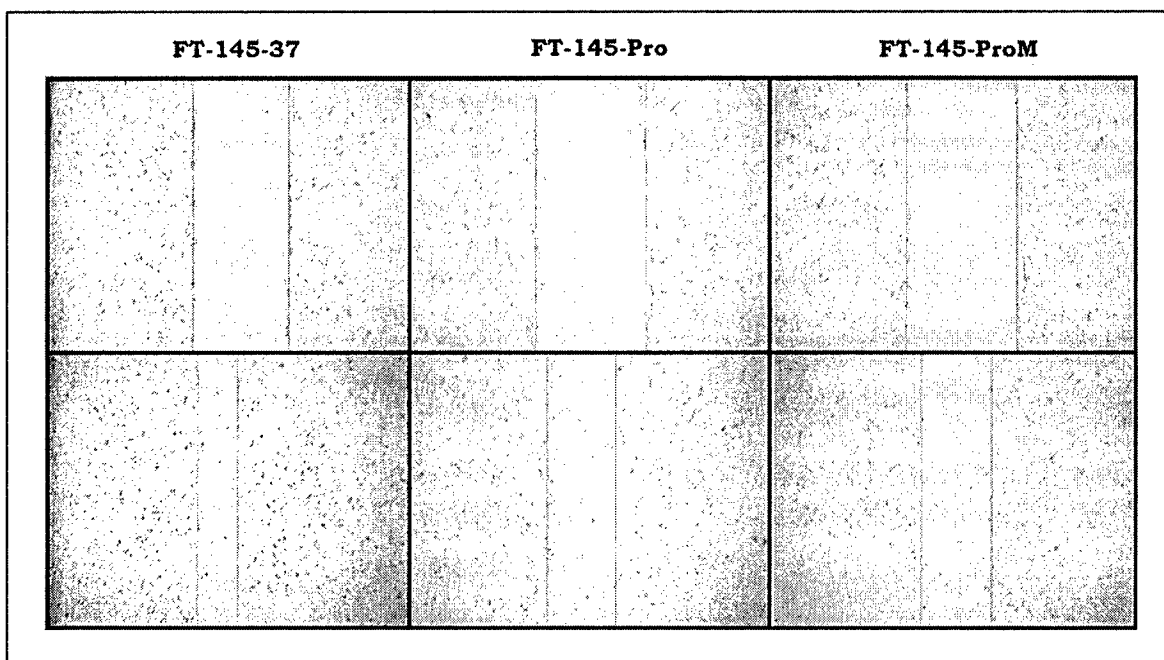


Figure 3 (below). Representative images of wound-healing of FT-145-37, FT-145-Pro, and FT-145-ProM in 24 hours. The upper panel shows the images of the wound at 0 hours, and the lower panel shows the wound images at 24 hours, for the cell lines indicated.



3. Prostaticin and EGFR:

We were unable to demonstrate specific prostaticin cleavage of epidermal growth factor receptor as described in the previous annual report. EGFR expressed in *E. coli* or lectin-purified from tissue-cultured cells was incubated with purified human prostaticin protein and the mixture was then examined by SDS-PAGE and western blot analysis using an EGFR antibody. No specific cleavage of EGFR at the predicted potential prostaticin cleavage site in the extra-cellular domain of the EGFR was observed (data not shown).

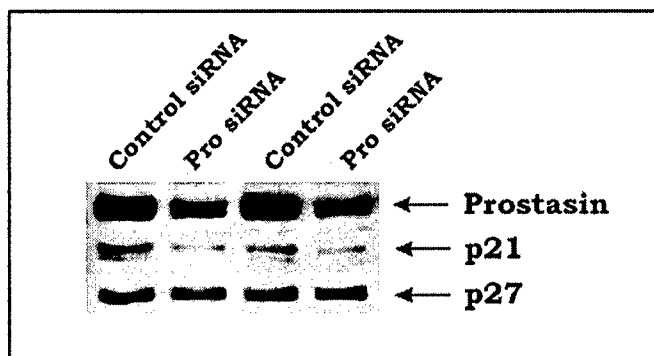
4. Prostaticin gene silencing in the LNCaP cells is associated with a down-regulation of p21:

The process of establishing the tet-inducible prostaticin expressing cell lines for DU-145 (completed) and PC-3 (ongoing) was unexpectedly time-consuming. We decided to use the time while we create these cell lines to pursue the role of prostaticin as a tumor suppressor from

the opposite direction. The experiment was to knock down the endogenous prostatic expression in the LNCaP cell line and inspect for cell cycle regulation or associated protein changes. Prostatic-specific short interfering RNA (siRNA) (2) and a random control siRNA (2) were synthesized by IDTDNA (Coralville, IA), and were transfected by lipofection into the LNCaP cells. Expression of the cyclin-dependent kinase inhibitors p21CIP1 and p27KIP1 were examined at the protein and the mRNA level.

The prostatic-specific siRNA was shown to reduce the expression of prostatic protein by ~50% in a western blot analysis, while the p21CIP1 protein expression was also reduced by ~80% (Figure 4). The p21CIP1 mRNA was measured by reverse-transcription followed by real-time PCR, and was found to be unaffected by the prostatic siRNA treatment (data not shown). The expression of p27KIP1 was also unaffected at either the protein or the mRNA level (Figure 4, and data not shown).

Figure 4 (right). Effect of prostatic siRNA on p21 and p27 protein expression in the LNCaP cells. Cells were transfected, *via* lipofection, with either a prostatic-specific siRNA or a random control siRNA, and assayed for protein expression of prostatic, p21, and p27 in a western blot analysis. The results of two representative experiments are shown.



Significance and Plans for Year 4 under No-cost Extension:

The establishment of the FT-145-37 cell line allowed the inducible and uniform expression of a potential tumor suppressor prostatic in the prostate cancer cells and the planned characterization of its impact on cell biology.

In Year 4 under no-cost extension, an FT cell line for the PC-3 prostate cancer cells will be established using similar procedures described above.

The confirmation of key cell-cycle regulator protein changes (*i.e.*, p21CIP1) as a result of prostatic expression knock-down in the LNCaP prostate cancer cells further suggests a role of prostatic as a tumor suppressor, despite the unsuccessful pursuit of EGFR as a target molecule for prostatic. In Year 4, other potential target signaling molecules will be investigated by reverse pathway mapping, *i.e.*, tracking the molecular changes upstream from the observed p21 protein but not mRNA change associated with prostatic gene silencing. Our immediate target areas are the pathways that affect p21CIP1 protein stability and turnover, *e.g.*, p38/JNK.

Specific Aim 2: We will examine whether prostatic re-expression resulted in a down-regulation of integrin using northern blot and/or RT-PCR-Southern blot, and western blot analyses and whether prostatic forms a direct link with β 1 and β 3 integrins using reciprocal immunoprecipitation/western blot analysis.

Approved Statement of Work:

- a). Determination of whether prostatic re-expression resulted in a down-regulation of integrin molecules at either the mRNA or protein level.

This task will be initiated at the onset of the project and is expected to be completed within months 1-12. The methods and reagents established in this task, however, will continue to serve the project in months 13-36.

b). Determination of whether prostasin forms a direct link with the $\beta 1$ and $\beta 3$ integrins

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

Overview and Results: In the first Annual Report, we had indicated two major outcomes in this specific aim. First, prostasin re-expression in DU-145 and PC-3 cells did not affect the mRNA or protein expression of $\beta 1$ and $\beta 3$ integrins, but the data were based on the previously established episomal prostasin expression in these cell lines. Second, using the HEK-293T cell line, we were able to immunoprecipitate the $\alpha 5$ integrin using a prostasin polyclonal antibody. A new paper published in the past year on integrin and EGFR signaling cross-talks (3) is very intriguing for the investigation of prostasin's signaling pathway(s). Specifically, adhesion of prostate epithelial cells to fibronectin was shown to have induced a p21 up-regulation by way of integrin-EGFR cross-talk. Based on this paper and with the establishment of a prostasin-inducible DU-145 cell line, the following experiments will be performed.

Plans for Year 4 under No-cost Extension:

- a). Analysis of mRNA and protein expression of $\beta 1$ and $\beta 3$ integrins in the FT-145-Pro or the FT-145-ProM cell lines.
- b). Reciprocal immunoprecipitation of $\alpha 5$ integrin with prostasin or prostasin serine active-site mutant (*i.e.*, pull-down of prostasin or mutant prostasin with an $\alpha 5$ antibody). This experiment was unsuccessful with the HEK-293T cell line, but will be tried with the FT-145-Pro or the FT-145-ProM cell lines.
- c). p21 and p27 changes in FT-145-Pro and FT-145-ProM during adhesion to fibronectin. Interestingly, this experiment is similar to those used to generate the preliminary data in the original grant application. In this regard, FAK tyrosine phosphorylation, part of the original research aim, will be revisited in our new cell lines capable of expressing prostasin uniformly.

Specific Aim 3: We will address the potential interactions between prostasin, Src, PKC α , and caveolin-1 by purifying caveolae and localizing the proteins biochemically by means of reciprocal immunoprecipitation/western blot analysis.

Approved Statement of Work:

a). Purification of caveolae and localization of prostasin in caveolae

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

b). Examination of interaction between prostasin and caveolar proteins

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

c). Interaction of prostaticin with caveolin-1, and/or Src, and/or PKC α in a time-dependent manner

This task will be initiated in month 16 and is expected to be completed within months 16-36.

Overview, previous results, and plans for Year 4 under no-cost extension:

In the first Annual Report, we had indicated two major outcomes relevant to this specific aim. First, prostaticin re-expression in DU-145 cells down-regulated both caveolin-1 and Src. Second, prostaticin is a proteolytic activator of the epithelial sodium channel (ENaC).

During Year-2 of the project, we found that the α subunit of ENaC is expressed in the prostate epithelial cells, and is clearly down-regulated in the highly invasive cell lines DU-145 and PC-3 as compared to the PrEC and the LNCaP cells. The β and γ subunits of the ENaC are not expressed in the prostate epithelial cells. The down-regulation of prostaticin and ENaC in the invasive prostate cancer cell lines may be causal and their relationship will be addressed using the FT-145-Pro and FT-145-ProM cell lines. In the previous report, we showed literature evidence linking the ENaC to a role in signal transduction. ENaC α is capable of binding to SH3-containing proteins (potentially, Src), *via* a C-terminal 18-amino acid proline-rich peptide.

We have previously stated that prostaticin down-regulation of caveolin-1 and Src in the DU-145 cell line suggests a role for prostaticin in the regulation and maintenance of the membrane composition and structure. In Year 4 under no-cost extension, we will perform the following key experiments to address the signaling of prostaticin through ENaC and Src:

- a). ENaC α -Src reciprocal immunoprecipitation from the DU-145 cells to show direct interaction of the two molecules.
- b). Examination of ENaC (all three subunits) mRNA expression changes in DU-145 cells expressing prostaticin or the active-site mutant (inducible).
- c). Localization of ENaC α on the membrane in LNCaP and DU-145 cells, in relation to the caveolae, and with Src.

Specific Aim 4: We will introduce into DU-145 and PC-3 cells a serine active site-inactivated prostaticin to determine whether the cells' invasiveness is still reduced or unchanged. The signaling pathways delineated from the investigations in the first three Specific Aims will be re-examined in cells expressing a serine active site-inactivated prostaticin.

Approved Statement of Work:

- a). Examination of invasiveness for cells (DU-145 and PC-3) that are transfected with a plasmid containing an active site-inactivated prostaticin cDNA
 - i). Construction of prostaticin mutant (Ala-prostaticin) cDNA plasmid: months 1-6.
 - ii). Establishment of DU-145 and PC-3 cells expressing Ala-prostaticin: months 7-12.
- b). Examination of the signaling pathways for cells (DU-145 and PC-3) expressing the Ala-prostaticin

This task will be initiated in month 16 and continue on through the project period (month 36)

Overview: The objective of this aim had been accomplished and the reagent generated (*i.e.*, ProM).

KEY RESEARCH ACCOMPLISHMENTS

- A tetracycline-inducible, prostaticin expressing DU-145 cell line has been established, this cell line will allow for proper assessment of prostaticin's role as an invasion suppressor and a tumor suppressor.
- Prostaticin serine protease and its serine active-site mutant were able to reduce the motility of DU-145 human prostate cancer cells.
- Prostaticin gene silencing by siRNA in the LNCaP human prostate cancer cells resulted in a down-regulation of the cyclin-dependent kinase inhibitor, p21CIP1 at the protein level but not the mRNA level, further suggesting a tumor suppressor role for prostaticin serine protease. The protein level change of p21CIP1 is a lead for pathway mapping of prostaticin signal transduction.

REPORTABLE OUTCOMES

- The Flp-In T-REx derivative of the human prostate cancer cell line DU-145, named as FT-145-37. This cell line, following further characterization, will be deposited at the American Type Culture Collection (ATCC) for distribution to researchers.

CONCLUSIONS

The major task undertaken during this reporting period was the establishment of inducible human prostate cancer cell lines expressing human prostaticin. This task was accomplished for the DU-145 cells and is underway for the PC-3 cells. The task was unexpectedly time-consuming but the outcome is very important to the continued studies of prostaticin's role as a tumor suppressor. We have also provided further evidence that prostaticin may be a tumor suppressor, in addition to its ability to suppress invasion by way of reducing cell motility. The LNCaP cells displayed a markedly reduced protein expression of the cyclin-dependent kinase inhibitor p21CIP1 when endogenous prostaticin expression was knocked down by a prostaticin-specific siRNA.

"SO WHAT": Confirmation of prostaticin's role as a tumor suppressor will be important for understanding how various growth factors, growth factor receptors, and other membrane signaling molecules act and interact in normal prostate, as well as the diseased prostate. Current and future treatment and prevention strategies for prostate cancer must address the role of a membrane-anchored extra-cellular signal modulator.

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