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14. ABSTRACT
During the 2008-12 funding period we have made significant progress on our project. We have published 22 papers and 28 abstracts. In this study, we revealed functional aspects of role of β-catenin signaling through PKD1 in prostate cancer. We have investigated novel mechanism of the suppression of β-catenin transcriptional activity by PKD1 and its modulators such as Bryostatin 1 and Curcumin. Our studies also suggest that, Bryostatin-1, a PKD1 modulator, efficiently attenuates β-catenin transcriptional activity and induces chemo-sensitization in prostate cancer cells. We identified the PKD1 domains involved in interaction and modulation of β-catenin activity. We, in collaboration with Dr. Chauhan, have also developed a xenograft mouse model system with the highly metastatic C4-2 cell lines. Our experiments suggest that PKD1 expression inhibited prostate tumor growth in xenograft mouse model. In addition, we have investigated the effects of PKD1 overexpression on gene transcription using PCR microarray techniques. To determine the correlation of PKD1 expression with prostate cancer progression, we have processed 60 prostate cancer samples for PKD1 and β-catenin staining. We have also revealed a novel curcumin pre-treatment strategy for inducing chemo/radio-sensitization of cancer cells. Based on clinical implications of this novel strategy, this study was published and selected for press release. All related studies have been presented at IMPACT and Annual American Association for Cancer Research (AACR) meetings. In addition to publications, we have obtained collaborative grants from the state (Governor's 2010 initiative), NIH (NCI RO1, NCRR COBRE) and pharmaceutical industries (Merck Pharmaceuticals, Investigator Initiated Grant).

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Final Progress Report 2008-2012
New investigator Award Proposal

Modulation of β-catenin activity with PKD1 in Prostate Cancer
(PC 073643)

(PI: MEENA JAGGI)

INTRODUCTION

Understanding the basic biology of prostate cancer will provide us with additional critical information necessary to improve existing treatments or to find newer treatments for patients suffering from prostate cancer. While several genetic alterations play specific functions in a cell, some of these genes can have multiple functions. One such gene encodes a protein called β-catenin, which plays a dual role in cancer cells by: a) playing a role in cellular adhesion via another protein called E-cadherin, and b) playing a role in causing cellular division through a set of proteins which cause cells to divide abnormally. In addition to these proteins which β-catenin is already known to associate with, we have discovered a new interaction with a protein called protein kinase D1 (PKD1) in prostate cancer cells. Unraveling this complex interaction of β-catenin with PKD1 in prostate cancer cells may hold the key to understanding the role of a single important protein in causing unregulated cellular division and loss of cellular adhesion – the two fundamental hallmarks of a cancer cell. We have previously made two important discoveries in this field: a) PKD1 levels are lower in advanced prostate cancer which are associated with more aggressive types of cancer, and b) PKD1 interacts with another important protein in cancer cells, β-catenin. These preliminary discoveries in prostate cancer have led us to put forth the current proposal. Our major objective in this proposal is to understand the consequences of binding of PKD1 to β-catenin in tumor development and to study the exact alteration of these proteins in human prostate cancer tissues.

Understanding the details of how cancer causing proteins communicate with each other in a cell will help us intervene in the disease process more effectively. To this end, we propose to study the effect of β-catenin and PKD1 interaction on the cancer cell. We plan to achieve these goals by increasing PKD1 activity in the cell by use of a drug called Bryostatin1, which has already been used in clinical trials in various types of cancers. During 2008-09 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). In brief, we investigated the effect of bryostatin on PKD1 expression, β-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mechanism to improve efficacy of Bryostatin 1 in clinical setting.

During the 2008-12 funding period we have made significant progress on our project. We have published 22 papers and 28 abstracts. In this study, we revealed functional aspects of role of β-catenin signaling through PKD1 in prostate cancer. We have investigated novel mechanism of the suppression of β-catenin transcriptional activity by PKD1 and its modulators such as Bryostatin 1 and Curcumin. We identified the PKD1 domains involved in interaction and modulation of β-catenin activity. We have generated retroviral constructs for the transfection of PKD1 mutants in prostate cancer cells. We, in collaboration with Dr. Chauhan, have also developed a xenograft mouse model system with the highly metastatic C4-2 cell lines. Our results are very encouraging and we have been able to generate highly vascularized tumors in nude mice. Our experiments suggest that PKD1 expression inhibited prostate tumor growth in xenograft mouse model. In addition, we have investigated the effects of PKD1

overexpression on gene transcription using PCR microarray techniques. To determine the correlation of PKD1 expression with prostate cancer progression, we have collected 60 prostate cancer samples and performed PKD1 and β-catenin staining. Our studies also suggest that, Bryostatin-1, a PKD1 modulator, efficiently attenuates β-catenin transcriptional activity and induces chemo-sensitization in prostate cancer cells. In addition, we have also discovered a new method of PKD1 activation suppression of β-catenin transcriptional activity and enhancement of β-catenin membrane localization by a natural compound curcumin. We have also revealed a novel curcumin pre-treatment strategy for inducing chemo/radio-sensitization of cancer cells. Based on clinical implications of this novel strategy, this study was published and selected for press release. All related studies have been presented at IMPACT and Annual American Association for Cancer Research (AACR) meetings. In addition to publications, we have obtained collaborative grants from the state (Governor's 2010 initiative), NIH (NCI RO1, NCRR COBRE) and pharmaceutical industries (Merck Pharmaceuticals, Investigator Initiated Grant).

BODY - Aim 1: Molecular Nature of PKD1 and β-catenin Interaction

Generation of retroviral PKD1 mutants to determine domain of PKD1 that are required for interaction with β-catenin:

We tried to generate stable clones of C4-2 cells with green fluorescent protein (GFP) fused PKD1 constructs in order to study the physiological significance of PKD1 in prostate cancer. We tried to isolate multiple colonies using standard techniques. However, repeated efforts at isolating colonies failed, since the colonies died very soon in isolation. We attempted transfection using multiple cationic reagents like Lipofectamine, Lipofectamine 2000, FuGENE HD, PolyFect and Effectene to raise stable transfection with little success. Among these various transfection reagents, we found FuGENE HD to produce maximal transfection efficiency. However because of low transfection efficiency, we have moved these PKD1 mutant constructs in retroviral system.

Generation of retroviral infected PKD1 mutant expressing C4-2 cells:

The DNA constructs (2μg) of PKD1 mutants tagged with GFP and cloned in pEGFP vector were obtained from our collaborator Dr. Angelika Hausser, University of Stuttgart, Germany. C4-2 prostate cancer cells were infected PKD1 mutant containing retrovirus. The cells were observed for GFP under a fluorescent microscope (Figure 1). After two days of incubation at 37°C/5%CO_2, the cells were trypsinized and used for further experiments. The retroviral constructs have shown higher transfection
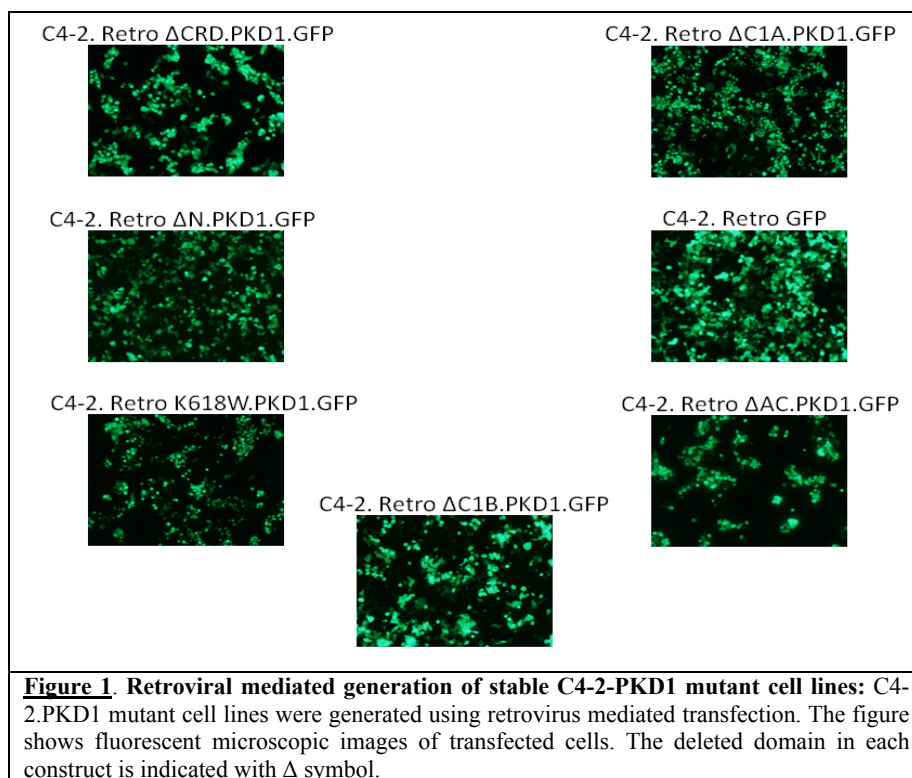

Figure 1. **Retroviral mediated generation of stable C4-2-PKD1 mutant cell lines:** C4-2.PKD1 mutant cell lines were generated using retrovirus mediated transfection. The figure shows fluorescent microscopic images of transfected cells. The deleted domain in each construct is indicated with Δ symbol.

efficiency in prostate cancer cells compared to conventional transfection methods. The stably transfected C4-2 cell lines were checked for growth, colony formation ability and motility characteristics.

Effect of PKD1mutant constructs on cell motility:
The effect of PKD1-GFP mutant constructs on cell motility was assayed using Boyden's chamber. In short, transfected cells were loaded into the chamber in media containing 1% FBS. A chemotactic gradient of 10% FBS was applied to by incubating the chamber in a 6 well plate containing media+10% FBS. Following, incubation of the plates for 24h, the motile cells on the membrane were fixed in methanol for 5min, stained with crystal violet for 30min, the membrane dried and mounted onto pre-labeled slides. Ten independent images were taken and the motile cells that migrated across the membrane were counted. Our results indicate major difference in

the migratory behavior of ΔN, ΔAP and PKD1.KD mutants, indicating the importance of these domains in cell motility (Figure 4). In the next cycle, we plan to generate stable transfects using retroviral system. Once the stable transfects are isolated, the interaction of PKD1-GFP mutant constructs with β-catenin will be analyzed by immunoprecipitation assays, using β-catenin and GFP specific antibodies as described previously. Additionally, the effect of the different constructs on β-catenin transcription will be analyzed.

Figure 2. Effect of mutant PKD1 overexpression on cell motility. Boyden's chamber was used to probe the effect of mutant PKD1 expression on C4-2 cell's motility. The cells were plated in 1% FBS onto the chaber and a chemotactic gradient of 10% FBS was was applied for 24h to facilitate cell motility. The cells were fixed, stained and images of ten random areas were captured and the number of cells was counted.

Since stable clones of PKD1 mutants generated in C4-2 cells using retroviral mediated transfection continued to lose mutant PKD1-GFP expression with subsequent passage of the cells, we used transiently transfected C4-2 cells for further experiments. In these experiments we investigated the effects of mutant PKD1 on subcellular localization of β-catenin and on nuclear β-catenin transcription activity.

Effect of PKD1 domain deletion on subcellular localization of PKD: The effect of domain deletion on the subcellular localization of PKD1 in C4-2 cells was analyzed by transiently transfecting PKD1-GFP mutant constructs into C4-2 cells. C4-2 cells were seeded overnight on glass slides and transiently transfected with various PKD1-GFP mutants constructs using FuGENE. The cells were incubated for 2 days at 37°C/5%CO$_2$. After 48h, the cells were observed for GFP fluorescence and phase contrast images of the transfected cells were captured (Figure 3). Our results indicate that the specific domains of PKD1 play essential role in subcellular localization of the protein. In C4-2 cells, our results reveal that the AC domain (acidic domain) is essential for nuclear import of PKD1, while the C1a domain of PKD1 is essential for nuclear export of
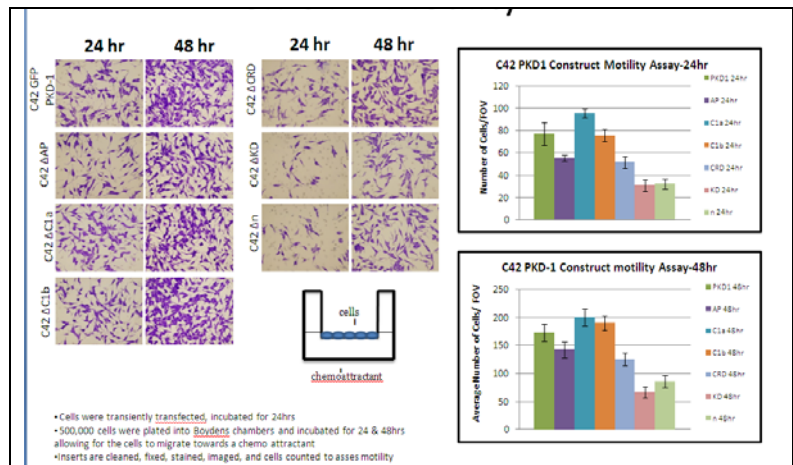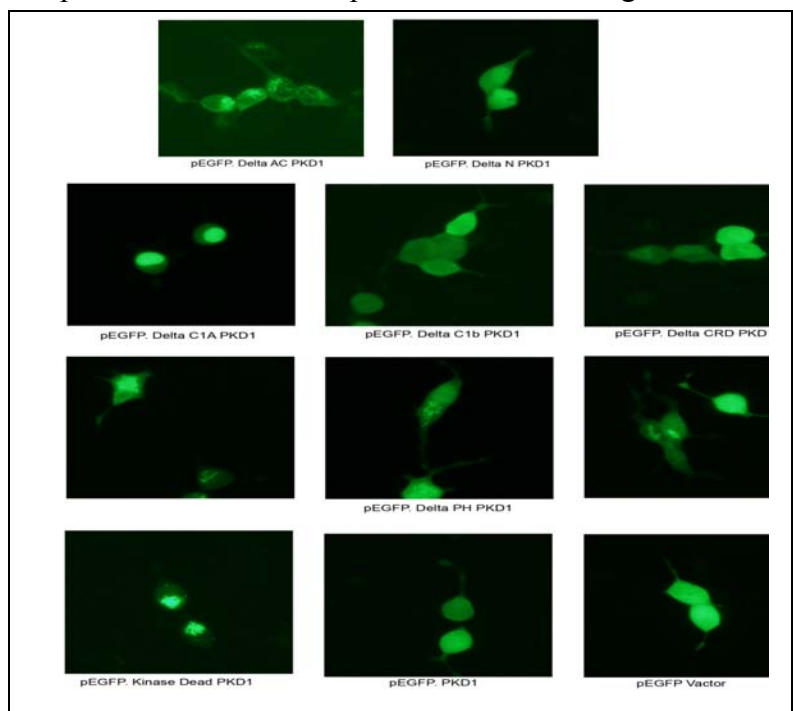

Figure 3. Subcellular localization of mutant PKD1 overexpression. C4-2 prostate cancer cells transfected with various mutants of PKD1 showed varying subcellular localization. The phase contrast images of the transfected cells are shown in the image above. While many of the mutant domains showed nuclear and cytoplasmic expression, similar to full length PKD1, the AC domain deletion (deltaAC.PKD1) showed predominantly cytoplasmic localization, while the C1A domain deletion (deltaC1A) showed predominantly nuclear localization within C4-2 cells, indicating the importance of these domain in nuclear import and nuclear export, respectively

PKD1 in cells.

Effect of PKD1 domain deletion on β-catenin transcription activity: We have previously demonstrated that PKD1 inhibits the transcription activity of β-catenin by interacting and phosphorylating the protein and decreasing the nuclear localization of β-catenin. In order to determine the domains of PKD1 that are essential for the inhibition of the transcription activity of β-catenin activity, we overexpressed PKD1-GFP mutant constructs in C4-2 cells and used reporter luciferase assay to estimate β-catenin transcription activity (Figure 4). C4-2 cells were transfected with mutant PKD1 and TCF luciferase reporter construct containing either TCF promoter binding sites (pTOP-FLASH) or mutant TCF promoter binding sites (pFOP-FLASH) along with internal control plasmid containing *Renilla* luciferase gene (pRL-TK). After 48h, the cells were assayed for β-catenin/TCF promoter activity and the results were expressed as a ratio of pTOP-FLASH/pFOP-FLASH activity, after normalizing to the transfection control (*Renilla* luciferase activity). Our results indicate that deletion of the cystein rich domains (CRD) or the deletion of pleckstrin homology domain (ΔPH) results in increased β-catenin activity compared to control cells, implicating an important role for these domains in interacting with β-catenin and inhibiting its nuclear transcription

Figure 4: **Effect of mutant PKD1 on inhibition of β-catenin /TCF transcription activity in C4-2 prostate cancer cells.** The β-catenin activity was measured using reporter luciferase assay. C4-2 cells were transfected with mutant PKD1 and TCF luciferase reporter construct containing either TCF promoter binding sites (pTOP-FLASH) or mutant TCF promoter binding sites (pFOP-FLASH) along with internal control plasmid containing *Renilla* luciferase gene (pRL-TK). After 48h, the cells were lysed and assayed for β-catenin/TCF promoter activity using Dual-Glo luciferase agent. The β-catenin transcription activity was first normalized to *Renilla* luciferase activity, and expressed as a ratio of pTOP-FLASH/pFOP-FLASH activity. The deletion of both cystein rich domains (ΔCRD) and the deletion of pleckstrin homology domain (ΔPH) resulted in increased β-catenin activity compared to control cells, implicating the important role these domains play in interacting with β-catenin and inhibiting its nuclear transcription activity. The domain C1A, which is predominantly present in the nucleus consistently showed lower β-catenin transcription activity than control PKD1 overexpressing cells, revealing the effect of presence of nuclear PKD1 in decreasing β-catenin activity.

activity. Interestingly the deletion of domain C1A, which resulted in the predominant presence of the PKD mutant in the nucleus consistently showed slightly lower β-catenin transcription activity than control cells overexpressing PKD1, thus revealing the effect of presence of nuclear PKD1 in decreasing β-catenin activity.
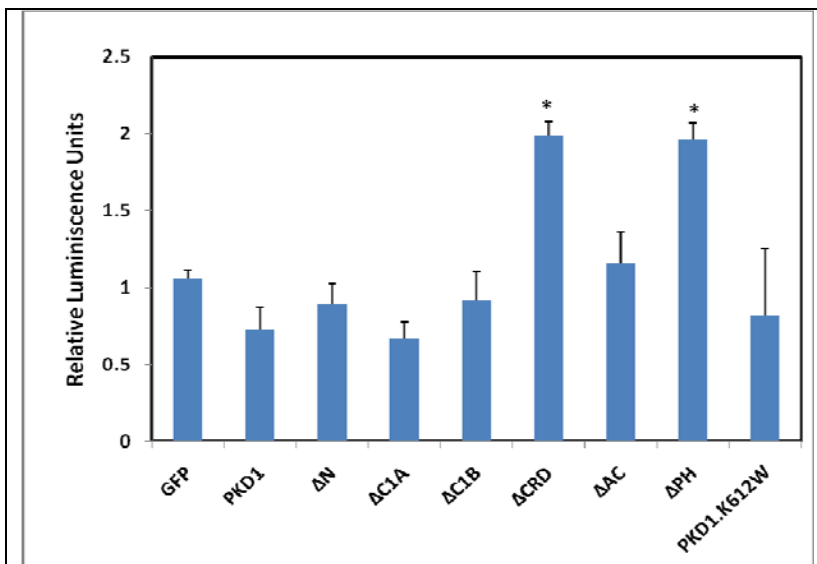
Effect of PKD1 domain deletion on subcellular localization of β-catenin: The effect of PKD1 domain deletion on the subcellular localization of β-catenin in C4-2 cells was analyzed by transiently transfecting PKD1-GFP mutant constructs into C4-2 cells. C4-2 cells were seeded overnight on glass slides and transiently transfected with various PKD1-GFP mutants constructs

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using FuGENE. The cells were incubated for 2 days at 37°C/5%CO_2. After 48h, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton-X-100, blocked with 10% normal growth serum. The cells were stained using anti-β-catenin antibody and cy3 conjugated secondary antibody and observed for immunofluoresnce using laser scanning confocal microscope (Figure 5). Our results indicate that the specific domains of PKD1 play essential role in subcellular localization of the protein.
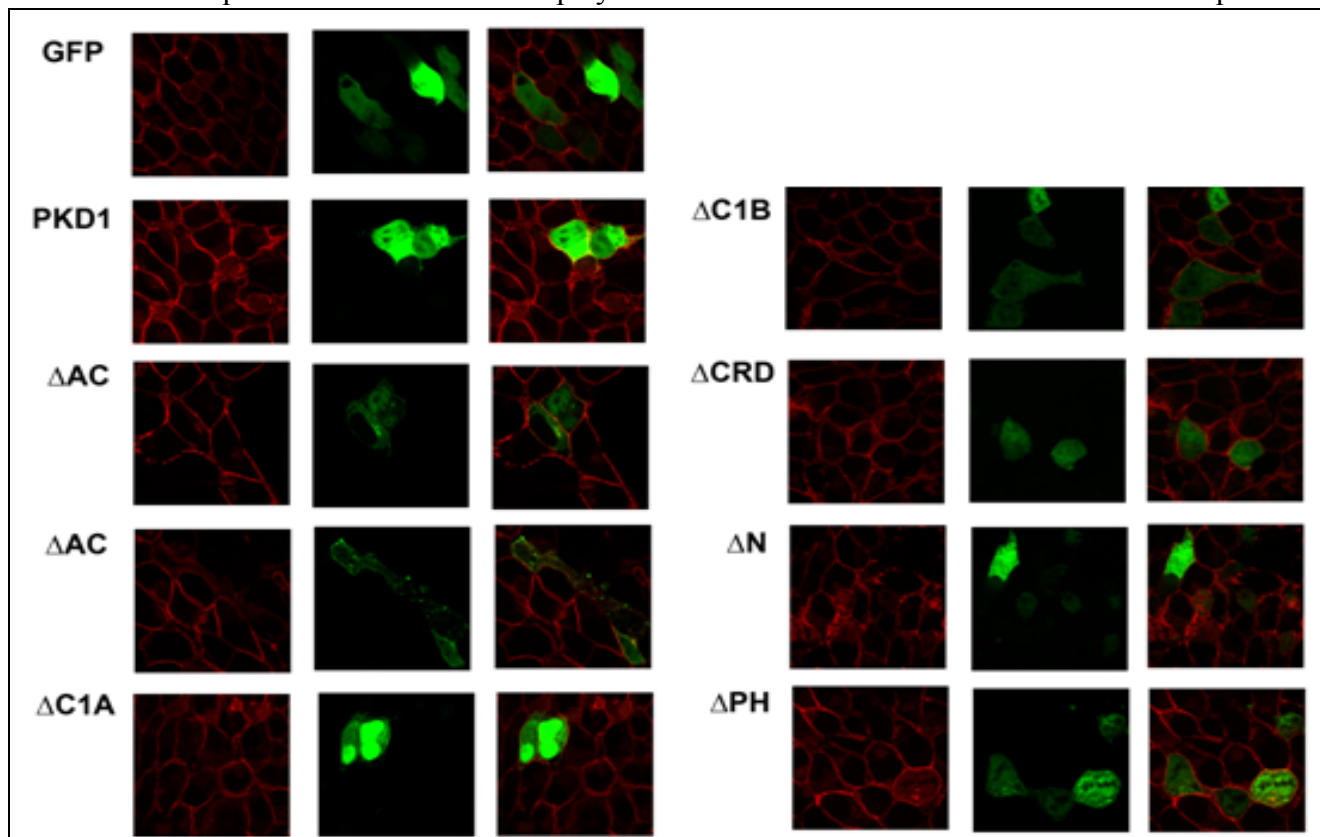

Figure 5: **Effect of mutant PKD1 on subcellular localization of β-catenin in C4-2 prostate cancer cells.** The C4-2 cells were transfected with mutant PKD1. After 48h, the cells were fixed and immunostained for β-catenin. While the deletion of most of the domains of PKD1 showed no change in β-catenin expression/localization on the membrane, the deletion of pleckstrin homology domain (ΔPH) resulted in enhanced cytoplasmic β-catenin compared to control cells, implying an important role for this domain in β-catenin subcellular localization.

Our results reveal that the deletion of the PH domain appeared to enhance the levels of cytoplasmic β-catenin within the cells (Figure 5), without changing the levels of β-catenin on the membrane. This correlates with higher activity of β-catenin transcription activity observed upon overexpression of PH domain deletion in C4-2 cells, implicating an important role for this domain in β-catenin interaction and subcellular localization.

Aim 2: To Demonstrate that Activation of PKD1 by Bryostatin 1 Influences the Cellular Phenotype in Prostate Cancer.

Bryostatin-1: Bryostatin-1, a natural macrocyclic lactone produced by a marine bryozoan, has shown potent anti-cancer properties. It is currently in clinical trials to assess its efficacy as an anti-cancer agent. Bryostatin-1 activates Protein Kinase C (PKC) and Protein Kinase D (PKD) pathways by binding to the phorbol ester binding cystein rich domains and induces effects that are quite different from phorbol ester binding, including biphasic dose response relationship, delayed kinetics and an ability to inhibit phorbol

ester mediated response. In this grant we propose to investigate the effect of Bryostatin-1 on prevention and treatment of prostate cancer. We believe that Bryostatin-1 might increase sensitization of the prostate cells to chemotherapeutic agents via altering the β-catenin axis (Figure 6). This approach will improve therapeutic efficacy of chemotherapeutic drugs for prostate cancer treatment.

Figure 6: **Schematic representation for molecular mechanisms of Bryosatin 1 in prostate cancer cell.** Bryostatin 1 (shown in inset) activates PKD1. Active PKD1 phosphorylates β-catenin and thus modulates the transcription and cell-cell adhesion functions of β-catenin. In addition, PKD1 also modulates AR function and transcription activity. PKD1 activation prevents cell migration and invasion through the phosphorylation and inhibition of proteins involved in actin remodeling and cell motility (Cortactin and slingshotphosphatase (SSH1L)). Thus the use of Bryostatin 1 will inhibit tumor growth, metastasis and chemo-sensitize cancer cells to anti-cancer drugs by activating PKD1.

Time and dose dependent activation of PKD1 by Bryostatin 1: Treatment of the prostate cancer cells stably transfected with PKD1-GFP with increasing concentrations (10-30 nM) of Bryostatin 1 for 3 h demonstrated increased transphosphorylation of ser738 and ser742 and autophosphorylation of ser910 residues of PKD1 (Figure 7 A and B). The activation of PKD1 is phosphorylation-dependent, and serine738 and 742 residues in human PKD1 (corresponding to serine744 and 748 in mouse) have been identified as crucial phosphorylation sites. These serine residues are located in the activation loop of the PKD1 catalytic domain. The C-terminal serine916 residue has been identified as an autophosphorylation site in PKD1 (1). Phosphorylation of these serine residues affects PKD1 activity and plays a role in modulation of PKD1 function *in vivo*. In order to exclude cell line specific effects we also confirmed that Bryostatin 1 activated and is associated with membrane translocation of PKD1 in androgen dependent LNCaP cells (data not shown).

Bryostatin-1 induces apoptosis in prostate cancer cells: The ability of Bryostatin-1 to induce apoptotic cell death in prostate cancer cells was examined by TUNEL staining followed
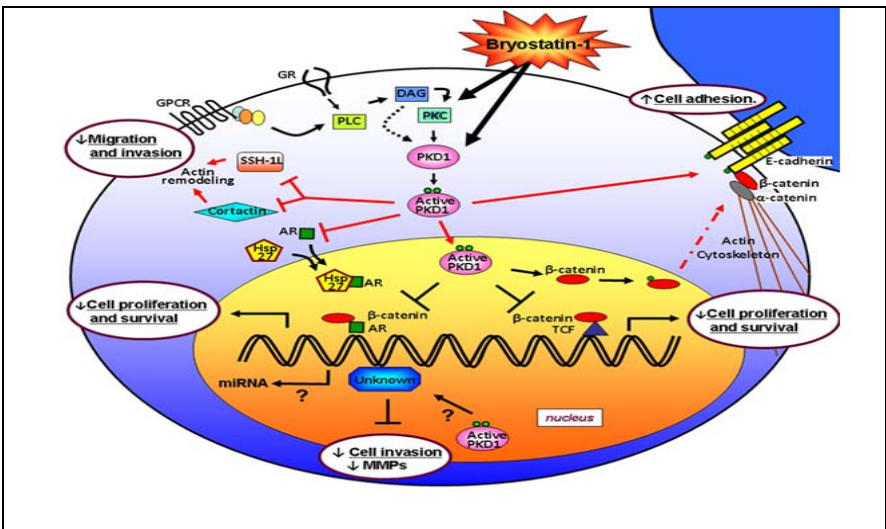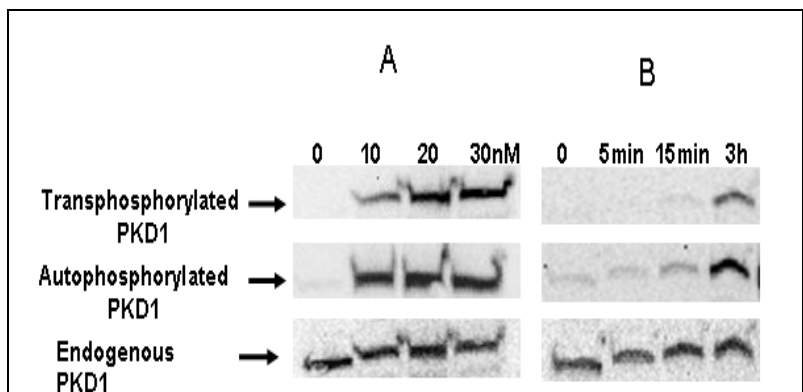

Figure 7: Time dependent phosphorylation of PKD1 in Bryostatin 1 treated cells, **A**, C4-2 cells stably transfected with GFP fused PKD1 (C4-2-PKD1-GFP) were treated with 0, 10, 20 and 30 nM Bryostatin 1. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note optimum phosphorylation at 10 nM. **B**, C4-2 -PKD1-GFP cells were treated with 10 nM Bryostatin 1 for 0-3 h. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note maximum phosphorylation at 3 h.

by flow cytometry. As shown in the figure, longer treatment of C4-2 cells with Bryostatin-1 resulted in higher levels of apoptotic cell death (Figure 8).

Effect of activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization

Subcellular localization of PKD1, E-cadherin and β-catenin in Bryostatin 1 activated C4-2-PKD1-GFP cells was analyzed by confocal microscopy. To examine PKD1 specific changes in subcellular localization of E-cadherin and β-catenin, we compared E-cadherin and β-catenin localization in Bryostatin 1 activated C4-2-GFP cells and C4-2-PKD1-GFP cells. In vector transfected C4-2 cells we did not detect any change in E-cadherin or β-catenin localization

Figure 8: Bryostatin-1 treatment induces apoptotic cell death in prostate cancer cells. C42 cells were treated with 10nM Bryostatin-1 for varying time points. The cells were washed, TUNEL labeled and analyzed by flow cytometry. Representative diagrams of each treatment is depicted on the left. Quantitative analysis of positive TUNEL labeled cells against their appropriate DMSO control is shown as a histogram.

after Bryostatin 1 activation (Figure 9). Our immunofluorescence study clearly revealed perinuclear and membrane localization of PKD1-GFP upon activation by Bryostatin 1 (Figure 6). The most striking observation was the colocalization of E-cadherin and β-catenin with PKD1-GFP in Bryostatin 1 activated C4-2-PKD1-GFP cells at perinuclear areas in addition to cell membranes (Figure 9, lane2, arrows). After 24 h of Bryostatin 1 treatment, strong membrane staining of E-cadherin/β-catenin and some perinuclear staining was also noticed (Figure 9, lane3). While the C4-2-GFP cells do not over-express PKD1-GFP (Figure 9), they do not show perinuclear localization of E-cadherin/β-catenin. This observation confirms that E-cadherin/β-catenin subcellular distribution is specifically mediated by PKD1 activation and not by other kinases activated by Bryostatin 1.

Bryostatin treatment decreases β-catenin transcriptional activity

We investigated the effect of PKD1 activation on β-catenin mediated

Figure 9: Activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization. Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells were stained for E-cadherin and β-catenin and analyzed by LSM. C4-2-GFP (**3.1**) and C4-2-PKD1-GFP (**3.2** and **3.3**) cells show differences in subcellular localization of E-cadherin (red) and β-catenin (blue). E-cadherin and β-catenin colocalizes with PKD1-GFP at perinuclear areas in addition to cell membranes (**3.2** arrows) in Bryostatin 1 activated C4-2-PKD1-GFP cells (**3.2** and **3.3**) but not in C4-2-GFP cells.

transcription activity and proliferation in prostate cancer cells. To investigate the effect of PKD1 on β-catenin mediated transcription activation of TCF, we transfected plasmids containing a wild type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla*
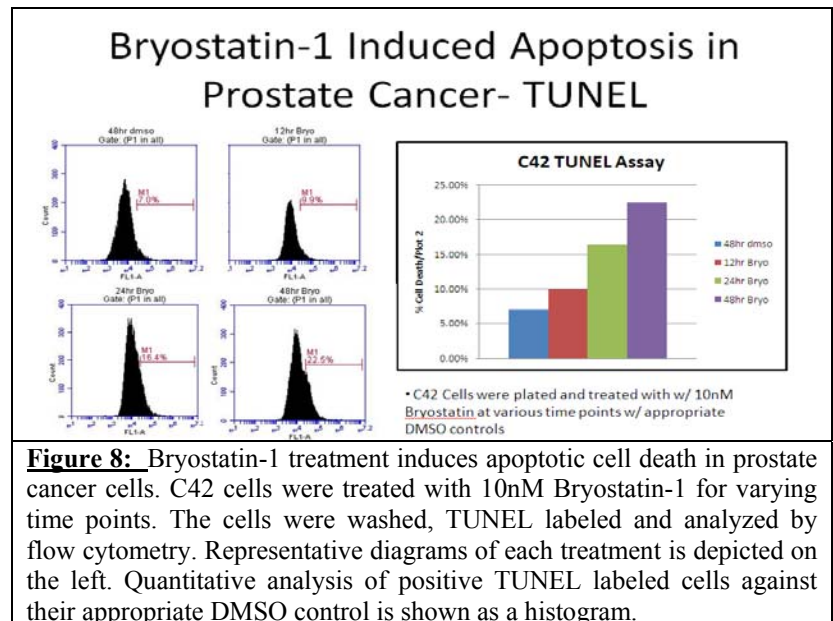
luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter (DLR) Assay System. After normalizing the firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. Bryostatin 1 activation in C4-2-PKD1-GFP cells led to a significant reduction (p value=0.019) in β-catenin reporter activity (Figure 10).

Figure 10: Bryostatin treatment decreases in β-catenin transcription activity, cell proliferation and increases cell-cell adhesion. **1)** Effect of Bryostatin treatment on β-catenin transcription activity**.** C4-2-PKD1-GFP cells were transfected with TOP or FOPFlash firefly luciferase reporter constructs and treated with 10 nM Bryostatin 1 or vehicle only. Data are expressed as fold induction normalized to the cotransfected Renilla luciferase-encoding pRL-TK plasmid. Treatment of C4-2-PKD1-GFP cells significantly decreased β-catenin mediated transcription activity as compared to vehicle only treated cells (p = 0.019). Error bars indicate standard error. **2)** Proliferation assay of Bryostatin 1 treated C4-2-PKD1-GFP cells. Proliferation assay after 48 h of 10 nM Bryostatin 1 treatment (see Materials and Methods) expressed as percent of control cells treated with vehicle only (p=0.001). Error bars indicate standard error. **3)** Bryostatin treatment increases cellular aggregation. Bryostatin 1 treated C4-2 -PKD1-GFP cells showed increased cellular aggregation (**B**) compared to vehicle only treated cells (**A**).

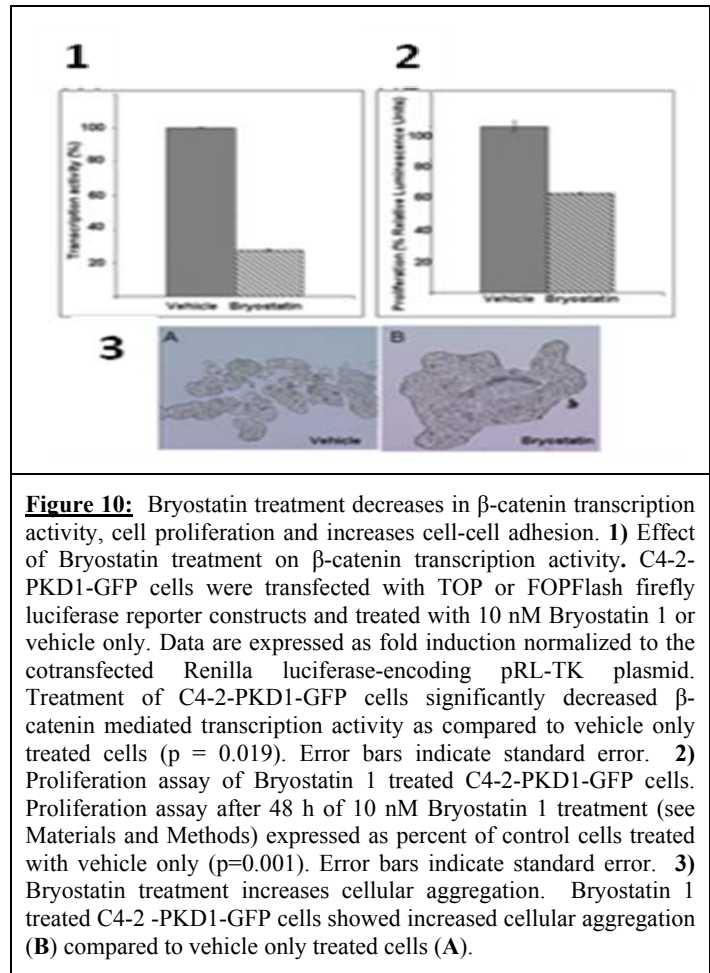### *Effect of activation of PKD1 by Bryostatin 1 proliferation*

The cell proliferation ability of Bryostatin 1 activated C4-2-PKD1-GFP cells was assayed by CellTiter-Glo. Bryostatin 1 activated C4-2-PKD1-GFP cells showed a 40% decrease in cell proliferation as compared to DMSO treated cells (Figure 10.2). A mixed ANOVA model will be used to compare the cell lines and doses. P-value < 0.05 was considered significant.

Effect of activation of PKD1 by Bryostatin1 on cellular aggregation

PKD1 is also known to be involved with altered cellular aggregation, which is required for a cancer cell to successfully complete the metastatic cascade (2). Because we have demonstrated that PKD1 activation with Bryostatin 1 is involved in trafficking of β-catenin, we sought to determine the effect of Bryostatin 1 activation on cellular aggregation in C4-2 cells over-expressing PKD1. Aggregation assays were performed on C4-2 cells expressing PKD1-GFP as described previously. Our experiments demonstrated increased cellular aggregation in Bryostatin 1 treated C4-2-PKD1-GFP cells compared to vehicle only treated cells (Figure 10.3).

Effect of PKD1 inhibition on β-catenin subcellular localization

To further demonstrate the specific function of PKD1 in mediating the subcellular redistribution of β-catenin, PKD1 expression was inhibited 90% by using small interfering RNA (siRNA) in C4-2-PKD1-GFP cells activated with Bryostatin 1. After inhibition of PKD1 expression, cells transfected with non-targeted siRNA were activated with Bryostatin 1, stained for β-catenin and trans Golgi network (TGN) specific (p230) antibody and analyzed by confocal microscopy. Non-targeted siRNA transfected and Bryostatin 1 activated cells showed perinuclear localization of PKD1 and β-catenin. Merging of PKD1, β-catenin and p230 images from these cells shows colocalization of these three proteins at the perinuclear region and colocalization of PKD1 and β-catenin at the cell junction. Immunofluorescence images of PKD1 siRNA transfected C4-2-PKD1-GFP cells shows inhibition of PKD1-GFP (Figure 8.E),

reduced staining of β-catenin at the membrane and lack of β-catenin localization at TGN (Figure11 F). Merging of PKD1, β-catenin and p230 images taken at the same confocal level in PKD1 siRNA transfected C4-2-PKD1-GFP cells (Figure11 H) do not show colocalization of the proteins at the perinuclear region. These results suggest that β-catenin subcellular localization is modulated predominantly by activated PKD1 and not by other kinases (PKC isoforms) activated by Bryostatin 1. Interestingly, down regulation of PKD1 by RNAi decreased β-catenin expression at the plasma membrane (Figure11 F), which further suggests that PKD1 plays a major role in membrane transport of β-catenin. We have previously published that down regulation of PKD1 in fact increases total cellular β-catenin. This provides further corroborative evidence for role of PKD1 in membrane trafficking of β-catenin because membrane β-catenin is decreased in spite of increased total levels of cellular β-catenin when PKD1 expression is reduced (3). However, the exact mechanism of regulation of β-catenin expression by PKD1 remains to be investigated.
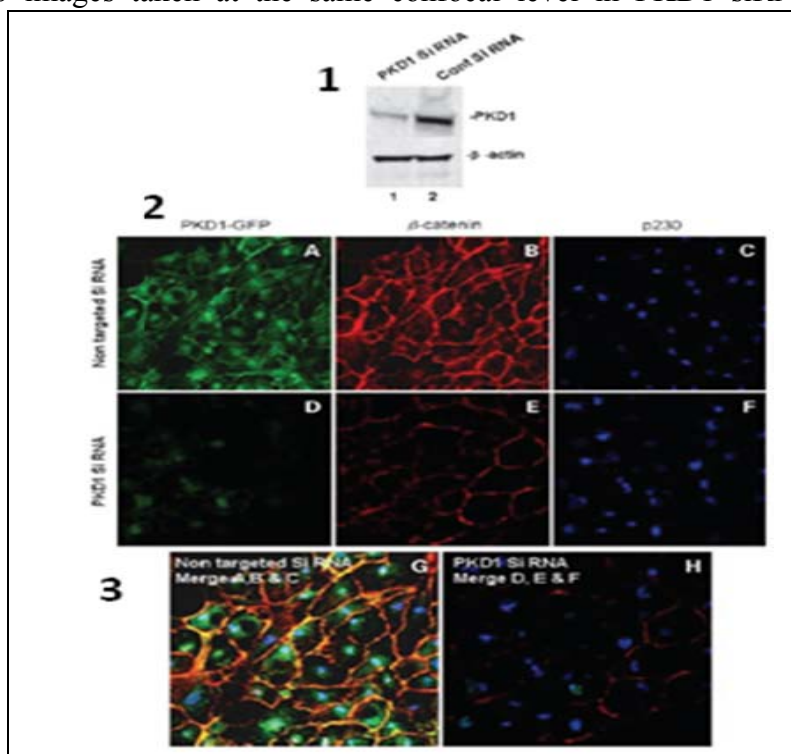

Figure 11: Activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization. Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells were stained for E-cadherin and β-catenin and analyzed by LSM. C4-2-GFP (**1**) and C4-2-PKD1-GFP (**2** and **3**) cells show differences in subcellular localization of E-cadherin (red) and β-catenin (blue). E-cadherin and β-catenin colocalizes with PKD1-GFP at perinuclear areas in addition to cell membranes (**2** arrows) in Bryostatin 1 activated C4-2-PKD1-GFP cells (**2** and **3**) but not in C4-2-GFP cells.

Effect of PKD1 expression on apoptosis: It has been shown that nuclear β-catenin forms a complex with TCF/LEF transcription factors and that this complex transactivates downstream targets such as *c-myc* and *cyclin D1*. These proteins have been implicated in cell cycle regulation. PKD1 overexpression in C4-2 cells decreases β-catenin/TCF transcription activity. Over expression of PKD1 causes increased cellular aggregation and decreased motility in prostate cancer cells. In order to determine the effect of PKD1 on cell cycle distribution Cell cycle distribution was assessed using BD FACSVantage SE pulse processing plus program for analysis of DNA content. C4-2-GFP vector and C4-2-GFP-PKD1 cells were stained with propidium iodide (PI). Each value represents percentage of cells in the noted cell cycle phase. Experiments were repeated three times and representative histograms are shown. The results show that PKD1overexpression resulted increase of cells in G1 phase and concomitant decrease in cell in G 2 phase, indicate
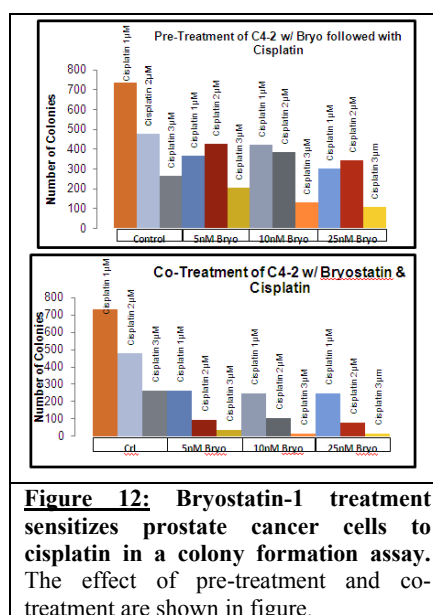

Figure 12: Bryostatin-1 treatment sensitizes prostate cancer cells to cisplatin in a colony formation assay. The effect of pre-treatment and co-treatment are shown in figure.
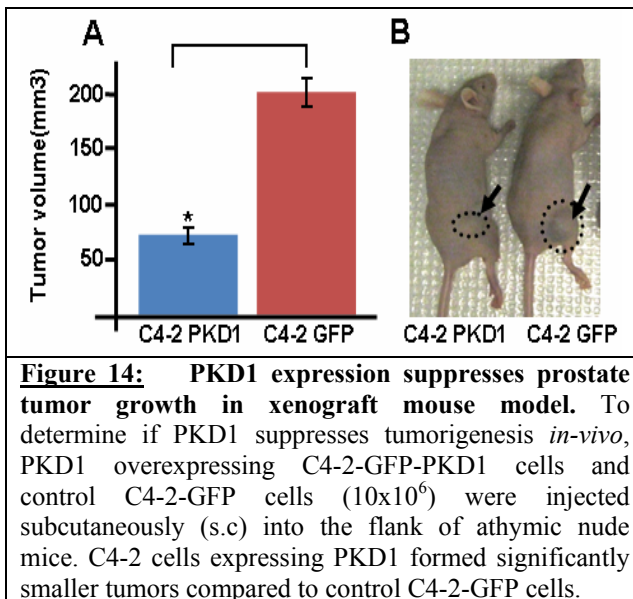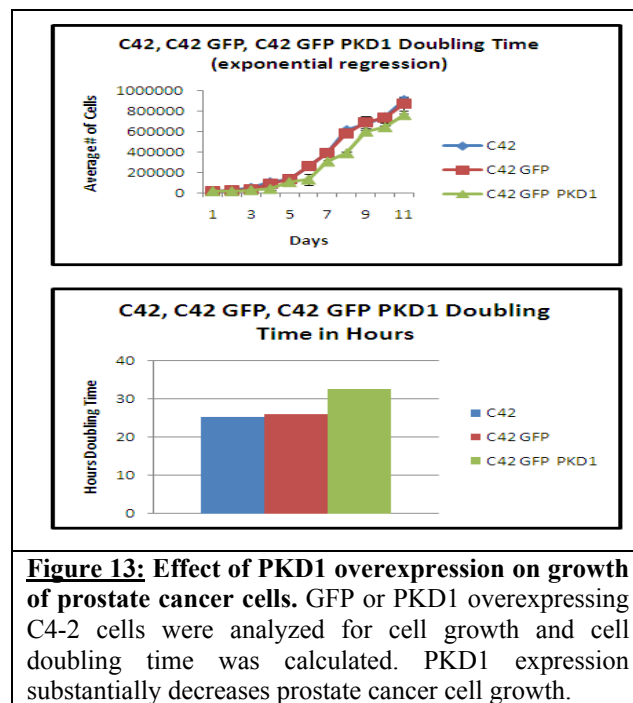
cell cycle arrest in G1 phase.

Bryostatin 1 induces chemo-sensitization in prostate cancer cells: Colony formation assay was used to assess the chemo-sensitizing ability of Bryostatin-1 in prostate cancer cell line model. Briefly, 600 cells were plated onto 60mm dishes. The cells were either pretreated or co-treated with Bryostatin-1 in combination with varying concentrations of cisplatin. Clearly, in this assay, we can see a chemo-sensitizing effect of Bryostatin-1 (Figure 12).

The inhibitory effect of PKD1 expression on tumorigenicityof prostate cancer cells:
To determine the ability of PKD1 to alter prostate cancer cell growth, cell proliferation and cell doubling time were analyzed. Briefly, 0.5×10^5 cells were plated in 35 mm dishes and analyzed for cell growth and proliferation by cell counting method (Figure 13). In addition to *in vitro* studies, we performed *in vivo* tumorigenicity assay using prostate cancer xenograft mouse model. Male athymic Swiss Webster nude mice, aged 6 to 8 weeks were obtained from Jackson laboratories. Prostate cancer cells with either vector or over-expressing PKD1 were used for tumor development (Figure 14). The tumor growth was monitored bi-weekly for fifty days. At the end of the experiment, all tumors were dissected, measured, weighed and specimen saved at $-80^{\circ}C$. PKD1 expression significantly reduced tumor volume (Figure 14). These data suggest a tumor suppressor function of PKD1 in prostate cancer.

In addition, we investigated the effect of PKD1 overexpression in a cancer cell line model on gene expression within these cells. Our results show modulation of a number of different genes, five of the highest modulated RNAs are shown (Figure. 15). It is very likely that similar sets of genes are modulated by PKD1 in prostate cancer cells. We would explore this in prostate cancer *in vitro* and *in vivo* model.

Figure 13: Effect of PKD1 overexpression on growth of prostate cancer cells. GFP or PKD1 overexpressing C4-2 cells were analyzed for cell growth and cell doubling time was calculated. PKD1 expression substantially decreases prostate cancer cell growth.

Figure 14: **PKD1 expression suppresses prostate tumor growth in xenograft mouse model.** To determine if PKD1 suppresses tumorigenesis *in-vivo*, PKD1 overexpressing C4-2-GFP-PKD1 cells and control C4-2-GFP cells (10×10^6) were injected subcutaneously (s.c) into the flank of athymic nude mice. C4-2 cells expressing PKD1 formed significantly smaller tumors compared to control C4-2-GFP cells.

Aim 3: To evaluate the expression of β-catenin and PKD1 proteins in progressive human prostate cancer: Our preliminary IHC studies demonstrate that, in addition to downregulation of β-catenin in human prostate cancer compared to benign glands, there is a decreased expression of β-catenin in prostatic intraepithelial neoplasia in a small subset of our study patients and an increased nuclear staining in high Gleason grade prostate cancer. This suggests an involvement of β-catenin and the Wnt signaling pathway

in prostate cancer. To determine a correlation between PKD1 and β-catenin expression, we evaluated the expression of these proteins in human prostate cancer tissues utilizing immunohistochemistry (IHC). The cases and controls, matched one-to-one on the basis of patient age and year of biopsy, were included in this study. We have successfully collected 60 prostate cancer patient samples for analysis by IHC. We have stained 60 human prostate cancer tissues for PKD1 and β-catenin protein expression with the Mach4 kit (Figure 16). The stained tissues were evaluated for intensity of staining and subcellular localization of PKD1 and β-catenin by a pathologist (Dr. M.R.D. Koch) and the mean composite score was calculated. The result was graphed (Figure 17 and 18). Our analysis reveal that β-catenin shows substantial increase in nuclear and cytoplasmic levels in Gleason grade 9-10 (high Gleason grade) cancer compared to low Gleason grade cancer (Gleason grade 6-8) (Figure 17). The figure above shows enhanced

Figure 15. PCR-micro array data: PKD1 overexpression modulates expression of some key tumor associated genes. PCR microarray analysis was done using PKD1.GFP overexpressing cancer cells. Incomparison to GFP vector control cells, we observe dramatic changes in some key genes as represented above.

significant decrease in membrane PKD1 staining in high Gleason grade (Gleason grade 9-10) cancer compared to low Gleason grade cancer (Gleason grade 6-8) Figure 18). No significant change in either the nuclear or cytoplasmic staining of PKD1 was observed between low Gleason grade and high Gleason grade prostate cancer. However, only the low Gleason grade cancer showed membranous PKD1 staining, implicating a role for decrease membrane PKD1 in progression of prostate cancer. Our preliminary IHC studies demonstrate that, in addition to downregulation of β-catenin in human prostate cancer compared to benign glands, there is a decreased expression of β-catenin in prostatic intraepithelial neoplasia in a small subset of our study patients and an increased nuclear staining in high Gleason grade prostate cancer. This suggests an involvement of β-catenin and the Wnt signaling pathway in prostate cancer. To determine a correlation between PKD1 and β-

Figure 16. Prostate cancer tissue samples. The samples were probed with PKD1 antibody. Figure shows suppression of PKD1 in Gleason grade 7 (4+3) cancerous areas compared to normal appearing glands. Original magnifications: 400x

Figure 17. β-catenin expression pattern in Prostate cancer samples. The patient samples were probed with anti-β-catenin antibody. The intensity and strength of staining and percentage of cancerous tissue stained were evaluated by a pathologist. The results were graphed. The figure above shows enhanced nuclear and cytoplasmic β-catenin in Gleason grade 9-10 (high Gleason grade) cancer compared to low Gleason grade cancer (Gleason grade 6-8).

catenin expression, we evaluated the expression of these proteins in human prostate cancer tissues utilizing IHC. The cases and controls, matched one-to-one on the basis of patient age and year of biopsy, were included in this study. We have successfully collected 60 prostate cancer patient samples for analysis by IHC. We have stained 60 human prostate cancer tissues for PKD1 and β-catenin protein expression with the Mach4 kit (Figure 16). The stained tissues were evaluated for intensity of staining and subcellular
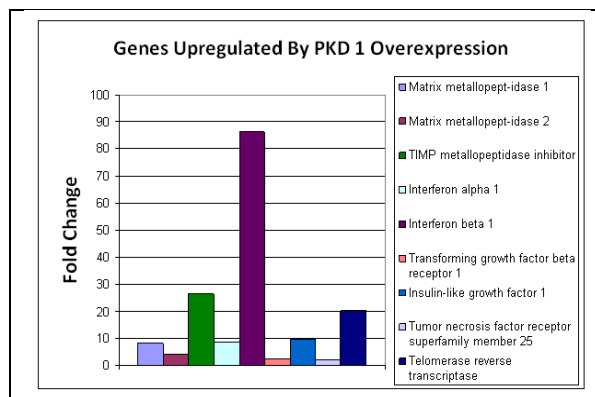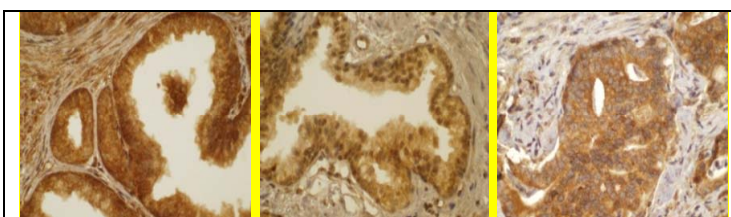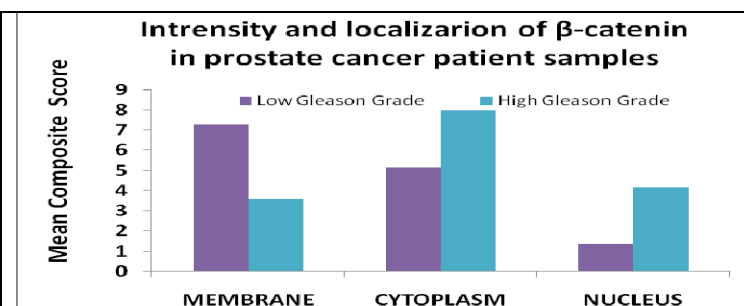
localization of PKD1 and β-catenin by a pathologist (Dr. Koch) and the mean composite score was calculated and data was analyzed and graphed (Figure 17 and 18).

Our analysis reveal that β-catenin shows substantial increase in nuclear and cytoplasmic levels in Gleason grade 9-10 (high Gleason grade) cancer compared to low Gleason grade cancer (Gleason grade 6-8) (Figure 17). Our results show an overall decreased membrane and cytoplasmic PKD1 staining in high Gleason grade (Gleason grade 9-10) cancer compared to low Gleason grade cancer (Gleason grade 6-8), PIN and BPH prostate glands (Figure 16). However there were no visible changes in the nuclear or cytoplasmic staining of PKD1 in early versus advanced Gleason grade prostate cancer samples (Figure 18). However, only the low Gleason grade cancer showed membranous PKD1 staining, implicating a role for decrease membrane PKD1 in progression of prostate cancer.
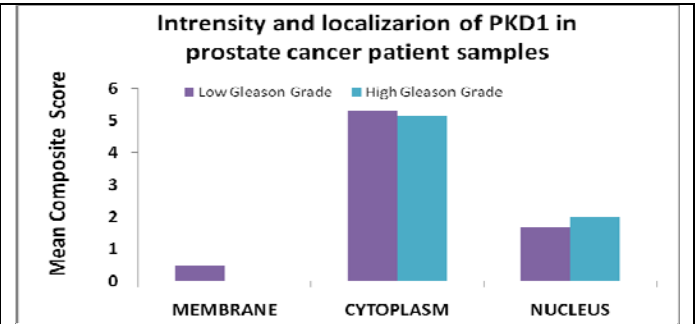

Figure 18. PKD1 localization in Prostate cancer tissue samples. The patient samples were probed with anti-PKD1 antibody. The intensity and strength of staining and percentage of cancerous tissue stained were evaluated by a pathologist. The results were graphed. The figure above shows enhanced significant decrease in membrane PKD1 staining in high Gleason grade (Gleason grade 9-10)cancer compared to low Gleason grade cancer (Gleason grade 6-8). No significant change in either the nuclear or cytoplasmic staining of PKD1 was observed between low Gleason grade and high Gleason grade prostate cancer.

Additional Investigation on PKD1- β-catenin modulators:

Curcumin a new PKD modulator: During this current year, we investigated the effect of many natural compounds that can modulate prostate cancer cells growth and investigated the effects of these on β-catenin transcription activity. Our investigation with curcumin demonstrated that it can attenuates β-catenin transcription activity in prostate cancer cells and can also modulate the expression/activation of PKD1 (Figure 19). These results suggest a novel molecular mechanism of curcumin related suppression of prostate cancer cells growth through modulation of PKD1.
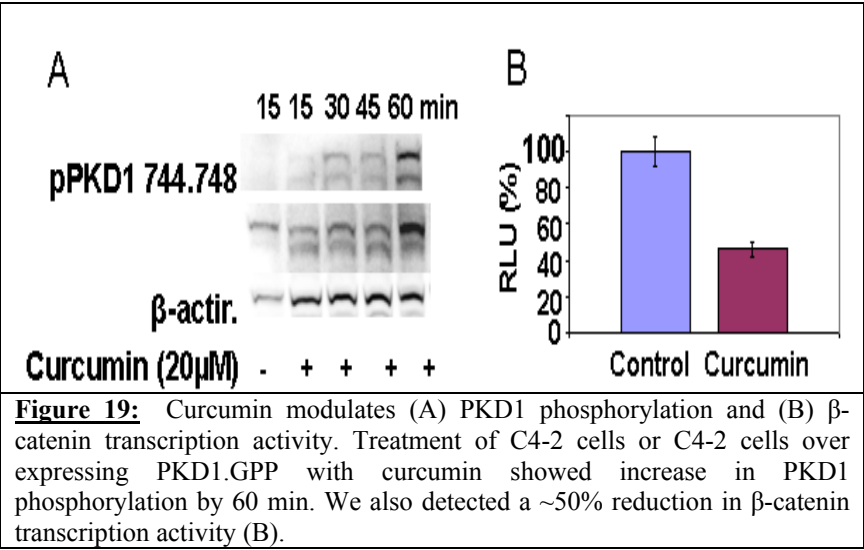

Figure 19: Curcumin modulates (A) PKD1 phosphorylation and (B) β-catenin transcription activity. Treatment of C4-2 cells or C4-2 cells over expressing PKD1.GPP with curcumin showed increase in PKD1 phosphorylation by 60 min. We also detected a ~50% reduction in β-catenin transcription activity (B).

Curcumin treatment enriches β–catenin localization at the cell membrane: β-catenin is an important cellular protein that is phosphorylated by PKD1 and we have previously shown PKD1 to increase the levels of membrane β-catenin and cell-cell interaction in prostate cancer cells. Therefore, to determine the effect of curcumin mediated activation of PKD1 on membrane β-catenin localization, curcumin treated C4-2 cells were immunostained for β-catenin and processed for confocal microscopy (Figure 20).

Curcumin treatment enriched membrane β-catenin localization in C4-2 cells within 1h of treatment compared to vehicle control treated cells. Similar results were also observed in LNCaP cells.

Curcumin mediated enhancement of membrane β-catenin is inhibited by PKD1 siRNA: We sought to investigate the role of PKD1 in curcumin mediated enrichment of membrane β-catenin. For this purpose, we used PKD1 specific siRNA to silence PKD1 in C4-2 cells. PKD1 siRNA effectively silenced PKD1 expression (over 95%) in C4-2 cells compared to scrambled (non-targeted) siRNA (Figure 21A). After inhibition of PKD1 expression, C4-2 cells were treated with curcumin and processed for confocal microscopy to determine β-catenin and PKD1 expression and localization (Figure 21B). In scrambled siRNA transfected cells, curcumin treatment efficiently enhanced β-catenin localization on the cell membrane (Figure 4B, A2-D2) compared to control cells (Figure 21B, A1-D1). However, in cells transfected with PKD1 silencing siRNA, curcumin treatment failed to enrich β-catenin on the membrane in C4-2 cells (Figure 21B, A4-D4). These data suggest that PKD1 plays a role in curcumin mediated enrichment of membrane β-catenin in prostate cancer cells.

Curcumin attenuates nuclear β-catenin signaling

PKD1 modulates the β-catenin signaling pathway by interacting, phosphorylating and modulating the subcellular localization and inhibiting the transcription activity of nuclear β-catenin. Transient activation
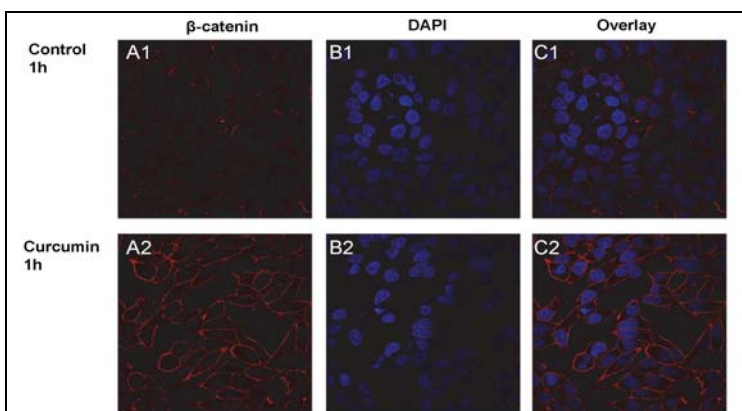

Figure 20: Curcumin treatment enhances membrane β-catenin. C4-2 cells were cultured on glass coverslips overnight and treated with DMSO (upper panel) or curcumin (20 μM) (lower panel) for 1h, washed, fixed and immunostained for β-catenin (red) and counter-stained with DAPI (blue). Higher β–catenin staining was observed on the cell surface at 1h of curcumin treatment, compared to DMSO control treated cells. Original Magnifications 600X.
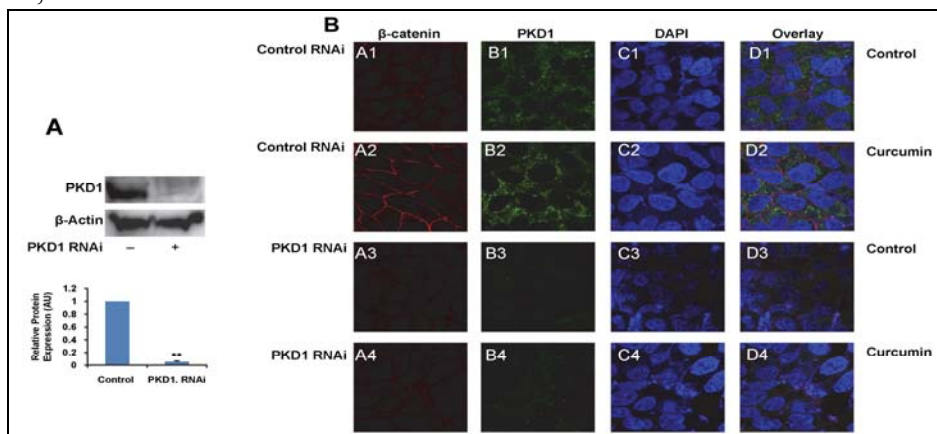

Figure 21: PKD1 is required for curcumin induced enrichment of β-catenin on the membrane. A). Silencing of PKD1 by PKD1 specific siRNA. C4-2 cells were transfected for 48h with 25nM control siRNA or PKD1 siRNA, lysed and immunoblotted for PKD1 and β-actin using specific antibodies. Immunoblotting shows over 95% suppression of PKD1 expression on transfection with PKD1 specific siRNA. (B). Suppression of PKD1 inhibits enrichment of membrane β-catenin levels. C4-2 cells were cultured on coverslips overnight. The cells were first transfected with either control siRNA (A1-D1; A2-D2) or PKD1 silencing siRNA (A3-D3; A4-D4) for 24h, followed by treatment with vehicle control (DMSO) (A1-D1; A3-D3) or curcumin (20μM) (A2-D2; A4-D4) for 1h. The cells were immunostained for β-catenin (red) or PKD1 (green) and the nucleus was counter stained with DAPI (blue). Higher β–catenin staining was observed on the cell surface of control siRNA cells at 1h of curcumin treatment (A2) compared to vehicle treatment (A1). However, siRNA mediated silencing of PKD1 (B3, B4) inhibited curcumin mediated enrichment of membrane β–catenin staining on the cell surface (A4 vs A3 and A2).

of PKD1 has been shown to have long term downstream cellular effects. Therefore, we further determined the effect of curcumin on membrane β-catenin localization after 24h of treatment using confocal microscopy (Figure 22). Higher membrane β-catenin localization along with reduced cytoplasmic β-
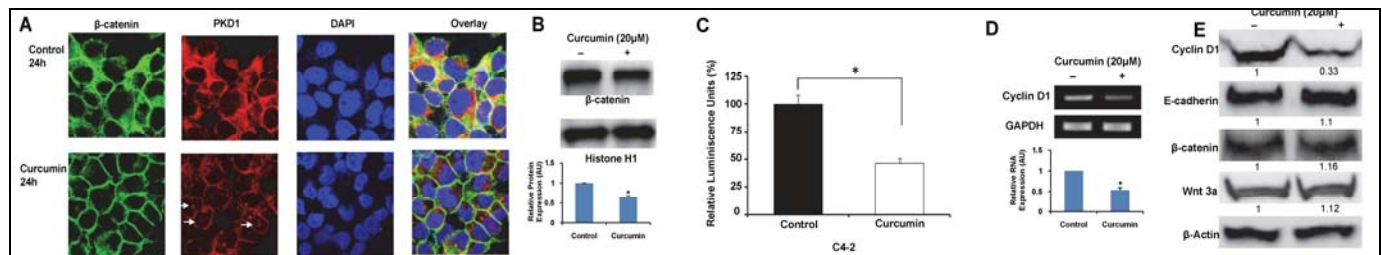

Figure 22: **Curcumin inhibits β-catenin transcription activity in prostate cancer cells.** A) Effect of curcumin treatment on the cellular localization of β-catenin and PKD1. C4-2 cells treated with curcumin (20 μM) or DMSO for 24h and immunostained for β-catenin (green) or PKD1 (red). Curcumin treated cells showed lower cytoplasmic and higher membrane β-catenin staining compared to control cells. In addition, curcumin treated cells exhibited PKD1 staining primarily on the cell membrane and in the nucleus (white arrows), with faint cytoplasmic staining. B) Effect of curcumin on nuclear β-catenin levels. Nuclear proteins isolated from C4-2 cells treated either with curcumin (20 μM) or DMSO were immunoblotted for β-catenin antibody. Histone H1 protein was used as loading control. Curcumin treatment markedly decreased the levels of nuclear β-catenin compared to vehicle treated cells. C) Effect of curcumin on β-catenin transcription activity in C4-2 prostate cancer cells. The β-catenin transcription activity was measured by transiently transfecting the cells with TCF luciferase reporter construct and after 3h, treating the cells with curcumin (20μM) or DMSO for 24h. Curcumin treatment significantly reduced β-catenin transcription activity in C4-2 cells compared to vehicle treated cells. Mean ± SE, n=3, *p<0.01. D). Effect of curcumin on transcription of cyclin D1. The transcription of cyclin D1 was analyzed by isolating RNA, converting to cDNA and PCR amplifying using cyclin D1 or internal control GAPDH specific primers. The amplified products were resolved on 1% agarose gel. Curcumin treatment reduced the expression of cyclin D1. E). Immunoblot analyses. Cell lysates from curcumin (20μM) or DMSO treated C4-2 cells were processed for immunoblotting using specific antibodies. Curcumin treatment markedly decreased cyclin D1 expression, whereas no effect was observed on the expression of total β-catenin, E-cadherin or Wnt 3a.

catenin was observed in cells after 24h of curcumin treatment (Figure 22). In addition, 24h curcumin treatment also altered the subcellular localization of PKD1 (Figure 22). While in control cells, PKD1 was primarily localized in the cytoplasm, curcumin treated cells exhibited PKD1 localization on the membrane and in the nucleus (Figure 22). β-catenin is a critical component of the Wnt signaling cascade. Nuclear β-catenin functions as a co-transcription factor by forming a complex with TCF and enhances the expression of a number of molecules with pro-oncogenic roles, including cyclin D1, c-myc and c-jun. Since PKD1 modulates the subcellular localization of β-catenin and since curcumin mediated PKD1 activation enhanced the membrane localization of β-catenin, we analyzed the effect of this activation on nuclear β-catenin expression using immunoblotting and on β-catenin transcription activity using a luciferase reporter system in C4-2 prostate cancer cells (Figures 22B and 22C). Curcumin treatment significantly reduced the nuclear expression of β-catenin compared to dimethyl sulfoxide (DMSO) treated cells (Figure 22B). The decrease in nuclear β-catenin levels was correlated with the attenuation of β-catenin transcription activity in C4-2 (Figure 22C). In order to determine the downstream effects of the suppressed β-catenin activity, the expression of cyclin D1 was analyzed in curcumin treated C4-2 cells. Interestingly, a marked decrease in the expression of cyclin D1 at mRNA (Figure 22D) and protein levels (Figure 22E) was observed in curcumin treated C4-2 cells. Similar results were also obtained by real-time PCR analysis (data not shown). However, no apparent change was observed in the expression of Wnt 3a, β-catenin and E-cadherin (Figure 22E). We also determined the effect of curcumin on β-catenin transcription activity in LNCaP cells. Similar to C4-2 cells, curcumin inhibited β-catenin transcription activity and markedly decreased the expression of cyclin D1 in LNCaP cells.

__Curcumin inhibits cell motility through PKD1 mediated cofilin phosphorylation__

We investigated the effect of curcumin on cell motility using a 'wound healing'. Compared to control, curcumin treatment inhibited cellular motility of C4-2 prostate cancer cells (Figure 23A). A similar effect on cellular motility was also observed in LNCaP cells. The highly coordinated process of actin remodeling underlies the process of cellular motility. This remodeling at the growing front requires the orchestrated action of a number of molecules involved in the F-actin reorganization. The actin related proteins (Arp) play an important role in the branching of the actin filament. The protein cofilin is an actin monomer generating molecule that is involved in actin remodeling. Cofilin is activated by slingshot (SSH) phosphatase mediated dephosphorylation reaction and is inactivated by LIM Kinase (LIMK) mediated phosphorylation reactions. PKD1 is intricately involved in inhibiting cell motility by interacting, phosphorylating and inhibiting the functions of many motility related proteins including a slingshot 1 like (SSH1L) phosphatase. Since curcumin activated PKD1, we sought to investigate the effect of curcumin on the activity and levels of cofilin and Arp3 protein by

Figure 23: **Curcumin treatment inhibits cell motility through phosphorylation of cofilin.** A). Scratch assay. C4-2 cells were grown, until confluent, in plates containing IBIDI inserts. The inserts were removed from the plates to generate gaps (solid white lines show width of the gap; dashed lines border the gap) and phase contrast images of the same area of the gaps were taken at varying time intervals in the presence or absence of 20 μM curcumin. Curcumin treatment inhibited motility of C4-2 prostate cancer cells. B). Effect of curcumin on the expression of actin remodeling proteins. Total cell lysates prepared from curcumin (20μM) or DMSO treated C4-2 cells were processed for immunoblotting using specific antibodies. Curcumin treatment induced a marked increase in the expression of inactive phospho-cofilin compared to DMSO treated control cells. Minor change was also observed in the expression of Arp3.

immunoblotting. Curcumin treatment increased the levels of inactive phospho-cofilin in C4-2 prostate cancer cells with little or no effect on the expression of the total protein (Figure 23B). Curcumin treatment also caused a slight decrease in the expression of Arp3 protein. These data suggest a potential role of PKD1 in curcumin mediated inhibition of cell motility *via* cofilin phosphorylation.
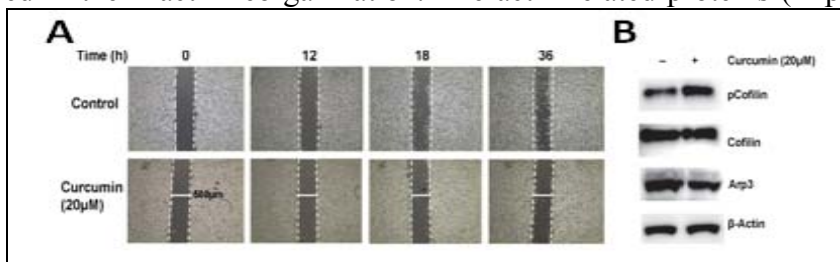
In vivo effects of curcumin on prostate cancer growth

A xenograft mouse model was used to examine the *in vivo* effect of curcumin on prostate tumor growth and β-catenin subcellular localization. Nude mice were subcutaneously inoculated with androgen-independent C4-2 cells. Following tumor development, the mice were administered intra-tumoral injections of curcumin or vehicle control. On day 7, the tumor volumes were measured and the rate of tumor growth following curcumin treatment was determined. Curcumin efficiently
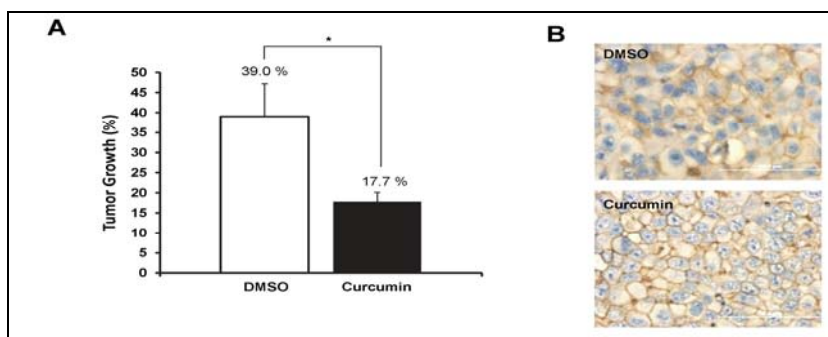
Figure 24: Curcumin inhibits prostate cancer growth in xenograft mouse model. A) C4-2 prostate cancer cells were used to generate xenografts in male nude mice. Following tumor development, the mice were treated intra-tumorally with curcumin (n=4) or DMSO (n=3). The rate of tumor growth was measured after 7 day and the percent tumor growth following treatment was graphed. Curcumin effectively inhibits prostate cancer growth. B) Effect of curcumin on β-catenin localization. Tumor tissues from curcumin or control treated mice were processed for IHC staining using anti-β-catenin antibody. Enhanced staining of membranous β-catenin was observed in curcumin treated mice compared to control mice. Original Magnifications 400X.

inhibited tumor growth by over two folds compared with the control-treated mice (*$p < 0.05$) (Figure 24). In addition, we observed change in β-catenin subcellular localization in curcumin treated tumor tissues (Figure 24), similar to *in vitro* observations. These results suggest that curcumin inhibits prostate tumor growth by modulating β-catenin functions. Based on these results, we propose a novel molecular mechanism for curcumin mediated inhibition of prostate cancer through the activation of PKD1 and subsequent attenuation of β-catenin signaling (Figure 25).

Reportable Outcomes:

Published one paper in ***PLoSONE*** and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). We were also invited to present this work as a talk in an international conference (Fourth International Conference on Translational Cancer Research. Rajasthan, India. *December 17, 2011).*

During the 2008-12 funding period we have made significant progress on our project. We have published 22 papers and 28 abstracts. In this study, we revealed functional aspects of role of β-catenin signaling through PKD1 in prostate cancer. We have investigated novel mechanism of the suppression of β-catenin transcriptional activity by PKD1 and its modulators such as Bryostatin 1 and Curcumin. We identified the PKD1 domains involved in interaction and modulation of β-catenin activity. We have generated retroviral constructs for the transfection of PKD1 mutants in prostate cancer cells. We, in collaboration with Dr. Chauhan, have also developed a xenograft mouse model system with the highly metastatic C4-2 cell lines. Our results are very encouraging and we have been able to generate highly vascularized tumors in nude mice. Our

Figure 25: **Schematic diagram showing possible signaling mechanisms modulated by curcumin mediated PKD1 activation.** Curcumin modulates a number of molecular pathways within the cancer cells including PKD1 signaling. Curcumin may suppress prostate cancer growth and metastasis by activating PKD1, which in turn may inhibit cell growth through the inhibition of β-catenin/TCF transcription activity, enhance cell-cell aggregation *via* enhanced translocation of β-catenin to the cell membrane and inhibit cell motility either directly by enhancing cell-cell aggregation and/or phosphorylating and inhibiting the function of sling shot 1 like (SSH1L) phosphatase or indirectly (dashed lines) by negatively regulating the expression of active cofilin *via* indirectly activating LIM kinase (LIMK).

experiments suggest that PKD1 expression inhibited prostate tumor growth in xenograft mouse model. In addition, we have investigated the effects of PKD1 overexpression on gene transcription using PCR microarray techniques. To determine the correlation of PKD1 expression with prostate cancer progression, we have collected 60 prostate cancer samples and performed PKD1 and β-catenin staining. Our studies also suggest that, Bryostatin-1, a PKD1 modulator, efficiently attenuates β-catenin transcriptional activity and induces chemo-sensitization in prostate cancer cells. In addition, we have also discovered a new method of PKD1 activation suppression of β-catenin transcriptional activity and enhancement of β-catenin membrane localization by a natural compound curcumin. We have also revealed a novel curcumin pre-treatment strategy for inducing chemo/radio-sensitization of cancer cells. Based on clinical implications of this novel strategy, this study was published and selected for press release. All related studies have been presented at IMPACT and Annual American Association for Cancer Research (AACR) meetings. In addition to publications, we have obtained collaborative grants
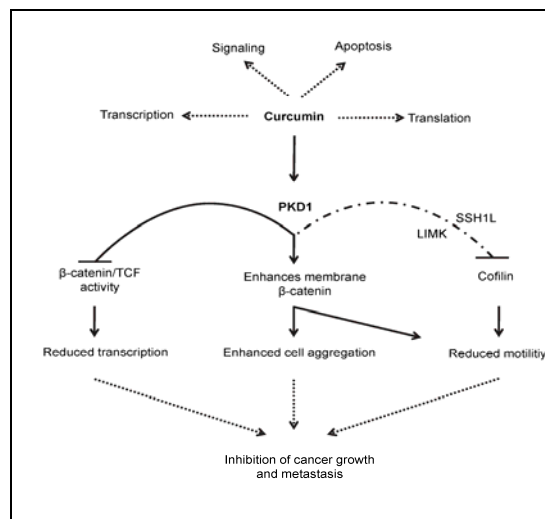
from the state (Governor's 2010 initiative), NIH (NCI RO1, NCRR COBRE) and pharmaceutical industries (Merck Pharmaceuticals, Investigator Initiated Grant).

- In 2008-2011, we have published total 22 papers and 28 abstracts.
- For the first time, we have identified Bryostatin-1 as well as curcumin to modulate β-catenin signaling through PKD1 and induces chemo-sensitization in prostate cancer cells.
- For the first time, we have shown that curcumin modulated PKD1 activation and subsequent β-catenin transcription activity.

Key Research Accomplishments: During this funding period we made considerable progress on our grant proposal and published one paper in **PLoS*ONE***. Along with this publication, a few more papers were published from our group. In brief, in 2008-12 funding period we have investigated the effect of Bryostatin 1 on PKD1 expression, β-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Initial activation of PKD1 with Bryostatin 1 leads to co-localization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mechanism to improve efficacy of Bryostatin 1 in clinical setting.

We have also demonstrated for the first time that curcumin activates PKD1, resulting in changes in β-catenin signaling by inhibiting nuclear β-catenin transcription activity and enhancing the levels of membrane β-catenin in prostate cancer cells. Modulation of these cellular events by curcumin correlated with decreased cell proliferation, colony formation and cell motility and enhanced cell-cell aggregation in prostate cancer cells. In addition, we have also revealed that inhibition of cell motility by curcumin is mediated by decreasing the levels of active cofilin, a downstream target of PKD1. The potent anti-cancer effects of curcumin *in vitro* were also reflected in a prostate cancer xenograft mouse model. The *in vivo* inhibition of tumor growth also correlated with enhanced membrane localization of β-catenin. Overall, our findings herein have revealed a novel molecular mechanism of curcumin action *via* the activation of PKD1 in prostate cancer cells.

We also explored the effect of transient overexpression of various PKD1 mutant constructs on proliferation, colony formation, cell motility, β-catenin transcription activity and effect of the mutants on subcellular localization of β-catenin. Our experiments suggest important role for C1b domain for proliferation and colony formation and the N-terminal region for cell motility. Our results also suggest a very important function for the PH domain in interacting with β-catenin, modulating the subcellular localization of β-catenin as well as in decreasing β-catenin transcription activity. The PKD mutant containing deletion of the CRD domain was also found to increase β-catenin transcription activity, implicating a role for this domain in interacting and inhibiting β-catenin transcription activity. The domain C1a mutant, which primarily localizes to the nucleus, has consistently shown lower β-catenin function, further confirming a negative regulation of β-catenin function by nuclear PKD1.

Selected Publications from year 2008-2012:

Sundram V, Chauhan SC, Ebeling M and **Jaggi M (*Corresponding Author)**. Curcumin regulates β-catenin activity in prostate cancer by activating protein kinase D1. **PLoS*ONE*** April 16 (2012).

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<u>Chauhan SC</u>, <u>Ebeling MC</u>, <u>Maher DM</u>, <u>Koch MD</u>, <u>Watanabe A</u>, <u>Aburatani H</u>, <u>Lio Y</u> and **Jaggi M.** MUC13 mucin augments pancreatic tumorigenesis. <u>**Mol Cancer Ther.**</u> 2011 Oct 25. (*Selected for cover illustration)*

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8. Yallapu MM., Othman SF., Curtis ET., Gupta BK., **Jaggi M**. and Chauhan SC. Multifunctional magnetic nanoparticles for theranostic applications. *102 AACR Annual Meeting 2011***,** Orlando, FL

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17. Yallapu MM., **Jaggi M**. and Chauhan SC. Design of β-cyclodextrin-curcumin self-assembly: A new approach for enhanced curcumin delivery and therapeutic efficacy in prostate cancer cells. *101 AACR Annual Meeting 2010,* Washington DC.

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19. Hughes JE., Chauhan SC., and **Jaggi M.** Protein kinase D1 attenuates tumorigenesis in SW 480 colon cancer cells by modulation β-catenin/T cell factor activity. 3rd *International Symposium on Translational Cancer Research* December 18-21, 2009, Bhubaneshwar, Orissa, India

Conclusions:

- Bryostatin-1 treatment modulates PKD1 expression, cell proliferation, and cellular aggregation and alters β-catenin translocation and transcription activity.

- Bryostatin-1 induces chemo-sensitization in prostate cancer cells.

- Initial activation of PKD1 with Bryostatin-1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking.

- Activation of PKD1 by Bryostatin-1 decreases nuclear β-catenin expression and thereby suppresses β-catenin/TCF transcription activity.

- For the first time, we have identified Bryostatin-1 modulates β-catenin signaling through PKD1.

- We have identified curcumin as molecule that can be effectively used for controlling prostate cancer. We have shown for the first time that curcumin modulated PKD1 activation and subsequent β-catenin transcription activity.

- We have demonstrated that curcumin mediated activation of PKD1 decreases nuclear β-catenin expression and thereby suppresses β-catenin/TCF transcription activity.

- Curcumin mediated activation of PKD1 decreases cell proliferation, motility and cellular aggregation via modulation of β-catenin activity and functions.

- PKD1 overexpression suppresses prostate tumor growth in xenograft mouse model.

- Staining of over 60 prostate cancer samples for PKD1 and β-catenin reveal significant differences in staining pattern of β-catenin between low Gleason grade sample and high Gleason grade sample. However, our initial analysis has not reveled any significant difference in the staining pattern of PKD1 in the cytoplasm or the nucleus of low Gleason grade sample and high Gleason grade sample.

- PKD1 shows decreased membrane localization in high Gleason grade samples compared to low Gleason grade samples.

- Transient transfection experiment with domain mutants of PKD1 reveal an important role for PH and CRD domain in decreasing β-catenin transcription activity.

- Our experiments suggest important role for C1b domain for proliferation and colony formation and the N-terminal region for cell motility.

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Appendices:

List of personnel
Meena Jaggi
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