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14. ABSTRACT Stromal-epithelial interactions are important in development and cancer of the prostate. Estrogen receptor functions as a transcription factor to regulate gene expression. One such ER-regulated gene is the protease cathepsin D (CathD). <i>This proposal has two immediate objectives. The first is to determine how overexpression of cyclin D1 (CD1) in the stroma induces the upregulation of the estrogen regulated gene CathD. The second objective is to determine how overexpression of CathD in the stroma can contribute to tumorigenesis in the epithelium.</i> Results show 1) CD1 interacts with the ER α to modify the expression of estrogen regulated genes like CathD in prostate fibroblasts. 2) ER signaling in the stroma contributing to CAFs induced tumorigenesis in adjacent epithelium. 3) Stromal specific overexpression of CathD promotes prostate tumorigenesis through activation of TGF β signaling pathways.					
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Introduction

Historically, the field of cancer biology has primarily been focused on studying the malignant cells comprising tumors (1). The emergence of the tumor microenvironment as a field of active study is providing some much needed insight into how non-malignant cells associated with cancer (cancer associated stroma) can promote or suppress tumorigenesis (2). The stromal phenotype has been shown to be a powerful prognostic indicator of cancer progression and of patient death underlining their importance in defining lethal versus indolent phenotypes (3).

Stromal-epithelial interactions are important in the development and cancer of the prostate (4-6). During carcinoma evolution, the stroma surrounding the nascent tumor undergoes phenotypic alterations that have been shown to enhance the invasive potential of the epithelial tumor (7-9). These stromal-epithelial interactions are mediated, in a large part, by paracrine signaling between epithelial tumor cells and neighboring stromal fibroblasts (9). In addition to receiving signals from malignant epithelial cells, the stromal fibroblasts stimulate tumorigenesis by releasing factors that act on adjacent epithelial tumor cells or exchange enzymes that modify local microenvironment promoting the proliferation and survival of the neoplastic cells (8-11).

Modifications to the local tumor microenvironment are accomplished through the actions of several different families of proteins, including proteases produced by either the tumor or the stroma (12). These enzyme families include matrix metalloproteases (MMP), cysteine, and serine proteases, which have been shown to play a role in the degradation of the basement matrix, promotion of angiogenesis, and the liberation of growth factors to stimulate tumor cell growth (13) (14).

Cathepsin D (CathD) is a ubiquitous lysosomal aspartic endoproteinase, CathD, has been shown to be involved in a number of physiological processes. CathD is known to play a critical role in barrier function, regulation of apoptosis, and epithelial differentiation (15-17). In cancer however, CathD is overexpressed and hypersecreted in various malignancies including PCa (18) (19). In breast cancer, CathD expression is associated with a poor prognosis and increased likelihood for the development of metastasis (20). Experimental evidence has shown CathD can stimulate the proliferation of PCa cell lines (21).

There are limited data defining CathD's function in prostate cancer progression. Some studies have concluded that CathD is overexpressed in the epithelium and stroma of PCa, and may promote proliferation (21, 22). Other studies have concluded that CathD produced by PCa may be inhibiting tumor growth (23, 24). Despite all the advances in basic and translational research the mechanisms underlying the development and progression of cancer to this date are still poorly understood.

Androgens and the androgen receptor have been shown to have an integral role in mediating proliferation, differentiation, and maintenance of the prostate epithelium (25). Not to be overshadowed by the role androgens play in PCa, estrogens also play a profound role in prostate carcinogenesis (26).

Estrogens act indirectly on the prostate gland by suppressing the release of leuteinizing and follicle stimulating hormones from the pituitary gland, a form of chemical castration, which prevents the production of testosterone by the testis. The lack of androgenic signaling in the prostatic stroma induces apoptosis in the adjacent epithelium. Exogenously supplied estrogens also act directly on the prostate mediated by the estrogen receptor in the stroma to induce squamous metaplasia (SQM) in the epithelium (27). Work, performed 69 years ago by Huggins and Hodges, showed the profound effects estrogens have on the prostate (27). In other animal models, intermittent exposure to estrogens during neonatal and/or perinatal development induces dysplastic changes in the adult prostates of mice (28, 29). Prolonged exposure to estrogens in combination with androgen in the NBL rat model produced a 100% incidence of PCa. Treatment with androgen alone induced PCa with a 40% incidence (30).

The actions of estrogen are mediated through two receptor subtypes estrogen receptor-alpha ($ER\alpha$) and estrogen receptor-beta ($ER\beta$). Expression of $ER\alpha$ in the prostate is localized to the stroma and becomes

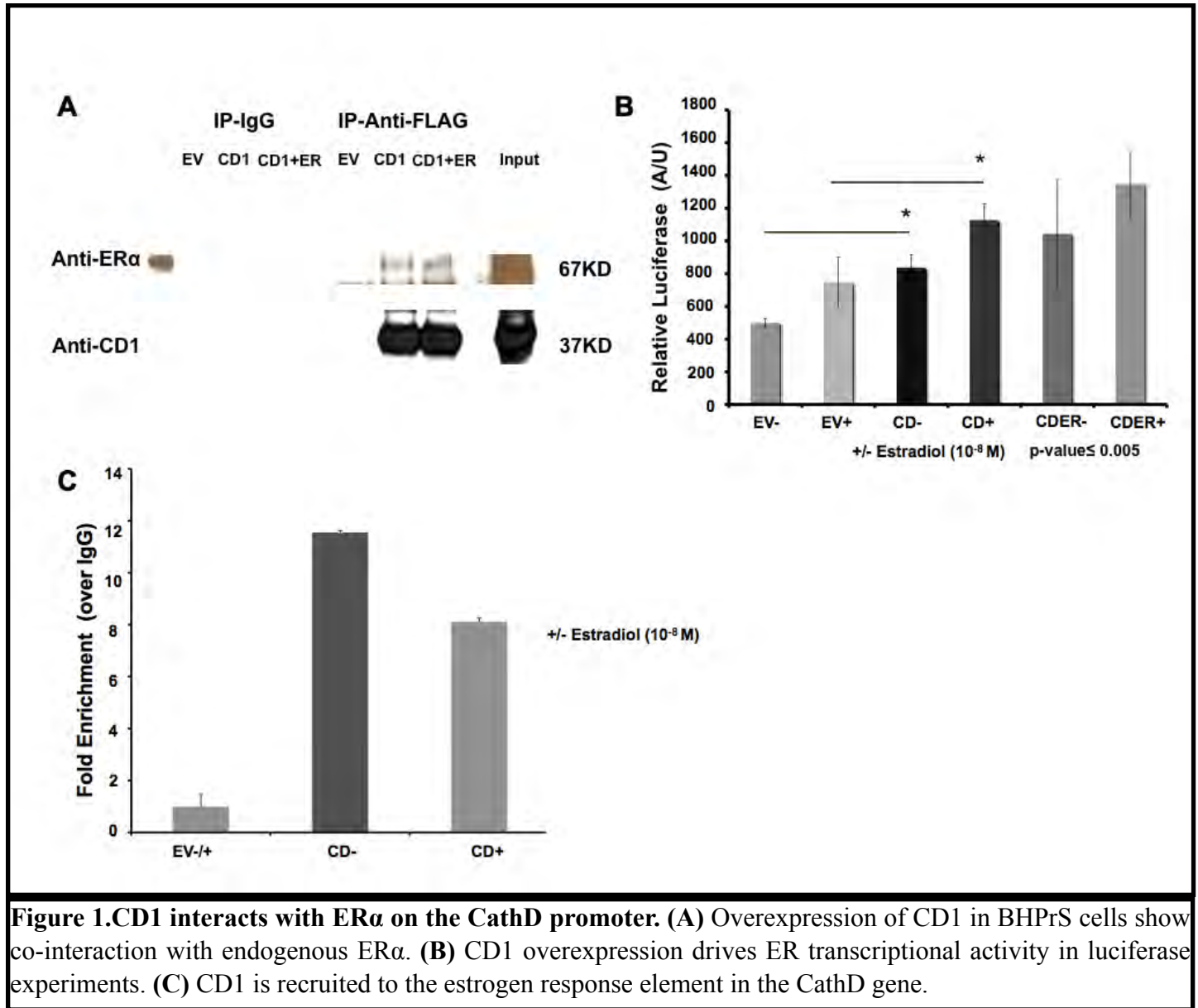
elevated during the progression of PCa. The expression of ER β in the prostate is localized to the epithelium and expression is lost during PCa progression (31). The estrogen receptor family function as transcription factors and regulate the expression of a number of different genes. One such ER-regulated gene is the aspartic endopeptidase cathepsin D (CathD) (32, 33). CathD is known to be involved in a number of physiological processes as well as in the regulation of apoptosis (34, 35). In various malignancies i.e. breast, and colon cancers CathD is overexpressed and hypersecreted (36) (37). Treatment of breast cancer cell lines with synthetic peptides corresponding to CathD induced increased expression of anti-apoptotic genes and cell cycle regulators (34). CathD is hypothesized to bind the mannose-6-phosphate receptor (M6PR)/insulin-like growth factor II receptor (IGFIIR) (21). However, blocking of the IGFIIR did not abrogate the pro-mitogenic effects of CathD (38).

Our lab has previously published on several molecules found to be aberrantly expressed in cancer associated fibroblast (CAFs) (including cyclin D1 (CD1), and stromal derived factor-1 (SDF-1) that induce tumorigenesis and malignant transformation in tissue recombination experiments (9, 39). Unpublished findings comparing CAF primary CD1-overexpressing-normal primary fibroblast (NPF^{cyclin D1}) and parental NPF revealed that the expression of CathD was overexpressed 7-fold in both CAF and NPF^{cyclin D1} in comparison to NPF. The cell cycle regulator CD1 and ER α are known to interact and can induce estrogenic gene transcription (40). This suggests that the overexpression of CathD in PCa associated stroma is due to the interaction of ER α and CD1. ***This proposal has two objectives. The first is to determine how overexpression of CD1 in the stroma induces the upregulation of the estrogen regulated gene CathD. The second objective is to determine how overexpression of CathD in the stroma can contribute to tumorigenesis in the epithelium.*** We believe that changes in the stroma result in alterations in stromal-to-epithelial paracrine signaling. This altered environment promotes the initiation and progression of tumorigenesis.

Body

The original stated goal in the approved statement of work for Task 1 was to determine if CD1 overexpression modifies estrogen regulated genes through interaction with endogenous ER α in prostate fibroblasts. To accomplish this task we developed a benign human prostate stromal cell line BHPrS to co-express CD1 and ER α to use as tool for examining the interaction of CD1 with ER α on the CathD promoter. In figure 1A, Co-immunoprecipitation experiments were performed to determine if CD1 could interact with ER α in prostate stromal cells. Co-overexpressing BHPrS^{CD1-ER α} cells demonstrate CD1 and ER α can interact when ectopically expressed. Overexpression of CD1 in BHPrS alone demonstrated interaction with endogenous ER α in prostate stromal cells. We next examined if CD1 overexpression modifies ER α transcriptional activity. In figure 1B, luciferase activity assays were performed with the use of estrogen responsive element fused with the luciferase gene. These experiments were performed in presence/absence of β -estradiol. In comparison to the empty vector control BHPrS^{EV}, CD1 overexpression induced increased ER transcriptional activity in the absence of hormone. This indicates that CD1 can induce the transcriptional activity of the ER with out the ligand bound in prostate stromal cells. This finding is in agreement with studies of ER and CD1 in breast cancer (40). Co-overexpression of ER α and CD1 in BHPrS cells induced greater luciferase expression compared to CD1 overexpression alone, both in the presence and absence of hormone. These data show that CD1 can interact with ER α to drive transcriptional activity of the estrogen receptor on non-chromosomal DNA. To determine if the CD1-ER α interaction binds chromosomal DNA we performed chromatin immunoprecipitation (ChIP) experiments with the BHPrS^{CD1} and BHPrS^{CD1-ER α} cell lines. Overexpression of CD1 in BHPrS showed greater than 11 fold recruitment over the IgG control of CD1 to the estrogen receptor element (ERE) in the CathD gene

in the absence of hormone. In the presence of hormone CD1 recruitment to the ERE in the CathD gene was only increased 8 fold over the IgG control.



We have previously shown that CD1 overexpression in benign prostate fibroblast cells (NPF^{CD1}) produces a phenotype similar cancer associated fibroblasts (39). A comparison of gene expression profiles from NPF^{CD1} and CAFs cells identified CathD to be overexpressed in both cell types in comparison to NPFs. Task 1 from the approved statement of work sought to determine if CD1 over expression modifies estrogen regulated genes through interaction with endogenous ERα in prostate fibroblasts. Our experimental approach showed that CD1 overexpression leads to aberrant ERα activity on the CathD gene.

Our original stated goal in the approved statement of work for Task 2 was to determine if inhibition of the ERα in signaling in the stroma abrogates the ability of CAF and/or NPF^{cyclin D1} to induce tumorigenesis. Our rational for this task is based on the understanding of the hormone levels in aging men. Levels of circulating testosterone in younger men are greater in comparison levels of estrogen. After 30 years of age, the hormone ratios in men start to change, with older men having lower levels of circulating testosterone and greater levels of

estrogen (41). Knockout animal models have been used to examine the role of the estrogen receptor in prostate. Recombination experiments with ER β knockout mesenchyme with wild type epithelium produced squamous metaplasia (SQM) when supplemented with a synthetic estrogen, however, SQM was not observed in ER α knockout mesenchyme was combined with wild type epithelium, indicating the importance of ER α in the stroma (42). In human PCa tissue, the expression of the ER α is up regulated in the stroma in comparison to benign prostate stroma (43) The co-administration of Testosterone (T) with Estrogen (E) stimulates cancer progression and malignant transformation in tissue recombination experiments of rat UGM (rUGM) and BPH-1, however, the single administration of T alone induces benign growth (26) (44). In the majority of models for hormonal carcinogenesis, estrogen along with the actions of the ER are required for maximal carcinogenic response to androgens.

To accomplish our approved second task we stated CAF and NPF^{CD1} cells would be engineered with shRNA specific to ER α . We attempted to transduce our different fibroblast cells with the shRNA constructs to make stable cell lines, however, CAFs and NPF^{CD1} cells under went replicative senescence. A downside to working with non-immortalized cells, is the limitation to the number of cell cycle replications. To overcome this pitfall and still address the second task we decided to use a pharmacologic approach and inhibit the ER in signaling with the use of Tamoxifen. In figure 2, we recombined CAFs with the initiated but non-transformed prostate epithelial cell line BPH-1 or NPF with BPH-1 and performed xenograft experiments in mice supplemented with T, E, T + E, T + Tamoxifen, and E + Tamoxifen for a period of 8 weeks. Recombinations of NPF + BPH-1 in mice supplemented with T produced benign growths with no malignant conversions. CAF + BPH-1 in mice supplemented with T induced a malignant conversions denoted by the BPH-1 cells invading into the mouse kidney. Recombinations of NPF+ BPH-1 in mice supplemented with T + E resulted in histologies resembling CAF + BPH-1 recombinations. The addition of estrogen in combination with T, drove a malignant conversion in the adjacent epithelium. This result is similar to published findings of tissue recombinations experiments of rUGM with BPH-1.

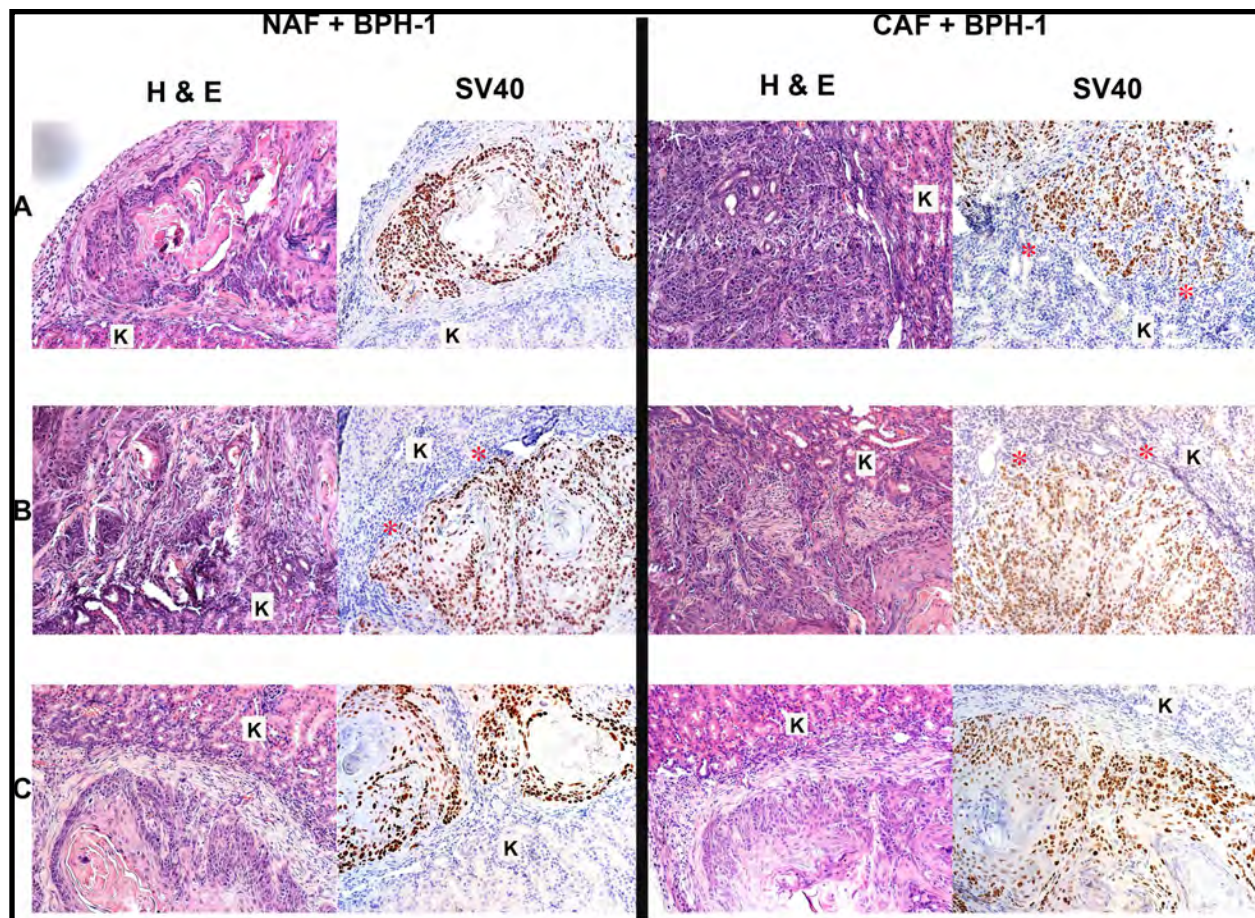


Figure 2. Inhibition of ER signaling in stroma prevents malignant transformation. Tissue recombination xenograft experiments in mice supplemented with (A) testosterone, (B) estrogen with testosterone, and (C) estrogen testosterone and tamoxifen. SV40 expressing BPH-1 cells that underwent a malignant transformation are denoted with asterisks.

As stated previously, the BPH-1 cell line is a prostate epithelial cell line, however, as a result of becoming immortalized the expression of the AR and ER were lost at the genomic level (45). In our model system the addition of hormones is specifically targeting the stroma and is not the epithelial cells. We asked the question how does inhibiting the ER in signaling in the stroma affect the adjacent epithelium. To address this question we recombined NAFs with BPH-1 cells and grafted under kidney capsule in mice supplemented with T + E + Tamoxifen. We previously observed the addition of T + E in this recombination induced a malignant conversion, however, when the ER was inhibited we observed no malignant conversion in the epithelium.

We know that the addition of testosterone is sufficient to induce a malignant conversion in recombination experiments of CAFs with BPH-1 cells. Inhibition of the ER with tamoxifen was sufficient at preventing the ability of CAFs to induce a malignant conversion in the adjacent epithelium in our model system. These results suggest that the activation of the ER in the stroma plays a critical role in prostate tumor progression. From our knowledge of the biosynthesis of sex steroid hormones, we know that testosterone can be directly converted into estrogen by the aromatase enzyme. We are currently investigating the role of the AR and ER in the stroma by using dihydro-testosterone, non-aromatizable form of testosterone. To ask the question which steroid hormone receptor is driving the prostate tumor progression.

Our final approved task was to determine how stromal production of CathD promotes tumorigenesis. The overexpression of CathD in neoplastic cells and neoplastic associated connective tissue was described as

long as 25 years ago, and is reported to play several roles in cancer progression (46, 47) (48, 49). To accomplish this task we engineered BHPoS cells to overexpress CathD (BHPoS^{CathD}) to perform *in vitro* and *in vivo* experiments. Sub-renal capsule xenograft experiments were performed using the BHPoS^{CathD} recombined with BPH-1 cells. After a period of 8 weeks, overexpression of CathD in the stroma induced a malignant transformation in the adjacent epithelium along with significantly larger tumors in comparison to recombinations with the empty vector control BHPoS cells. The reverse experiment was performed using BPH-1 cells made to overexpress CathD (BPH-1^{CathD}) and recombined with parental BHPoS cells and grafted under the kidney capsule. After a period of 8 weeks, epithelial overexpression of CathD failed to induce significant differences in growth or a malignant transformation. Our model identifies that stromal specific expression of CathD plays a role in promoting tumorigenesis. Our model is further supported by our findings of CathD knockdown expression in CAFs abolishes the ability to induce a malignant transformation in adjacent epithelium (Pruitt et al. - in press-).

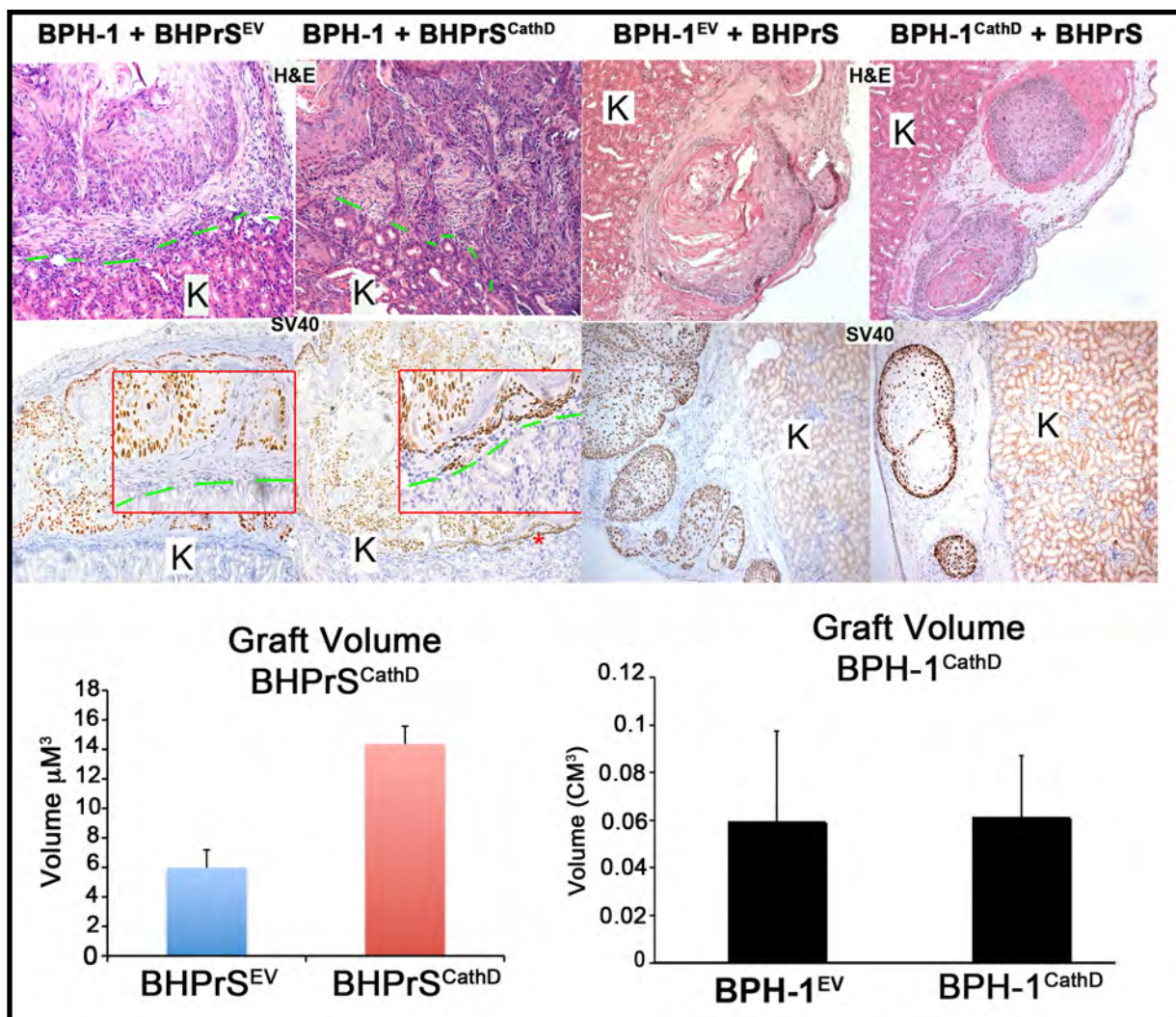


Figure 3. Stromal specific expression of CathD promotes malignant transformation. Tissue recombination experiments of A)BHPoS overexpressing CathD recombined with BPH-1 cells, B) BPH-1 cells overexpressing CathD recombined with parental BHPoS cells. Green hashmarks delineate graft-kidney interface. Red asterisk denotes enlarged inlay showing SV40 expressing BPH-1 cells invading into mouse kidney.

To delve deeper into understanding the role of stroma specific expression of CathD promoting tumorigenesis, we examined growth factor signaling pathways in the BPH-1 cells treated with conditioned media from BHP^{rS}^{CathD}. Conditioned media collected from BHP^{rS}^{CathD} and BHP^{rS}^{EV} was added to monolayer cultures of BPH-1 cells prior to isolation of protein lysates. Western blot analysis was performed on BPH-1 cell lysates for changes in the growth factor signaling pathways MAPK, and Akt/PKB. No differences were observed in the phosphorylation states of MAP kinases p38 or ERK1/2, however, we did observe increased p-Akt levels in BPH-1 cells supplemented with BHP^{rS}^{CathD} conditioned medium.

Our laboratory has previously published a study identifying a possible mechanism for CAFs to induce tumorigenesis in the adjacent epithelium. The study identified two molecules, transforming growth factor-beta (TGF-β) and stromal cell derived factor-1 (SDF-1) as being overexpressed in CAF cells. These factors were acting in a paracrine manner on the epithelial cells resulting in Akt hyperactivation (9). We questioned whether the increased levels of p-Akt seen in our experiments with the conditioned media isolated from BHP^{rS}^{CathD} cells

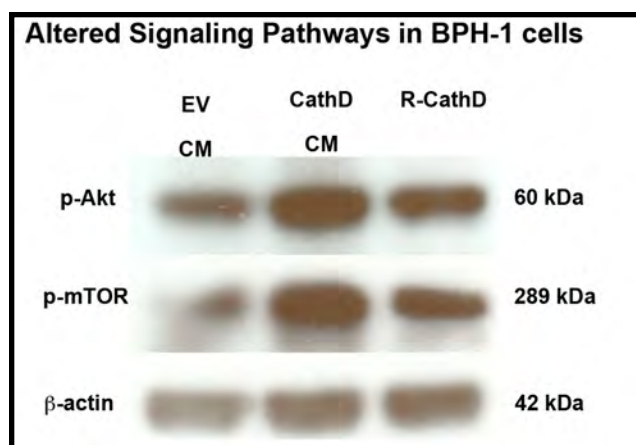


Figure 4. Stromal derived CathD alters Akt activity in epithelial cells. Media conditioned from BHP^{rS}^{CathD} added to BPH-1 cells increases p-Akt and downstream p-mTOR levels. Similar increases are seen when recombinant CathD is added to BPH-1 cells.

was related to TGF-β activity. It has been previously shown in *in vitro* experiments that CathD can liberate TGF-β from the latency inhibitor complex, allowing for activation of the TGFβ receptor (TGFBR) complex (50). In vivo experiments using the CathD prozyme, showed CathD can be fully activated in the extracellular environment and cleave substrates in the mouse prostate (51). We examined our tissue recombinations of BHP^{rS}^{CathD} with BPH-1 cells for increased TGF-β signaling. Immunohistochemical staining for p-SMAD2/3, the immediate downstream substrate of TGFBR activation, showed significantly greater p-SMAD2/3 staining in CathD overexpressing recombinants. We also examined differences in the TGF-β responsive gene collagen type 4 α2 (ColIVα2). Staining for showed increased deposition of ColIVα2 in recombinations overexpressing CathD. Masson's trichrome staining of tissue xenografts from the CathD overexpressing stromal cells also revealed increased production of collagen in comparison to recombinations with the EV control stromal

cells. Collectively we concluded that stromal derived CathD was promoting tumorigenesis through the activation of TGF-β signaling pathways.

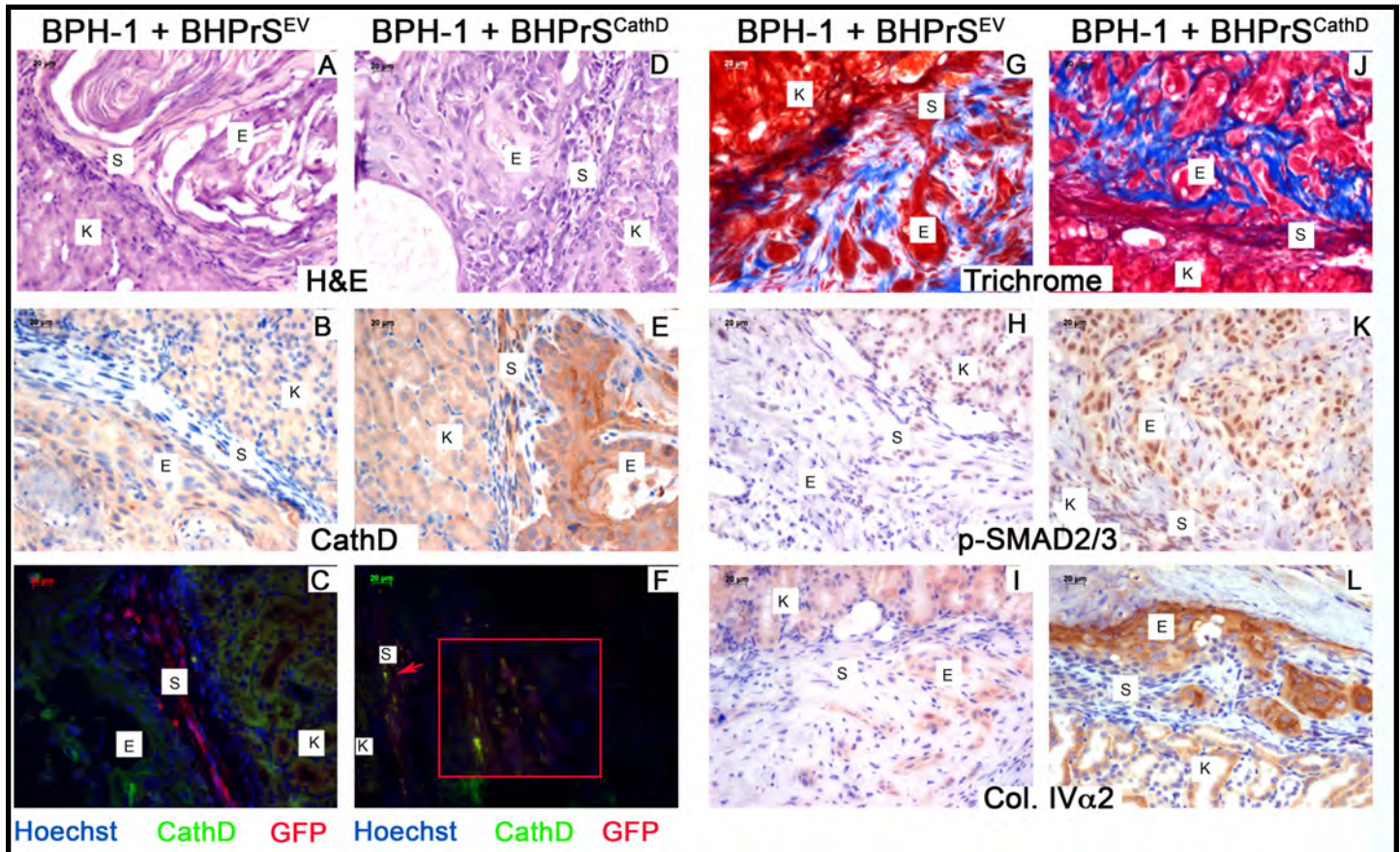


Figure 5. Stromal expression of CathD leads to activation of TGF- β . Overexpression of CathD in BHPPrS (E) results in malignant transformation (D) through increased TGF- β signaling leading to increased p-SMAD 2/3 activation (K), and downstream TGF- β responsive genes like collagen type IV α 2 (L). Masson's trichrome staining reveals a reactive stromal phenotype, denoted by increased collagen deposition in BHPPrS^{CathD} recombinations (J).

The second line item under the approved third task was to determine if the conversion of pro-CathD to the active protease is dependent on the presence of glycosaminoglycans (GAGs) on the surface of PCa cells. To accomplish this task we treated a series of PCa cell lines with heparinase to remove heparin sulfate containing proteoglycans from the cell surface prior to the addition of pro-CathD. The heparin sulfate proteoglycans have been previously shown to convert the CathD zymogen to the active state(50). Completion of the CathD activity assay after treatment with heparin lyase did not show any inhibition in the conversion of pro-CathD to the active protease. We also performed this experiment with 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), a specific inhibitor for Na(+)/H(+) antiporters. It has been shown that human PCa and PCa cell lines have lower extracellular pH due to increased proton pump expression (52). We hypothesized that CathD activity was dependent on the activity of proton pumps present on the surface of PCa cells. PCa cell lines treated with EIPA show drastic decreases in extracellular CathD activity similar to levels when of cell lines treated with the CathD inhibitor pepstatin A. Our results show that extracellular CathD activity is not dependent on heperan sulfate containing proteoglycans, however is dependent on the extracellular pH.

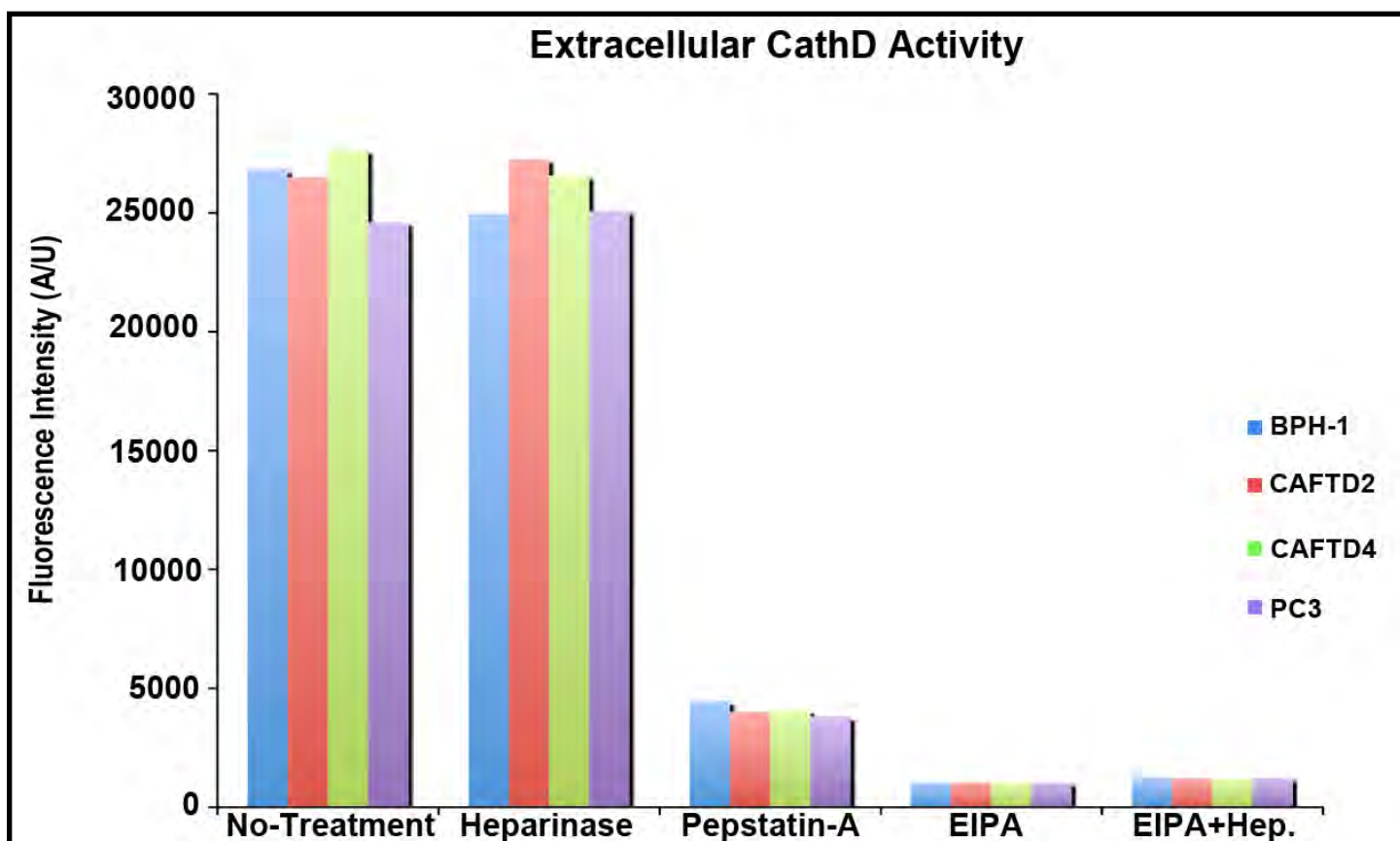


Figure 6. Extracellular CathD activity is dependent on proton pump exchangers. Deactivation of proton exchangers on benign and malignant prostate cell lines with EIPA inhibits CathD proteolytic cleavage on fluorescent substrate.

In summary we have shown that CD1 interacts with the steroid hormone receptor $ER\alpha$ to modify the expression of estrogen regulated genes like CathD in prostate fibroblasts. We have also highlighted the importance of the ER in signaling in the stroma in contributing to CAFs induced tumorigenesis in adjacent epithelium. We have also identified a possible mechanism to explain how stromal derived CathD overexpression can promote tumorigenesis through TGF- β signaling. Further work on determining if the inhibition of the ER in signaling in the stroma abrogates CAF ability to induce tumorigenesis is currently being performed.

Task 1. To determine if CD1 over expression modifies estrogen regulated genes through interaction with endogenous $ER\alpha$ in prostate fibroblast (months 1-12):

1. Develop BHPs cells that co-express CD1 and $ER\alpha$ (Months 1-3). **Completed**
2. Characterize BHPs ^{$ER\alpha$ -CD1} stable cell lines (Months 4-6). **Completed**
 - a. Determine expression of CD1 and $ER\alpha$ by western blot.
 - b. Quantify proliferation by crystal violet assay.
 - c. Quantify luciferase expression after transient transfection with luciferase reporter construct fused with an estrogen responsive element.

3. Perform biochemical studies examining CD1 and ER α physical interaction on CathD promoter (Months 7-12). **Completed**
 - a. ChIP assays

Task 2. To determine if inhibition of the ER α signaling in the stroma abrogates CAF and/or NPF^{cyclin D1} ability to induce tumorigenesis (months 8-24).

1. Engineer a ER α specific shRNA expression construct for stable expression (Months 8-12). Establish ER α knockdown CAF and NPF^{cyclin D1} cell lines (Months 10-15).
 - a. Determine knockdown efficiency by western blot.
 - b. Determine effects of knockdown on cell proliferation by crystal violet assay.
2. Perform xenograft studies using ER α knockdown stroma cell lines recombined with BPH-1 and BHPRE1 (Months 14-24).
 - a. Evaluation of tumorigenesis by measuring tumor volume.
 - b. Evaluation of malignancy and invasion by IHC.
 - c. Quantification of proliferation by IHC.

Changed experimental approach to use pharmacologic inhibitor to target Estrogen Receptor- Work is still ongoing.

Task 3. To determine how stromal production of CathD promotes tumorigenesis (months 20-36).

1. Characterize the effects of conditioned media from BHPRE1 on BPH-1 and BHPRE1 cell lines (Months 20-24). **Completed**
 - a. Quantify CathD induced proliferation of epithelial cell lines.
 - b. Examine cell signaling pathways i.e. phospho-Akt, phospho-p38 MAPK by western blot analysis.
2. Examine the conversion of pro-CathD (inactive) to CathD (active) by GAG present on the surface of PCa cell lines (Months 22-36). **Ongoing**
 - a. Perform activity assays using purified pro-CathD added to the PCa cell lines LNCaP, C4-2B, and PC3.
 - b. Perform activity assays for CathD using the BHPRE1 conditioned media added to PCa cell lines.
 - c. Perform CathD activity assays using purified pro-CathD and BHPRE1 conditioned media after protein glycosylation is inhibited using soluble inhibitor.

Task 4. Prepare manuscript(s) for publications (month 36). One Completed

Key research accomplishments

- Prostate stromal cell lines were generated and characterized.
- CD1 can interact with endogenous ER α to direct transcriptional activation (**figure 1**).
- Possible mechanism uncovered to explain how stromally expressed CathD can promote prostate tumorigenesis (**figure 5**).
- Extracellular CathD proteolytic activity is dependent on proton pump activity (**figure 6**).

Reportable outcomes

The following publication was referenced by this training grant during the last year.

1. Freddie L. Pruitt, Yue He, Omar E. Franco, Ming Jiang, Justin M. Cates, Simon W. Hayward. Cathepsin D acts as an essential mediator to promote malignancy of benign prostatic epithelium. *The Prostate*. Currently in press

Conclusions

Significant progress has been made towards achieving the stated goals considering the technical limitations experienced in the second aim. This work has produced one publication with the possibility of producing a second.

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Cathepsin D Acts as an Essential Mediator to Promote Malignancy of Benign Prostatic Epithelium

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BACKGROUND. Stromal–epithelial interactions are important in both development and prostate cancer. Stromal changes have been shown to be powerful prognostic indicators of prostate cancer progression and of patient death helping to define lethal versus indolent phenotypes. The specific molecular underpinnings of these interactions are incompletely understood. We investigated whether stromal cathepsin D (CathD) overexpression affects prostate tumorigenesis through a paracrine mechanism.

METHODS. Normal prostate fibroblasts (NPF) were retrovirally transduced to overexpress cyclin D1 (CD1) and were designated NPF^{CD1}. Cathepsin D expression was knocked down using shRNA in cancer associated fibroblasts (CAF) and NPF^{CD1}. We analyzed these stromal cell lines using immunohistochemistry, Western blot, and tissue recombination.

RESULTS. An examination of human prostate tissue revealed significantly increased stromal staining of CathD in malignant prostate tissue. Overexpression of CD1 in normal prostate fibroblasts (NPF^{CD1}) produced a phenotype similar to, but more moderate than, CAF in a tissue recombination model. Knockdown studies revealed that CathD is required for NPF^{CD1} motility and invasive growth in vitro. BPH-1 cell proliferation was found to be induced when cultured with NPF^{CD1} conditioned medium, this effect was inhibited when CathD was knocked down in NPF^{CD1} cells. Overexpression of CathD in prostate stromal cells induced malignancy in adjacent epithelium, and this transformation was inhibited when stromal CathD expression was knocked down in CAF.

CONCLUSIONS. The study presented here demonstrates increased CathD expression is seen in human CAF. The upregulation of CD1 results in concomitant increases in CathD expression. Elevated CathD expression in the stroma contributes to tumor promotion.

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KEY WORDS: cathepsin D; stromal–epithelial interactions; prostate cancer

INTRODUCTION

Historically, the field of cancer biology has primarily been focused on studying the malignant tumor epithelium [1]. The emergence of the field of tumor microenvironment is providing some much needed insight into how non-malignant cells associated with cancer (cancer associated stroma) can promote or suppress tumorigenesis. The stromal phenotype has been shown to be a powerful prognostic indicator of cancer progression and of patient death underlining the

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importance of local stromal cells in defining lethal versus indolent phenotypes [2].

Stromal–epithelial interactions are important in both the development of the prostate, and in prostate cancer (PCa) [3–5]. During carcinoma evolution, the stroma cells adjacent to the pre-malignant or malignant epithelium experience phenotypic alterations that have been shown to enhance the invasive potential of the epithelial tumor [6–8]. These stromal–epithelial interactions are mediated, in large part, by paracrine signaling between epithelial tumor cells and neighboring stromal fibroblasts [8]. We have previously published on several molecules found to be aberrantly expressed in cancer-associated stroma that induce tumorigenesis and malignant conversion [7–13]. These intercellular interactions are clearly complex and there are likely a number of molecular routes which can either promote or suppress tumor-inducing activity. One purpose of pursuing these studies is to start to determine the identity of pathways which are either sufficient or necessary to induce transformation and to examine how such pathways might interact. We showed that the up-regulation of a cell cycle regulator known as cyclin D1 (CD1) in normal prostate fibroblasts mimics aspects of the phenotype of malignant conversion seen in cancer associated stroma. In addition to receiving signals from malignant epithelial cells, the stromal fibroblasts stimulate tumorigenesis by releasing factors that act on adjacent epithelial tumor cells or exchange enzymes that modify local microenvironment promoting the proliferation and survival of the neoplastic cells [7,8,14,15].

One mechanism by which modifications to the local tumor microenvironment are accomplished is via the actions of several different families of proteases produced by either the tumor or the stroma [16]. These enzyme families include matrix metalloproteases (MMP), cysteine, and serine proteases, which have been shown to play a role in the degradation of the basement matrix, promotion of angiogenesis, and the liberation of growth factors to stimulate tumor cell growth [17,18].

Cathepsin D (CathD) is a ubiquitous lysosomal aspartic endoprotease. CathD has been shown to be involved in a number of physiological processes, playing a critical role in barrier function, regulation of apoptosis, and epithelial differentiation [19–21]. In cancer, CathD is overexpressed and hypersecreted in various malignancies including PCa [22,23]. In breast cancer, CathD expression is associated with a poor prognosis and increased likelihood for the development of metastasis [24]. Experimental evidence has shown CathD can stimulate the proliferation of PCa cell lines [25].

There are limited data defining CathD's function in prostate cancer progression. Some studies have concluded that CathD is overexpressed in the epithelium and stroma of PCa, and may promote proliferation [25,26]. Other studies have concluded that CathD produced by PCa may be inhibiting tumor growth [27,28]. In the present study, we highlight CathD as a mediator of cancer associated stromal promotion of prostate tumorigenesis.

METHODS

Cells

BPH-1 (a non-tumorigenic human prostatic epithelial cell), and its tumorigenic derivatives BPH^{CAFTD} were from our own stocks [29,30]. CAF cells were isolated from human prostate tumor samples and their activity validated in a tissue recombination model. The technique for the isolation of CAF is described in Olumi et al. [4] which also describes a bioassay which was used to confirm the tumor-inducing activity of the CAF used in the present study. NPF^{CD1} cells, which we have shown in the past to overexpress CathD, were generated as previously described [9]. Benign human prostate stromal cells (BHPPrS1) were isolated from a prostate surgical sample and immortalized with hTERT as previously described [10]. Cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA) with 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY), and 5% Cosmic Calf Serum (CCS-HyClone, Logan, UT).

Generating Genetically Modified Cell Lines

The pSuper.Retro-control (PSR-OligoEngine, Seattle, WA) and pSuper.Retro-CD1 shRNA (PSR-CD1sh) were kindly provided by Drs. Rene Bernards and Daniel Peeper from the Netherlands Cancer Institute. The two plasmids were engineered into CAF by retroviral transduction as previously described [9]. Positively transduced cells were selected for resistance to puromycin (5 µg/ml) to generate two cell strains (CAF^{scram} and CAF^{CD1sh}). The pSilencer 2.1-CathD1shRNA vector was kindly provided by Dr. Daniel E. Johnson from the University of Pittsburgh Cancer Institute. PSR-cathepsin Dsh was generated by removing the CathD1sh coding sequence from pSilencer 2.1-CathD1shRNA with *HindIII* and *BamHI* and ligated into the PSR construct. The PSR and PSR-CathD sh were engineered into CAF by retroviral infection as described previously [9]. The positive transduced cells were selected for resistance to puromycin (5 µg/ml) to generate the cell line (CAF^{CathDsh}). BPH-1^{NPF}, BPH-1^{CAFTD1}, and BPH-1^{NPFCD1} cells were re-isolated from resulting growths as previously described [9].

BHPPrS1 cells were engineered to overexpress CathD by lentiviral transduction (Genecopoeia, Inc., Rockville, MD). Viral supernatant was generated, centrifuged at 3,000 rpm for 5 min and passed through a 0.45 μ m filter before frozen at -80°C until used. Polybrene (Sigma-Aldrich, St. Louis, MO) was added to the viral suspension at 5 $\mu\text{g}/\text{ml}$ to increase the efficiency of the transduction. GFP-expressing cells were selected by fluorescence-activated cell sorting (FACS) to establish the BHPPrS^{CathD} and BHPPrS^{EV} as an empty vector control.

Western Blotting Analysis

Cell lysates were prepared and Western blotting was performed as previously described [31]. Membranes were incubated with mouse primary antibody to PTEN (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), Cdk2 (1:1,000, Santa Cruz), Cdk4 (1:1,000, Santa Cruz), Cdk6 (1:1,000, Santa Cruz), cyclin E (1:1,000, Santa Cruz), CD1 (1:1,000, BD Biosciences Pharmingen, San Jose, CA), β -actin (1:5,000, Sigma) or CathD (1:1,000, Cell Signaling, Denver, MA) overnight and washed with PBS-Tween 20 for 1 hr, and incubated with horseradish-Peroxidase linked anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ, 1:10,000 dilution) for 1 hr. Bound antibodies were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

Tissue Recombination and Xenografting

Rat urogenital mesenchyme (rUGM) was obtained from 18-day embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by tryptic digestion, as described previously [32]. BPH-1 + rUGM, BPH-1 + NPF, BPH-1 + NPF^{CD1}, BPH-1 + CAF, BPH-1 + CAF^{CyclinD1sh}, BPH-1 + CAF^{CathDsh}, BPH-1 + BHPPrS^{CathD}, and BPH-1 + BHPPrS^{EV} tissue recombinants were made as previously described [33]. 1.0×10^5 epithelial cells and 2.5×10^5 stromal cells combined in type I rat tail collagen were used to make the recombinants. After overnight incubation, the tissue recombinants were grafted under the kidney capsule of adult male severe combined immunodeficient (SCID) mice (Harlan, Indianapolis, IN) supplemented with 25 mg testosterone pellets (PCCA, Houston, TX). All the experiments were repeated six times. Mice were sacrificed at 8 weeks and grafts were harvested, fixed, and paraffin embedded. Graft dimensions were measured using the formula: volume = width $\times$ length $\times$ depth $\times \pi/6$ as described previously [10].

Wound Healing Assays

Confluent monolayers of NPF and NPF^{CD1} cells were grown in 6-well plates. Confluent cell monolayers were wounded by scratching with a pipette tip. Specific points on the wounds were identified and marked. These open areas were then inspected microscopically over time as the cells migrated in and filled the damaged area. Wounds were imaged at 0, 3, 6, and 8 hr post wounding and the cell migration rate into the wound was calculated. Experiments were performed in triplicate.

Outgrowth Assay

1.0×10^5 NPF, NPF^{CD1}, NPF^{CD1-CathD} control or NPF^{CD1-CathDsh} were resuspended at 4°C in Matrigel (0.2 ml, 10 mg/ml; Becton and Dickinson), and overlaid to a previously solidified layer of Matrigel in 24-well plates. The top Matrigel layer was solidified at 37°C for 30 min and covered with culture medium containing 10% FCS (0.5 ml).

Conditioned Medium

NPF or NPF^{CD1} were seeded with 5% FCS in RPMI 1640 at a density of 5.0×10^5 per 75-cm² flask, allowed to grow, and attached overnight. Confluent cultures of NPF or NPF^{CD1} were rinsed twice in PBS and incubated for 3 days in RPMI + 0.5% FCS. The medium was collected, centrifuged, passed through a 0.45- μ m filter (Millipore), and stored at -80°C for later use. Conditioned medium was thawed and diluted 1:1 with fresh DMEM + 0.5% FCS before use. BPH-1 cells were seeded at 2.0×10^4 per well in 6-well plates in conditioned medium. The cultures were incubated for 3 days and the total number of cells was determined by direct counting in a hemacytometer.

Human Prostate and Prostate Tissue Microarray

Human prostate tissue array (PR806) was obtained from US Biomax, Inc. The array contained duplicates from 30 cases of adenocarcinoma ranging in Gleason scores and 10 cases of normal prostate tissue. Normal human prostate tissue was also obtained from the Vanderbilt University Medical Center Department of Pathology.

Histochemical and Immunohistochemical Staining

Masson's trichrome stain was performed as previously described using Diagnostics Accustain Masson trichrome stain kit (Sigma), Bouin's solution (Sigma) and Weigert's Iron Hematoxylin set (Sigma) [10]. Immunohistochemical staining was performed following a protocol that was described previously [31].

Tissue slides were then incubated with the primary antibody against CD1 (1:200, BD Biosciences Pharmingen), CathD (1:200, Santa Cruz), p-SMAD2/3 (1:400, Santa Cruz), Col4A2 (1:200, Santa Cruz). The polyclonal rabbit or mouse immunoglobulins/biotinylated anti-mouse secondary antibody (DAKO, Carpinteria, CA) was incubated for 60 min after the slides were washed with PBS buffer for 1 hr. After washing the slides in PBS extensively, slides were incubated in ABC-HRP complex (Vector Laboratories) for 30 min. Bound antibodies were then visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Slides were then rinsed extensively in tap water, counterstained with hematoxylin, and mounted.

Immunofluorescence

For histological analysis, 5 μ m tissue sections were dewaxed, and the antigen was unmasked by heating samples in unmasking solution (Vector Laboratories). Slides were blocked in 12% BSA in PBS for 30 min at room temperature before incubating with primary antibodies against CathD and GFP (1:200, Santa Cruz). After 1 hr washing in PBS buffer, slides were incubated with secondary antibodies (1:200; Alexa-Fluor 488 anti-Rabbit IgG and AlexaFluor 594 anti-mouse IgG2a) for 30 min at room temperature. Slides were incubated in Hoechst 33258 (4 mg/L) for 5 min. Tissue sections were washed for 30 min in PBS, mounted, and visualized.

Quantitative Image Analysis

Immunostained slides were analyzed using the Ariol SL-50 automated slide scanner (Applied Imaging, San Jose, CA) to quantitate the amount of staining for CathD in the stroma of benign and malignant human prostate tissue sections. Positive staining was calculated by applying two thresholds, with one recognizing weaker brown-positive cells, and another recognizing stronger brown-positive cells. The intensity of the stain was calculated by masking out all non-stromal areas from the tissue section and calculating the integrated optical density of brown within the remaining area. This value was divided by the area in pixels of the brown mask to calculate the average intensity of the tissue section.

Statistical Analysis

Data from in vitro and in vivo are presented as the mean $\pm$ standard deviation (SD). The data was analyzed using GraphPad PRISM software (La Jolla, CA). *P*-values less than 0.05 were considered statistically significant. Quantitated intensity of CathD expression

in human prostate samples were compared with analysis of variance followed by post hoc analysis of significant means by Mann-Whitney's *U*-test was used in comparison of normal to tumor tissue. Post hoc analysis of significant means by Dunn's multiple comparison test was used for the comparisons of normal tissue with low grade and high grade malignant prostate tissue. *P*-values less than 0.05 were considered statistically significant.

RESULTS

CathD Expression is Upregulated in Prostate Clinical Samples, and CathD Is Overexpressed in the Stroma of Tumorigenic Tissue Recombinants

We examined the expression patterns of CathD in human prostate clinical samples using a tissue microarray containing 30 cases of adenocarcinoma, and 10 cases of normal prostate tissue. The tissue microarray contained duplicate cores per case. Quantification of positive CathD staining in stromal regions of prostate tissue showed significantly greater areas of CathD expression in tumor tissue in comparison to normal prostate tissue. When tumor tissue was stratified between low and high grades, a significant difference was only observed in high grade tumors compared with normal tissue with a non-significant elevation of expression in low grade tumors (Fig. 1). A similar trend with no significant difference was also observed in comparisons of low and high grade tumors. It was noteworthy that the expression of CathD apparently corresponded to areas which stained a light red color in the adjacent trichrome-stained sections. This likely indicates the presence of myofibroblastic cells in this area, which would correspond to the source of our experimental CAF. Examination of CathD expression in tissue recombinations of BPH-1 + NPF, BPH-1 + rUGM, BPH-1 + NPF^{CD1}, and BPH-1 + CAF was performed by IHC. The recombinations of BPH-1 + NPF and BPH-1 + UGM isolated after 8 weeks of growth produced small growths overall which displayed solid epithelial cord structures surrounded by a muscular stroma. IHC staining displayed minimal expression of CathD in the stroma with some epithelial expression seen in the BPH-1 + NPF recombinants (Fig. 2A1 and A2). In marked contrast, recombinations of BPH-1 + NPF^{CD1} and BPH-1 + CAF isolated after 8 weeks produced poorly differentiated carcinoma along with areas of squamous metaplasia similar to previously published results (Fig. 2A3 and A4) [9]. Recombinations of BPH-1 + NPF^{CD1} and BPH-1 + CAF displayed strong CathD staining in the stroma and epithelium (Fig. 2A3 and A4). These results are consistent with the observations of CathD overexpression in the stroma of human PCa clinical tissues.

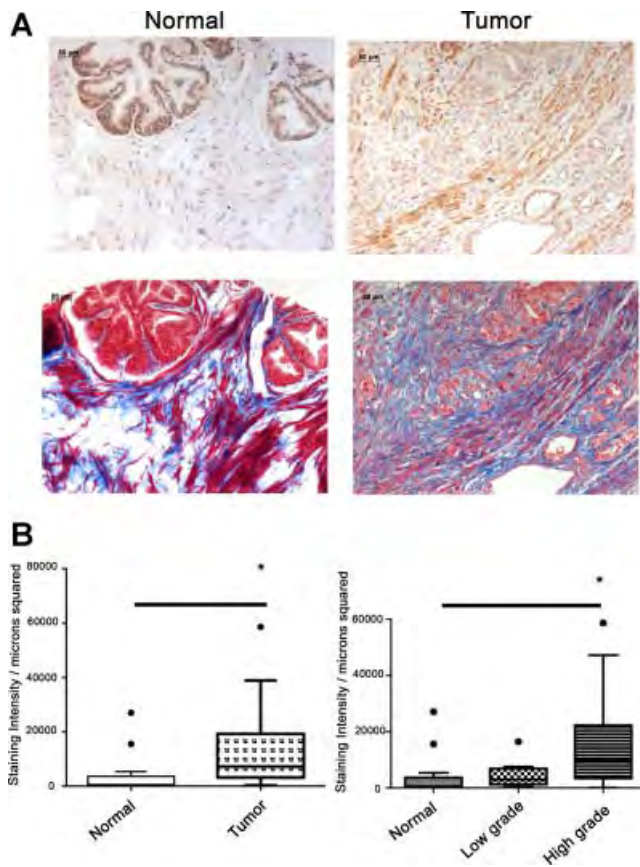


Fig. 1. Cathepsin D is overexpressed in malignant prostate clinical samples. **A:** Representative images from immunohistochemical (top) analysis of CathD expression in normal (left) $n = 18$ and tumor (right) $n = 30$ human prostate tissues. Representative images of Masson's tri-chrome staining (bottom) from normal (left) and tumor (right). Scale bar is equal to $50 \mu\text{m}$. **B:** Quantitation of CathD expression in the prostate comparing normal to malignant tissue. Data are presented as means $\pm$ SD.

These data raised the question of whether the upregulation of CathD protein is a passive result of prostatic tumorigenesis or plays an active role as a paracrine mediator required to induce a malignant transformation in the adjacent prostatic epithelium.

Stromal Expression of CD1 Affects Cell Cycle Regulators in Adjacent Epithelium

Fluorescence-activated cell sorting of BPH-1 cells isolated from the xenografts of BPH-1 + NPF and BPH-1 + NPF^{CD1} tissue recombinants showed striking differences in cell population distributions. Previously published DNA flow cytometric analysis showed a majority (55%) of BPH-1 cells isolated from the tissue xenografts of BPH-1 + NPF^{CD1} were hyperploid along with another large population of BPH-1 cells (23.1%) that were polyploid [9]. In order to gain

some insight as to what signaling pathways in BPH-1 cells were being affected by NPF^{CD1} we performed Western blot analysis for several proteins involved in cell cycle regulation. Epithelial cells were isolated and cultured from xenografts of BPH-1 + NPF, BPH-1 + CAF, and BPH-1 + NPF^{CD1}. The resulting cells were designated BPH-1^{NPF}, BPH-1^{CAFTD}, and BPH-1^{NPF-CD1} respectively. Densitometric analysis of band intensities from Western blots revealed that overexpression of CD1 in the local stromal cells increased the expression of the cell cycle related proteins CD1, cyclin dependent kinases-6 (CDK6) and 2 (CDK2), and CathD in BPH-1^{NPF-CD1} over BPH-1^{NPF} (Fig. 2B), consistent with increasing proliferative activity in these cells. Similar results were observed in BPH-1^{CAFTD1}. No changes in the expression CDK4, cyclin E were observed.

CathD Is a Critical Component in NPF^{CD1} Motility and 3D Outgrowth

We have previously reported the abilities of NPF^{CD1} and CAF to induce tumorigenesis in tissue recombination experiments [4,9]. In order to further investigate mechanisms underlying this malignant transformation we characterized the effects of knocking down CathD expression in NPF^{CD1}. NPF^{CD1} displayed enhanced motility in wound healing assays compared with control NPFs (Fig. 3A). The enhanced motility displayed by NPF^{CD1} was significantly abrogated when CathD expression was knocked down, with the use of CathD specific shRNA (NPF^{CD1}-CathDsh) (P -value < 0.005). These findings demonstrate that CathD plays a role in NPF^{CD1} migration in vitro. Western blot analysis was performed to confirm the knockdown of CathD expression in CD1 overexpressing fibroblast (Fig. 3D). Overexpression of CD1 in NPF results in increased CathD expression as previously published [9]. Stable expression of stable hairpin RNA (shRNA) specific for CathD results in 66% knockdown in CathD expression. Expression of non-specific shRNA in NPF^{CD1} does not alter CathD expression.

To further characterize the requirement for CathD expression in NPF^{CD1}, we examined fibroblast outgrowth in 3D matrices. As shown in Figure 3B, overexpression of CD1 promoted outgrowth of normal prostatic fibroblasts embedded into Matrigel. After 14 days of culture, NPF^{CD1} cells had adopted a stellate morphology and formed invasive colonies with protrusions sprouting into the surrounding matrix (Fig. 3B2). In contrast, normal prostatic fibroblasts presented a well-delineated spherical appearance of quiescent and/or dying cells and grew poorly, neither invading nor forming protrusions to the surrounding

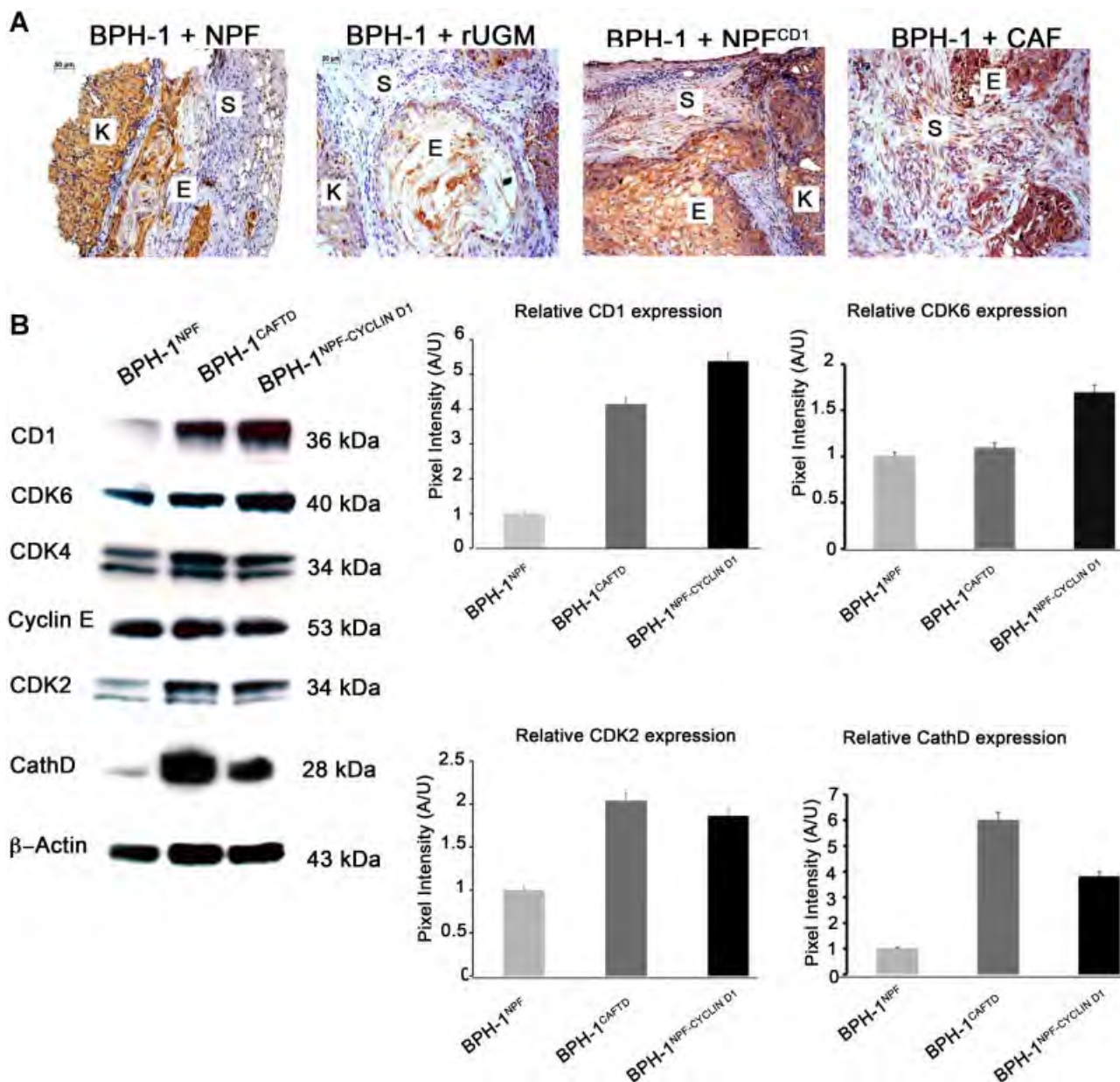


Fig. 2. Evaluation of Cathepsin D as a paracrine mediator of neoplastic epithelial cell growth in tissue recombinants in vivo. **A:** Immunohistochemical analysis of CathD expression in recombinations of (1) BPH-I + NPF, (2) BPH-I + rUGM, (3) BPH-I + NPF^{CD1}, and (4) BPH-I + CAF. Scale bar is equal to 50 μ m (Letters K, S, E, refer to kidney, stroma, and epithelium). **B:** Evaluation of densitometric analysis of cell cycle regulators and CathD in BPH-I^{NPF}, BPH-I^{CAFTD}, and BPH-I^{NPF^{CD1}} cells. Expression of Beta-actin was used for a loading control. Graphical representation of the mean $\pm$ SD of band intensities.

matrix (Fig. 3B1). NPF^{CD1-CathDsh} cultured in 3D matrix failed to form invasive colonies that protruded into the surrounding matrix (Fig. 3B3), unlike NPF^{CD1-control}, which retained the ability to form invasive growth feature (Fig. 3B4). These data strongly imply a role for CathD as a factor in promoting the invasive growth of NPF^{CD1} cells in vitro.

CathD Is a Paracrine Mediator of Neoplastic Epithelial Cell Growth In Vitro

To investigate the role of CathD as a paracrine mediator of prostate epithelial cell growth, we generated conditioned media from NPF and NPF^{CD1} cells, and measured BPH-1 cell numbers after growth for 3 days

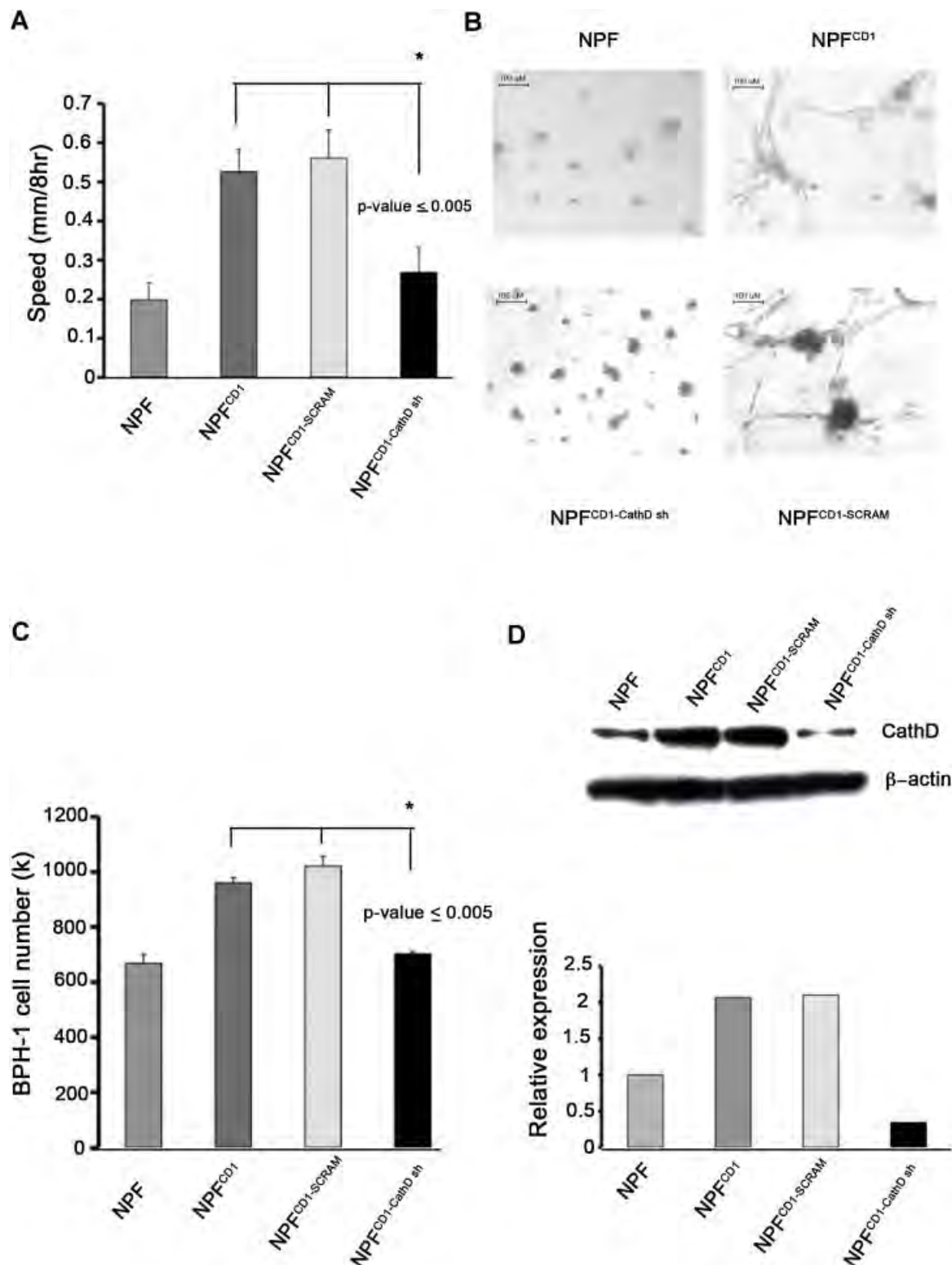


Fig. 3. Cathepsin D is a critical mediator between BPH-I cells and NPF^{CDI} in vitro. **A:** Wound healing assay. Confluent monolayers of NPF, NPF^{CDI}, NPF^{CDI}-control, and NPF^{CDI}-CathD sh were scratched with a pipette tip. Bar graphs represent the mean $\pm$ SD of rate of wound closure over 8 hr period. Significance determined by ANOVA, P -value ≤ 0.005 , $n = 3$. **B:** 3D outgrowth assays. NPF cell lines were embedded in matrigel and cultured for 14 days, scale bar = 100 μ m. Images taken at 10 $\times$ (3B, b). **C:** Evaluation of CathD as a paracrine mediator of growth. BPH-I cells were treated with conditioned media collected from NPF cell lines for 3 days. Cell numbers were quantitated by direct counting, graphical representation of the mean $\pm$ SD of the experiment is shown, significance determined by ANOVA, P -value ≤ 0.005 , $n = 3$. **D:** Western blot confirming knockdown of CathD in NPF, NPF^{CDI}, NPF^{CDI}-control, and NPF^{CDI}-CathD sh cells (Top). Densitometric analysis of band intensities performed to determine knockdown efficiency (Bottom).

in the conditioned medium. Conditioned medium from NPF^{CD1} increased the proliferation of BPH-1 cells by 1.7-fold, when compared with medium conditioned by parental NPF (Fig. 3C). The pro-mitogenic effects from NPF^{CD1} conditioned medium were abrogated when CathD expression was knocked down in NPF^{CD1-CathDsh} (P -value ≤ 0.005). These results suggest that a significant component of NPF^{CD1} proliferative influence toward epithelium is mediated through secreted CathD.

CathD Is an Essential Mediator of CAF Induced Tumorigenicity In Vivo

To elucidate the role of CD1 and CathD in CAF's ability to induce tumorigenesis of BPH-1, we took a knockdown expression approach. CAF were engineered to express shRNA vectors specific for either CD1 or CathD. Western blotting was used to assess knockdown efficiency. CD1 expression was knocked down 50% and CathD expression was knocked down 95% in CAF cells (Fig. 4C). Based on gross morphology it was found that BPH-1 + CAF^{CyclinD1sh} and BPH-1 + CAF^{CathDsh} recombinants formed significantly smaller grafts compared with BPH-1 + CAF grafts (p -value ≤ 0.05) (Fig. 4A and C). Histologically, BPH-1 + CAF recombinants formed adenosquamous carcinoma as previously described [4]. Knockdown recombinants formed benign, small cord structures with no tumorigenic response (Fig. 4B). These findings establish CathD to be a key mediator in prostate stroma-epithelial interaction in the development of tumorigenesis.

To further clarify the role of stromal derived CathD in promoting tumorigenesis we engineered BHPPrS, a benign human prostate stromal cell line, to overexpress CathD (BHPPrS^{CathD}) by lentiviral transduction. In comparison to recombinations of BPH-1 + BHPPrS^{EV}, BPH-1 + BHPPrS^{CathD} recombinants exhibited a malignant transformation. Based on the H&E staining, recombinations of BPH-1 + BHPPrS^{EV} exhibited thick stromal regions delineating BPH-1 cells from the kidney interface (Fig. 5A). The opposite was observed in recombinations of BPH-1 + BHPPrS^{CathD}, where BPH-1 cells are directly adjacent to kidney interface (Fig. 5D). IHC staining for CathD indicates strong stromal expression of CathD in the recombinations of BPH-1 + BHPPrS^{CathD} (Fig. 5E). IF staining for GFP positive stromal cells (red) and CathD expression (green) show strong stromal specific expression of CathD seen in the yellow overlay (Fig. 5F).

Masson's trichrome staining was performed on tissue sections from recombinations of BPH-1 + BHPPrS^{EV} and BPH-1 + BHPPrS^{CathD} (Fig. 5G and J). Heavy analine blue stains indicated increased

deposition of newly synthesized collagen fibrils in the CathD overexpressing recombinations (Fig. 5J). IHC staining was performed to examine the phosphorylated-SMAD2/3 (p-SMAD2/3), a surrogate reporter of transforming growth factor-beta (TGF- β) activity (Fig. 5H and K). CathD overexpressing recombinations (Fig. 5K) shows increased nuclear p-SMAD2/3 staining. Quantitation of p-SMAD2/3 positive cells indicated a significant difference in the CathD overexpressing recombinations. Type IV α 2 collagen (Col.IV α 2) is a known TGF- β responsive gene. We performed IHC staining for Col.IV α 2 and the CathD overexpressing recombinations (Fig. 5L) displayed strong expression for Col.IV α 2, in comparison to recombinations with the empty vector construct (Fig. 5I).

DISCUSSION

Tumor stroma has been implicated in the regulation of cell growth, determining metastatic potential, and impacting the outcome of therapy. Stromal-epithelial interactions in cancer have implicated as promoting several malignancies including prostate, breast, colon, and pancreatic cancers [1,4]. The stroma is often radically changed around malignant tumors and such changes both predict prognosis and may actually contribute to disease progression [1,4]. We have previously examined the role of several molecules found to be aberrantly expressed in cancer associated stroma that induce tumorigenesis and malignant conversion [8]. We reported that CD1-overexpressing BPH-1 cells are non-tumorigenic in the presence of rUGM in tissue recombination experiments, but in contrast, the overexpression of CD1 in prostate fibroblasts induces a strong tumorigenic response in the non-malignant but genetically initiated BPH-1 cells [9]. The tumor-promoting abilities of NPF^{CD1} produce changes very similar to published descriptions of tissue recombinations of CAF with BPH-1 [4]. A comparison of the genetic profiles from CAF and NPF^{CD1} identified CathD as being upregulated sevenfold in comparison to NPF [9]. From this finding we hypothesized that CathD may be a mediator of stromal-epithelial interactions contributing to prostate tumorigenesis.

CathD overexpression in neoplastic cells and neoplasia-associated connective tissue was described as long as 24 years ago, and is reported to play several roles in cancer progression [34–37]. Cathepsins have recently been shown to be upregulated in a pancreatic tumor model and also contribute to invasive breast tumor growth [38–40]. We previously reported that CathD is upregulated in both NPF^{CD1} cells (which mimic CAF) as well in CAF. Microarray analysis

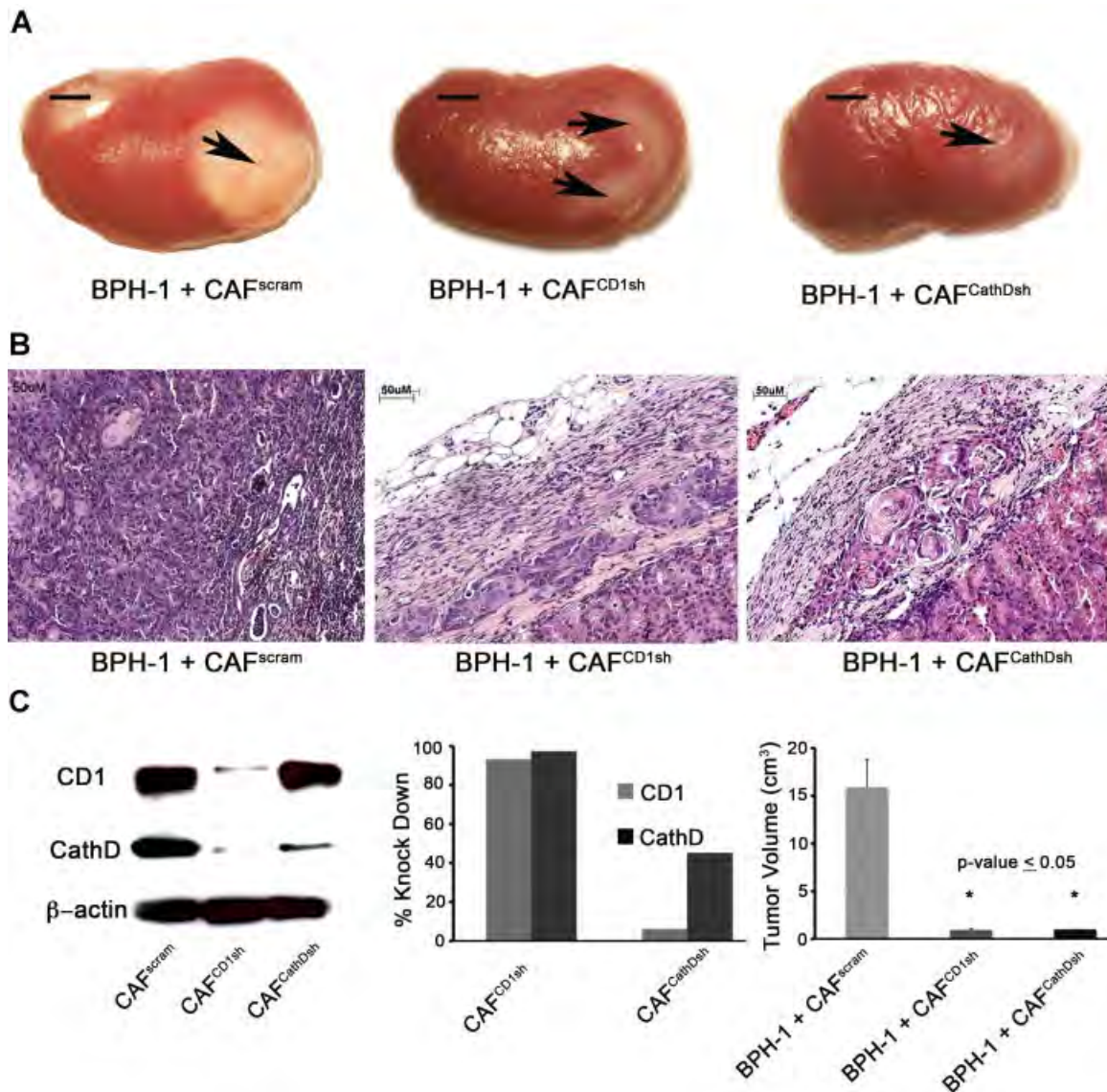


Fig. 4. Cyclin D1 and Cathepsin D are required for CAF induced tumorigenicity in vivo. **A:** Gross morphology of 2 month grafts of BPH-I + CAF^{PSR}, BPH-I + CAF^{CD1sh}, and BPH-I + CAF^{CathDsh}, scale bar equal to 5 cm. **B:** H&E staining of BPH-I + CAF^{PSR}, CAF^{CD1sh}, or CAF^{CathDsh}. BPH-I + CAF^{PSR} recombinants formed adenosquamous carcinoma as previously described. Scale bar equal to 50 μ m. **C:** Western blot confirming knockdown of CD1 and CathD in CAFs (left). Knockdown efficiency determined by performing densitometric analysis of Western blot represented by bar graph (middle). Quantitation of tumor volume of 2 month grafts of BPH-I + CAF^{PSR}, BPH-I + CAF^{CD1sh}, and BPH-I + CAF^{CathDsh} (right), graphical representation of the mean $\pm$ SD of the grafts is shown in $n = 6$. Significance determined by ANOVA, P -value ≤ 0.05 .

revealed a sevenfold increase in CathD resulting from CD1 expression in NPF. NPF^{CD1} cells display increased motility in comparison to control NPFs in a wound healing assay which was shown to depend upon the expression of CathD [9]. Here we show that the ability of NPF^{CD1} to survive and invade into 3D matrices was also dependent on CathD. The overexpression of CD1 in NPF produced invasive colonies with protrusions sprouting into the surrounding matrix. This invasive growth was inhibited in NPF^{CD1}

cells when CathD expression was knocked down. This finding is supported by similar results from Laurent-Matha [22] where CathD was critical for outgrowth of human fibroblast in 3D matrices. Our findings were consistent with a model in which CD1-induced overexpression of CathD resulted in increased fibroblast motility and invasion.

An immunohistochemical examination of clinical specimens revealed low levels of expression of CathD in normal prostate stromal tissue. Malignant areas

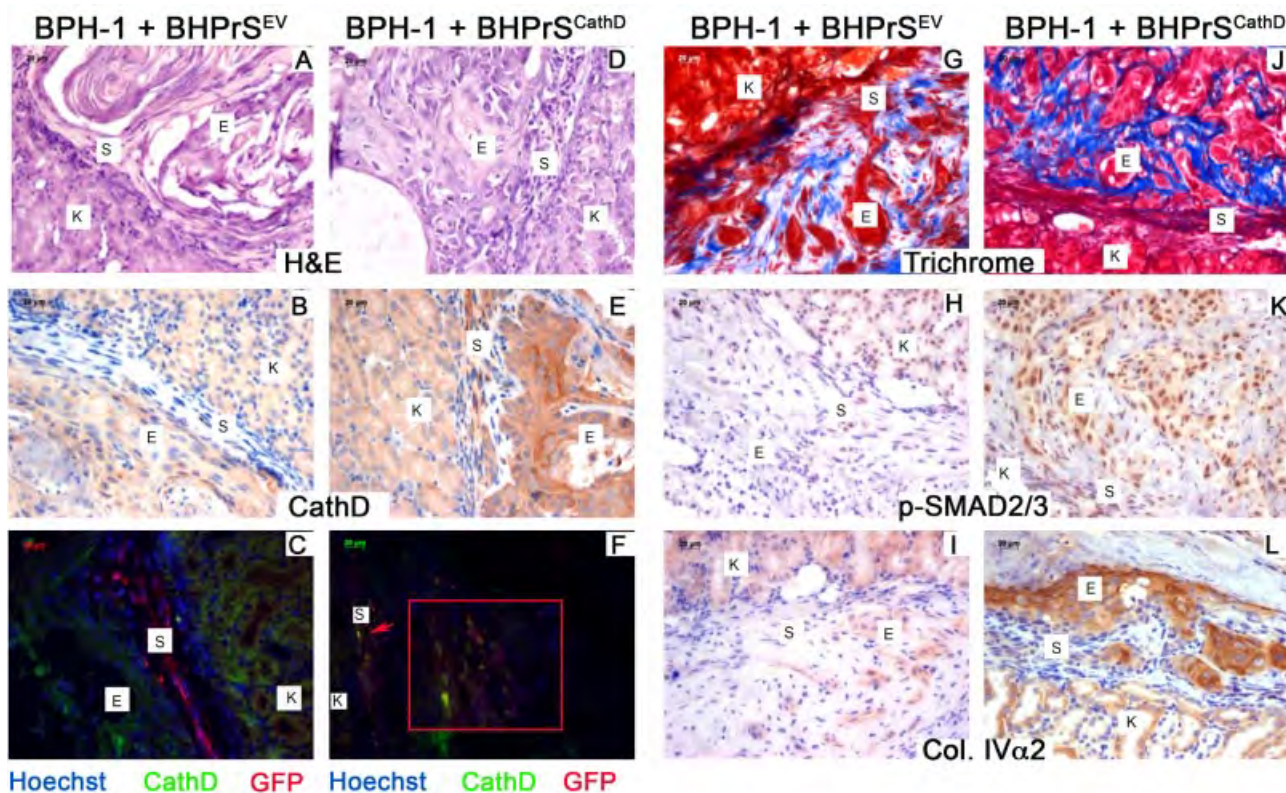


Fig. 5. Overexpression of Cathepsin D induces a malignant transformation through activation of TGF β signaling. **A:** Characterization of CathD overexpressing grafts. H&E staining (top) of BPH-1 + BHPPrS^{EV} and BPH-1 + BHPPrS^{CathD}. Recombinations of BPH-1 + BHPPrS^{CathD} produced malignant transformations. IHC for CathD (middle) strong expression visible in the stroma of recombinations of BPH-1 + BHPPrS^{CathD}. Immunofluorescence for CathD (green) and GFP (red) show co-localization for GFP and CathD (yellow) in recombinations of BPH-1 + BHPPrS^{CathD}. **B:** Masson's trichrome staining (top) of recombinations of BPH-1 + BHPPrS^{EV} and BPH-1 + BHPPrS^{CathD}. IHC for p-SMAD2/3 (middle) and Collagen IV α 2 (lower) in recombinations of BPH-1 + BHPPrS^{EV} and BPH-1 + BHPPrS^{CathD}. Scale bar equal to 20 μ m.

showed prominent stromal expression of CathD, with the significantly greater stromal CathD expression in high grade tumor samples. IHC analysis of CathD expression in tissue recombinations of BPH-1 + CAF and NPF^{CD1} also revealed strong stromal staining in comparison to recombinations of BPH-1 + NPF and BPH-1 + rUGM. CathD expression in human prostate cancer stroma correlates with shorter survival and recurrence-free periods [41]. Our experimental data establishing a link between the overexpression of CD1 with the up-regulation of CathD in prostate CAF, coupled with the similar findings in human disease, indicates a strong association between cell cycle regulation and protease expression in prostate tumorigenesis. The cell cycle regulator CD1 and the estrogen receptor alpha (ER α) are known to interact and can induce estrogenic gene transcription [42]. This suggests the possibility that the overexpression of CathD in PCa associated stroma is due to the interaction of ER α with CD1.

Further investigation into the role of CathD in the tumor microenvironment showed that CathD expression is necessary for NPF^{CD1} cells to promote epithelial growth under in vitro conditions. The pro-mitogenic effect of NPF^{CD1} conditioned medium on BPH-1 cells was inhibited when CathD expression was knocked-down in NPF^{CD1}. This result mirrors published findings showing that CathD is mitogenic to PCa cell lines [25]. These data do not, of course, imply that CathD is a direct mitogen, merely that its presence results in a mitogenic environment. Given the possibility that this protease may activate latent growth factors associated with extracellular proteoglycans, an indirect mechanism is not only possible but likely.

To further pursue an underlying mechanism, we engineered BHPPrS cells to overexpress CathD and combined these BHPPrS^{CathD} cells with BPH-1 and performed renal grafting experiments. Recombinations of BPH-1 cells with BHPPrS resulted in benign solid

epithelial cords similar to recombinations of NPF with BPH-1 cells [10]. However, the overexpression of CathD in BHP1S in recombination experiments with BPH-1 cells induced a malignant transformation with invasion into the mouse kidney. This is consistent with our findings with the CathD knockdown approach in experiments with the CAFs. A feature of the prostate tumor microenvironment in human disease is the expansion of myofibroblast-like cells with increased deposition of extracellular matrix proteins [18]. Masson's trichrome staining of tissue xenografts from the CathD overexpressing stromal cells revealed increased production of collagen in comparison to recombinations with the EV control stromal cells. These staining patterns were similar to previous publications that pointed towards altered TGF- β signaling. We have previously shown that the overexpression of TGF- β in BHP1S cells resulted in the development of poorly differentiated adenocarcinoma with increased deposition of collagen in tissue recombination experiments [10]. TGF- β is expressed by most cultured cells in an inactive form due to binding with latent complex, and activation requires the proteolytic degradation of this complex. CathD derived from fibroblast conditioned media has been shown to liberate active TGF- β from the latent complex [43]. Investigation of altered TGF- β signaling in our model revealed increased p-SMAD2/3 staining, a surrogate marker for TGF- β response, in the CathD overexpressing recombinations. Examination of Col.IV α 2, a direct TGF- β responsive gene, expression in the tissue xenografts revealed increased staining for Col.IV α 2. Collectively, the differences in stromal composition observed from trichrome staining can be linked to increased TGF- β signaling and responsive gene expression as a result of stromal derived CathD. The overexpression of CathD in the stroma resulted in a somewhat minor, all though sufficient, malignant transformation of initiated epithelial cells similar to the tumor inductive properties of CAF.

We previously demonstrated that NPF^{CD1} cells and CAF elicited permanent malignant transformation of BPH-1 cells [9,30,44]. Data from IHC of clinical tissue showed increased CathD in the stroma adjacent to malignant regions of the prostate. To address the contribution of CathD in CAF-induced tumorigenesis of BPH-1, we engineered CathD knock-down CAF. The ability of CAF to induce tumorigenesis in BPH-1 recombinations was abolished when CathD expression was knocked-down. Similar results were observed when CD1 expression was knocked down in CAF. These data indicated that CathD is not only an important mediator of stroma-epithelial cross talk in vitro, but also an essential component in promotion of tumorigenesis in vivo, at least in this model.

In summary, the study presented here demonstrates that CathD can play a role as a paracrine mediator contributing to prostate tumorigenesis. The identification of key players, such as CathD, that participate in the promotion of the tumor microenvironment contributes to our understanding of the molecular mechanisms underlying this process and may prove to be valuable for the development of novel anti-cancer therapies. Current anti-cancer therapies target the malignant epithelial cells, which progressively acquire genetic alterations during the progression of the disease [45–47]. The biggest obstacle facing clinicians treating people with cancer in general is the toxicity of treatments combined with the development of resistance to therapy. The tumor microenvironment has been shown to be more genetically stable and therefore less likely to develop resistance to novel anti-cancer therapeutics [48,49]. Since tumor promotion by the microenvironment is a function of many different signaling molecules, it should be possible to develop therapeutic strategies which appropriately modify several pathways simultaneously rather than simply attempting to totally block a single signal. This is likely to be both more effective and better tolerated, since the normal biological effects of the molecules concerned will be less affected. Further investigation is needed to explain in detail how CathD is acting. A better understanding of the complexities of CathD in the tumor microenvironment may provide targets for suppressing lethal PCA phenotypes.

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