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14. ABSTRACT To explore the role of cyclin D1 in prostate cancer progression, we manipulated cyclin D1 expression in both epithelium and stroma by retroviral infection and examined the role of cyclin D1 using a tissue recombination model. The data showed that over expression of cyclin D1 in stroma elicited a permanent malignant transformation of adjacent initiated epithelium by paracrine manner, instead of direct manner <i>in vivo</i> . The highly concordant gene expression pattern between prostatic carcinoma associated fibroblasts and cyclin D1 over expressing fibroblasts allows for the rational design of therapies aimed at inhibiting prostate tumor growth by manipulating cyclin D1 expression in the relatively stable stroma.					
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The role of Cyclin D1 in altering stromal-epithelial interactions in prostatic carcinogenesis

Introduction.

Prostate development is controlled by steroid hormones that induce and maintain a complex cross-talk between the stromal and epithelial cells. The process of prostatic carcinogenesis includes aberrations in the interactions of the prostatic epithelium and its local microenvironment resulting in reciprocal dedifferentiation of both the emerging carcinoma cells and the prostatic smooth muscle.

Cyclin D1 encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein and promotes progression through G₁ to S phase of the cell cycle. Overexpression of cyclin D1 plays important roles in the development of human cancers, including breast, colon, and melanoma. Increased cyclin D1 expression occurs relatively early during tumorigenesis; however, its role in prostate cancer is not well understood. Immunostaining studies indicated that primary prostate carcinoma samples displayed moderate or strong expression of cyclin D1 protein in the epithelial compartment compared with normal epithelium. Little is known about the role of cyclin D1 in the stromal compartment of tumors, especially in adenocarcinomas. One study of cyclin D1 expression in esophageal carcinomas indicated that cyclin D1 is strongly expressed in stromal fibroblasts.

In this study, we examined the consequences of targeted regulation of cyclin D1 expression in epithelial or stromal cells to investigate the effects of cyclin D1 in prostate cancer progression.

Body and Key achievement.

(1) To determine if Cyclin D1 expression levels are elevated in malignant human prostatic epithelial cell lines. Cyclin D1 expression was examined by Western blotting in the prostate cancer cell lines, DU145, LNCaP, BPH^{CAFTD1} and BPH^{CAFTD2} and in a subset of non-tumorigenic prostatic cells, PrE1, 957E/hTERT, PrE3 and BPH-1 cell line. Cyclin D1 expression was found to be higher in all of the cancer cells as compared to the non-tumorigenic prostatic cells. Cyclin D1 expression was higher in all of the cancer cells compared with the nontumorigenic prostatic cells (Figure 1).

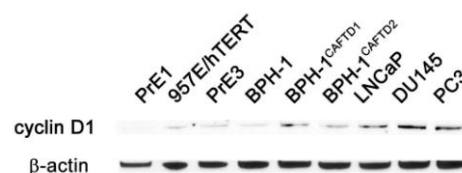


Figure 1. Cyclin D1 expressions in different cell lines

(2) To determine the consequence of overexpression of cyclin D1 in epithelium. We overexpressed Cyclin D1 in BPH-1 cells by retroviral infection to generate BPH-1^{C7-cyclin D1} cell lines. The consequences of cyclin D1 expression was examined by wound healing, transwell migration, boydem chamber and growth curve analysis. We found that overexpression in BPH-1 cells can increase cell proliferation rate, migration, and invasive ability in vitro compared with control BPH-1^{C7-Δ} cells (Figure 2).

(3) To determine whether cyclin D1 could exert a tumorigenic effect on prostate cells *in vivo*. 100k BPH-1^{C7-cyclin D1} cells or control cells were recombined with 300k rUGM and grafted under the kidney capsule of SCID mice. The grafts were harvested after 4, 8, 12 and 16 weeks. The results showed that BPH-1^{C7-cyclin D1} cells formed significantly larger and more vascularized grafts under the induction of rUGM, compared with BPH-1^{C7-Δ} cells. To Consistent with our in vitro

experiments, which showed that BPH-1^{C7-cyclin D1} cells proliferate faster than controls *in vivo* in our tissue recombination model. Although cyclin D1 can increase BPH-1 cell motility and promote cell proliferation *in vitro*, overexpression of the gene did not induce BPH-1 cells to undergo malignant transformation with associated invasion (Figure 3).

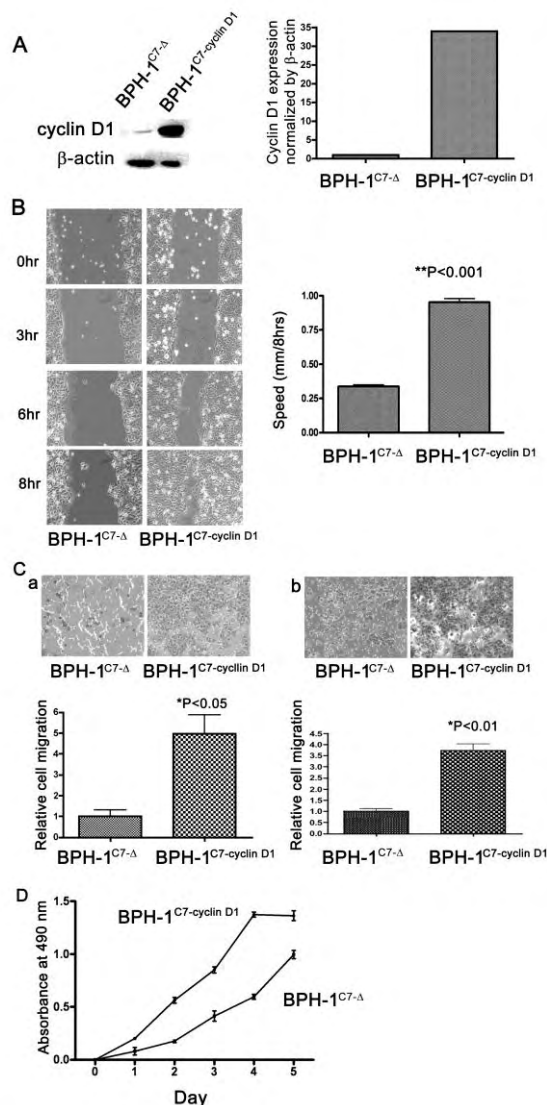


Figure 2. *In vitro* comparison of BPH-1^{C7-Δ} and BPH-1^{C7-cyclin D1} cells.

(A) Cyclin D1 overexpression in BPH-1^{C7-cyclin D1} was confirmed by western blotting and band intensity quantitated.

(B) Wound healing assay. BPH-1^{C7-cyclin D1} closed wounds in the confluent monolayer significantly faster than BPH-1^{C7-Δ} cells. Images (left) and quantitation (right) shown. Student's t-test, p<0.001.

(C) Transwell migration study and invasion assay. BPH-1^{C7-cyclin D1} migrated through the uncoated Boyden chamber significantly faster than BPH-1^{C7-Δ}. Representative phase-contrast optical photomicrographs after overnight culture shown (a, above) and quantitated (a, bottom). Student's t-test, p<0.05. BPH-1^{C7-cyclin D1} cells had increased invasive ability in a Matrigel coated Boyden chamber invasion assay compared to BPH-1^{C7-Δ}. Representative phase-contrast optical photomicrographs after overnight culture shown (b, above) and quantitated (b, bottom). Student's t-test, p<0.01.

(D) Proliferation assay. Cyclin D1 overexpression promoted BPH-1 cell proliferation significantly over control growth rate. (Student's t-test, p<0.001).

(4). To determine the consequence of overexpressing cyclin D1 in stromal cell.

Since the stroma is viewed as an important

active contributor to tumor growth, and in order to understand whether cyclin D1 performs different functions in stromal and epithelial tissues, we generated NPF^{cyclin D1} cells by overexpressing cyclin D1 in primary cultures of normal prostate stromal cells. We have found that NPFs acquired a prolonged life span as a consequence of upregulated cyclin D1. Our experiments also showed that CAFs expressed a much higher level of cyclin D1 protein than either NPFs or fibroblasts isolated from BPH patients.

(5). To investigate the *in vivo* consequences of overexpression of cyclin D1 in NPFs. 100k BPH-1

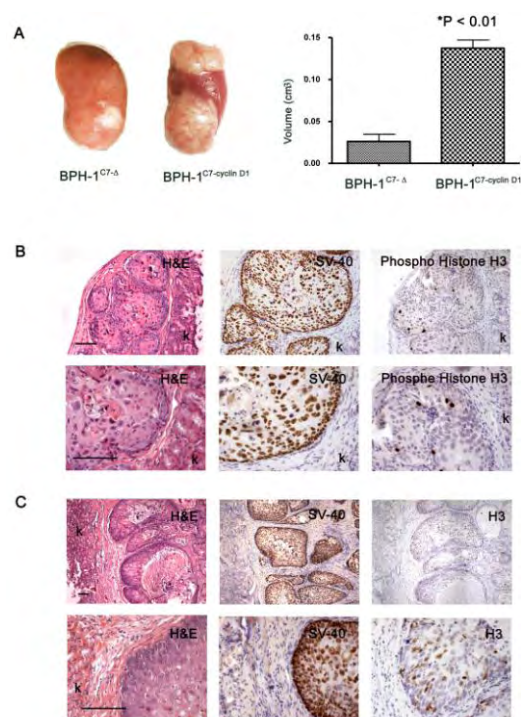


Figure 3. Overexpression of cyclin D1 in epithelium was insufficient to induce malignant transformation in BPH-1 cells as determined by *in vivo* assays.

(A) BPH-1^{C7-cyclin D1} cells were not tumorigenic under the influence of the inductive rat UGM in the tissue recombination model. Gross morphology of 2 months grafts of BPH-1^{C7-Δ} + UGM (left) and BPH-1^{C7-cyclin D1} + UGM (right). The volume of grafts containing BPH-1^{C7-cyclin D1} was significantly larger than controls. Student's t-test, $p < 0.01$. (B) H & E staining of BPH-1^{C7-Δ} + UGM grafts showed that the recombinants formed solid cord structures with no sign of invasion to the host kidney. SV40 T-antigen staining confirmed the cell origin of the epithelium. Phospho histone H₃ staining identified few positive cells in the solid cords. Higher magnification pictures were shown in bottom. (C) H & E and SV-40 T antigen staining of BPH-1^{C7-cyclin D1} + UGM grafts. Histology is similar to that seen in the control groups. The basement membrane between kidney and graft was intact and there was no sign of invasion into the host kidney was seen. Phospho histone H₃ staining identified significantly more positive cells in the solid cords compared in B. Higher magnification pictures were shown in bottom.

cells were recombined with either 300k NPFcyclin D1 or NPF cells. After 5 months of incubation in the kidney capsule, BPH-1

cells formed larger tumors with clear kidney invasion. Small kidney tubes intermingled with tumor cells and there were no clear margins between the kidney and grafts. Control BPH-1 + NPF recombinants only showed minimal growth (Figure 5).

(6). To investigate if Epithelial cells isolated from BPH-1 + NPF^{cyclin D1} grafts (BPH-1^{NPF-cyclin D1}) are tumorigenic. After cell culture and G418 selection, two cells strains

were derived from BPH-1 + NPF and BPH-1 + NPF^{cyclin D1} grafts, designated BPH-1^{NPF} and BPH-1^{NPF-cyclin D1}. The two strains were grafted in collagen gels beneath the renal capsule of male SCID mice. Grossly, after 3 months, the BPH-1^{NPF-cyclin D1} cells formed significantly larger grafts than the control group (Figure 6A). The BPH-1^{NPF-cyclin D1} cells formed large fused nests generally with a broad pushing margin to the host kidney (Figure 6C,a-arrow). Many smaller nests with irregular shapes were scattered throughout the tumor and intermingled with stroma (Figure 6C, d and e). Some infiltrative areas recapitulated prostatic carcinoma (Figure 6C, b-arrow). Minimally invasive growth was found in some areas (Figure 6C, f). Our data indicated that epithelial cells isolated from BPH-1 + NPFcyclin D1 grafts (BPH-1NPF-cyclin D1) are tumorigenic.

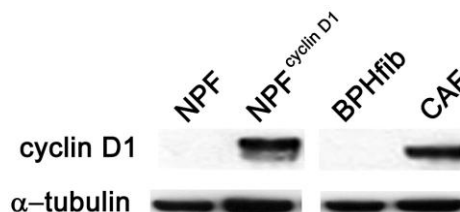


Figure 4. Cyclin D1 overexpressing NPF cells (NPF^{cyclin D1} cells), human prostatic carcinoma-associated fibroblasts (CAF) cells expressed elevated levels of cyclin D1 protein as compared to NPF or to BPH-derived fibroblasts.

Reportable Outcome.

The work so far done in this project has been presented in the department seminar at Vanderbilt University. We have published one paper in Cancer research last year based on this study (Tissue-Specific Consequences of Cyclin D1 Overexpression in Prostate Cancer Progression. *Cancer Research* 67, 8188-8197, September 1, 2007). A more comprehensive outcome can be found in the publication.

Conclusion.

The concept of stroma as a contributor to, and potentially an initiator of, carcinogenesis have led to altered perceptions of the development and progression of epithelial malignancies. Not only stromal-epithelial interactions play an important role in normal development and adult growth quiescence of the prostate, but also changes in these interactions can promote a malignant progression of initiated epithelium and result in tumorigenesis.

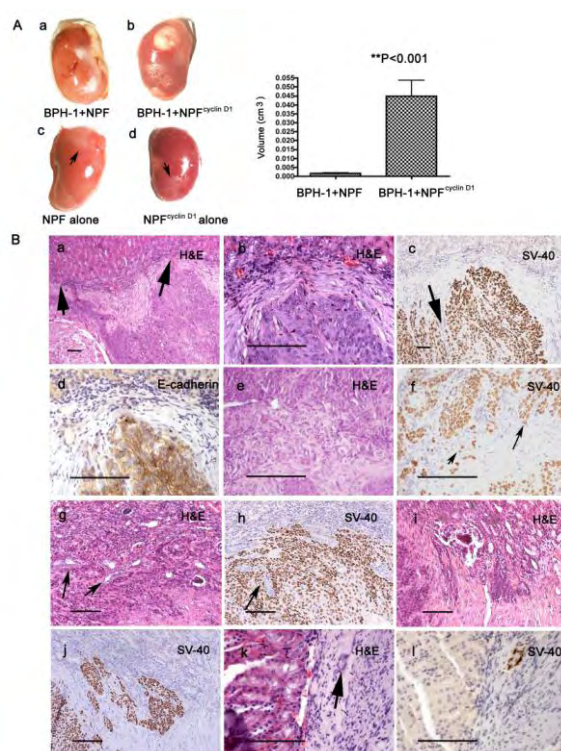
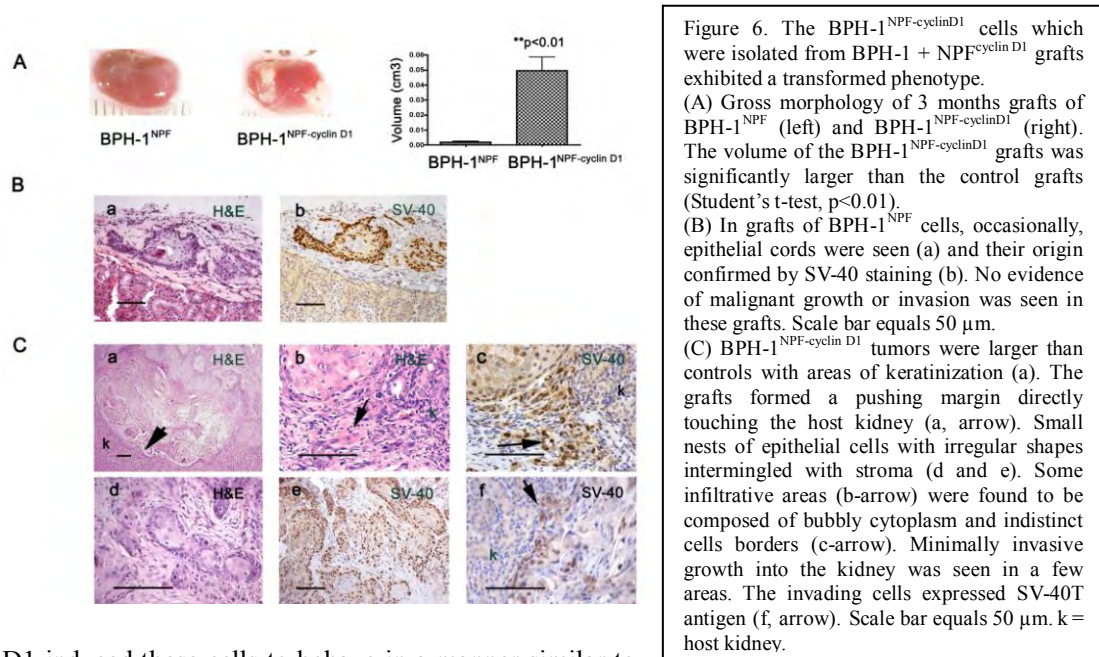


Figure 5. Effects of NPF^{cyclin D1} cells on BPH-1 epithelium *in vivo*.

(A) Gross morphology of one month grafts of tissue recombinants composed of BPH-1 + NPF(a), BPH-1 + NPF^{cyclin D1} cells (b), NPF alone (c) and NPF^{cyclin D1} alone (d). The graft volume of BPH-1 + NPF^{cyclin D1} was significantly larger than that of BPH-1 + NPF. (B) Staining of BPH-1 + NPF^{cyclin D1} recombinants revealed organization resembling a poorly differentiated carcinoma. Some epithelium fused to form large nests (a, b) with keratinization and a broad pushing margin to kidney (a, arrow). Tumors contained irregular epithelial cords and epithelial cells intermingled within a fibrous stroma (c, e and f-right arrow). Single epithelial cells were intermixed with fibrous stroma in other areas (c-arrow and f-left arrow). Immunohistochemical localization of SV-40 T antigen confirmed the origin of the tumors (c and f). E-cadherin staining was patchy (d). After 5 months incubation in kidney capsule, BPH-1 cells formed larger tumors and invaded the host kidney (g, h, i, j). Tumor cells surrounded and intermingled with kidney tubes (Figure 4B, g and h-arrows). There were no clear margins between kidney and grafts. H & E staining of BPH-1 + NPF grafts revealed small grafts with minimal epithelial growth consistent with previous observations (k, arrow and l).

Cyclin D1 is an important oncogene in many human cancers, but its function in prostate cancer is not clear. We show in this study that cyclin D1 is up-regulated in prostate cancer cell lines, indicating that it might be associated with prostate tumorigenicity. We have observed that BPH-1 cells, in which cyclin D1 was overexpressed, did not become tumorigenic under the influence of inductive rUGM in the tissue recombination model when grafted to SCID mice. This underlines the important point that increased proliferation per se is insufficient for malignant transformation.

In marked contrast to the effects in epithelial cells, overexpression of cyclin D1 in primary cultures of benign human prostatic fibroblasts extended the life span and altered the behavior of the stromal cells, nonetheless falling short of directly inducing malignant transformation. Cyclin



D1 induced these cells to behave in a manner similar to CAFs, imparting an ability to elicit malignant transformation in BPH-1 epithelial cells in a tissue recombination model. By expressing cyclin D1 in stromal cells, we showed that benign stromal cell behavior can be modified to mimic that of cancer stromal cells. NPFcyclin D1 cells have a potential to transform BPH-1 cells similar to that seen with CAFs although with a reduced intensity. Tissue architecture in recombinants showed irregular epithelial cords and epithelium infiltrating into the stroma. This observation indicated that the presence of altered stromal cells in proximity to an initiated epithelium has an important biological effect on prostatic carcinogenesis. Expression of this single oncogene in the stroma may mimic the effects of CAFs on epithelium by modifying the local microenvironment. Specifically altering the expression of growth factors and ECM proteinases results in expansion and malignant progression of the initiated epithelial cells. BPH-1 cells form tumors after recombination with CAFs and epithelial cells derived from these tumors (BPH-1CAFTD) are tumorigenic without the stimulation of stromal cells when regrafted to mice (23). The present study shows that the tumorigenic behavior of BPH-1NPF-cyclin D1 cells (derived from recombination of BPH-1 + NPFcyclin D1 cells) also resulted in a permanent malignant transformation of epithelial cells similar to that seen with CAF. It is important to note that CAFs have elevated expression levels of cyclin D1 protein; therefore, many of their characteristics could be linked to the downstream consequences of this change. Microarray comparison of the NPFcyclin D1 and CAFs versus NPF showed highly concordant gene expression profiles. The same 118 unique genes were up-regulated and 51 unique genes were down-regulated in NPFcyclin D1 cells and CAFs when compared with NPFs. Relatively few significant differences in transcript abundance measurements between NPFcyclin D1 cells and CAFs were identified. These data indicate that cyclin D1 expression in stroma can critically affect paracrine interactions with adjacent epithelial cells in a manner resembling CAFs.

In summary, the present study showed for the first time the importance of cyclin D1 as a potential regulator of paracrine interactions in prostate cancer progression. The cyclin D1-overexpressing fibroblasts have an increased life span and share many commonalities with CAFs making them a potentially useful research tool. Traditional therapy for all epithelial malignancies, including

prostate cancer, has been targeted at the epithelial cells that progressively acquire genetic changes. The stroma may provide a more stable target at which to direct treatment because the gene expression profile differs from that seen in normal tissues.

Future directions. In the future, we will work on the second aim in the project to determine if the cathepsin D is one of the mediators of the crosstalk between BPH-1 cells and cyclin D1 overexpressing NPF cells. Since cathepsin D is overexpressed in NPF-cyclin D1 cells and CAFs (based on microarray data, not shown), we will make cathepsin D overexpressing NPF cells and recombine them with BPH-1 cells to investigate if it can produce same phenotype as BPH-1 + NPF-cyclin D1 recombinants. We will also make NPF-cyclin D1-cathepsin Dsh cells (in which cathepsin D is knocked down by shRNA) and recombine with BPH-1 cells to determine if can reverse the phenotype.

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