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13. ABSTRACT (Maximum 200 Words)

Prostate specific membrane antigen (PSMA) is a well-studied, highly restricted prostate epithelial cell membrane antigen. In contrast to other highly restricted prostate-related antigens such as prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), which are secretory proteins, PSMA is an integral membrane protein. In addition to its prostate specificity, PSMA is expressed by a very high proportion of prostate cancers (Pca), expression is further increased in higher grade cancers, in metastatic disease, and in hormone-refractory PCa. Among reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. Although PSMA is primarily localized to the plasma membrane immunofluorescence analysis also revealed its localization to a membrane compartment around the centrosome. Consistent with its localization around the centrosomes PSMA expressing cells rapidly exited mitosis. We have now determined that the association of the cytoplasmic tail of PSMA with key regulators of cell cycle progression is involved the aberrant mitotic exit of PSMA expressing cells.

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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., in press, see enclosed manuscript).

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. We also 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. Accordingly the goal of this proposal is to understand the mechanism by which PSMA is involved in cell cycle progression and its consequence in prostate cancer cells.

Body:

1. Cytoplasmic domain of PSMA interacts with APC

Since CDC27 is a cytoplasmic protein we reasoned that the cytoplasmic domain of PSMA might associate with CDC27. To test this possibility we generated a mutant construct in which the cytoplasmic tail of PSMA was deleted and generated a cell line stably expressing this mutant. This cell line progressed through the cell cycle like control cells that do not express PSMA indicating that the cytoplasmic tail is critical for PSMA function in cell cycle progression (data not shown). Consistent with this idea, coimmunoprecipitation analysis revealed that this mutant does not bind CDC 27 (Fig.1A). To further confirm that the cytoplasmic tail of PSMA is sufficient for CDC27 association we generated a GST-fusion chimera of the PSMA cytoplasmic tail (GST-PSMA-CD)

and performed affinity precipitation assays. This analysis clearly revealed that the cytoplasmic tail of PSMA is sufficient for CDC27 association (Fig.1B). Now that we unambiguously confirmed that the cytoplasmic tail of PSMA associates with CDC27 we have started to characterize the critical amino acids involved in PSMA binding to CDC27. These experiments are in progress.

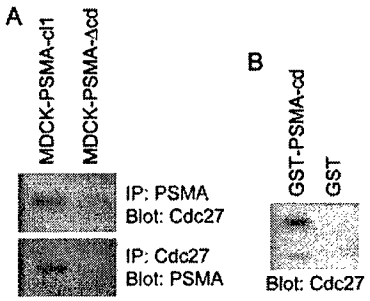

Figure 1. PSMA cytoplasmic tail associates with CDC27. A. MDCK cells expressing full length PSMA and cytoplasmic tail deletion mutant of PSMA was generated and used for coimmunoprecipitation experiments. CDC27 coimmunoprecipitated with PSMA and vice versa as revealed by immunoblot analyses. Whereas CDC 27 did not coimmunoprecipitate with cytoplasmic tail deletion mutant of PSMA (PSMA-Δcd). B. Affinity precipitation of CDC27 by GST-PSMA-cytoplamic tail chimera.

2. Standardization of in vitro APC assay:

The next important question to be addressed was whether PSMA association with CDC27 alters APC activity. APC is an ubiquitin-protein ligase involved in the ubiquitinylation of cyclin B during mitosis. Destruction of cyclin B allows cells to exit from mitosis. Therefore, ubiquitinylation of cyclin B has been used as an assay to determine APC activity. This assay is complex and expensive since it requires purified enzymes such as ubiquitin conjugating enzyme E2, purified ubiquitin and several compounds such as ATP-γ-S, ubiquitin aldehyde and MG132, a proteosome inhibitor. To test APC activity we cloned the N-terminal region comprising 102 amino acids of cyclin B into a pCDNA3 vector. This region of cyclin B is ubiquitinylated by APC and the pCDNA3 vector allows in vitro transcription of cyclin B from a T7 promoter. Cyclin B was transcribed and translated in the presence of ^{35}S-methione in vitro using a commercially available kit (Promega). The radiolabeled cyclin B was then incubated with APC assay cocktail and cell lysates containing APC from PC3 and PC3-PSMA. If APC is active then cyclin B will be ubiquitinylated and since proteosome activity is inhibited under our assay conditions, cyclin B will not be degraded and can be observed as high molecular mass bands in the autoradiogram. As shown in figure 2 APC is more active in PC3-PSMA cells than in PC3 cells (compare lanes 4-6 with lanes 1-3). Strikingly, APC was active in nocodazole blocked PC3-PSMA cells indicating incomplete inactivation of APC these cells (Fig. 2, lane 4).

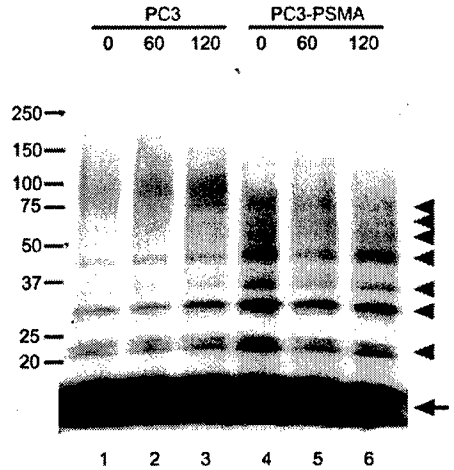

Figure 2. In vitro APC assay to monitor ubiquininylation of cyclin B. Arrow indictes the in vitro translated cyclin B. Arrow head indicates ubiquitinylated cyclin B. Note increased amount of ubiquitinylated cyclin B in nocodazole blocked PC3-PSMA cells (lane 4) compared to PC3 cells Lane 1.

3. Role of α-catenin in β-catenin mediated oncogenic signaling in prostate cancer

β-catenin is a well studied molecule localized to adherens junction. β-catenin is also found in the cytoplasm and is involved in the Wnt signaling pathway, which is activated during normal embryonic development. Aberrant β-catenin signaling has been strongly implicated in the cell proliferation of various forms of cancer including prostate cancer. The mechanisms by which free β-catenin levels are regulated have been an intensive area of research in cancer biology. It has long been speculated that adherens junctions might play a role in the regulation of free β-catenin levels and the subsequent signaling processes. However, a model system to show this has been lacking.

By reintroducing α-catenin expression by microcell transfer of chromosome 5 in a α-catenin null prostate cancer cell line, we showed for the first time that the adherens junction has an important role in the sequestration of free β-catenin and regulation of its transcriptional activity in prostate cancer cells. We also showed that in the absence of adherens junction the APC/GSK3β scaffolding complex involved in β-catenin degradation is not sufficient to suppress β-catenin transcriptional activity and cell proliferation in prostate cancer cells (Inge et al., submitted; see enclosed manuscript). Based on our results we proposed a new model for the adherens junction and α-catenin in the regulation of β-catenin signaling in prostate cancer cells. We strongly believe that this study should generate new directions for the role of adherens junctions in oncogenic signaling in prostate and probably other epithelial cancers.

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**

Progress: Using a stable cell line that expresses the cytoplasmic tail deletion mutant of PSMA we have determined that the cytoplasmic tail of PSMA is necessary for cell cycle progression and association with APC. We have also generated stable cell lines expressing a PSMA construct in which most of the extracellular domain is deleted. This cell line will be used to confirm whether the enzyme activity, which is localized to the extracellular domain of PSMA has any role in cell cycle progression. We are in the process of generating stable cell lines with constructs harboring point mutations in the cytoplasmic tail of PSMA to determine critical amino acids involved in cell cycle progression and APC association.

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**

Progress: We have standardized the APC assay and have determined that APC is active in nocodazole-blocked cells. We are in the process of comparing the Mad2 and BubR1 levels associated with APC in PSMA expressing cells by coimmunoprecipitation analysis.

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

- ✓ **Generate PSMA expressing PEAZ 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.**

Progress: We have generated a lentiviral vector harboring PSMA and have transduced the PEAZ cell line. We have confirmed that PSMA is localized to a membrane compartment around the centrosome in this cell line. Although it has been reported that this cell line has normal chromosomal complement our initial cytogenetic analysis revealed that this cell line shows several chromosomal abnormalities and cannot be used for further experiments. We therefore generated PSMA expressing HCT 116 cells. This cell line has normal chromosomal complement and has been used for studies related to aneuploidy in cancer. We are in the process of characterizing this cell line for PSMA expression and cell cycle progression and passaging this cell line to test whether PSMA expression induces aneuploidy.

Key Research Accomplishments so far:

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.
- ✓ A new role for α-catenin in the establishment of adherens junction β-catenin signaling in prostate cancer cells was uncovered.

Other progress:

Jason Christiansen, was awarded a PhD degree for his work on the functional analysis of PSMA.

Reportable outcomes so far (publications):

1. Rajasekaran, A.K, Anilkumar, G. and J. Christiansen (2005). Is Prostate specific membrane antigen a multifunctional protein? (American Journal of Physiology, Cell Physiology, invited review, in press).

2. Inge, L.J., Ryazantsev, S., Ewing, C.M., Isaacs, W.B., and A.K. Rajasekaran: α-catenin expression restores adherens junction and suppresses β-catenin transcriptional activity in prostate cancer cells (in review, Cancer Research).

Conclusions: We have made considerable progress in this proposal and have accomplished key experiments of each task described in the Statement of Work. For Task 1 we have confirmed a role for the cytoplasmic tail of PSMA in the cell cycle progression as well its association with APC. For Task 2 we have standardized the in vitro APC assay and for Task 3 we have generated the PSMA expressing HCT cell line. We hope to complete a significant portion of this proposal in the next 6 months and write a manuscript on this work.

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7. Inge, L.J., Ryazantsev, S., Ewing C.M., Isaacs W.B., and A.K. Rajasekaran: α-catenin expression restores adherens junction and suppresses β-catenin transcriptional activity in prostate cancer cells (submitted).

Appendix: 1. Copies of one accepted and one submitted manuscript are enclosed.

Is Prostate Specific Membrane Antigen A Multifunctional Protein?

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Running head: Is PSMA a multi-functional protein?

ABSTRACT

Prostate Specific Membrane Antigen (PSMA) is a metallopeptidase predominantly expressed in prostate cancer (PCa) cells. PSMA is considered as a biomarker for PCa and is under intense investigation for use as an imaging and therapeutic target. Although the clinical utility of PSMA in the detection and treatment of PCa is evident and is intensely pursued, very little is known about its basic biological function in PCa cells. The purpose of this review is to highlight the possibility that PSMA might be a multifunctional protein. We suggest that PSMA may have the function of a receptor internalizing a putative ligand, an enzyme playing a role in nutrient uptake, and a peptidase involved in signal transduction in prostate epithelial cells. Insights into possible functions of PSMA should improve the diagnostic and therapeutic values of this clinically important molecule.

prostate cancer; receptor; peptidase; endocytosis

OVERVIEW

Prostate specific membrane antigen (PSMA) was originally defined by the monoclonal antibody (mAb) 7E11 derived from immunization with a partially purified membrane preparation from the LNCaP prostatic adenocarcinoma cell line (26). A 2.65 kb cDNA fragment encoding the PSMA protein was cloned and subsequently mapped to chromosome 11p11.2 (28, 43).

Initial analysis of PSMA demonstrated the widespread expression within the cells of the prostatic secretory epithelium. Immunohistochemical staining demonstrated that PSMA was absent to moderate in hyperplastic and benign tissues, while malignant tissues stained with the greatest degree of intensity (26). Subsequent investigations have recapitulated these results and evinced PSMA expression as a universal feature in practically every prostatic tissue examined to date. These reports further demonstrate that expression of PSMA increases precipitously in a manner proportional to tumor agressiveness (9, 13, 14, 30, 34, 50, 55, 64, 66, 71).

Consistent with the correlation between PSMA expression and tumor stage, increased levels of PSMA are associated with androgen-independent prostate cancer. Analysis of tissue samples from prostate cancer patients demonstrated elevated PSMA levels following physical castration or androgen-deprivation therapy. Unlike expression of PSA, which is downregulated following androgen ablation, PSMA expression is significantly increased in both primary and metastatic tumor specimens (30, 71). Consistent with the elevated expression in androgen-independent tumors, PSMA transcription is also known to be downregulated by steroids, and administration of testosterone mediates a dramatic reduction in PSMA protein and mRNA levels (27, 71). PSMA is also highly expressed in secondary prostatic tumors and occult metastatic disease. Immunohistochemical analysis revealed relatively intense and homogeneous expression

of PSMA within metastatic lesions localized to lymph nodes, bone, soft tissue and lungs, as compared to benign prostatic tissues (14, 40, 64).

Some reports have also indicated limited PSMA expression in extraprostatic tissues, including a subset of renal proximal tubules, some cells of the intestinal brush border, and rare cells in the colonic crypts (13, 26, 27, 36, 66). However, the levels of PSMA in these tissues are generally two to three orders of magnitude less than those observed in the prostate (58). PSMA is also expressed in the tumor-associated neovasculature of most solid cancers examined, yet is absent in the normal vascular endothelium (13, 34, 55). Although the significance of PSMA expression within the vasculature is unknown, the specificity for tumor-associated endothelium makes PSMA an intriguing potential target for the treatment of many forms of malignancy.

The highly restricted expression of PSMA and the upregulation in advanced carcinoma and metastatic disease portend a promising role for PSMA as a clinical biomarker for the diagnosis, detection, and management of prostate cancer. Furthermore, as an integral membrane protein, PSMA can be exploited as an antigenic target for a variety of clinical applications (19).

Immunoscintographic scanning using an ^{111}In labeled form of mAb 7E11 has shown promise for the detection and *in vivo* imaging of PSMA expressing tumor cells. This antibody has received FDA approval for the detection and imaging of metastatic prostate cancer in soft tissues and is currently marketed under the name of ProstaScint (Cytogen, Philadelphia, PA) (24, 36, 49). However, positive signals detected with this technology is likely ascribed to immunoreactivity of this antibody in dead or dying cells within a tumor mass, as the mAb 7E11 recognizes an intracellular epitope and is incapable of binding to viable cells (67). This observation provides a rationale to explain why ProstaScint is more effective at identifying metastases in well-vascularized soft tissues than in bone, in which metastatic lesions tend to be

relatively small and do not characteristically have a high percentage of necrotic or apoptotic cells. Development and application of antibodies that recognize epitopes encoded within the extracellular domain of PSMA substantially enhanced the sensitivity and should improve the usefulness of PSMA-based *in vivo* imaging techniques (24, 34).

In addition to *in vivo* imaging strategies, the use of PSMA specific antibodies is also being assessed for therapeutic purposes. PSMA specific mAbs have been conjugated to radionuclides and cytotoxic drugs (5, 6, 17, 38, 41). Such mAbs can be exploited as a vehicle with which to deliver concentrated doses of therapeutic agents directly to the site of prostate tumor cells, while sparing damage to normal tissues. These antibodies can induce cell death specifically in PSMA expressing cells and reduce the size of LNCaP spheroids, *in vitro* (57). Furthermore, administration of a single dose of radioactively labeled PSMA-specific mAbs was able to achieve a 15-90% reduction in mean tumor volume in xenograft bearing mice, concomitant with a 2 to 3 fold increase in the median survival time, relative to untreated control mice (69).

Despite the potential use of PSMA for immunotherapy of PCa, a paucity of information exists regarding the physiological functions of this protein. The purpose of this review is to highlight the possible functions of PSMA based upon its structural and enzymatic properties, cellular localization, trafficking route, interacting partners, and information gathered from related proteins.

STRUCTURE OF PSMA

The PSMA gene consists of 19 exons that span approximately 60 kb of genomic DNA. This gene encodes a type II transmembrane protein with a short, N-terminal cytoplasmic tail (19

amino acids), a single hydrophobic transmembrane domain (24 amino acids), and a large extracellular domain (707 amino acids) at the C-terminus (Figure 1) (28, 43).

The extracellular domain of PSMA is highly glycosylated, with N-linked oligosaccharides accounting for up to 25% of the molecular weight of the native protein (25). Regions within this domain share modest degrees of homology with the transferrin receptor (TfR) (28) and with members of the M28 family of co-catalytic aminopeptidases (47). Although the TfR has only a vestigial catalytic site, PSMA is known to possess both N-acetylated alpha-linked acidic dipeptidase (NAALADase) and folate hydrolase activities (11, 45). These two related peptidase activities hydrolyze gamma-peptide bonds between N-acetylaspartate and glutamate in the abundant neuropeptide N-acetylaspartylglutamate (NAAG) and the gamma-glutamyl linkages in pteroylpolyglutamate, respectively. Thus, this enzyme has been alternatively referred to as both glutamate carboxypeptidase II (GCPII) and folate hydrolase I (FOLH1). The enzymatic activity of PSMA is largely inhibited phosphate, even at millimolar concentrations (56), and is dependent upon glycosylation and dimerization for proper function (18, 53). In contrast to the large extracellular domain, the cytoplasmic tail of PSMA consists of just 19 amino acids. In spite of its diminutive stature, the cytoplasmic domain interacts with a number of proteins and has a major impact on the localization and molecular properties of PSMA (2, 46).

Evidence using RT-PCR suggests the existence of alternative PSMA isoforms, including PSM' and recently described PSM-B and PSM-C. In contrast to the integral transmembrane orientation of full length PSMA, these variants are believed to exist within the cytosol and are thought to be the consequence of alternative splicing of the PSMA gene (52, 60). Although reports have suggested that the ratio of transmembrane to cytosolic PSMA transcript increases

with cancer in a manner proportional to advancing disease grade, little is known regarding the significance of alternatively spliced PSMA mRNA.

A murine homologue to PSMA has also been identified, and is referred to as either GCP-II or Folh1. This protein shares over 80% amino acid identity within the extracellular domain and possesses the same enzymatic peptidase activities as human PSMA (3). Interestingly, while Tsai and colleagues reported that ablation of Folh1/GCP-II resulted in embryonic lethality, Bacich and colleagues reported that Folh1/GCP-II knockout mice experienced no detectable detriment (4, 68). The reasons for this discrepancy are not completely clear, highlighting the fact that caution must be taken when attempting to extrapolate data from mouse models to human PSMA. Additionally, these two homologues display disparate profiles of tissue expression (3) and deletion of Folh1/GCP-II gene expression did not result in loss of NAALADase activity, suggesting the functional redundancy of this enzyme in murine cells (4). Furthermore, while PSMA and Folh1/GCP-II share significant homology within their respective extracellular domains, these proteins have minimal conservation of sequence homology within their cytoplasmic domains, including domains involved in mediating PSMA endocytic traffic and binding of interacting partners, such as filamin a (FLNa) (2, 3, 46).

DIMERIZATION OF PSMA

Homodimerization is a fundamental feature of many transmembrane receptors. Induction of homodimer formation is often induced by ligand binding, which is in turn necessary for mediating the cellular response of the receptor (51). The TfR is an archetypal example of one such receptor. This type II transmembrane protein is involved in regulating cellular iron homeostasis through binding and internalization of iron-laden transferrin (1).

PSMA shares homology with the TfR, both at the level of amino acid identity and at the level of domain organization (37). Like the TfR, PSMA is expressed as a non-covalently linked homodimer on the cell surface (33, 53). This dimerization is apparently mediated by epitopes within the large extracellular domain, as truncated versions of PSMA lacking the cytoplasmic and transmembrane domains are still capable of interacting. PSMA dimerization is critical to maintain the conformation and enzymatic activity of PSMA (53). Although the possibility has yet to be fully addressed, the similarity between PSMA and TfR at the amino acid and structural level, combined with the common dimerization requirement, may suggest that these proteins share similar receptor and ligand transport functions.

RESEMBLANCE OF PSMA CELLULAR TRAFFICKING WITH MEMBRANE RECEPTORS

A variety of transmembrane receptors and membrane components are internalized from the plasma membrane and trafficked through the endocytic system. This endocytic trafficking allows cells to maintain homeostasis and internalize vital nutrients, lipids, and proteins. For example, binding of iron bound transferrin to the TfR results in an induction of receptor internalization and iron transport into the cell (31). Additionally, endocytosis of membrane receptors is also an established mechanism to down-regulate signal transduction cascades. One classical example is the regulation of epidermal growth factor receptor (EGFR) signaling. Binding of epidermal growth factor (EGF) induces EGFR endocytosis and signal attenuation (12).

Like the transferrin and EGF receptors, PSMA undergoes endocytosis from the plasma membrane. This endocytosis occurs through clathrin-coated pits and involves the first five N-

terminal amino acids of the cytoplasmic tail. This motif of MWNLL appears to constitute a novel endocytic-targeting signal and likely interacts with the AP-2 adaptor protein complex (46). Although PSMA is constitutively internalized from the cell surface, binding of antibodies or related antibody fragments to the extracellular domain increases the rate of PSMA internalization (35). These antibodies may be acting like a natural ligand, thus indicating that, like the TfR, PSMA may have a receptor function involved in endocytosis of a putative unknown ligand.

Interestingly, the NAALADase activity of PSMA is inhibited by the millimolar concentration of phosphate present in culture media (65). Therefore, since internalization assays are done under normal culture conditions, it appears that NAALADase activity is not required for the internalization function of PSMA. In addition, N-acetyl-aspartyl-glutamate (NAAG), a well-known substrate of PSMA did not increase the rate of PSMA internalization in PCa cells (our unpublished data).

Following endocytosis, a number of receptors are recycled back to the plasma membrane surface. While some proteins are recycled directly from early endosomes, other receptors are first targeted to a tubulovesicular membrane structure proximal to the centrosomes referred to as the recycling endosomal compartment (REC) (39, 48). The TfR is one of the best-studied markers of the REC. Following internalization, PSMA is targeted to the REC with similar kinetics to the TfR (2, 46).

The antibody induced, clathrin mediated internalization of PSMA and the accumulation in the REC supports the hypothesis that PSMA might function as a receptor internalizing a putative ligand. Whether PSMA acts in a manner analogous to the TfR in the transport or metabolism of specific elements, or like the EGFR in the regulation of signal transduction has

yet to be addressed. However, future studies to identify the ligand of PSMA are crucial to answer this question.

ASSOCIATION OF PSMA WITH FILAMIN A (FLNa), AN ACTIN FILAMENT CROSS-LINKING PROTEIN

FLNa is a dimeric actin cross-linking phosphoprotein that plays a vital role in the stabilization of many receptors at the plasma membrane (59). It is known that many membrane receptors, like the metabotropic glutamate receptor, dopamine receptor, calcitonin receptor, tumor necrosis factor receptor and insulin receptor, interact with FLNa. The interaction between FLNa and these receptors plays a crucial role in modulating receptor function (16, 23, 54).

Using the N-terminal 19 amino acids as bait, the cytoplasmic domain was shown in to interact with the 23rd to 24th repeat of FLNa in a yeast two-hybrid assay. When expressed in a filamin-negative cell line, PSMA was rapidly internalized from the cell surface; however, ectopic expression of filamin A in these cells resulted in a 50% reduction in the rate of PSMA internalization. These data suggest that filamin A may stabilize PSMA at the cell surface by tethering it to the actin cytoskeleton, likely preventing AP-2 from binding. Interestingly, expression of FLNa also reduced the NAALADase activity of PSMA at the cell surface, perhaps by inducing a conformational change in the extracellular domain (2). These data suggest that competitive binding of AP-2 and FLNa to the PSMA cytoplasmic tail regulate endocytosis, recycling, and enzymatic activities of PSMA. Furthermore, the fact that glutamate receptor, a protein that transports glutamate, and PSMA, an enzyme that releases glutamate, both bind to FLNa raises the intriguing possibility that PSMA and glutamate receptor exist as a multi-protein

complex on the plasma membrane. This interaction would potentially facilitate the generation and transport of glutamate into the cell.

FLNa is also known to play a role in cell adhesion and motility. Calderwood and colleagues have evinced an interaction between β.integrin and FLNa, and further demonstrated that this interaction is inhibitory to cell migration (10). Another binding partner for FLNa is RalA, a small GTP binding protein known to play a role in filopodia formation (42). We have seen an accumulation of PSMA in filopodial structures (our unpublished data) and it is not known whether this observation points towards a role for PSMA in cell migration.

RESEMBLANCE OF PSMA TO MULTIFUNCTIONAL PEPTIDASES

Numerous examples suggest a role for enzymatic peptidases in mediating cell migration by affecting signaling cascades. For example, the interaction of the neutral endopeptidase (NEP) cytoplasmic tail with Lyn kinases blocks the activation of PI3-kinase. This inhibition of PI3-kinase prevents FAK phosphorylation-mediated cell migration (62). NEP is also known to inhibit the proliferation of prostate epithelial cells by its direct association with PTEN (61). PTEN is a lipid and protein phosphatase, which inhibits PI3-kinase mediated activation of Akt, a kinase involved in cell survival. A catalytic mutant of NEP was also able to block the cell proliferation and migration, suggesting that the enzymatic activity of NEP was not necessarily required. Mutational analysis of the cytoplasmic tail of NEP identified a basic amino acid rich motif containing five lysine and arginine residues proximal to the transmembrane domain that mediates the interaction between NEP and PTEN (61). The cytoplasmic tail of PSMA also has a stretch of three basic arginine residues proximal to the membrane-spanning domain, thus raising a possibility that PSMA may also interact with PTEN.

CD26 is another interesting example of a multifunctional type II cell surface glycoprotein with important roles in cell signaling. This molecule is expressed on a wide variety of cells and possesses dipeptidyl peptidase IV (DPIV) activity (7, 8, 21). CD26 has been shown to regulate cell migration and proliferation independent of its enzymatic activity (20). CD26 is known to bind adenosine deaminase, an enzyme involved in irreversible deamination of adenosine, and this association has been shown to be essential for the promotion of cell proliferation and cytokine production (29). CD26 has also been shown to affect the migratory behavior of T cells through interactions with extracellular matrix proteins such as collagen and fibronectin (22, 44). These examples clearly indicate that although PSMA is a peptidase, it could have multiple roles, not only as an enzyme but also as a protein with cell survival and migratory functions.

POTENTIAL ROLE OF PSMA ENZYME ACTIVITY IN PCa

Increased PSMA enzymatic peptidase activity is associated with metastatic PCa (32). However, the significance of these enzymatic activities in the context of benign and malignant prostatic cells remains to be elucidated.

The prostate gland is mainly composed of stromal, epithelial, and neuroendocrine cells. The dynamic balance of cell proliferation, differentiation, and apoptosis in general maintains the cellular and tissue homeostasis. This balance is generated by the continuous cross talk among these cell populations (63). For this purpose, epithelial and stromal cells secrete various types of growth factors, chemokines, and neuropeptides (70). Deregulation in this paracrine communication can result in derangement of the prostate gland such as benign prostate hyperplasia and prostate carcinoma (15). For example, the peptidase NEP normally acts to inhibit the migratory properties of prostate epithelial cells. NEP achieves the inhibition of

prostatic epithelial cell migration by cleaving critical neuropeptides such as bombesin and endothelin and thereby prevents the relay of signal transduction mediated by G-protein coupled receptors (62). CD26 is also involved in regulation of paracrine signaling by promoting cleavage of growth factors, chemokines, neuropeptides and hormones and thus contributes to the regulation of T-cell and monocyte migration (20).

Like NEP and CD26, PSMA is also a type II transmembrane glycoprotein with co-catalytic metallopeptidase activity. The increased expression of PSMA in prostatic adenocarcinoma may indicate a role in the cleavage of signaling molecules involved in maintaining prostate gland architecture and function. Although neuropeptide and growth factor substrates that influence signaling mechanisms have yet to be identified, the overexpression of PSMA could potentially disturb the growth balance of the prostate gland. Future investigation along these lines should provide important insights into the role of PSMA in prostate cancer.

CONCLUSION

In conclusion, we suggest that PSMA could be a multifunctional protein. We have summarized the potential multifunctional nature of PSMA in figure 2. Dimerization, structural similarity to TfR, and localization to REC indicate a potential receptor function for PSMA. NAALADase and folate hydrolase activities of PSMA are consistent with a role in enzyme activities involved in nutrient uptake. Interaction of PSMA with FLNa and its localization to filopodia indicates a possible role in cell migration. Finally, PSMA as a peptidase might also activate signaling cascades involved in cell survival, proliferation, and cell migration. Future investigations into these areas should provide valuable information regarding the possible

functions of this clinically important molecule. Improved understanding of PSMA function should allow the fulfillment its therapeutic and diagnostic potential.

GRANTS

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FOOTNOTES

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FIGURE LEGENDS

Fig.1. Schematic diagram of PSMA structure. PSMA is a type II transmembrane protein with a short N-terminal cytoplasmic domain (CD), a hydrophobic transmembrane region (TM), and a large extracellular domain (ED). The CD contains an endocytic targeting motif and filamin A binding site (A). The large ED is highly glycosylated with 9 predicted N-glycosylation sites (Y). The ED contains 2 domains of unknown function that span amino acid residues 44-150 (B) and 151-274 (D), proline- and glycine-rich regions that span amino acid residues 145-172 and 249-273, respectively (C and E), a catalytic domain that spans amino acid residues 274-587 (F), and a final domain of unknown function (amino acids 587-750) to which a helical dimerization domain (amino acids 601-750) is localized (G).

Fig. 2. Diagram illustrating hypothetical functions of PSMA. Evidence suggests that PSMA may perform multiple physiological functions within the cell. The peptidase activity and potential interaction with signaling molecules alludes to a role in signal transduction. Homodimerization of PSMA, homology to the transferrin receptor, and intracellular trafficking to the REC implies a role for PSMA as a receptor for an unidentified ligand. The peptidase activity of PSMA suggests a possible role in nutrient uptake, particularly related to glutamate or folate absorption. The interaction of PSMA with FLNa and the potential localization to filapodial structures suggests a role in cell motility.

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Figure 1

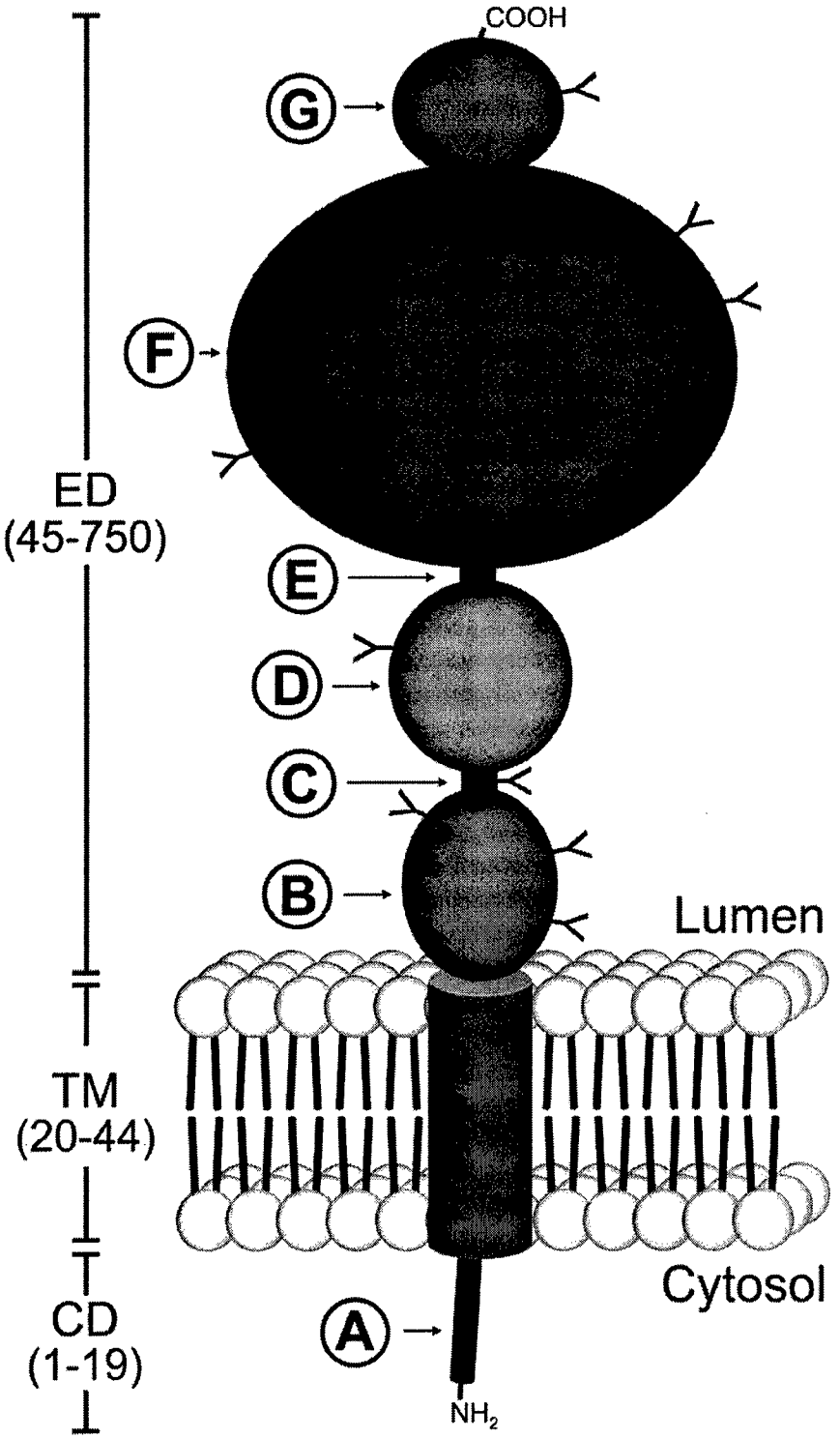
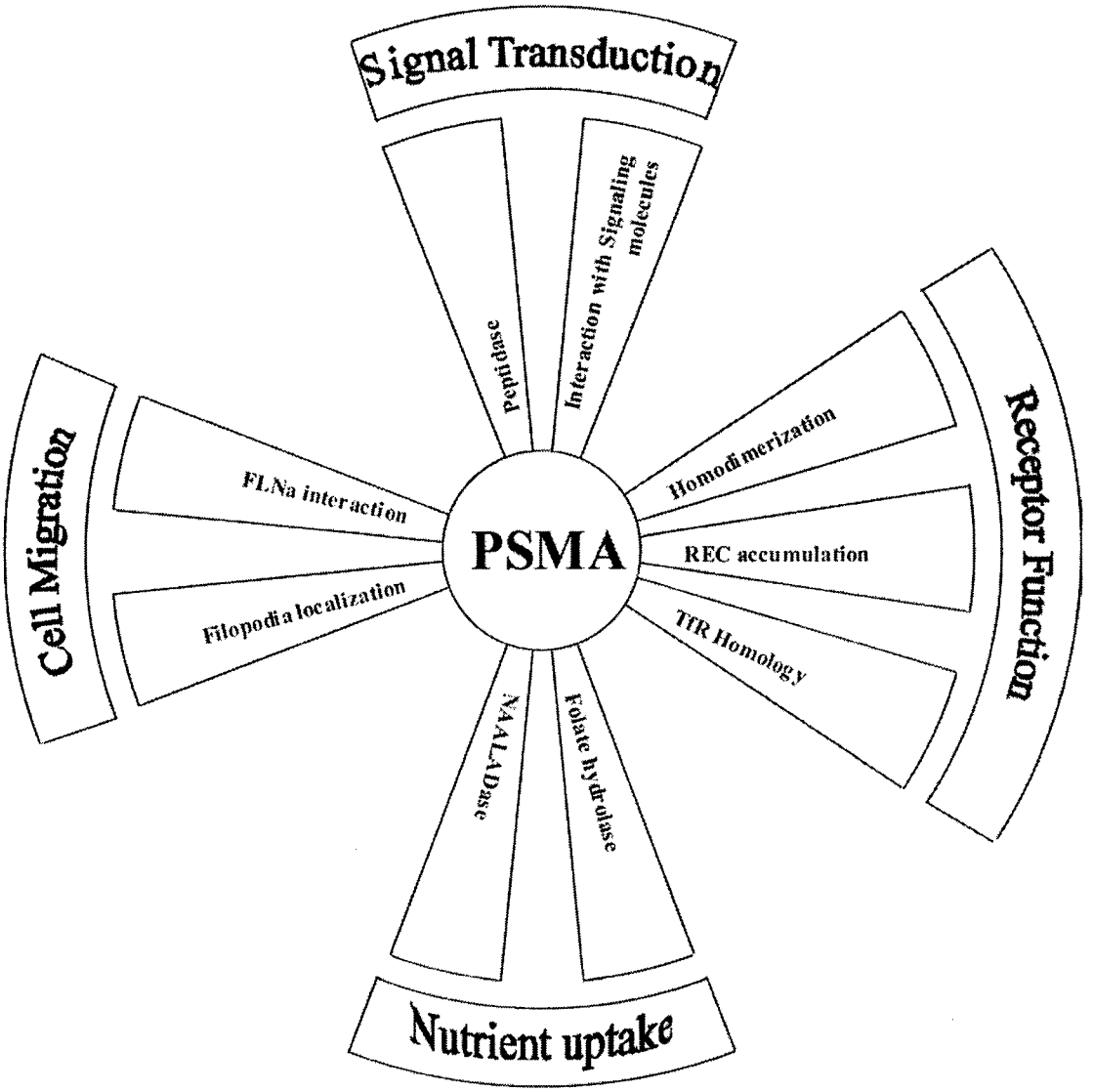


Figure 2


Signal Transduction
peptidase
Interaction with Signaling molecules
Receptor Function
Homodimerization
REC accumulation
TfR Homology
Cell Migration
FLNa interaction
Filopodia localization
PSMA
NAALADase
Folate hydrolase
Nutrient uptake

α-catenin expression restores adherens junction and suppresses β-catenin transcriptional activity in prostate cancer cells

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Key Words: β-catenin, adherens junction, α-catenin, TCF/LEF transcription, APC, GSK-3β, prostate cancer

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Abstract

The α, β, γ and p120 catenins bound to the cytoplasmic tail of E-cadherin play a central role in the regulation of cell-cell adhesion and signaling in epithelial cells. Altered expression of catenins is associated with events involved in cancer progression. α-Catenin binds to β-catenin and links E-cadherin complex to the actin cytoskeleton. β-catenin also functions as a transcriptional activator and has roles in cell proliferation, embryonic development and carcinogenesis. In this study, we present evidence that in PC3 cells, a α-catenin null prostate cancer cell line, increased β-catenin mediated transcriptional activity, is associated with lack of the adherens junction, elevated cyclin D1 levels and increased cell proliferation. The α-catenin expression in this cell line, restored the adherens junction, reduced β-catenin mediated transcription, cell proliferation and cyclin D1 levels. In addition, the APC/GSK-3β scaffolding complex involved in the degradation of free β-catenin is active in α-catenin null and α-catenin expressing PC3 cells. These results are consistent with a model in which α-catenin and the adherens junction play a critical role in the sequestration of free β-catenin and suggest that loss of α-catenin in prostate cancer activates β-catenin signaling and could contribute to the development and or progression of prostate cancer.

Introduction

Cell-to-cell adhesion plays an important role in both the establishment and maintenance of junctional complexes and thus, in the formation and maintenance of the well-differentiated phenotype of epithelial cells. The calcium dependent cell adhesion molecule, E-cadherin plays a crucial role in this process. The extracellular domain of E-cadherin mediates homophilic interaction and the cytoplasmic tail associates with the catenins (α,β,γ and p120 catenins) [1-4]. Both these interactions are essential for the cell-cell adhesive function of this protein. The linkage of E-cadherin to the actin cytoskeleton by the catenins is crucial for stabilizing E-cadherin at the plasma membrane.

α-Catenin is an actin binding protein that links E-cadherin-catenin complex to the actin cytoskeleton [5]. Cell lines deficient in α-catenin expression showed reduced cell-cell adhesion [6-9], which can be reversed by repletion of α-catenin [7-9]. In addition to its role in cell-cell adhesion, α-catenin function has also been implicated in cell proliferation [6, 7] and suppression of tumorigenicity in *in-vivo* xenograft experiments [8, 9]. However, the mechanism by which α-catenin is involved in the control of cell proliferation or tumor growth suppression is not well understood.

β-Catenin localizes to the adherens junction, where it binds directly to the cytoplasmic tail of E-cadherin and plays a critical role in the cell-cell adhesion function of E-cadherin [1, 2, 10]. However, in addition to its role in cell-cell adhesion, the regulation of β-catenin levels plays a major role in the Wnt

signaling pathway, which is involved in the regulation of cell proliferation, embryonic development and carcinogenesis [11, 12]. The levels of cytoplasmic (free) β-catenin is regulated by its association with Adenomatous Polyposis Coli (APC)[7] protein, which targets β-catenin for phosphorylation by the protein kinase, Glycogen Synthase Kinase-3 β (GSK-3β), marking β-catenin for degradation via a ubiquitin/proteosome pathway [12]. Activation of the Wnt receptors, Frizzled and LRP5/6, inhibits APC/GSK-3β function and results in the inhibition of β-catenin degradation [11-13]. This inhibition allows β-catenin to translocate to the nucleus, where it associates with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of activators, and induces transcription of target genes, such as myc and cyclin D1, proteins involved in cell growth and proliferation [14-16]. Mutations to either the APC protein or the GSK-3β phosphorylation sites of β-catenin increases level of free β-catenin and prevents its degradation in carcinoma cells [12, 17, 18].

In this study, we show that repletion of α-catenin expression in α-catenin null PC3 cells formed extensive adherens junction and significantly reduced β-catenin mediated TCF/LEF transcription of cyclin D1 and cell proliferation. Our results are consistent with a model that adherens junctions are involved in sequestering free β-catenin and loss of this junction overwhelms the APC- GSK-3β scaffolding complex leading to increased transcriptional activity of β-catenin in prostate cancer cells. These results suggest that loss of α-catenin in prostate

cancer cells increases the transcriptional activity of β-catenin and may contribute to events associated with prostate cancer progression.

Materials and Methods

Cell Lines and Cell Culture

The Prostate Cancer cell line, PC3 was obtained from the American Type Culture Collection. The PC3 cells expressing α-catenin were generated by microcell transfer of chromosome 5 (PC3-α) and a revertant cell line that lost expression of α-catenin (PC3-Rev) have been previously described [8]. All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, MEM nonessential amino acid solution (Invitrogen, Grand Island NY) and penicillin/streptomycin.

Antibodies and Reagents

Monoclonal antibodies against E-cadherin (Zymed, CA), cyclin D1 (Cell Signaling Technology, MA), α-Catenin (BD Transduction Laboratories, NJ), β-catenin (BD Transduction Laboratories, NJ), Adenomatous Polyposis Coli (APC) (Oncogene, CA) and polyclonal antibodies against β-catenin (Chemicon, CA), phosphorylated-β-catenin (Cell Signaling Technology, MA), and polyclonal ZO-1 (Zymed, CA) were obtained from indicated vendors. FITC and Texas Red labeled anti-mouse and anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories (PA) and horseradish peroxidase conjugated anti-mouse and anti-rabbit were obtained from Cell Signaling Technology (MA). The TOPFLASH and FOPFLASH reporter plasmids were kindly provided by Dr. Marian Waterman (University of California, Irvine, Irvine CA).

Immunoblotting

Cell lysates were prepared using either a lysis buffer containing 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM Sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM Sodium Vanadate, 1mM phenylmethylsulfonyl fluoride and 5μg/ml of antipapain, leupeptin and pepstatin or a lysis buffer containing 25mM Tris-HCl (pH 7.4), 95mM NaCl, 3mM EDTA, 2% SDS, 1mM phenylmethylsulfonyl fluoride and 5μg/ml of antipapain, leupeptin and pepstatin. 100μg of cell lysates were separated by SDS-PAGE (all proteins except APC were separated on 10% gels, APC was separated on a 8% gel) and transferred to nitrocellulose membrane. For immunoblotting, blots were blocked in 5% nonfat Milk in TBS (Tris buffered saline) /0.1% Tween 20 (TBST). Primary antibodies were diluted in 5% nonfat milk/TBST and incubated overnight at 4^{O}C. Secondary antibodies were diluted in 5% nonfat milk in TBST and blots were developed with ECL or ECL plus (Amersham, NJ). For phosphorylated-β-catenin, 5% Bovine Serum Albumin (Sigma, MO) in TBST was used instead of milk.

Immunofluorescence and Confocal Microscopy

Immunofluorescence and confocal microscopy were performed as described earlier [19]. Co-localization of both β-catenin and PI (propidium iodide) or cyclin D1 and PI was performed with a Fluoview laser scanning confocal microscope (Olympus America Inc., Melville, NY). Images where generated using the Fluoview software (version 2.1.39).

Transmission electron microscopy (TEM)

Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2-4 h at room temperature and processed for transmission electron microscopy as described previously [20]. Samples were examined for junctional complexes with a Philips 301 electron microscope (Phillips, Einthoven, Holland).

Triton X-100 solubility

PC3 and PC3-α cells were plated to 80% confluency, washed with cold PBS-CM and incubated for 10 minutes at 4°C with 500μl of extraction buffer (10mM PIPES, pH 6.8, 50mM NaCl, 3mM $MgCl_2$, 0.5% Triton X-100, 300mM Sucrose, 1mM phenylmethylsulfonyl fluoride and 5μg/ml of antipapain, leupeptin and pepstatin). Cells were scraped, transferred into eppendorf tubes, spun at 13,000 rpm for 30 minutes and the supernatant (soluble fraction) was transferred to a fresh tube. The pellet (insoluble fraction) was resuspended in 500μl 2XSDS sample buffer and sonicated. For immunoblotting, the soluble fraction (30μl of supernatant + 30μl of 2X SDS sample buffer) and the insoluble fraction (30ul of pellet fraction + 30ul of extraction buffer) were separated by SDS-PAGE and blotted with a monoclonal anti-β-catenin antibody as described above.

TOPFLASH/FOPFLASH reporter assay

$1X10^5$ cells per well were plated onto 6-well plates. 250ng of either TOPFLASH or FOPFLASH were transfected using LIPOfectamine PLUS (Invitrogen, Grand Island, NY) according to manufacturer's instructions. 0.05ng of Renilla control luciferase plasmid was co-transfected to normalize for transfection efficiency. 48

hours post transfection, cells were lysed with 1X passive Lysis Buffer (Promega, NJ) and the luciferase assay was performed using the Dual-Luciferase reporter Assay kit (Promega, NJ) as described previously [21]. TOPFLASH and FOPFLASH values were normalized to Renilla and fold induction for each cell line was calculated as normalized RLU (relative light units) of TOPFLASH divided by normalized RLU of FOPFLASH.

Measurement of Growth Curve

$1X10^5$ or $5X10^4$ cells per well of PC3 or PC3-α cells were plated onto 6-well plates. At time points of 24, 48, 72, 96 and 120 hours, media was removed, cells washed once with PBS, trypsinized, and counted using a hemocytometer. The cell doubling time was calculated according to the equation: $Td = 0.693(t)/\ln(N/No)$; t = time (in hours), N = cell number at time t, and No = cell number at initial time.

H^3-Thymindine Incorporation Assay

$5X10^4$ PC3 or PC3-α cells per well were plated onto 6 well plates. 6μCi/ml of H^3-Thymindine was added to each well and cells were pulsed for 4 hours. Cells were washed 3 times with cold PBS-CM, 3 times with cold 5% TCA and lysed with 300ul of 0.125N NaOH/0.05% SDS lysis buffer. Lysates were transferred to 10ml of scintillation fluid and counted in a Beckman LS 6500 scintillation counter.

Results

Restoration of tight junctions, adherens junctions, and desmosomes in PC3-α cells

PC3-α and PC3 cells expressed similar levels of E-cadherin, whereas α-catenin was absent in the parental PC3 line (Fig.1A). To investigate if re-expression of α-catenin induced formation of epithelial junctional complexes, PC3 and PC3-α cells were analyzed by immunofluorescence using antibodies against markers for the tight junction (ZO-1) and the adherens junction (E-cadherin). As shown in figure 1B (top Panels), both E-cadherin and ZO-1 revealed intense staining at cell-to-cell contacts, typical of cells expressing these junctional complexes [19]. However, PC3 cells, despite E-cadherin staining at the plasma membrane, did not form epithelial junctions (Fig.1B-bottom Panels). In addition, ZO-1 staining was predominantly intracellular (Fig.1B-bottom panels). Consistent with the immunofluorescence data, transmission electron microscopy revealed tight junctions (arrowhead) and adherens junctions (arrow) in PC3-α cells (Fig.1C). PC3-α cells also showed microvilli (Fig.1C, asterisk) and desmosomes (Fig.1D-white arrowhead). These junctional complexes were absent in PC3 cells (Fig.1E and F).

Reduced β-catenin transcriptional activity in PC3-α cells

A quantitative immunoblot analysis of the β-catenin levels in the detergent soluble (S) and insoluble (In) fractions revealed 11-fold more β-catenin in the

detergent insoluble fraction in PC3-α cells compared to PC3 cells (Fig.2A), indicating that a large fraction of β-catenin is associated with the cytoskeleton in PC3-α cells. Consistent with this data, confocal microscopy revealed intense β-catenin staining at the regions of cell-to-cell contact in PC3-α cells, with low levels in both the cytoplasm and nucleus (Fig.2B). In contrast, PC3 cells revealed increased β-catenin staining in both the cytoplasm and the nucleus (yellow) compared to PC3-α cells (Fig.2B). The increased detergent solubility of β-catenin (Fig.2A) combined with its cytoplasmic localization (Fig.2B) indicates the presence of free β-catenin in PC3 cells.

Nuclear localization of β-catenin suggested that PC3 cells might have increased β-catenin TCF/LEF transcriptional activity [16]. The TOPLASH and FOPFLASH luciferase assay is a well-established quantitative reporter assay for β-catenin/TCF/LEF transcriptional activity [22]. We transiently transfected either TOPFLASH (positive control containing TCF/LEF binding sites for β-catenin) or FOPFLASH (negative control containing mutated TCF/LEF binding sites) plasmids into PC3 and PC3-α cells and measured the luciferase reporter activity. As expected, PC3 cells showed 2.91-fold more TOPFLASH activity over FOPFLASH, while PC-3α cells showed minimal (0.67 fold) TOPFLASH activity over FOPFLASH (Fig.2C). This result indicated that α-catenin expression in PC3-α cells significantly reduced the transcriptional activity of β-catenin (Fig.2C).

Decreased cyclin D1 levels and cell proliferation in PC3-α cells

Activation of β-catenin/TCF/LEF transcription has been shown to induce expression of cyclin D1, a protein that is involved in cell proliferation [14, 15]. Immunoblot analysis of cyclin D1 revealed that PC3 cells have 6-fold more cyclin D1 compared to PC3-α cells (Fig.3A). In addition, confocal microscopy revealed nuclear localization of cyclin D1 in PC3 cells (Fig.3B) indicating activation of cyclin D1 [23]. In PC3-α cells, nuclear staining of cyclin D1 was not detected (Fig.3B).

Increased expression of cyclin D1 is associated with increased cell proliferation [24, 25]. Consistent with cyclin D1 levels, the PC3 cells showed a doubling time of 25 hrs compared to 41 hours in PC-α cells (Fig.3C). As an independent test to verify cell proliferation, we compared the H^3-Thymindine incorporation of PC3 and PC3-α cells. Consistent with the increased doubling time PC3-cells revealed a 3-fold increase in the levels of H^3-Thymindine incorporation compared to PC-3α cells (Fig.3D). Taken together, these data demonstrate that re-expression of α-catenin in PC-3 cells decreased cyclin D1 levels and cell proliferation.

Loss of α-catenin expression restores parental PC3 phenotype in PC3-α cells

PC3-α cells were generated by microcell transfer of an entire copy of chromosome 5, suggesting that other genes located on chromosome 5 might also contribute to formation junctional complexes, reduced β-catenin

transcriptional activity and cell proliferation. Initially, we attempted to rule out this possibility by generating PC3 cells expressing α-catenin alone using conventional transfection methods. However, the constitutive expression of α-catenin by a viral promoter caused growth arrest (data not shown) and hindered the isolation of a stable cell clone. Therefore, we resorted to a revertant clone of PC3-α cells to investigate the specific role of α-catenin. As described previously, PC3-Rev cells are null for α-catenin expression (Fig.4A), yet contain both the copy of chromosome 5 and are resistant to neomycin [8]. Transmission electron microscopy of PC3-Rev cells did not reveal tight junctions, adherens junctions and desmosomes (Fig.4C). Further, immunofluorescence analysis of β-catenin revealed both cytoplasmic and nuclear localization similar to PC3 cells (Fig.4B). The β-catenin transcriptional activity in PC3 and PC3-Rev cells showed comparable activation of TOPFLASH over FOPFLASH (1.79 for PC3 versus 2.04 for PC3-Rev) (Fig.4E). Cyclin D1 levels were comparable in PC3-Rev cells to PC3 cells (Fig.4D). Additionally, the growth rate and doubling time (26 hrs) of PC3-Rev cells were comparable to PC3 cells (Fig.4F). Taken together, these results are consistent with a role for α-catenin in the induction of epithelial junctional complexes and suppression of β-catenin/TCF/LEF signaling in PC3-α cells.

Reduced GSK-3β phosphorylated β-catenin in PC3-α cells

One of the several genes localized onto chromosome 5 with α-catenin is the APC protein. Therefore, introduction of an additional copy of APC could

increase the levels of APC and might contribute to increased β-catenin degradation in PC3-α cells. To test this possibility, we immunoblotted protein lysates from PC3, PC3-α and PC3-Rev cells with an antibody for APC that detects the full-length protein. As shown in figure 5A, the levels APC were comparable between PC3, PC3-α and PC3-Rev cells indicating microcell transfer of chromosome 5 does not alter the level of APC in PC3-α cells.

Binding of β-catenin to Adenomatous Polyposis Coli (APC) protein leads to phosphorylation by GSK-3β kinase at serines 33,37 and theronine 41 leading to its degradation through an ubiquitin-mediated mechanism [12, 18]. Since the levels of APC are comparable in all these cell lines, we sought to test the levels of GSK-3β phosphorylated β-catenin in these three cell lines. Protein lysates from PC3, PC3-α and PC3-Rev cells were immunoblotted with an antibody specific to GSK-3β phosphorylated β-catenin (Fig.5B). In addition, to control for the specificity of GSK-3β phosphorylation, we treated PC3, PC3-α and PC3-Rev cells with LiCl, a specific inhibitor of GSK-3β [26]. As shown in figure 5B, treatment with 20mM LiCl considerably decreased the intensity of phosphorylated β-catenin in all the three cell lines. The levels of GSK-3β phosphorylated β-catenin were similar in PC3 and PC3-Rev cells, whereas there was a 3.17-fold reduction in PC3-α cells (Fig.5B). Although the total levels of β-catenin were comparable in all these cell lines (Fig.5A), the drastic reduction in the levels of GSK-3β phosphorylated β-catenin in PC3-α cells indicates that there is less cytoplasmic β-catenin available for GSK-3β phosphorylation. Furthermore, these

results also indicate that APC and GSK-3β functions are not significantly altered in PC3, PC3-Rev and PC3-α cell lines.

Discussion

In this study, we demonstrated that repletion of α-catenin expression by microcell transfer of chromosome 5 in PC3 cells induced epithelial junctional complexes such as tight junctions, adherens junctions, and desmosomes. These junctional complexes were absent in PC3-Rev cells, which have lost the expression of α-catenin portion of the transferred chromosome, indicating that α-catenin expression is critical for the re-establishment of junctional complexes in PC3-α cells. PC3-α cells showed reduced β-catenin mediated TCF/LEF transcriptional activity, as confirmed by TOPFLASH/FOPFLASH reporter assays, as well as by determining the levels and localization of cyclin D1, a target gene activated by β-catenin signaling. Furthermore, we have presented evidence that increased cyclin D1 levels were associated with the increased cell proliferation of parental PC3 and PC3-Rev cells. Although total levels of β-catenin were comparable in PC3, PC3-α and PC3-Rev cells, the levels of GSK-3β phosphorylated β-catenin was highly reduced in PC3-α cells, indicating that in PC3-α cells there is less cytoplasmic β-catenin available for GSK-3β phosphorylation. These results strongly indicate that α-catenin plays a crucial role in modulating free β-catenin levels by sequestering β-catenin in the adherens junction in prostate cancer cells.

PC3-α cells showed tight junctions, adherens junctions and desmosomes, whereas these junctional complexes were absent in both PC3 and PC3-Rev cells. Such induction of junctional complexes following α-catenin repletion has been

shown in PC9, a α-catenin null lung cancer cell line [7] and in the human ovarian carcinoma cell line, OV2008, which contains a mutated α-catenin that is unable to bind to β-catenin [9]. Similarly, keratinocytes from α-catenin knock out mice lacked adherens and tight junctions [6], demonstrating a critical role of α-catenin in the establishment of adherens and other junctional complexes in epithelial cells. We have previously shown that optimal levels of E-cadherin and Na, K-ATPase β-subunit expression are important for the establishment of junctional complexes in epithelial cells [21, 27]. The levels of both these proteins were comparable in PC3, PC3-Rev, and PC3-α cells (unpublished data). Since α-catenin is an actin binding protein [5], repletion of α-catenin expression might result in the recruitment of more actin and other actin binding proteins to the subplasma membrane region, thus facilitating stabilization of E-cadherin and Na, K-ATPase on the plasma membrane leading to the induction of adherens junction and other junctional complexes in epithelial cells.

Our results on the decreased doubling time and ^{3}H-thymidine uptake in PC3-α cells, as compared to PC3 and PC3-Rev cells, demonstrate that repletion of α-catenin reduced cell proliferation in PC3 cells. This result is consistent with a previous study in PC3 cells [8]. We now provide evidence that decreased β-catenin/TCF/LEF transcriptional activity and cyclin D1 levels in PC3-α cells is a possible mechanism for reduced cell growth in these cells. Interestingly, keratinocytes obtained from α-catenin knockout mice exhibited hyperproliferation, yet in this case, β-catenin transcriptional activity was not

altered [6]. In contrast, expression of wild type α-catenin in OV2008 cells, reduced cell growth, however the mechanism of the reduced cell growth is not known [9]. Taken together these results indicate that α-catenin has a role in suppression of cell proliferation in cancer cells. Our results indicate that in prostate cancer cells, loss of α-catenin is associated with increased transcriptional activity of β-catenin, leading to increased cell proliferation. Since the expression of α-catenin in PC3 cells is achieved via microcell transfer, α-catenin expression is regulated by its own promoter elements in this system. Therefore the observed results are not due to over expressing α-catenin from a heterologous promoter. However, future research is needed to further explore the role of α-catenin in cell proliferation.

The total levels of β-catenin were similar in PC3, PC3-Rev and PC3-α cells indicating that loss of α-catenin or repletion of α-catenin do not significantly alter the total levels of β-catenin. Similarly, the levels of APC were also similar in these cell lines. However, the levels of GSK-3β phosphorylated β-catenin in PC3 and PC3-Rev cells were 3-fold higher than PC3-α cells. In addition, PC3-α cells showed 11-fold more β-catenin in the detergent insoluble fraction. Based upon these results, we suggest that in PC3-α cells, formation of adherens junction sequesters β-catenin and prevents β-catenin from accumulating in the cytoplasmic pool and translocating into the nucleus (see Fig.6). A large reduction of both the β-catenin transcriptional activity and cyclin D1 levels in PC3-α cells further supports this notion. In the absence of α-catenin and adherens junction,

the APC/GSK-3β mediated degradation system might be saturated with β-catenin, allowing β-catenin to escape the degradation mechanism and enter the nucleus and activate transcription of target genes such as cyclin D1 (Fig.6). A recent study suggested that β-catenin exists in two distinct conformations binding to E-cadherin and TCF respectively, responsible for either cell adhesion or cell signaling. Interestingly, binding of α-catenin to β-catenin was found to enhance β-catenin's affinity for E-cadherin [28], while monomeric β-catenin preferentially binds to TCF. It is possible that in PC3 cells, loss of α-catenin might result in decreased β-catenin affinity for E-cadherin and increased affinity for TCF, leading to β-catenin transcriptional activation. Thus these studies illuminate that loss of α-catenin in prostate cancer might result in the activation of β-catenin signaling activity providing a proliferative advantage to prostate cancer cells. In fact, a potential prognostic value for α-catenin in prostate cancer has been suggested [29-32] and chromosome 5 (which contains the α-catenin gene) has been shown to be lost in high-grade prostate cancer [33].

Based on our results we suggest that β-catenin exist in distinct structural and signaling pools. When adherens junctions are present (Fig.6A), the structural pool is stabilized with E-cadherin and α-catenin at the adherens junction, while the signaling pool is targeted for degradation by the proteosome by the APC/GSK-3β-scaffolding complex. Normally, Wnt signaling stabilizes the signaling pool of β-catenin and allows transcriptional activation by β-catenin. In the absence of adherens junction (Fig.6B), the level of β-catenin within the

structural pool decreases with a concomitant increase in the cytoplasmic pool (indicated by thick arrow). A large transition of β-catenin to the cytoplasmic pool overwhelms the APC/GSK-3β scaffolding complex mediated degradation of β-catenin (indicated by dotted lines). The surplus β-catenin is then able to enter the nucleus and activate transcription. Thus this model provides evidence for a crucial regulatory role for the adherens junction and α-catenin in controlling the availability of β-catenin for signaling mechanisms.

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Figure Legends

Fig. 1. Expression of α-catenin in PC3 cells restores epithelial junctional complexes. A: Immunoblot of PC3 and PC3-α SDS protein lysates for E-cadherin and α-catenin. Both cell lines express equivalent amounts of E-cadherin, while α-catenin is expressed only by PC3-α cells. β-actin was used as a loading control. Representative blot from three independent experiments is shown. B: Immunofluorescence analysis of PC3 and PC3-α cells. Top panels: PC3-α cells revealed intense staining of both E-cadherin (green) and ZO-1 (red) at sites of cell-to-cell contact. Bottom Panels: PC3 cells revealed E-cadherin (green) staining at the cell membrane, but did not show cell-cell contact. ZO-1 (green) was localized to the cytoplasm. C, D: Transmission electron microscopy of PC3-α cells. PC3-α cells revealed tight junction (arrowhead, C), adherens junction (arrow, C) and desmosome (white arrowhead, D), as well as microvilli (asterisk, C). E, F: Transmission electron microscopy of PC3 cells. PC3 cells did not reveal epithelial junctional complexes. F shows a magnified region highlighted in E by a rectangle.

Fig. 2. Expression of α-catenin affects β-catenin localization and transcriptional activity. A: Triton X-100 soluble (S) and insoluble (In) fractions were extracted from PC3 and PC3-α cells. Equal volumes of soluble and insoluble fractions were separated by SDS-PAGE and immunoblotted for β-catenin. Representative blot from three independent experiments is shown. B: Immunofluorescence staining of β-catenin in PC3 and PC3-α cells. β-catenin (green) localized to sites of cell-to-cell contact in PC3-α cells. Nuclei are stained with propidium iodide (red). Note the localization of β-catenin in the nucleus (yellow) in PC3 cells. C: TOPFLASH/FOPFLASH reporter assay in PC3 and PC3-α cells. Results are from two independent experiments done in triplicate. Bars indicate standard error.

Fig.3. Expression of α-catenin affects cell proliferation in PC3 cells. A. Protein lysates from PC3 and PC3-α cells were separated by SDS-PAGE and immunoblotted for cyclin D1. β-actin was used as a loading control. Representative blot from three independent experiments is shown. B. Immunofluorescence of cyclin D1 in PC3 and PC3-α cells. Top Panels: PC3-α cells. Bottom Panels: PC3 cells. Note the localization of cyclin D1 in the nucleus (yellow) in PC3 cells. Nuclei are stained with propidium iodide (red). C. Growth Curve of PC3 and PC3-α cells. Results are from two independent experiments done in triplicate. Bars indicate standard error. D. Thymindine uptake in PC3

and PC3-α cells. Data is presented as counts per minute (CPM). Results are from two independent experiments done in triplicate. Bars indicate standard error.

Fig.4. Loss of α-catenin expression in PC3-α cells, results in reversion to parental PC3 phenotype. A. Immunofluorescence staining of α-catenin (green) in PC3-Rev, PC3 and PC3-α cells. Nuclei are stained with propidium iodide (red). B. Immunofluorescence staining of β-catenin in PC3 and PC3-Rev cells. Both cell lines revealed cytoplasmic and nuclear staining of β-catenin (FITC-green). Nuclei are stained with propidium iodide (red). C. Low (left panel) and high (right panel) magnification of transmission electron microscopy of PC3-Rev cells. Epithelial junctional complexes are absent in PC3-Rev cells. D. Immunoblot analysis of cyclin D1 in PC3 and PC3-Rev cells. Note both cell lines reveal similar levels of cyclin D1. β-actin was used as a loading control. Representative blot from three independent experiments is shown. E. TOPFLASH/FOPFLASH reporter assay in PC3 and PC3-Rev cells. Note similar transcriptional activity of β-catenin in these cells. F. Growth Curve of PC3 and PC3-Rev cells. Results are from two independent experiments done in triplicate. Bars indicate standard error.

Fig. 5. α-catenin expression does not affect APC levels or GSK-3β phosphorylation of β-catenin. A. Immunoblot analysis of PC3, PC3-α and PC3-Rev cells showing total levels of APC and β-catenin. β-actin was used as a loading control. Representative blot from three independent experiments is shown. B. Immunoblot of PC3, PC3-α and PC3-Rev cell for GSK-3β phosphorylated β-catenin. Cells were lysed and immunoblotted with an antibody specific to phosphorylated β-catenin. Activity of GSK-3β was inhibited with 20mM LiCl. Samples were treated for two hours before lysis. β-actin was used as a loading control. Representative blot from three independent experiments is shown.

Fig. 6. A schematic model illustrating the roles of α-catenin and adherens junction in the regulation of β-catenin transcriptional activity. (see text for details).

Fig. 1

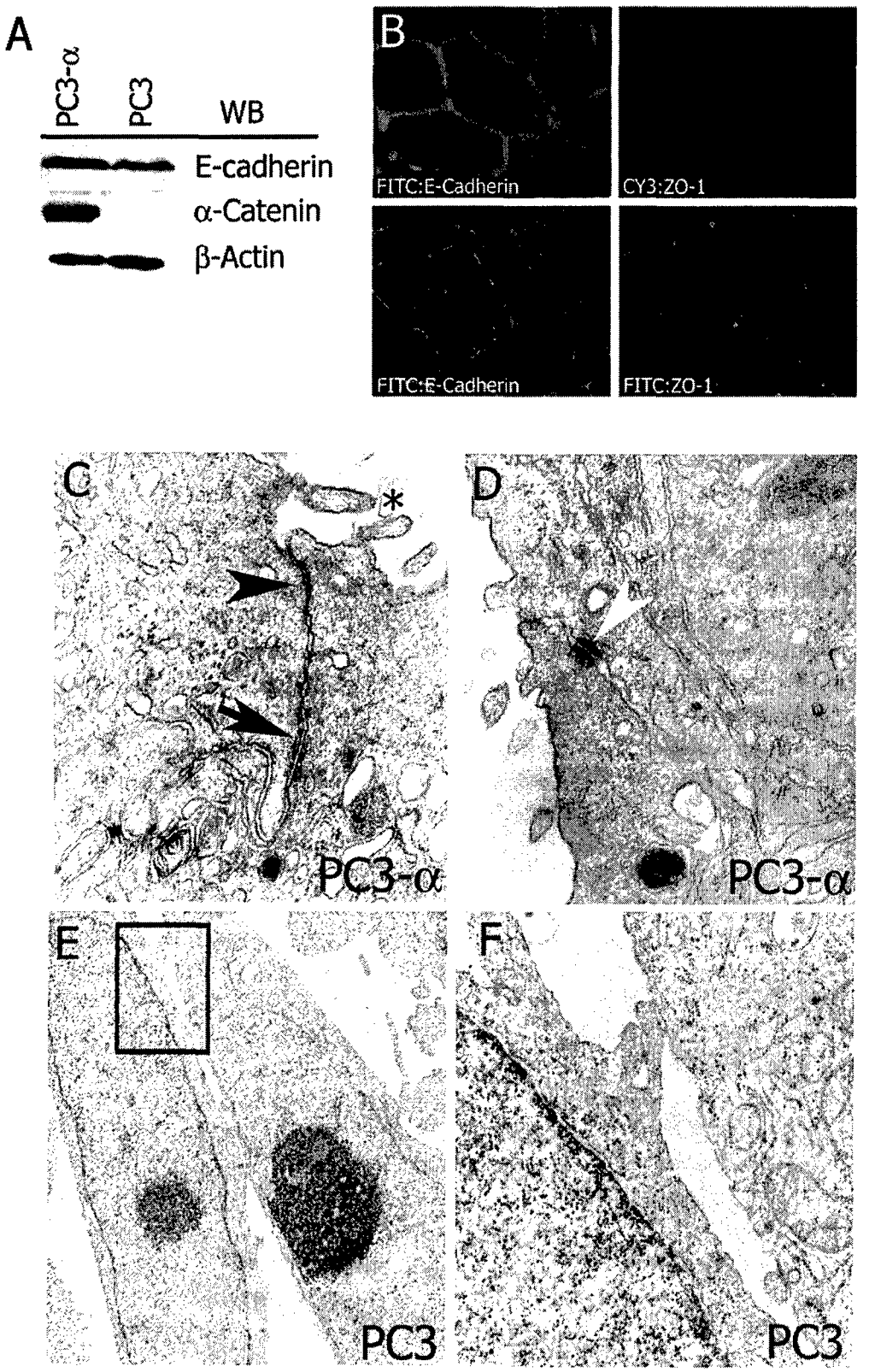
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Fig. 2

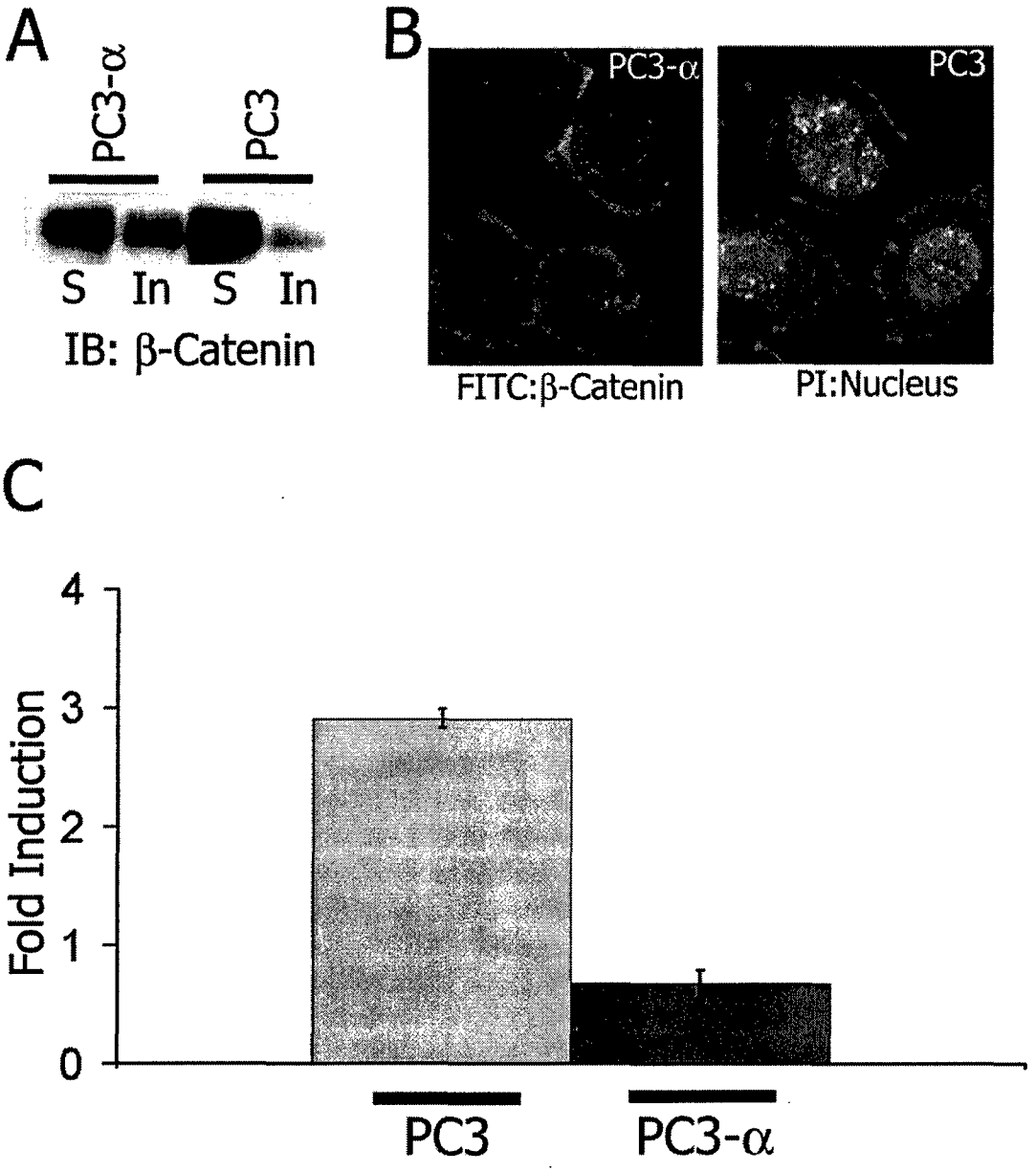
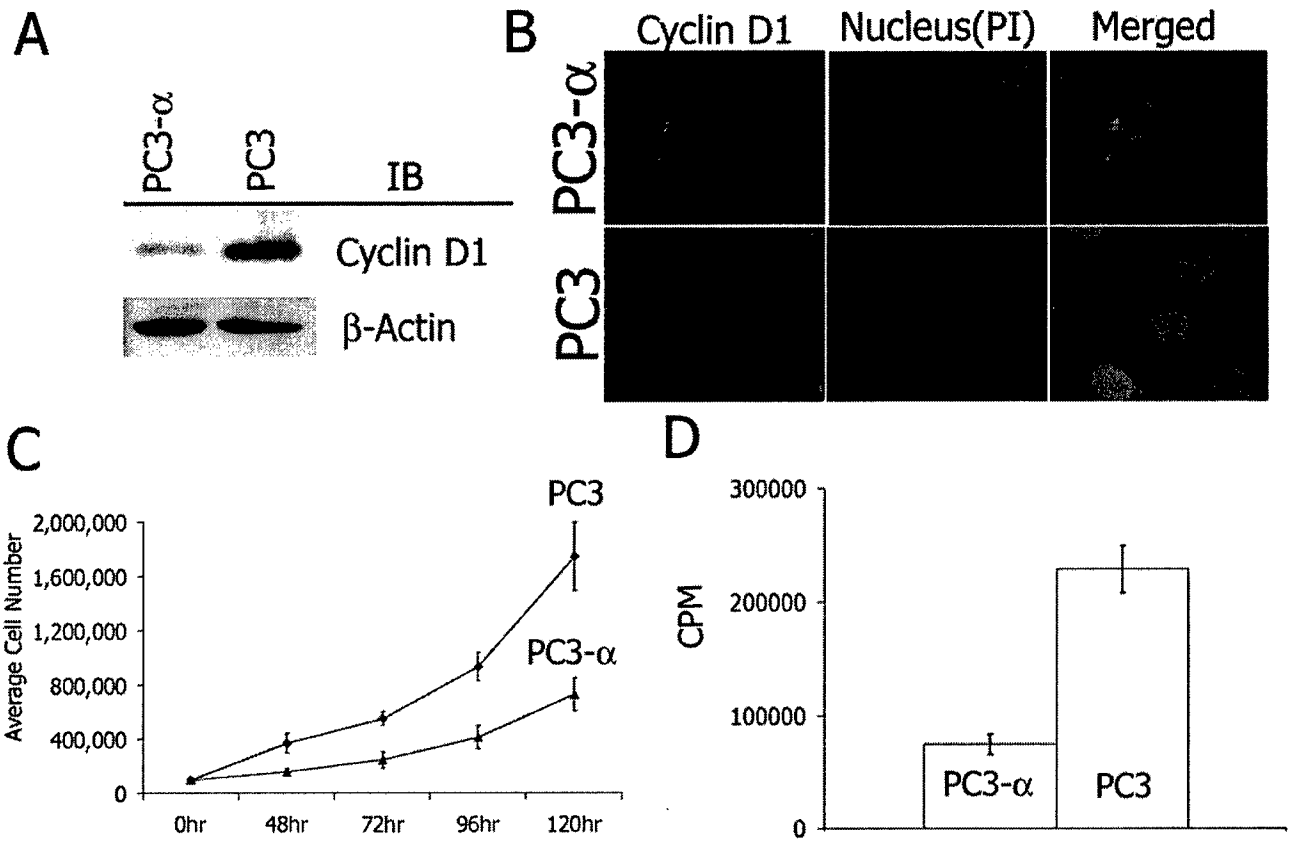

A
PC3-α
PC3
S
In
S
In
IB: β-Catenin
B
PC3-α
PC3
FITC:β-Catenin
PI:Nucleus
C
Fold Induction
0
1
2
3
4
PC3
PC3-α

Fig. 3


A
PC3-α
PC3
IB
Cyclin D1
β-Actin
B
Cyclin D1
Nucleus(PI)
Merged
PC3-α
PC3
C
PC3
PC3-α
Average Cell Number
2,000,000
1,600,000
1,200,000
800,000
400,000
0
0hr
48hr
72hr
96hr
120hr
D
CPM
300000
200000
100000
0
PC3-α
PC3

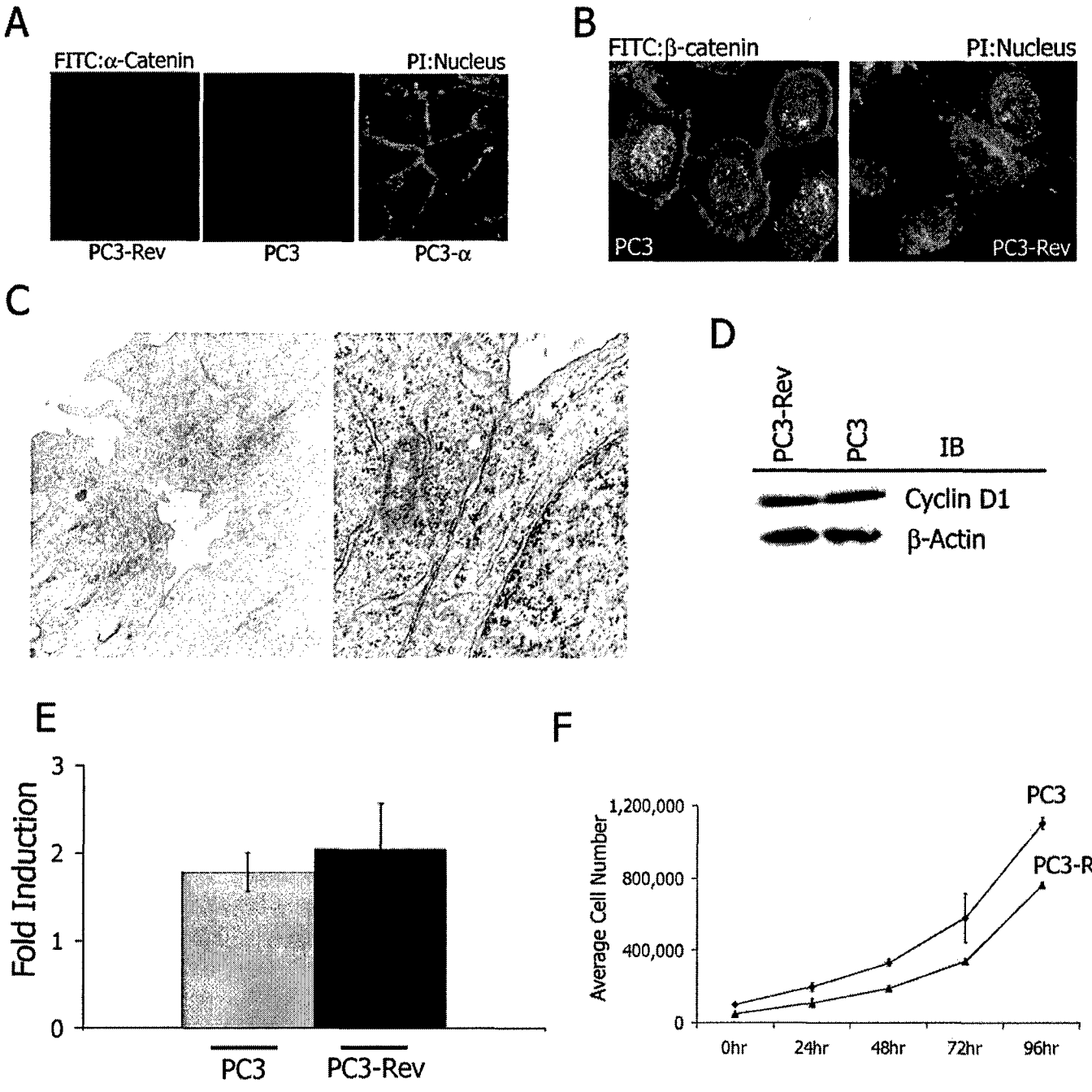

A
FITC:α-Catenin
PI:Nucleus
PC3-Rev
PC3
PC3-α
B
FITC:β-catenin
PI:Nucleus
PC3
PC3-Rev
C
D
PC3-Rev
PC3
IB
Cyclin D1
β-Actin
E
Fold Induction
3
2
1
0
PC3
PC3-Rev
F
Average Cell Number
1,200,000
800,000
400,000
0
PC3
PC3-Rev
0hr
24hr
48hr
72hr
96hr

Fig. 4

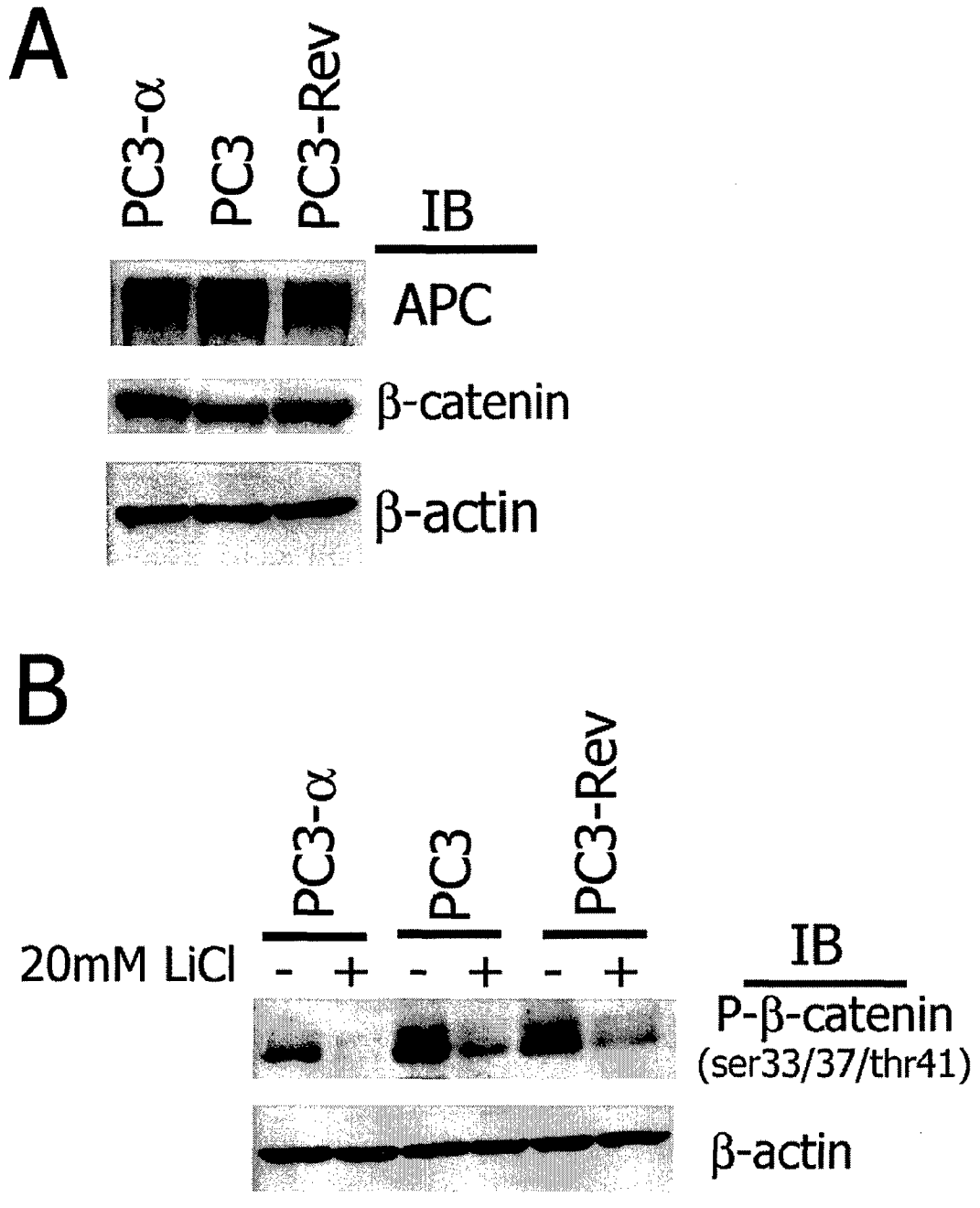
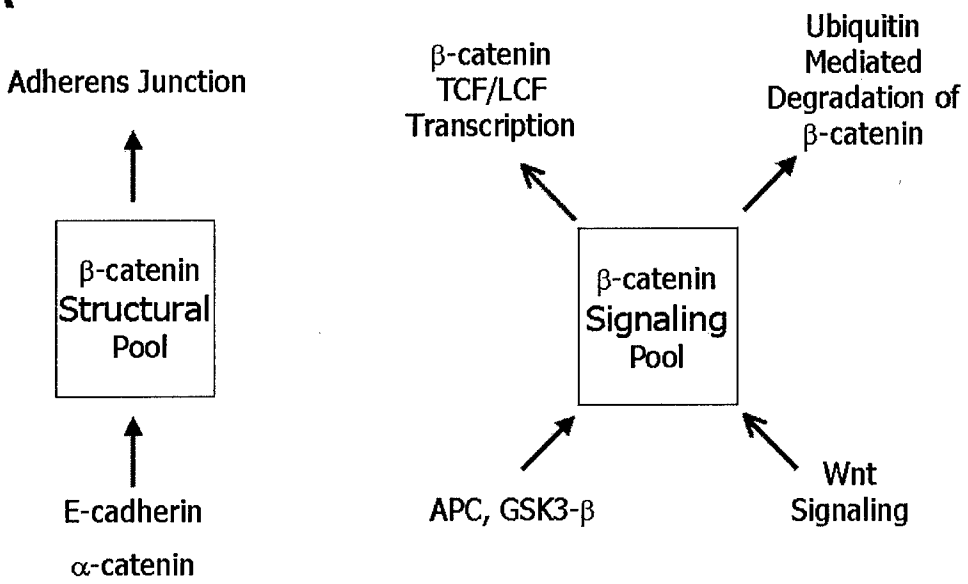

A
PC3-α
PC3
PC3-Rev
IB
APC
β-catenin
β-actin
B
PC3-α
PC3
PC3-Rev
20mM LiCl
- + - + - +
IB
P-β-catenin
(ser33/37/thr41)
β-actin

Fig. 5

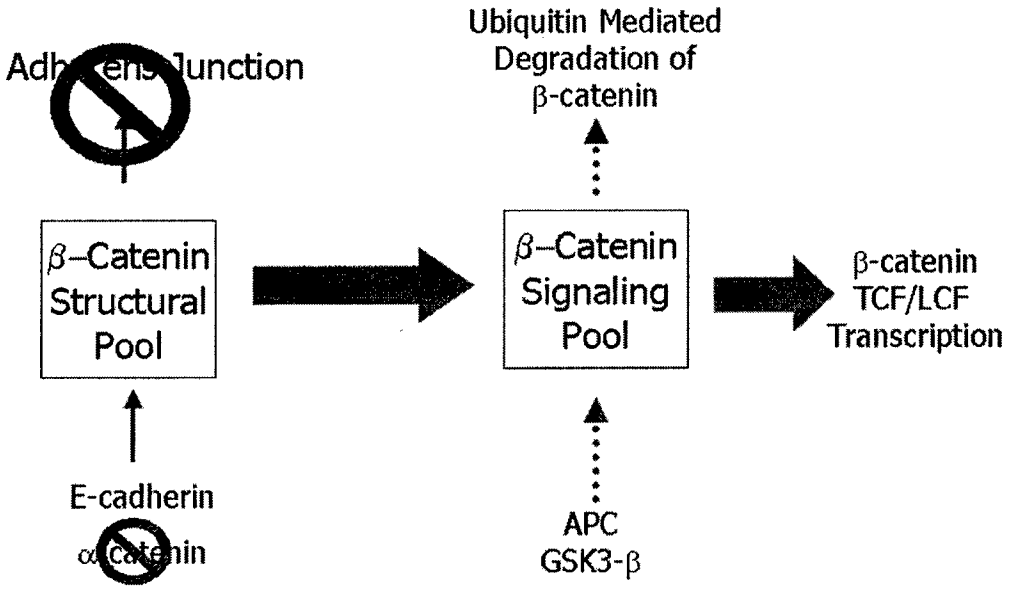
Fig. 6

A



Presence of Adherens Junction

B



Loss of Adherens Junction Function