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β-Catenin Mediates Alteration in Cell Proliferation, Motility and Invasion of Prostate Cancer Cells by Differential Expression of E-Cadherin and Protein Kinase D1

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Abstract We have previously demonstrated that Protein Kinase D1 (PKD1) interacts with E-cadherin and is associated with altered cell aggregation and motility in prostate cancer (PC). Because both PKD1 and E-cadherin are known to be dysregulated in PC, in this study we investigated the functional consequences of combined dysregulation of PKD1 and E-cadherin using a panel of human PC cell lines. Gain and loss of function studies were carried out by either transfecting PC cells with full-length E-cadherin and/or PKD1 cDNA or by protein silencing by siRNAs, respectively. We studied major malignant phenotypic characteristics including cell proliferation, motility, and invasion at the cellular level, which were corroborated with appropriate changes in representative molecular markers. Down regulation or ectopic expression of either E-cadherin or PKD1 significantly increased or decreased cell proliferation, motility, and invasion, respectively, and combined down regulation cumulatively influenced the effects. Loss of PKD1 or E-cadherin expression was associated with increased expression of the pro-survival molecular markers survivin, β-catenin, cyclin-D, and c-myc, whereas overexpression of PKD1 and/or E-cadherin resulted in an increase of caspases. The inhibitory effect of PKD1 and E-cadherin on cell proliferation was rescued by coexpression with β-catenin, suggesting that β-catenin mediates the effect of proliferation by PKD1 and E-cadherin. This study establishes the functional significance of combined dysregulation of PKD1 and E-cadherin in PC and that their effect on cell growth is mediated by β-catenin. J. Cell. Biochem. 104: 82–95, 2008.

Key words: proliferation; β-catenin; adhesion; E-cadherin; PKD1

Prostate cancer (PC) is the most commonly diagnosed noncutaneous malignancy among American men and approximately 30,000 men die from the disease in the United States each year [ACS, 2007]. Death from PC is primarily due to its metastasis to distal sites [Morrissey and Vessella, 2007]. The metastatic process consists of detachment of cancer cells from the primary tumor, adhesion to and degradation of the extracellular matrix, the invasion of blood and lymphatic vessels, and finally the establishment of metastases at secondary sites [Woodhouse et al., 1997]. A prerequisite for invasion into surrounding tissue is a change in the overall cellular machinery controlling these processes. A salient feature in the progression of PC is dysregulated expression of the E-cadherin and ß-catenin complex which play a pivotal role in epithelial cell–cell adhesion and in the maintenance of differentiated adult epithelia [Kemler, 1993; Hinck et al., 1994]. Perturbation of the expression or function of cadherin–catenin complex is widely involved in tumor progression and metastasis [Shimoyama et al., 1992; Kanai et al., 1994; Oyama et al., 1994; Yoshida et al., 2001].

E-cadherin is the dominant epithelial cell adhesion molecule and a member of a large family of cell–cell adhesion molecules. It is

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present in adheren junctions, where it undergoes calcium dependant homophilic binding [Berx et al., 1995; Shapiro et al., 1995]. E-cadherin interacts with a group of cytoplasmic proteins including α, β, and γ catenin. While β and γ-catenin bind directly to the E-cadherin cytoplasmic tail, α-catenin binds to either β or γ-catenin in a mutually exclusive complex. α-catenin links the E-cadherin complex to the cytoskeleton through its interaction with actin filaments [Aberle et al., 1994; Nathke et al., 1994; Berx et al., 1995; Shapiro et al., 1995]. Decreased E-cadherin expression is considered to be a central event in the disruption of the normal cell–cell adhesion required for the acquisition of the invasive and metastatic phenotype of various epithelial neoplasms, including PC [Bussemakers et al., 1992; Paul et al., 1997]. Aberrant E-cadherin expression was associated with a larger tumor size [Rubin et al., 2001], a higher tumor grade and stage, and an increased risk of disease recurrence after prostatectomy [Kuefer et al., 2005].

We have previously published a novel interaction between E-cadherin and Protein Kinase D1 (PKD1), which is associated with cell aggregation and motility in PC [Jaggi et al., 2005b]. PKD1 is a member of an evolutionarily conserved serine/threonine kinase family of proteins [Johannes et al., 1994; Valverde et al., 1994] and it is well positioned in cells, to regulate membrane, cytoplasmic, and nuclear events. Indeed, it is emerging that the PKD1 is involved in the regulation of a remarkable array of fundamental biological processes, including signal transduction, membrane trafficking, cell survival, migration, differentiation, and proliferation [Jamora et al., 1999; Liljedahl et al., 2001; Baron and Malhotra, 2002; Hausser et al., 2002; Storz et al., 2003; Yeaman et al., 2004; Rozengurt et al., 2005]. PKD1 plays a direct role in the proliferation of keratinocytes, endothelial cells, pancreatic cancer cells, and T cells either by increasing telomerase activity or up regulating anti-apoptotic proteins c-FLIP and survivin [Yancopoulos et al., 2000; Liljedahl et al., 2001; Hausser et al., 2002; Carmeliet, 2003].

Interestingly, PKD1 also interacts with β-catenin and is associated with its membrane trafficking of β-catenin (unpublished data). β-catenin is a unique member of the cadherin–catenin complex because of its dual role in cell adhesion and proliferation. Dismantling of adheren junctions frees ß-catenin from the membrane and, in the presence of Wnt signaling, ß-catenin escapes proteosomal degradation by binding the adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3ß (GSK-3ß) complex. It then translocates to the nucleus and binds members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factor family. The ß-catenin/TCF complex subsequently regulates the transcription of many genes involved in cell function, including survival, migration, differentiation, and proliferation. Cyclin D1 and c-myc are two key cell cycle proteins whose expression is regulated by ß-catenin/TCF signaling in cancer cells [Shtutman et al., 1999; Tetsu and McCormick, 1999; Kamei et al., 2003].

Although there is ample information in the literature about the functional roles of E-cadherin and PKD1 in cells, there is paucity of information regarding the combined influence of PKD1and E-cadherin on cell growth and motility of PC cells. To fill this gap in knowledge, we employed gain-and loss-of-function approaches to assess the combined role of PKD1 and E-cadherin in PC cell lines. Our findings demonstrate that suppression of E-cadherin and PKD1 increased cell proliferation independently and cumulatively. The effect is at least in part mediated by β-catenin, which upregulates Wnt signaling target genes, c-myc and cyclin-D1. Importantly, ectopic expression of E-cadherin/PKD1 leads to apoptosis of cells as evident by an increase in caspase-3. Furthermore, suppression of ß-catenin largely rescues the phenotype resulted from knockdown of PKD1 and E-cadherin and overexpression of ß-catenin rescues the phenotype resulted from overexpression of PKD1 and E-cadherin in PC cells, suggesting that β-catenin is a functional mediator of proliferative effects of PKD1 and E-cadherin in PC cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human PC cell lines LNCaP, C4-2 (derived from LNCaP cells [Liu et al., 2004]), ALVA-41 (derived from PC3 cells [van Bokhoven et al., 2003]), were cultured in RPMI-1640 (ATTC, Manassas, VA) and DU-145 was cultured in Minimum Essential Medium (ATTC) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) with 1% penicillin/streptomycin (Life Technologies, Gaithersburg,

MD). The cells were maintained at 37°C in 5% CO_2.

Expression Plasmids

The vectors containing PKD1 and ß-catenin were kindly provided by Dr. F.J. Johannes (Fraunhofer Institute for Interfacial Engineering, Stuttgart, Germany) and Dr. Rolf Kemler (Max-Planck Institute of Immunobiology Freiburg, Germany), respectively. Full length human E-cadherin (2.6 kb) was amplified by PCR using specific forward 5′-CACAGCTCGAGATGGGCCCTT GGAGCCGCAG and reverse 5′-CTCTAAGCTTGTCGTCCTCGCCGCCTCCGT primers. The PCR product was inserted into pEGFP-N1 by *Xho*I and *Bam*HI digestion.

Construction of shRNA Expression Vectors

Construction of an anti-PKD1 shRNA and anti-β-catenin shRNA expression vector was performed as described previously [Du et al., 2006]. For shRNA targeted to PKD1 Oligos: 5′-GATCCGGAAGGAAATATCTCATGATTCAAGAGA TCATGAGATATT TCCTTCC TTTTT A and 5′-AGCTT AAAAAGGAAG GAAATATCTC ATGATCTCTTGAATCATGAGATATTTCCTTCC G were annealed and for ß-catenin, Oligos 5′-GATCCCCACAAATCCAGTGAACAATTCAAGAGATTGTTCACTGGATTTGTGGTTTTTA and 5′-AGCTTAAAAACCACAAATCCAGTGAACAATCTCTTGAATTGTTCACTGGATTTGTGGG were annealed and ligated to KSU6 vector that had been digested with *Bam*HI and *Hin*dIII [Du et al., 2006]. For down regulation of E-cadherin transient transfection with siRNA was done as described below.

Transfection of PC Cells With Expression Vectors or siRNAs

The prostate cells (1×10^5 cells) were transfected with the pSV-β-galactosidase vector (Promega, Madison, WI) and the expression vector (1 μg/ml) containing a transgene or its respective empty control vectors or SMARTpool small interfering RNAs (5.0 nM, siRNA Dharmacon, Lafayette, CO) targeted to human E-cadherin (RNAi sequence 5′-GAUUGCACCGGUCGACAAAdTdT-3′) or nontargeting siRNA #1 using LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. Some of the transfected cells were stained for X-Gal to estimate transfection efficiency, which ranged from 50% to 60%. Seventy-two hours after transfection, total RNA or protein was extracted to confirm altered gene expression by RT-PCR or Western blotting. For soft agar and cell invasion assays, cells were plated on soft agar or Matrigel invasion chambers respectively 24 h after transfection.

Cell Proliferation Assay

PC cells (LNCaP, DU-145, C4-2, and ALVA-41) were seeded at a density of 1,000 cells/well in 96-well plates in medium containing 10% serum. Following 24-h growth, the cells were transfected with PKD1, E-cadherin, β-catenin, shRNA-PKD1, shRNA β-catenin, siRNA directed against E-cadherin or their respective controls using Lipofectimine 2000. Following 72 h of transfection, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution (Promega) was added to each well, and plates were incubated for 4 h in a humidified atmosphere. The absorbency of the product formazan, which is considered to be directly proportional to the number of living cells in the culture, was measured at 490 nm in a spectrophotometer. Relative cell growth of transfected cells with a target gene was expressed as percent change compared to cells transfected with respective control.

Invasion Assay

The invasion assay was done using 24-well Biocoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). The transfected cells and their respective control cells were suspended in medium at a concentration of 1×10^5 cells/ml, and 0.5 ml of each was added to the invasion chambers in triplicate. Medium (0.75 ml) supplemented with 10% fetal bovine serum was added to each well of the plate to act as a chemoattractant and the plates were placed in an incubator. After 18 h of plating, noninvading cells were removed from the upper surface of the membrane by scrubbing. The cells on the lower surface of the membrane were fixed for 2 min in 100% methanol and stained with 1% toluidine blue in 1% sodium borate for 2 min. Cells that invaded through the insert were counted in five random fields per slide. All slides were coded to avoid biased counting.

Soft Agar Colony-Formation Assay

The effects of suppression or ectopic expression of E-cadherin and PKD-1 on anchorage-

independent growth were measured as their abilities to form colonies on soft agar. LNCaP, C4-2, ALVA-4, and DU-145 cells were transiently transfected with E-cadherin, or PKD1 expression vectors (1 μg/ml) or with siRNA directed against E-cadherin or shRNA directed against PKD1 (5 nM), or with respective control vectors and control siRNAs as described above. After 24 h, transfected cells were plated on soft agar. A soft agar colony formation assay was done using six-well plates. Each well contained 2 ml of 0.6% agar in complete medium as the bottom layer, 1 ml of 0.3% agar in complete medium and 3,000 cells in the feeder layer, and 1 ml complete medium as the top layer. Cultures were maintained under standard conditions. The number of colonies was determined with an inverted phase-contrast microscope at 100× magnification; a group of >10 cells was counted as a colony.

Western Blot Analysis

To obtain cellular extracts, cells were lysed in lysis buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 0.5% Nonidet P-40), and proteins were quantified using protein determination kit (Pierce, Rockford, IL). Proteins (20 μg) from control and transfected cells were separated by sodium dodecyl sulfate–polyacrylamide (SDS) gel electrophoresis in 10% gels and transferred to PVDF membranes (BioRad, Hercules, CA). Blots were blocked with 5% nonfat dry milk in Trisbuffered saline/0.1% Tween 20 for 1 h and were probed with anti-E-cadherin antibody (1:2,500, BD Biosciences), anti-PKD1 antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin antibody (1:1,000 dilution; Santa Cruz Biotechnology), c-myc antibody (1:200 dilution; Santa Cruz Biotechnology), survinin antibody (1:500 dilution, BioLegend, San Diego, CA), cyclin-D1 antibody (1:1,000 dilution; Cell Signaling, Beverly, MA), caspases 9, 8, and 3 (1:1,000 dilution; Cell Signaling) or β-actin antibody (1:5,000 dilution; Sigma, St. Louis, MO) overnight at 4°C and then incubated with a secondary antibody (1:5,000 dilution) in 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 for 1 h at room temperature. After washing, bound antibodies were detected by modified chemiluminescence with enhanced chemiluminescence (ECL) reagents (Amersham, GE Health care life sciences, Piscataway, NJ) and visualized by autoradiography.

RNA Isolation, Semiquantitative RT-PCR

Total RNA was isolated from transfected and control cultures using the Tri-reagent (Sigma) according to the manufacturer's protocol. RNA (1 μg) from each sample was reverse-transcribed using GeneAmp RNA PCR kit (Perkin Elmer, CT). Complementary DNAs derived from RNA samples were stored at −20°C until used. Relative expression level of proliferating cell nuclear antigen (PCNA) in cell cultures was determined by semiquantitative RT-PCR. The PCNA primers were 5′-GGCGTGAACCTCACCAGTAT-3′ (forward) 5′-GTGTCCCATATCCGCAATTTT-3′ (reverse). Specific primers for GAP-DH were 5′-ATCACCATCTTCCAGGAGC-3′ (forward) and 5′-GGATGATGTTCTGGAGAGCC-3′ (reverse). Aliquots of 2 μl of cDNA were subjected to PCR amplification. Hot-start PCR using AmpliTaq Gold DNA polymerase (Perkin–Elmer) was used in all amplification reactions to minimize nonspecific product amplification. The annealing temperature for amplification of PCNA and GAPDH was 60°C. The number of amplification cycles was 35 for PCNA and 25 for GAP-DH. The other conditions were the same, with initial denaturation for 6 min at 94°C, cycle denaturation for 1 min at 94°C, annealing for 1 min, and extension at 72°C for 1 min. The PCR products were fractionated on 2% agarose gel.

Assays of Caspase Activity

LNCaP cells were transfected with shRNA of PKD1, siRNA E-cadherin or PKD1, E-cadherin expression vectors and following 72 h of incubation, the detached cells and the adherent cells were collected from each culture and suspended in 500 ml of ice-cold lysis buffer provided with the Caspases Assay kit (MLB International, Watertown, MA). After sonification, the cell lysate was centrifuged for 20 min at 14,000*g* at 4°C. The resulting supernatants were analyzed for protein concentrations using protein determination kit (Pierce) and stored at −20°C until use. Colorimetric enzymatic activity assays for caspases were performed according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Data were analyzed by ANOVA. Statistical significance was inferred at P-value <0.05.

RESULTS

Knockdown of PKD-1 and E-Cadherin Enhances Growth of PC Cells

Jaggi et al. [2005a,b] have reported reduced expression of PKD1 and E-cadherin in PC specimens. To understand the role(s) of PKD1 and E-cadherin separately or conjointly on PC cell growth, we knocked down the expression of these genes using shRNA and siRNA for PKD1 and E-cadherin, respectively, in four PC cell lines. A significant loss of PKD1 and E-cadherin protein expression was observed in PC cell cultures transfected with siRNAs (Fig. 1A). The expression level of E-cadherin in DU-145 cells was below detectable levels. No change in PKD1 and E-cadherin expression was observed in control cultures (Fig. 1A). Reduction in E-Cadherin and PKD1 expression was associated with a significant enhancement of cell growth in all four PC cell lines (Fig. 1B). Combined suppression of PKD1 and E-cadherin in PC cells resulted in a greater increase in cell growth compared to individual suppression, suggesting that a common pathway may be involved in the PKD1 and E-cadherin mediated effect on cell growth. The increase in cell growth was corroborated by examining the expression of cell proliferation marker, PCNA after normalization of the PCNA transcript with GAPDH (Fig. 1B).

PKD1 and E-Cadherin Suppression Increases the Ability of PC Cells to Grow in Soft Agar and Migrate Through Matrigel

Next, we investigated the effect of PKD1 and E-cadherin siRNA treatment on anchorage-independent growth by assaying colony formation on soft agar, an indicator of malignant potential. shRNA of PKD1 and siRNA of E-cadherin caused a significant increase in the number of soft agar colonies compared to non-silencing siRNA transfected cells (Fig. 2A). Combined suppression of PKD1 and E-cadherin was more effective than individual suppression in increasing the number of colonies formed in C4-2 and LNCaP cell lines (Fig. 2A). Because decreased E-cadherin expression is associated with poorer clinical outcomes and PKD1 is characteristically down regulated in advanced

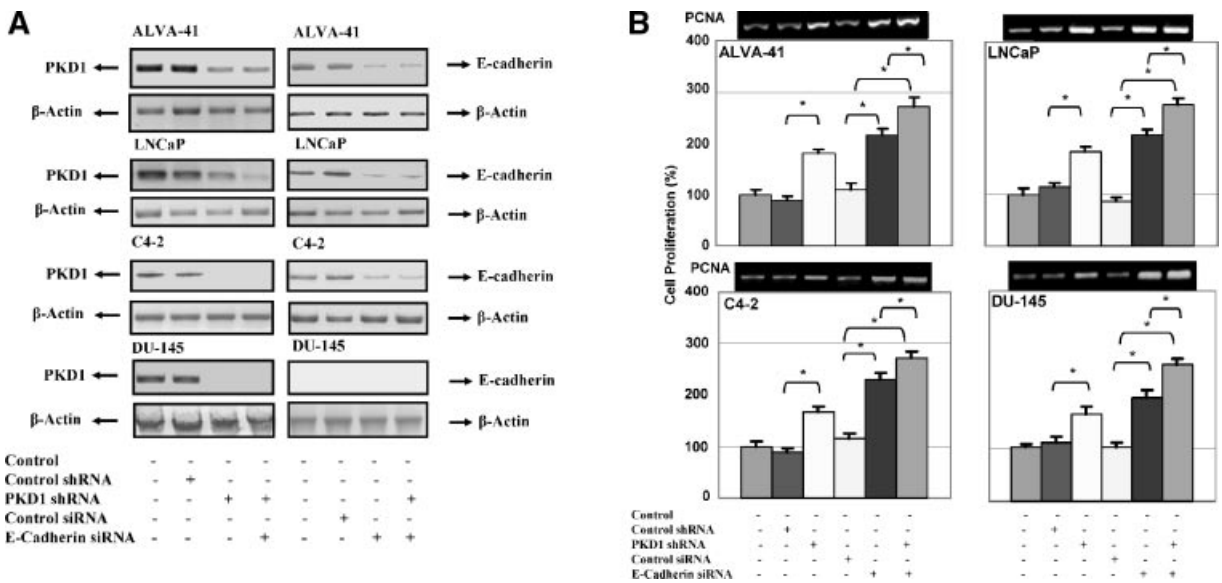


Fig. 1. Enhancement of prostate cancer (PC) cell proliferation by down-regulation of PKD1 and E-cadherin. **A**: Western blot analysis of E-cadherin/PKD1 following transfection with shRNA PKD1 or siRNA E-cadherin into PC cell lines (ALVA-41, LNCaP, C4-2, and DU-145). Nontransfected cells or empty vector transfected cells were used as controls. Successful protein down regulation is demonstrated by gene silencing. **B**: Effect of PKD1 and E-cadherin down regulation on the proliferation of PC cell lines. Four PC cell lines (ALVA-41, LNCaP, C4-2, and DU-145) were transfected with shRNA PKD1 and/or siRNA E-cadherin. Control cells were transfected with respective empty vector or control siRNA and additional control included untransfected cells. After 72 h of transfection, cell proliferation was measured by MTS assay, and proliferation of transfected cells was expressed as a percentage increase of proliferation of empty vector transfected control cells. Data are the means of three experiments with triplicate samples. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$). Expression of cell proliferation marker PCNA is shown on the top on each panel, which is consistent with MTS assay results.

PC, we hypothesized that PKD1 and E-cadherin function may be critical for cell migration, which is essential for invasion and metastasis. We evaluated the effect of siRNA-mediated PKD1 and E-cadherin depletion on cell migration on Matrigel. PKD1 and E-cadherin suppression increased the ability of PC cells to migrate through the Matrigel compared to control cells (Fig. 2B). However, unlike the effect on cell proliferation, combined suppression of PKD1 and E-cadherin did not enhance cell migration compared to individual suppression (Fig. 2B).

Restoration of PKD-1 and E-Cadherin Expression Attenuates Proliferation of PC Cells

Having established that PKD-1 and E-cadherin suppression promotes a malignant phenotype of PC, alterations in tumorigenicity following forced expression of PKD-1 and E-cadherin in PC cell lines were assessed as changes in cell growth in culture, colony formation efficacy in soft agar, and cell invasiveness through Matrigel. Overexpression of PKD-1 and E-cadherin was accomplished by transfection of expression vectors containing PKD-1 and E-cadherin into each PC cell line. Successful induction of higher levels of protein expression compared to controls was confirmed by Western blotting (Fig. 3A). Overexpression of PKD-1 and E-cadherin significantly reduced cell proliferation by more than 40–60%, respectively (Fig. 3B). Concurrent oversuppression of PKD-1 and E-cadherin further suppressed growth in C4-2 and DU-145 cell lines but not in ALVA-41 and LNCaP cells. In all cell lines, growth suppression showed an association with increased caspase-3 expression and decreased PCNA expression (Fig. 3B).

Colony-Forming Potential and Invasiveness of PC Cells Is Reduced by the Ectopic Expression of PKD-1 and E-Cadherin

Anchorage independent growth and invasive potential are important characteristics of malignant cells. Cells transfected with an expression vector carrying either PKD1 or E-cadherin showed greatly reduced capacity to form colonies on soft agar compared to controls (Fig. 4A). Number of colonies was reduced by about 20% in PKD1 over-expressing cells and about by 50–60% in E-cadherin ectopically expressing cells (Fig. 4A). Co-suppression of PKD-1 and E-cadherin further inhibited colony

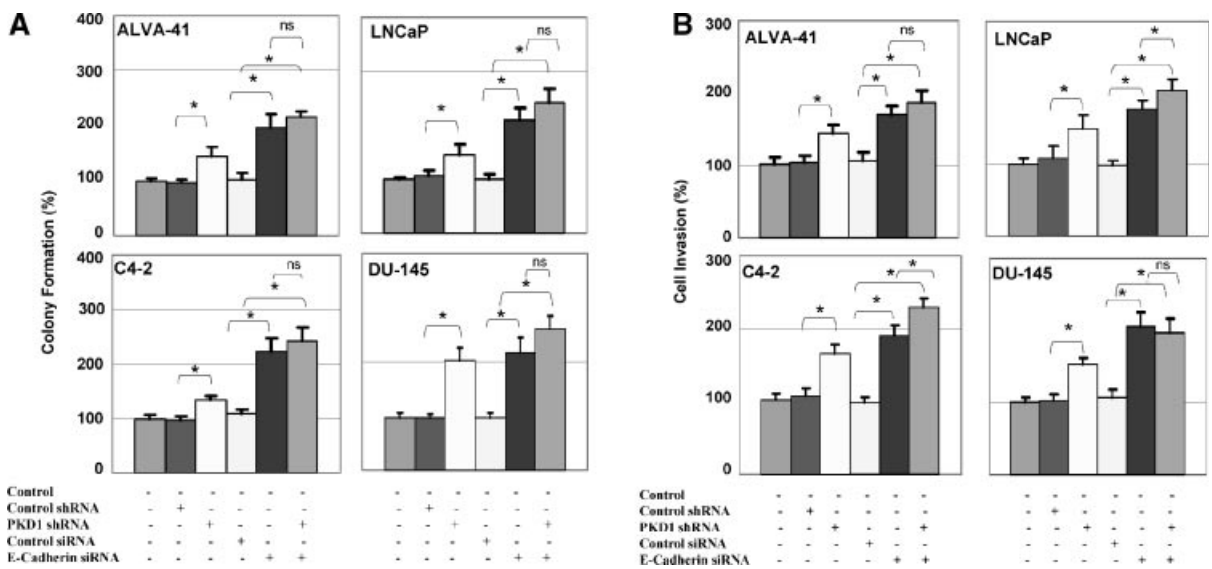


Fig. 2. Knockdown of PKD1 and E-cadherin increases cell motility and invasiveness. Effects of E-cadherin and PKD1 down regulation on (**A**) colony-forming ability and (**B**) invasion of ALVA-41, LNCaP, C4-2, and DU-145 cells were examined. Cells were transfected with shRNA PKD1, siRNA E-cadherin or respective controls, as indicated and additional control included untransfected cells. After 24 h, cells were cultured on soft agar to examine colony-forming ability or on Matrigel chambers to evaluate migration. Number of colonies were counted 21 days later and cells that migrated through the Matrigel were counted following 22 h of platting. Data are the means of three experiments with triplicate wells or plates. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$).

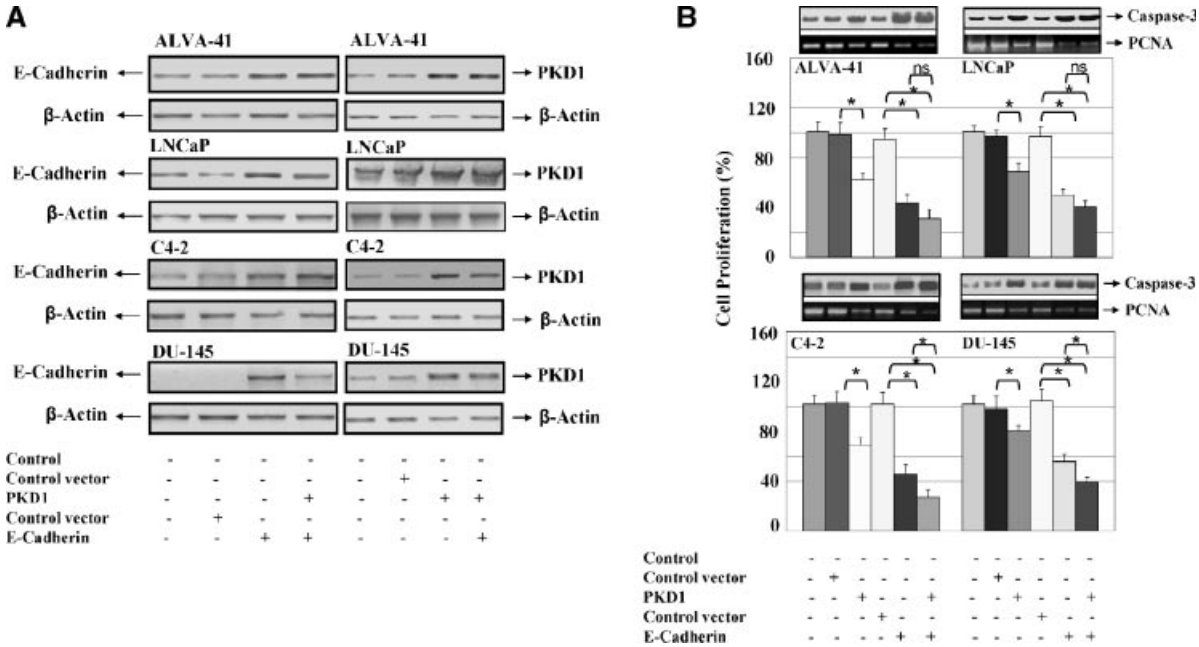


Fig. 3. Inhibition of PC cell proliferation by over expression of PKD1 and E-cadherin. **A**: Western blot analysis of PKD1 and E-cadherin after ectopic expression of PKD1 and E-cadherin in PC cell lines (ALVA-41, LNCaP, C4-2, and DU-145). A significant increase in PKD1 and E-cadherin levels in transfected cell lines compared to controls is shown in the figure. **B**: Effect of PKD1 and E-cadherin over expression on the proliferation of PC cell lines. Four cell lines (ALVA-41, LNCaP, C4-2, and DU-145) were transfected with PKD1 and/or E-cadherin. Control cells were transfected with respective empty vector or left untransfected. Three days later, cell proliferation was measured by MTS assay, and proliferation of transfected cells was expressed as a percentage decrease of proliferation of empty vector transfected control cells. Data are the means of three experiments with triplicate samples. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$). Expression of cell proliferation marker PCNA and cell apoptosis marker caspase C is shown on the top on each panel, which is consistent with MTS cell growth and viability assay.

formation only in ALVA-41 cells (Fig. 4A). The effects of ectopic expression of PKD-1 and E-cadherin on the invasiveness of ALVA-41, LNCaP, C4-2, and DU-145 cell lines were determined by measuring the ability of the transfected cells to pass through a layer of the Matrigel using invasion chambers. Figure 4B shows that transfection of prostate cell lines with expression plasmids containing PKD-1 and E-cadherin produced significant decreases (20–40%, PKD1 and 60–70%, E-cadherin) in the invasiveness of these cell lines compared to control. Additionally, cells transfected with both PKD-1 and E-cadherin showed a significantly greater decrease in invasiveness when compared to PKD1 or E-cadherin individually transfected cells.

Knockdown of PKD1 and E-Cadherin Results in Over Expresison of ß-Catenin in PC Cells

After establishing that suppression of PKD1 and E-cadherin promotes malignant phenotype of PC cells and over expression of PKD1 and E-cadherin is associated with apoptosis, we analyzed the expression of proapoptotic and anti-apoptotic genes in LNCaP cells transfected with shRNA of PKD1, siRNA of E-cadherin or expression vectors of PKD1 and E-cadherin. A marked increase in the expression of β-catenin, survivin (pro-survival proteins) and c-myc and cyclin-D (β-catenin target transcriptional proteins) was demonstrated in LNCaP cells in which PKD1 and E-cadherin expression was knocked down. LNCaP cells overexpressing PKD1 and E-cadherin showed increased expression of caspases associated with apoptosis and had no effect on the expression of survivin, β-catenin, c-myc, and cyclin-D (Fig. 5A). We next investigated whether caspases activity levels were altered by overexpression of PKD1 and E-cadherin. We measured the enzymatic activity of caspase-3, -8, and -9 against synthetic caspase subtype-specific tetrapeptide substrates DEVD p-NA (for caspase-3), IETD p-NA (for caspase-8), and LEHD p-NA (for caspase-9), after 72 h of transfection of LNCaP with shRNA of PKD1, siRNA of E-cadherin or expression vectors of PKD1 and E-cadherin.

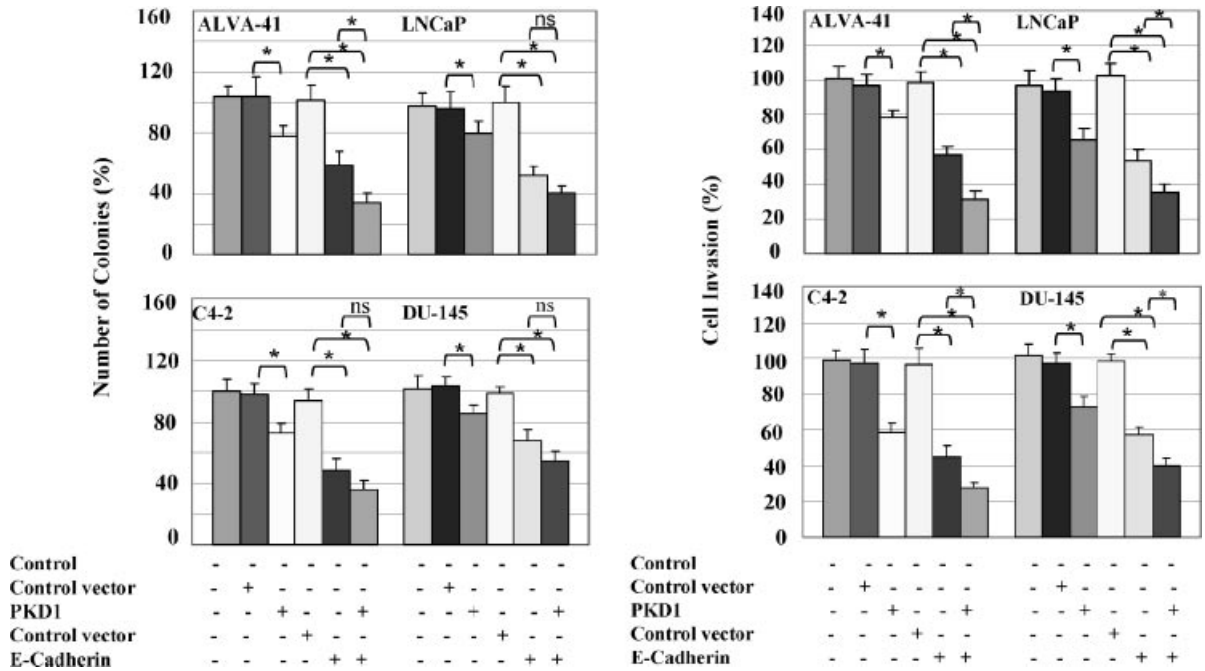


Fig. 4. Inhibition of anchorage independent growth and invasiveness of PC cells by ectopic expression of PKD1 and E-cadherin. **A**: Cells transfected with PKD1 and/or E-cadherin or mock transfected were harvested 24 h after transfection and plated in media containing soft agar. Colonies were allowed to form for 21 days and then counted. **B**: PKD1, E-cadherin, or mock transfected cells were plated on Matrigel chambers to evaluate migration. Cells that migrated through the Matrigel were counted. Data are the means of three experiments with triplicate wells/plates. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$).

The activities of caspase-8 and -3 in cultures of LNCaP cell lines transfected with E-cadherin and PKD1 alone or conjointly were significantly higher (3 to 4.5-fold) than activities found in control cultures (Fig. 5B). Similarly, activity of caspase-9 resulted in a twofold increase in E-cadherin and PKD1 transfected LNCaP cells compared with controls. No significant changes in the activities of caspase-9, -8, and -3 were detected in LNCaP cells transfected with siRNA of E-cadherin or shRNA of PKD1 (Fig. 5B).

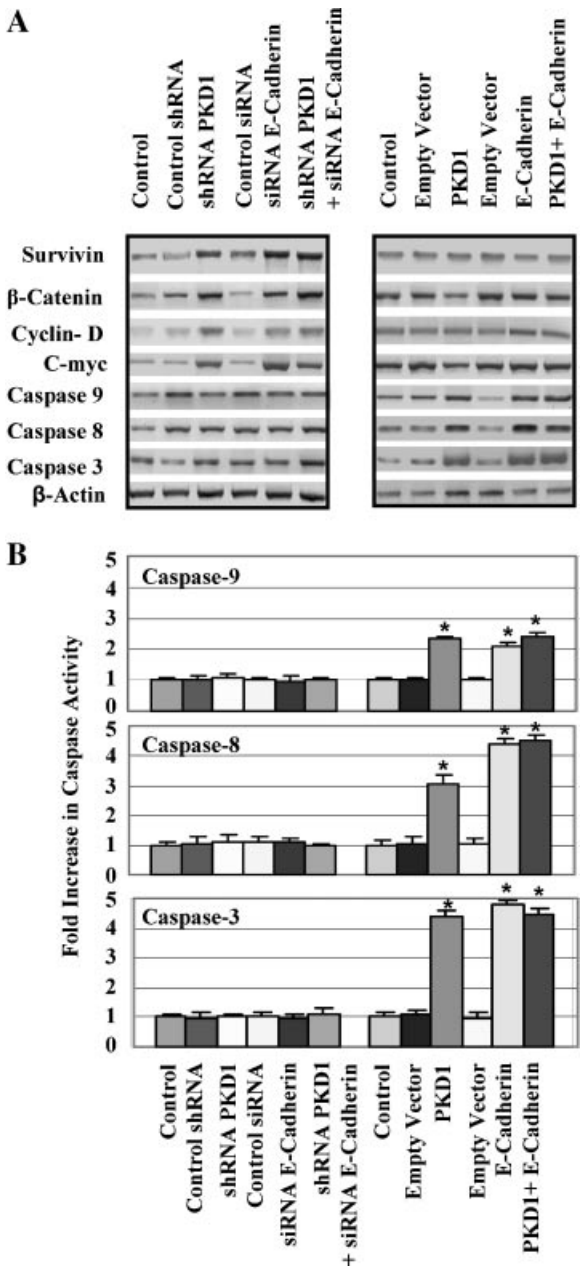
PC Cells Invasive Phenotype Induced by Knockdown of PKD1 and E-Cadherin Can be Rescued by Suppression of ß-Catenin

Our results demonstrate increased expression of β-catenin and β-catenin target genes, c-myc and cyclin-D in PC cells following down regulation of PKD or E-cadherin expression. To determine if enhanced ß-catenin expression is responsible for increased cell growth, all four study PC cell lines were co-transfected either with shRNA of PKD1 and shRNA of β-catenin or siRNA of E-cadherin and shRNA of β-catenin and cell proliferation was assessed by MTS assay. In all the cell lines tested, PKD1 and E-cadherin suppression showed significant increase in cell growth compared to cells co-transfected with shRNA of β-catenin (Fig. 6A) and transfection of shRNA of β-catenin alone suppressed cell growth in two out of four cell lines (Fig. 6A). Co-transfection of cells with β-catenin abrogated PKD1 and E-cadherin induced cell growth inhibition (Fig. 6B) and over expression of β-catenin significantly enhanced cell proliferation in all cell lines (Fig. 6B). These experiments demonstrate that alteration in cell growth by PKD1 and E-cadherin is at least partly mediated by ß-catenin.

DISCUSSION

Prostate carcinogenesis involves a multistep progression from precancerous cells to cells that proliferate locally and then metastasize. Studies have shown that the regulation of E-cadherin expression may play a critical role in the transition from noninvasive to invasive phenotype in PC [Isaacs et al., 1994, 1995]. In

normal epithelial tissues, E-cadherin complexes with the actin cytoskeleton via cytoplasmic catenins (α-, β-, and γ) to maintain the functional characteristics of epithelia [Morita et al., 1999; Jamora and Fuchs, 2002]. Disruption of this complex, primarily due to loss or reduced expression of E-cadherin, is frequently observed in advanced and poorly differentiated cancers and can be correlated with poor prognosis in several cancers including prostate [Umbas et al., 1992; Richmond et al., 1997; Luo et al., 1999; Morita et al., 1999].



We previously demonstrated that [Jaggi et al., 2005b] another protein, PKD1, interacts with E-cadherin and is associated with altered cellular aggregation and motility in PC. A major goal of this research was to fill a data gap regarding the lack of information on functional interactions of PKD1 and E-cadherin. Experimental alterations of PKD1 and E-cadherin levels in cells disrupt their functions. We were able to suppress or over-express PKD1 and E-cadherin protein expression in PC cells and subsequently relate their effects on cellular functions such as proliferation, anchorage-independent growth and migration. Our data demonstrate that ectopic expression of PKD1 and E-cadherin in PC cell lines significantly inhibited cell growth, elevated caspase-3 activity, and reduced both colony formation in soft agar and migration of cells through Matrigel. These findings are consistent with the notion that E-cadherin suppresses tumor invasion and metastasis and thus has tumor suppressor actions in cancer cells [Frixen et al., 1991; Takeichi, 1991; Vleminckx et al., 1991]. Anti-proliferative action of PKD1 has been reported in PC cells [Jaggi et al., 2005b]. To our knowledge, this is the first report extending and *elaborating* anti-tumogenic effects of PKD1 as evidenced by anchorage independent growth and cell migration.

Our results demonstrate that siRNA-mediated downregulation of PKD1 and E-cadherin expression increased cell growth, colony formation in soft agar, and invasiveness in PC cell lines, thus linking reduced expression levels of PKD1 and E-cadherin to oncogenic events in PC

Fig. 5. Loss of PKD1 and E-cadherin expression in LNCaP cells is associated with increased expression of survivin, cyclin-D and c-myc and gain of PKD1 and E-cadherin expression is associated with increased expression of caspases. **A**: LNCaP cells were transfected with shRNA of PKD1, siRNA E-cadherin or PKD1, E-cadherin expression vectors as indicated. Following 72 h of incubation, lysates were analyzed by Western blot with indicated antibodies. Loss or gain of PKD1 and E-cadherin is associated with increased expression of pro-survival proteins (survivin, β-catenin, and c-myc) or proapoptotic caspases, respectively. **B**: Enzyme activities of cell lysates toward tetrapeptide caspase substrates in LNCaP cells (LNCaP cells transfected with shRNA of PKD1, siRNA E-cadherin or PKD1, E-cadherin expression vectors as indicated) after 72 h of incubation. The chromogenic substrates were DEVD-pNA (caspase-3), IETD-pNA (caspase-8), and LEHDpNA (caspase-9). The caspase activity is expressed as fold relative to untreated controls and represented as the means of two experiments carried out in triplicate. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$).

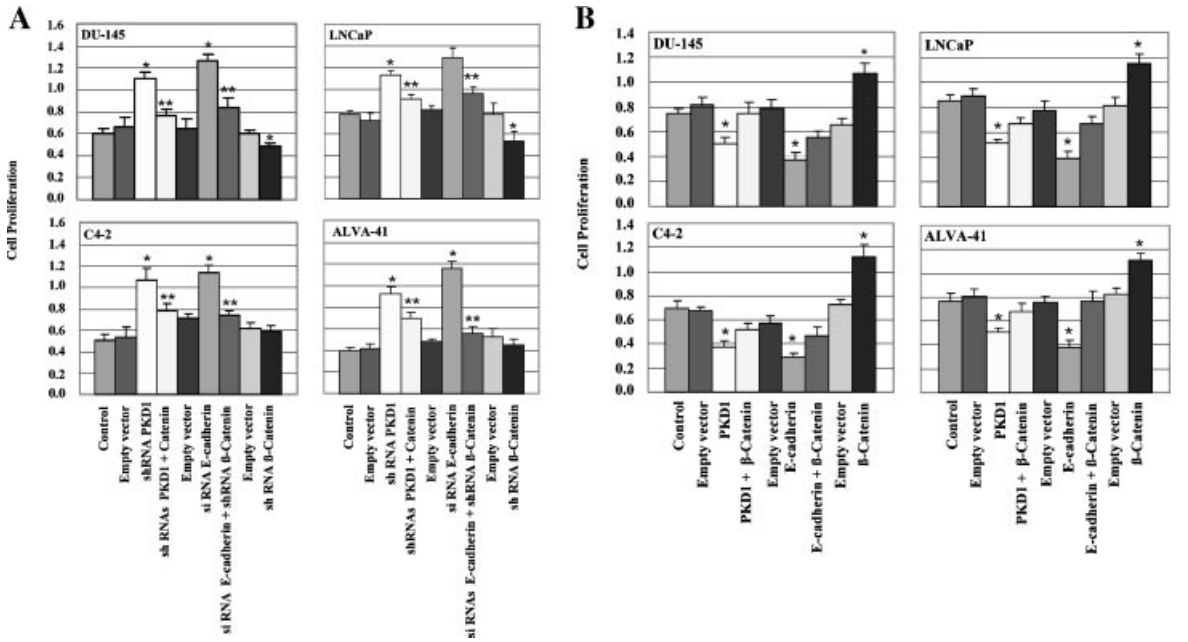


Fig. 6. **A**: Suppression of β-catenin rescues the increased cellular proliferative effect induced by knockdown of PKD1 and E-cadherin; four PC cell lines (ALVA-41, LNCaP, C4-2, and DU-145) were transfected with shRNA PKD1, siRNA E-cadherin or cotransfected with shRNAi of ß-catenin as indicated. Control cells were transfected with respective control constructs. **B**: Co-transfection with β-catenin abrogates the proliferation inhibitory effect of ectopic expression of PKD1 and E-cadherin. PC cells were transfected with PKD1, E-cadherin or cotransfected with ß-catenin as indicated. Control cells were transfected with respective control constructs. Three days later, cell proliferation was measured by MTS assay. Data are the means of two experiments with triplicate samples. Bars are mean ± SEM and * indicates statistically significant difference ($P < 0.05$).

cells. While increased cell migration following down regulation of E-cadherin was anticipated, a similar effect following down regulation of PKD1 was surprising. The highlight of this study is that we have provided the first evidence that PKD1 down-regulation directly promotes cell growth and favors anchorage independent growth and cell invasion. Interestingly, the combined suppression of PKD1 and E-cadherin in PC cells resulted in a greater increase in cell proliferation compared to PKD1 or E-cadherin alone, implicating that combined down regulation of E-cadherin and PKD1 may have a cumulative effect on cancer progression. Because PKD1 is known to be involved in multitude of cellular functions, it is conceivable that PKD1 causes alteration in cell proliferation by alternate pathways independent of E-cadherin as well.

Surprisingly, we observed the cumulative effect of PKD1 and E-cadherin knockdown on proliferation of DU-145 cells as well, which did not have detectable levels of E-cadherin by Western blot. Although DU-145 cells were considered to be E-cadherin negative, recent publications demonstrate that DU-145 cells do express E-cadherin, albeit at low levels [Kubota et al., 2000; Kwok et al., 2005]. The results of these experiments suggest that low levels of E-cadherin may be sufficient to influence cellular phenotype. Our results are in agreement with studies showing an association of low expression of E-cadherin with tumor invasion and metastasis [Vleminckx et al., 1991; Oka et al., 1993], an effect that can be reversed by restoring E-cadherin expression in invasive cells. From a cancer biology perspective, the fact that low levels of E-cadherin in cells can still influence malignant phenotype may be very important. Although down regulation of E-cadherin in several solid tumors is well established, the functional value of remaining E-cadherin in cells may be critical and can be potentially targeted to influence cellular phenotypic behavior.

Since β-catenin is a unique member of cadherin–catenin complex of protein that plays a dual role in cell proliferation and adhesion, we evaluated whether β-catenin has any role in mediating the effects of PKD1 and E-cadherin in PC cells. Others have demonstrated that E-cadherin plays a role in cell proliferation

through β-catenin in other cell line models [Kapitanovic et al., 2006; Koenig et al., 2006]. Our study clearly demonstrates that the β-catenin expression could clearly rescue the effect on proliferation induced by PKD1 and E-cadherin. While the E-cadherin-β-catenin mediated effect on cell proliferation is known, the role of PKD1-β-catenin in cell proliferation is novel. Ongoing work in our laboratory shows that PKD1 interacts with β-catenin and is involved in the golgi to membrane trafficking of β-catenin. Furthermore, down regulation of PKD1 is associated with decreased membranous β-catenin and increased β-catenin transcriptional activity (unpublished data). Conversely, overexpression of PKD1 may be associated with increased membranous β-catenin thus inhibiting β-catenin stimulated growth and invasion of cancer cells. We can hypothesize that both E-cadherin and PKD1 influence subcellular distribution β-catenin, which in turn influences cell proliferation. Clearly, more detailed experimentation is needed to study the mechanism by which PKD1 can modulate the transcriptional activity of β-catenin in PC cells.

Beta-catenin plays a role in cellular functions in number of ways. β-catenin acts as a coactivator with LEF/TCF transcription factors to stimulate transcription of a variety of target genes. Signaling through beta-catenin is regulated by modulating its degradation and nuclear translocation. In the absence of an activating signal, phosphorylation of beta-catenin by GSK3β acting in conjunction with APC and axin/conductin causes beta-catenin to interact with the beta-transducin repeat-containing protein which results in its ubiquitination and degradation. Signaling from the Wnt pathway activates dishevelled which, inhibits the activity of GSK3β resulting in an increase in the cytoplasmic free pool of beta-catenin, and translocation into the nucleus. In addition to these pathways, β-catenin interacts with AR. Free β-catenin may be able to interact with AR to augment ligand-dependent transcription. It is interesting to note that the effects exhibited by β-catenin are very similar in both androgen dependent (LNCaP and C4-2) and androgen independent (DU145 and ALVA41) PC cells suggesting that the effects elicited by β-catenin on the cellular phenotypic behavior of PC cells are may be independent of AR.

Our study demonstrates that ectopic expression of PKD1 and E-cadherin in PC cell lines diminished the growth rate compared with mock-transfected controls. Reduced growth is attributed to elevated expression of caspases 9, 8, and 3. Most importantly, we found that induction of apoptosis by PKD1 and E-cadherin in these cells utilized both the death receptor/caspase-8 cascade and the mitochondrion/caspase-9 pathway. Our results are in agreement with previous studies showing association between overexpression of E-cadherin and apoptosis in number of cancer cells [Lowy et al., 2002; Escaffit et al., 2005]. A link between PKD1 overexpression and caspases activation reported in the present study has not been investigated. However, it is suggested that PKD1 may be involved in apoptosis through JNK activation [Zhang et al., 2005]. Although overexpression of PKD1 and E-cadherin inhibit growth of PC cells, it does not have any effect on the expression of survivin, β-catenin, c-myc, and cyclin-D. This observation may suggest that different genes are activated by gain or loss of function.

It is intriguing that dysregulation of one or two proteins such as PKD1 or E-cadherin is sufficient to significantly alter multiple malignant phenotypic features including proliferation, motility and invasion. It is conceivable that the intricate signaling circuitry of a tumor cell may be profoundly altered by the inactivation or loss of expression of tumor suppressor genes, and as a consequence tumor cells activate different signaling pathways that may ultimately result in influencing malignant phenotype. Reintroducing PKD1/E-cadherin in PC cells where the respective endogenous gene expression was reduced or inactive caused a marked inhibition of growth, induction of apoptosis and inhibition of tumorigenesis. This effect of "independence from tumor suppressor" in a cancer cell may be considered the opposite of "oncogene addiction," a recently described novel concept wherein altered signaling circuitry makes cancer cells depend on a single oncogenic event [Weinstein, 2002], without which cells would not survive. The results from our experiments clearly demonstrate that similar to oncogenic addiction, alteration in expression in one or two tumor suppressor proteins is sufficient to cause PC progression.

The potential biological significance of our study is that dysregulation of two proteins either synergistically or in succession could lead to PC progression. Our data clearly

confirmed that combined dysregulation of PKD1 and E-cadherin alters cell proliferation compared to dysregulation of either protein alone, which suggest that combined dysregulation of PKD1 and E-cadherin can lead to PC progression.

E-cadherin and PKD1 demonstrate potential for biomarker evaluation or therapeutic targeting because their expressions are dysregulated in human PC samples. Down regulation of E-cadherin is an early event in PC and therefore, study of E-cadherin expression in apparently localized PC biopsy specimens may help in identifying patients at higher risk of progression [Kuniyasu et al., 2003; Ohmori et al., 2006]. On the contrary, PKD1 is apparently unaltered in early PC is however down regulated in advanced PC [Jaggi et al., 2003]. While current understanding of PKD1 does not make it an ideal marker for biomarker evaluation, its interaction and ability to influence E-cadherin mediated functions establishes PKD1 as a potential therapeutic target. Because kinases in general have proved to be most susceptible to rationale base drug designing [Mikalsen et al., 2006; Ventura and Nebreda, 2006], targeting PKD1 may provide a novel means to influence E-cadherin function. This is particularly attractive because unlike oncogenes, targeting genes that are down regulated is several human diseases including cancer using gene therapy strategies such as viral vectors and vaccines have met with limited success [Woo et al., 2006]. An alternate strategy of targeting up stream regulators of genes that are down regulated such as E-cadherin may prove to be an effective method of regaining protein function and reinstating cellular homeostasis. Our current study has identified PKD1 as a novel kinase that could be targeted to restore E-cadherin mediated cell functions.

In conclusion, we have established that down-regulation of PKD1 and E-cadherin expression in PC potentiates cellular malignant characteristics and supports tumor progression that is at least in part mediated by β-catenin. On the contrary, over expression of PKD1 and E-cadherin has potent anti-tumorgenic affects on PC cells. Our study demonstrates that combined dysregulation of PKD1 and E-cadherin contributes to the malignant phenotype of PC cells by influencing cell proliferation, motility, and invasion. The effect of cell proliferation can be rescued by ß-catenin suggesting an important role of ß-catenin in PKD1/E-cadherin mediated effects.

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