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Progress report

Introduction:

PSMA, a transmembrane glycoprotein of approximately 100kD, is expressed in prostate epithelial cells (Horoszewicz et al., 1987; Israeli et al., 1993). In addition to being expressed in non-neoplastic prostate epithelium, PSMA is expressed by a very high proportion of prostate cancers. Expression is further increased in higher-grade cancers and metastatic disease, and in hormone-refractory prostate cancers (Wright et al., 1995). PSMA is a type II membrane protein with a short N-terminal cytoplasmic tail and a large C-terminal extracellular domain (Israeli et al., 1993). The extracellular domain of PSMA has several potential N-glycosylation sites and shows homology (54% at nucleic acid level) to the transferrin receptor. PSMA is homologous to glutamate carboxypeptidase II (85% at nucleic acid level) and has been suggested to have folate hydrolase activity and N-acetylated α -linked acidic dipeptidase (NAALADase) activity (Pinto et al., 1996; Luthi-Carter et al., 1998). Abundance of PSMA expression in prostate cancer cells suggests that PSMA expression might be associated with events involved in prostate cancer progression (Rajasekaran et al., 2005).

In this proposal we presented evidence that PSMA is localized to membrane compartments around the centrosomes in interphase cells (Anilkumar et al., 2003, Rajasekaran et al., 2003) and at the spindle poles in mitotic cells. This localization suggested a potential role for PSMA in cell cycle regulation. The overall goal of this proposal was to identify the molecular mechanism by which PSMA is localized to the mitotic spindle pole and regulate cell cycle progression. We have provided evidence that PSMA expressing cells exit mitosis rapidly compared to cells that do not express PSMA. We finally showed interaction of PSMA with CDC27 a core subunit of the anaphase promoting complex (APC), which is involved in regulating the timing event during mitosis. Fidelity of proper chromosome segregation during mitosis is primarily governed by a spindle checkpoint that becomes activated upon a defect in chromosome segregation. This checkpoint acts to restrain cells from entering anaphase, the chromosome segregation step in mitosis, until all replicated chromatids have formed proper attachments to a functional bipolar spindle. The checkpoint pathway transduces a signal that ultimately halts the action of APC, an enzyme required to drive cells into anaphase. Based on these results we proposed that increased PSMA expression might be associated with aberrant cell cycle progression and aneuploidy in prostate cancer cells. As indicated in this proposal we now provide data that PSMA expression interferes with APC activity and induces aneuploidy in prostate cancer cells. A manuscript on these results was recently published in *Molecular Cancer Therapeutics* (Rajasekaran et al., 2008, see enclosed reprint). In addition to accomplishing these primary goals of this project funds from this award were also used to complete eight more publications (see enclosed reprints).

Body:

1. PSMA is localized to the spindle poles in mitotic cells

In addition to its plasma membrane localization (Anilkumar et al., 2003, Rajasekaran et al., 2003) PSMA was distinctly evident at mitotic spindle poles and colocalized with pericentrin,

a centrosomal marker (Fig. 1). Localization to the mitotic spindle poles was also observed in stable clones of Madin Darby Canine Kidney cells expressing PSMA (MDCK-PSMA-1) (Fig. 1). Strikingly, cells with multiple centrosomes showed PSMA localized around each additional centrosome in both PC3-PSMA and MDCK-PSMA cells (Fig. 1, inserts). Spindle pole localization was contingent upon the cytoplasmic tail of PSMA, as removal of this domain (PSMA- Δ CD) resulted in the loss of pericentrin co-localization with expression primarily limited to the plasma membrane and trans Golgi network (Fig. 1). Furthermore, a PSMA deletion mutant lacking most of its extracellular domain (MDCK-PSMA- Δ 103-750) showed distinct localization at the mitotic spindle poles (Fig. 1) indicating that the glutamate carboxy peptidase activity of PSMA which is localized to the extracellular domain is not necessary for its spindle pole localization.

Figure 1

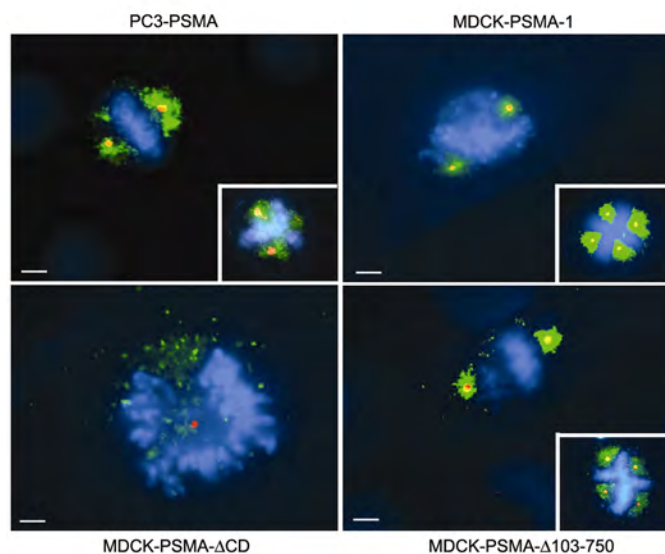


Figure 1 Localization of PSMA at the mitotic spindle poles. Note distinct colocalization of PSMA (green) with centrosomes (red) at the mitotic spindle poles in PC3-PSMA, MDCK-PSMA-1, and MDCK-PSMA- Δ 103-750 cells but not in MDCK-PSMA- Δ CD cells. Scale bars, 8 μ m.

2. PSMA expressing cells exit mitosis in an accelerated manner

Given the function of spindle poles in cell division, we hypothesized that localization of PSMA to the spindle poles is associated with a role in mitosis. Cells were synchronized in mitosis by nocodazole and cell cycle progression after its removal was monitored by flow cytometry. Nocodazole treatment blocked over 90% of cells in G₂/M with a DNA content of 4N, for both PC3 and MDCK cells, irrespective of PSMA expression, suggesting efficient synchronization of cells in G₂/M (Fig. 2). PC3-PSMA cells (Fig. 2b) and two independent clones of MDCK-PSMA cells (clone 1 and 2) (Fig. 2d-e) exited mitosis in an accelerated manner compared to control cells that do not express PSMA (Fig. 2a and c, respectively). Strikingly, MDCK-PSMA- Δ CD cells exited mitosis slower than either MDCK-PSMA clone, but with an almost identical rate to MDCK-pCDNA3 (empty vector) cells (Fig. 2c, f). Furthermore, MDCK-

PSMA-Δ103-750 cells (Fig. 2g) exited mitosis faster than MDCK-pCDNA3 cells and similar to MDCK-PSMA cells. Taken together, these results demonstrate that the cytoplasmic tail is essential for PSMA localization to the mitotic spindle poles and accelerated exit from mitosis.

Figure 2

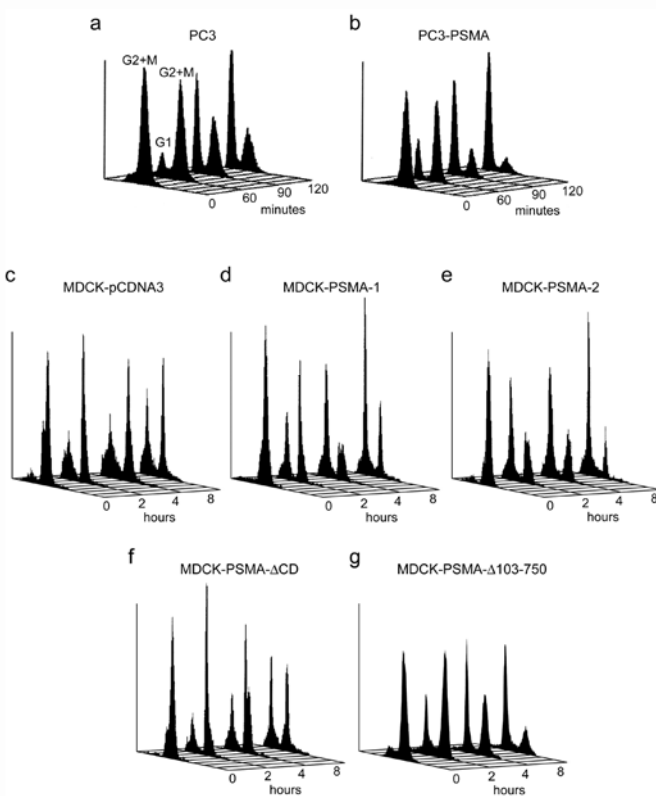


Figure 2 Analysis of the cell cycle progression in PSMA expressing cells after release of the mitotic block. **(a, b)** After 60 minutes 25 ± 2.14 % of PC3-PSMA cells and 3 ± 0.81 % of control PC3 cells exited mitosis. At 90 and 120 minutes, 52% and 79 ± 0.68 % of PC3-PSMA cells and 43% and 69 ± 2.83 % of PC3 cells exited mitosis. Data shown represent the means $\pm$ SEM of two independent experiments. **(c-e)** After 2 hours, 43 ± 0.50 % of MDCK-PSMA-1 **(d)** and 57 % of MDCK-PSMA 2 **(e)** cells and only 25 ± 1.48 % of MDCK-pCDNA3 cells **(c)** exited mitosis. At 4 hours, 53 ± 3.16 % of MDCK-PSMA-1 and 64 % of MDCK-PSMA-2 cells had exited mitosis compared to 29 ± 0.27 % of MDCK-pCDNA3 cells. At 8 hours, 64 ± 0.55 % of MDCK-PSMA-1 and 81 % of MDCK-PSMA-2 cells had exited mitosis compared to 41 ± 1.69 % of MDCK-pCDNA3 cells. **(f)** MDCK-PSMA-ΔCD cells exited mitosis similar to MDCK-pCDNA3 cells, with 25 ± 1.48 %, 39 ± 1.10 %, and 52 ± 0.70 % of cells exiting mitosis by 2, 4, and, 8 hours, respectively. **(g)** MDCK-PSMA-Δ103-750 cells exited mitosis similar to the MDCK-PSMA clones, with 22 ± 0.74 %, 43 ± 0.23 %, and 68 ± 2.97 % of cells having exited mitosis after 2, 4, and 8 hours, respectively. Data shown in **(c,d, f, g)** represent the means $\pm$ SEM of three independent experiments.

3. PSMA expressing cells have increased APC activity

Entry and exit from mitosis are regulated primarily through control of (cyclin dependent kinase) CDK1 activity via ubiquitin-mediated proteolysis of cyclin B. As the chromosomes attach to spindle microtubules and become properly aligned at the metaphase plate, APC becomes activated and targets key substrates, including cyclin B, for degradation. The mitotic spindle checkpoint halts the action of APC and acts to restrain cells from entering anaphase. We hypothesized that accelerated exit from mitosis of PSMA expressing cells is due to a defect in the spindle checkpoint with increased APC activity leading to premature degradation of cyclin B. During nocodazole arrest and following mitotic release, cyclin B1 levels of PC3-PSMA cells were consistently lower than in control PC3 cells (Fig. 3a), suggesting increased activity and/or incomplete inactivation of the APC in PSMA expressing cells. An *in vitro* APC assay demonstrated that APC ubiquitin ligase activity for cyclin B was substantially higher at 0, 60, and 120 minutes after release from nocodazole block in PC3-PSMA cells relative to PC3 cells (Fig. 3b). Increased APC activity in nocodazole blocked PC3-PSMA cells indicate that APC is incompletely inactivated by the mitotic spindle checkpoint, and confirms our hypothesis that PSMA expression leads to a compromised spindle checkpoint function in prostate cancer cells.

APC is a large protein complex consisting of >10 subunits and is subjected to a complex pattern of regulation. Mad2 and BubR1 are well-characterized negative regulators of APC, while Cdc20 activates APC function. However, immunoblot analysis revealed that asynchronous populations of PC3 and PC3-PSMA cells expressed similar levels of the APC regulators Mad2, BubR1, and Cdc20, and of the core subunit of the APC, Cdc27 (Fig. 3c). The levels of BubR1, Cdc20, and Cdc27 were all increased to a similar extent in mitotically active PC3 cells independent of PSMA expression. Due to phosphorylation, Cdc27 and BubR1 showed multiple bands with the banding pattern and intensity of these bands being comparable in PC3 and PC3-PSMA cells (Fig. 3c). Co-immunoprecipitation analysis using anti-Cdc27 antibody revealed similar levels of Mad2, BubR1, and Cdc20 associated with APC in both PC3 and PC3-PSMA cells (Fig. 3d) and in addition, Mad2, BubR1 or Cdc20 were not detected in PSMA immunoprecipitates (data not shown), making it unlikely that PSMA activates APC by altering association of these regulators with APC.

We then tested whether PSMA associates with the core APC complex. We observed that in both PC3-PSMA and MDCK-PSMA cells Cdc27 co-immunoprecipitated with PSMA (Fig. 3e) but not with the cytoplasmic tail deletion mutant of PSMA in MDCK-PSMA-ΔCD cells confirming that the cytoplasmic tail mediates the association of PSMA with APC. These results were further verified by *in vitro* pull-down assays from PC3-PSMA cell lysates using a GST-fusion protein containing the cytoplasmic tail of PSMA (GST-PSMA-CD) (Fig. 3f). We were also able to co-immunoprecipitate PSMA and Cdc27 in lysates made from six PSMA-positive tumor tissue samples (Fig. 3g). Taken together, these results demonstrate that the cytoplasmic tail of PSMA associates with Cdc27 in both cultured cells and in prostate tumor tissues and this association results in increased APC activity in PSMA expressing cells.

Figure 3

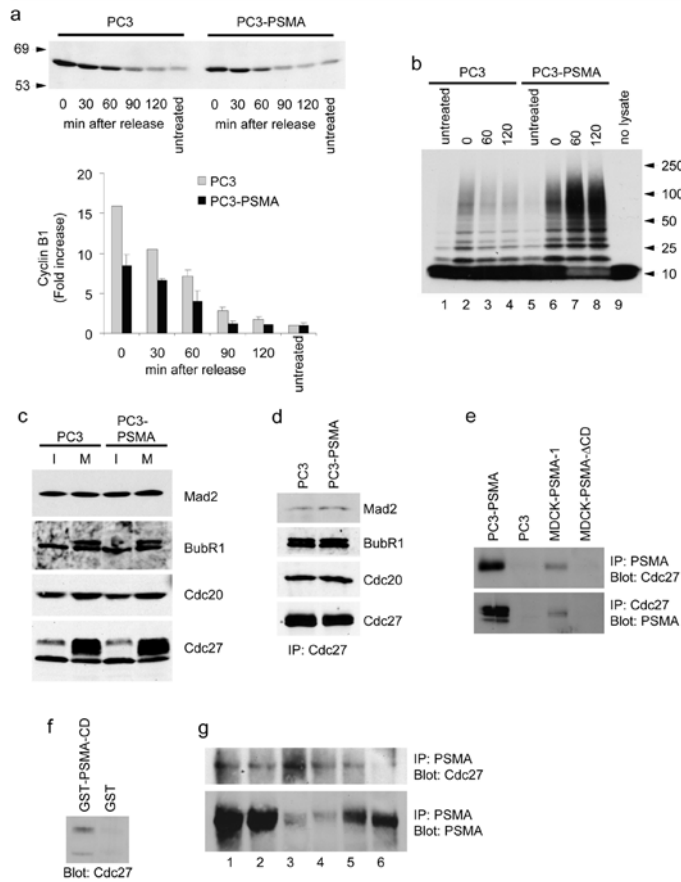


Figure 3 Analysis of APC activation and association with PSMA. **(a)** Immunoblot analysis of cyclin B₁ in PC3 and PC3-PSMA cells after release of the mitotic block. Data represent the means $\pm$ SD of two independent experiments. **(b)** In vitro APC ubiquitin ligase activity assay in PC3 and PC3-PSMA cells after release from nocodazole block. **(c)** Immunoblot analysis of Mad2, BubR1, Cdc20, and Cdc27 in interphase (I) and Mitotic (M) cells. **(d)** Co-immunoprecipitation of Mad2, BubR1, and Cdc20 with Cdc27 in PC3 and PC3-PSMA cells. Equal amounts of Cdc27 used in the immunoprecipitation (IP) was confirmed by immunoblotting. **(e)** Co-immunoprecipitation of PSMA with Cdc27 (top panel) and Cdc27 co-immunoprecipitating PSMA (bottom panel) in PC3-PSMA and MDCK-PSMA cells. Note Cdc27 does not co-immunoprecipitate with PSMA- Δ CD. **(f)** Affinity precipitation of Cdc27 by GST-PSMA from PC3 cell lysate. **(g)** Co-immunoprecipitation of Cdc27 with PSMA in prostate cancer tissues (top panel). PSMA levels in same tissues are shown.

5. Induction of aneuploidy in PSMA expressing cells

The elevated APC activity in PSMA expressing cells resulting in premature degradation of cyclin B₁ would provide less time for chromosome segregation, increasing the likelihood of aneuploidy. In order to assess the significance of PSMA expression on genomic stability, we

expressed PSMA in HCT-116 cells (HCT-PSMA). Derived from colorectal carcinoma, the HCT-116 cell line possesses a nearly diploid karyotype and is an established model for studying chromosomal instability. This cell line was used in such analysis since no karyotypically stable prostate cancer cell line was available. We confirmed the diploid karyotype of this cell line by standard karyotype analysis (data not shown). PSMA co-localized with centrosomes in HCT-PSMA cells, and these cells exited mitosis faster than control HCT-GFP cells. Fluorescence *in situ* hybridization (FISH) was done using probes specific for chromosomes 3, 7, 17 centromeres, and 9p21 region, respectively. No chromosomal abnormalities were observed between HCT-PSMA and HCT-GFP cells at passage 2 (Fig. 4b). However, at higher passage numbers of 15, 34, and 45, all these chromosomes showed various degrees of aneuploidy in HCT-PSMA cells (Fig. 4c-g). The frequency of aneuploidy increased with passage number, and by passage 45 there was significant increase in the number of aneuploidy in PSMA expressing cells ($P < 0.001$, chi square = 13.25; $n = 1000$). In addition clear evidence of micronuclei formation was observed (Fig. 4e, arrow, and f) which provides further evidence for chromosomal instability.

Figure 4

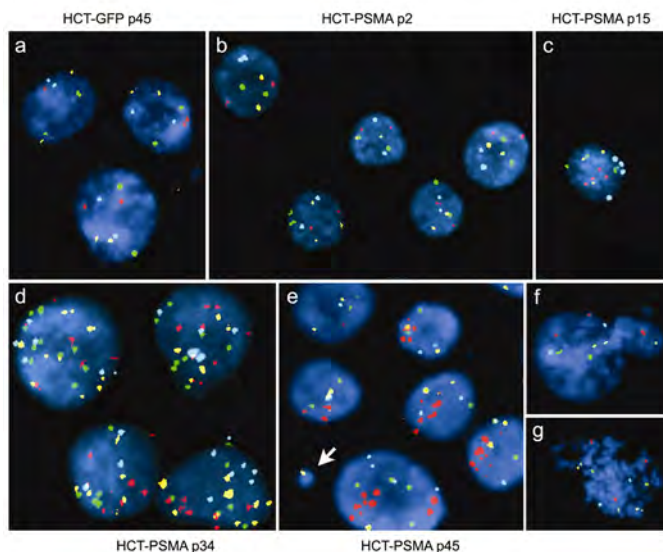


Figure 4. PSMA expressing human cells are genetically unstable. HCT-PSMA and HCT-GFP cells were analyzed by multicolor FISH with Chromosome 3 labeled with Spectrum Red (CEP3), Chromosome 7 with Spectrum Green (CEP7), Chromosome 17 with Spectrum Aqua (CEP 17), and 9p21 region (p16 gene) with Spectrum Gold. Control HCT-GFP cells at passage 45 (a) and HCT-PSMA cells at passage 2 (b) are diploid whereas HCT-PSMA cells at passage 15 (c), 34 (d), and 45 (e) show aneuploidy. Micronucleus formation (e, arrow and f) and abnormal metaphase (g) in HCT-PSMA cells at passage 45 are shown.

Additional data obtained at Nemours following the move from UCLA

Since the goals of the funded project were completed we utilized the remaining funds for additional new studies. Epidemiologic and preclinical studies suggest that diet plays an important role in prostate cancer (PCa) development and progression. While a high-fat diet has

been linked to prostate cancer the identity of other food products contributing to PCa development and progression remains controversial. Processed or cured meats have been strongly associated with increased risk of advanced or metastatic PCa. One of the critical ingredients of processed meat is the high level of sodium from salt, food preservatives, and flavoring agents (<http://topnews.net.nz/content/22163-high-sodium-diets-hazardous-health>). A high sodium diet has been linked to a variety of human illnesses including cancer. In the USA, 150,000 people die each year due to diseases associated with the consumption of high amounts of dietary salt (<http://www.collectivewisdom.com/150000DieFromTooMuchSalt.html>). However, whether dietary salt has any role in increasing PCa risk or alters the prognosis of an already existent cancer is not known. In this novel study we evaluated whether castration resistant prostate cancer cells PC3 has growth advantage in vivo when grown in the presence of high salt.

We tested whether high dietary sodium promotes tumor growth in *in vivo* xenografts of PC3 cells, an androgen-independent, highly aggressive metastatic PCa cell line. Once the subcutaneous xenografts were established in immunocompromised mice, the control group was fed with normal salt diet and the experimental group with high salt diet. Strikingly, in mice that received high salt diet the tumors grew twice as fast compared to mice fed with normal salt diet (Fig. 5). Hematoxylin and eosin (H&E) staining of tumor tissues revealed that tumors from high salt diet mice had 3-4 fold more mitotically active cells (primarily in the easily discernable metaphase and anaphase) (Fig. 6) indicating that signaling mechanisms leading to increased cell proliferation are activated in mice fed with high salt diet. A comprehensive analysis of signaling pathways in the tumor tissues suggested that Wnt signaling might be activated in high salt diet fed mice (see below).

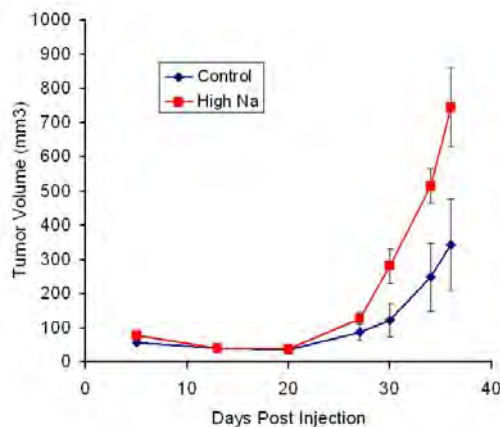
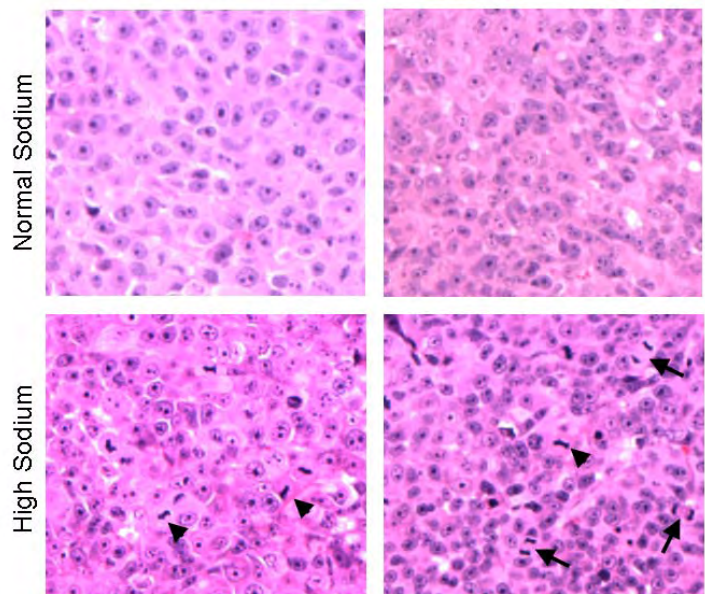


Fig. 5. High salt diet increases tumor growth of PC3 xenografts. 20 male Hairless SCID mice were randomized by weight and divided into two groups of 10 mice each. One group received a normal salt diet (0.49% NaCl) and the other group a high salt diet (4% NaCl). One week after diet start PC3 cells were injected subcutaneously. The tumor size was measured with calipers twice weekly. Note the increased growth of tumors in mice on high salt diet ($P < 0.05$; paired student's t-test).

Fig. 6. H&E staining of PC3 xenografts from mice fed with normal and high sodium diet. Note that in xenografts of mice fed with high salt diet mitotic cells including the easily discernable cells in metaphase (arrowheads) and anaphase (arrows) were increased 4-fold. Images are taken from tumors of two independent mice of each group.



Castration-resistant PCa and Wnt signaling: Recent studies have shown that aberrantly activated Wnt signaling is commonly observed in castration-resistant PCa. The canonical Wnt/ β -catenin pathway comprises soluble Wnt ligand binding to its receptors Frizzled and LRP5/6 activating the downstream component Dishevelled, which in turn inhibits glycogen synthase kinase (GSK)-3 β , axin, and adenomatous polyposis coli (APC), a protein complex that phosphorylates β -catenin and targets it for degradation. Activation of Wnt signaling thus stabilizes β -catenin that can bind the LEF/TCF transcription factors in the nucleus and activate transcription of downstream targets such as cyclin D1 and Myc. Interestingly, β -catenin also binds to ligand-engaged androgen receptor (AR) and activates its transcriptional activity. Furthermore, LEF binds to the AR promoter and increases its expression in castration-resistant PCa cells. Since Wnt signaling increases cell proliferation and since it is activated in castration-resistant PCa we hypothesized that high salt diet activates Wnt signaling in PC3 cells.

In support of this hypothesis we found increased serine phosphorylation of GSK-3 β and increased total β -catenin (Fig. 7) and cyclin D1 (data not shown) levels in tumors from mice fed with high salt diet, suggesting the activation of Wnt signaling in these tumors. These data are consistent with the model that high dietary salt activates Wnt signaling in NaK- β_1 deficient, androgen-independent xenografts and promotes PCa progression. To our knowledge this is the first observation that relates high salt diet to the activation of Wnt signaling.

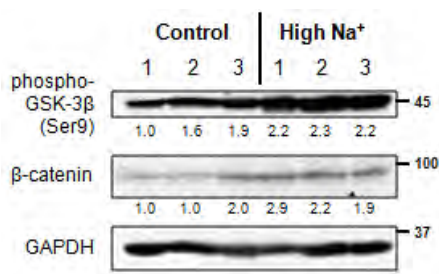


Fig. 7. High salt diet fed mice show increased phosphorylation of GSK-3 β and increased levels of total β -catenin. Immunoblots shown are from tumors of three mice (1-3) from each category of control and high Na⁺ diet. Numbers below blots indicate the relative protein levels as compared to the levels in the control tumor #1. GAPDH is used as loading control.

Statement of Work

Task 1. Identify crucial cytoplasmic tail amino acid essential for APC association (months 1-12)

- ✓ Generate stable cell lines expressing cytoplasmic tail mutants of PSMA
- ✓ Test APC association with PSMA by co-immunoprecipitation experiments
- ✓ Perform FACS analysis to test the effect of mutant PSMA on cell cycle progression
- ✓ Compare cyclin B1 levels in cells expressing full-length PSMA and cytoplasmic tail mutants of PSMA

Task 2. Determine whether PSMA association to APC alters APC and spindle checkpoint functions (months 1-18)

- ✓ Determine whether APC is active in nocodazole blocked PSMA expressing cells

- ✓ **Compare the levels of Mad2 and BubR1 associated with APC in control and PSMA expressing cells**
- ✓ **Standardize an assay for APC and compare the APC activity in control and PSMA expressing cells**

Task 3. Test the role of PSMA expression in aneuploidy (months 12-36)

- ✓ **Generate PSMA expressing PEA3 and HMEC cell lines**
- ✓ **Perform FACS analysis to test cell cycle progression every five passages for up to 20 passages**
- ✓ **Perform molecular cytogenetic analysis to determine aneuploidy in control and PSMA expressing cells every five passages for up to 20 passages.**

Key Research Accomplishments:

Research:

- ✓ Established that cytoplasmic tail of PSMA is critical for accelerated exit of cells from the cell cycle.
- ✓ Confirmed that the cytoplasmic tail is essential for APC association by coimmunoprecipitation experiments in cells expressing a cytoplasmic tail deletion mutant of PSMA.
- ✓ Confirmed that the cytoplasmic tail of PSMA is sufficient for APC association by GST affinity precipitation experiments.
- ✓ Standardized an in vitro assay for APC activity.
- ✓ Confirmed that APC is active in nocodazole treated cells.
- ✓ Generated a HCT 116 cell line stably expressing PSMA.
- ✓ Confirmed our initial hypothesis that PSMA expression has a causal effect in inducing aneuploidy in prostate cancer cells.
- ✓ Identified recycling endosomal components at the spindle pole

Reportable outcomes (9 publications):

1. **Rajasekaran A.K.**, G. Anilkumar, and J.J. Christiansen (2005). Is Prostate specific membrane antigen a multifunctional protein? *Am. J. Physiol. Cell Physiol.* 288: C975-C981.
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Conclusions: PSMA is not expressed at high levels in normal prostatic epithelial cells. However, expression is significantly elevated during the progression of cancer, with the greatest levels observed in high-grade tumors, metastatic lesions, and androgen independent disease. Thus, increased expression of PSMA directly correlates with the aggressiveness of the disease. Low expression of PSMA *per se* might not constitute a factor leading to aneuploidy, since low levels of PSMA are found in benign prostate tumors and some normal non-prostatic tissues. Our data strongly suggest that upon increased expression of PSMA, the ability of its cytoplasmic tail to associate with the APC complex dysregulates APC function leading to aneuploidy in cancer cells. Thus, PSMA function might not be required for normal cell cycle progression but its elevated expression and mislocalization at the spindle pole region and association with APC is an *accidental and pathological consequence* in cancer cells leading to aneuploidy. An understanding of the putative oncogenic role of PSMA could have major implications for the management and therapy of prostate cancer. Targeted therapies against PSMA expressing prostate cancer cells are already being evaluated for their potential to treat

prostate cancer. Since PSMA expression is closely associated with aneuploidy, such anti-PSMA therapeutic strategies might have the advantage of specifically targeting the most aggressive and aneuploid cells.

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