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Functional Analysis of Human Beta Defensin-1: Novel Insight into Its Role in Prostate Cancer

Rebecca S. Bullard
Medical University of South Carolina

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novel insight into its role in prostate cancer

Rebecca S. Bullard

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Approved by:

Chairman, Advisory Committee

To my Abba.

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AMP	antimicrobial peptide
AO	acridine orange
AR	androgen recptor
bp	base pair
BPH	benign hyperplasia
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
Da	dalton
DEFB1	human beta defensin-1 gene
DIC	differential interference contrast
DMEM	dulbecco modified eagles medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRE	digital rectal exam
DTT	1,4-dithio-DL-threitol
ECL Plus	enhanced chemiluminescence plus
EtBr	ethidium bromide
F-12	ham's F-12 medium
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
H_2O_2	hydrogen peroxide
hPREC	human prostate epithelial cells

HRP	horseradish peroxidase
hBD-1	human beta defensin-1 peptide
iDC	immature dendritic cell
ICC	immunocytochemistry
kDa	kilodalton
LCM	laser capture microscopy
LOH	loss of heterozygosity
LSM	laser scanning microscopy
$MgSO_4$	magnesium sulfate
mRNA	messenger ribonucleic acid
MSR1	macrophage scavenger receptor 1
MTT	metabolic 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
NGM	normal growth media
O/N	over night
PBS	phosphate buffered saline
PI	propidium iodide
Pon A	ponasterone A
PSA	prostate specific antigen
QRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	RPMI-1640 medium

RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
tBID	truncated BID
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween 20
TSG	tumor suppressor gene

ABSTRACT

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States. The molecular pathogenesis of this disease includes genetic alterations, infection, and exposure to inflammatory or dietary oxidants. Recently, research has focused on the role of host defense peptides in tumor immunity. Defensins, a highly conserved multigene family of proteins that possess antimicrobial and antiviral properties, play an essential role in innate and adaptive immunity. These peptides are categorized into alpha- and beta-defensins based upon the spacing and connection between the six conserved cysteine residues of the mature peptide. Human Beta Defensin-1 (hBD-1), an important component of the innate immune system, is frequently lost in malignant prostate tissue, while expression is maintained in adjacent benign regions. Several studies indicate there may be multiple tumor suppressor genes present within the 8p22-23 region in prostate carcinoma, an area where frequent genetic alterations occur. The high incidence of loss of hBD-1 expression in prostate cancer, along with its chromosomal location of 8p23.2, raised the possibility that it may play a role in tumor suppression. Our studies demonstrated that induction of hBD-1 expression results in significant changes in cell viability, membrane permeability, and the activation of caspase-mediated apoptosis in DU145 and PC3 human prostate cancer cell lines. However, no effect was observed following the induction of hBD-1 in LNCaP. To gain insight into the caspase-mediated cell death, we analyzed the activation of specific caspases within the intrinsic and extrinsic pathway following the induction of hBD-1 expression. In

DU145 and PC3 prostate cancer cell lines the activation of both apoptotic pathways was observed, specifically the activation of caspases 3, 8 and 9. However, no caspase activation was observed in the LNCaP prostate cancer cell line. Although our studies suggest that within our expression system and employed methodologies, hBD-1 does not exhibit a 'bystander effect' on neighboring cells, the results indicate a possible upward trend suggesting that hBD-1 may exhibit a 'bystander effect' under different conditions than those used in this study. Collectively, this data indicates the loss of hBD-1 in the area surrounding prostate cancer cells may create an environment that promotes the progression of prostate cancer.

CHAPTER 1

INTRODUCTION

Prostate cancer continues to be a major burden on public health with high incidence of diagnosis and cancer-related deaths as compared to other forms of cancer. In South Carolina, it is the most commonly diagnosed cancer among males regardless of race[1]. However, the incidence rate is almost two times higher in black males as white males[1]. In addition, prostate cancer is the second leading cause of cancer-related deaths among South Carolina men with mortality rates due to prostate cancer being three times higher in black males[1]. The current methods of early detection include the prostate specific antigen test (PSA), the digital rectal exam (DRE), and the diagnostic tissue biopsy. Although together these methods have helped diagnose prostate cancer at earlier stages in the general population over the years[2, 3], they have marked deficiencies. Consequently, a better understanding of the molecular pathogenesis of prostate cancer may lead to the identification of pre-malignant molecular biomarkers that accurately predict neoplastic transformation within the prostate.

The pathobiology of prostate cancer is multifaceted due to the altered expression of numerous genes[4], as well as environmental factors such as inflammation and diet[5] allowing for a multitude of potential biomarkers. A therapeutic target and potential biomarker of prostate cancer we are exploring is human Beta Defensin-1 (hBD-1). We previously demonstrated the cancer-specific loss of innate immune peptide hBD-1, with a chromosomal location of 8p23.2, in prostate cancer[6]. Several cytogenetic studies have demonstrated deletions on the short arm of chromosome 8 in a variety of cancers, including carcinomas of the bladder[7], liver[8], ovary[9], and prostate[10-16]. Since the location of

tumor suppressor genes (TSGs) is suggested by the homozygous deletion of chromosomal regions[17], it has been proposed that the 8pter-8p23 region contains at least three separate tumor suppressor genes[18-20]. The high incidence of loss of hBD-1 expression in prostate cancer, along with its chromosomal location, raised the possibility that it may be a tumor suppressor gene.

In addition to the loss of TSGs, prostate tumor progression has also been associated with an increase in immune suppression in patients[21]. In addition, it has been suggested that a tumor is capable of escaping destruction by the immune system via disruptions in the activation and suppression of immunity during tumor growth[22]. Even though little is known about specific components of the immune system involved in tumor suppression, the loss of hBD-1 expression suggested that it may be a component of a tumor surveillance system that functions specifically through tumor immunity. Therefore, a better understanding of the expression pattern and role of hBD-1 in prostate cancer will give a more complete picture of precisely how this antimicrobial peptide is involved in the progression of prostate cancer.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE:

A REVIEW OF THE LITERATURE

The Prostate Gland

The prostate gland is a walnut-sized organ that functions as part of the male reproductive system. It is located in the pelvic area in front of the rectum and underneath the bladder (Figure 2-1). In addition, the prostate encompasses a portion of the urethra coming from the bladder known as the prostatic urethra that merges with two ejaculatory ducts.

Heterogenous in nature, the prostate is divided into glandular and non-glandular tissues. The glandular components include the ducts and glands, while the non-gladular components consist of fibromuscular tissue. Pathologically the prostate is classified by four zones: the peripheral zone, the central zone, the transition zone, and the anterior zone[23]. The peripheral zone is the closest to the rectum and accounts for the largest portion of prostatic volume. This zone is where the largest percentage of prostate cancers originate[23]. Comprising the second largest portion of the prostate volume, the central zone surrounds the ejaculatory ducts. The smallest and innermost glandular component of the prostate, the transition zone, surrounds the prostatic urethra and is rarely associated with cancer. The anterior zone, closest to the abdomen and composed mainly of fibromuscular tissue, consists of the non-glandular components of the prostate.

In younger males the prostate is generally healthy, however, as a man ages changes begin to occur within the gland (Figure 2-2). These changes mainly present in a clinical setting as one of three main prostate

Figure 2-1: Illustration of the location of the prostate gland. The prostate is a part of the male reproductive system that encompasses a portion of the prostatic urethra. (adapted from www.cancer.gov)

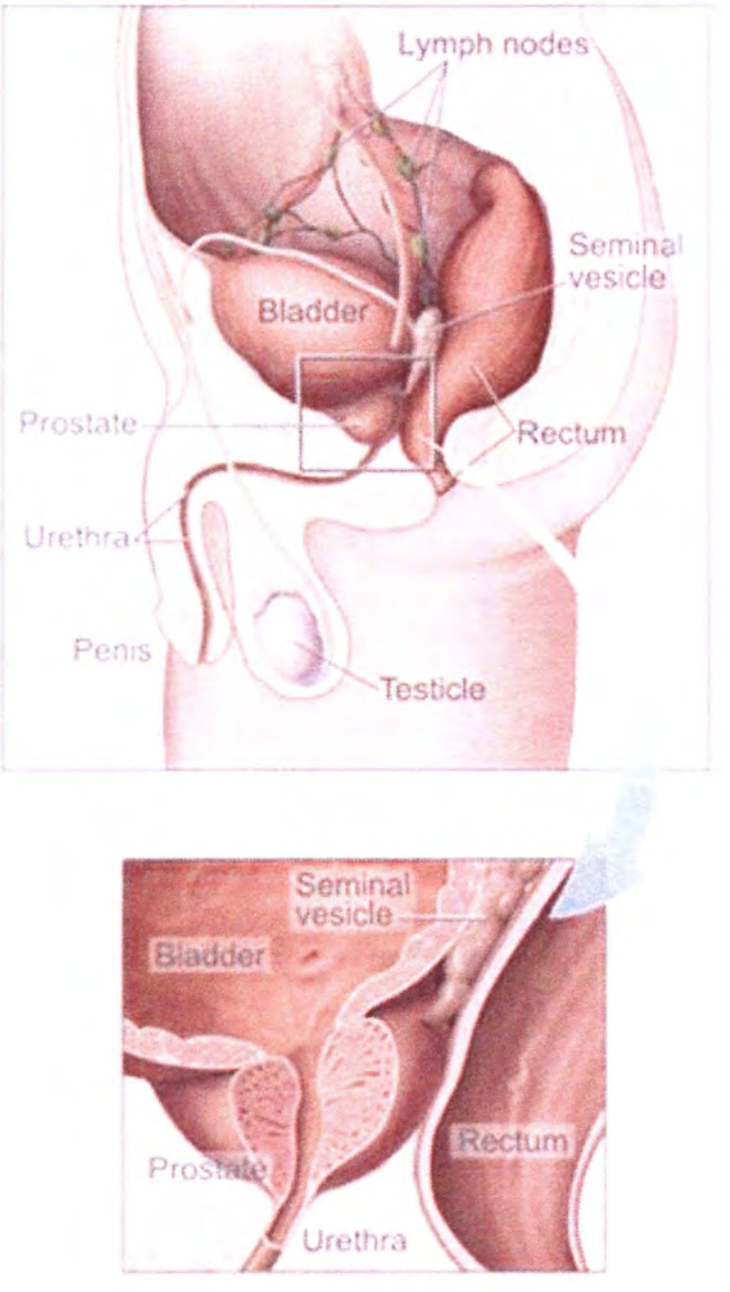


Figure 2-1: Illustration of the location of the prostate gland. (adapted from www.cancer.gov)

Figure 2-2: Schematic of changes from normal prostate to prostate cancer. A histologic account for the changes from a healthy prostate typical of a young male that lead to prostate cancer. (adapted from Nelson et al 2003[26])

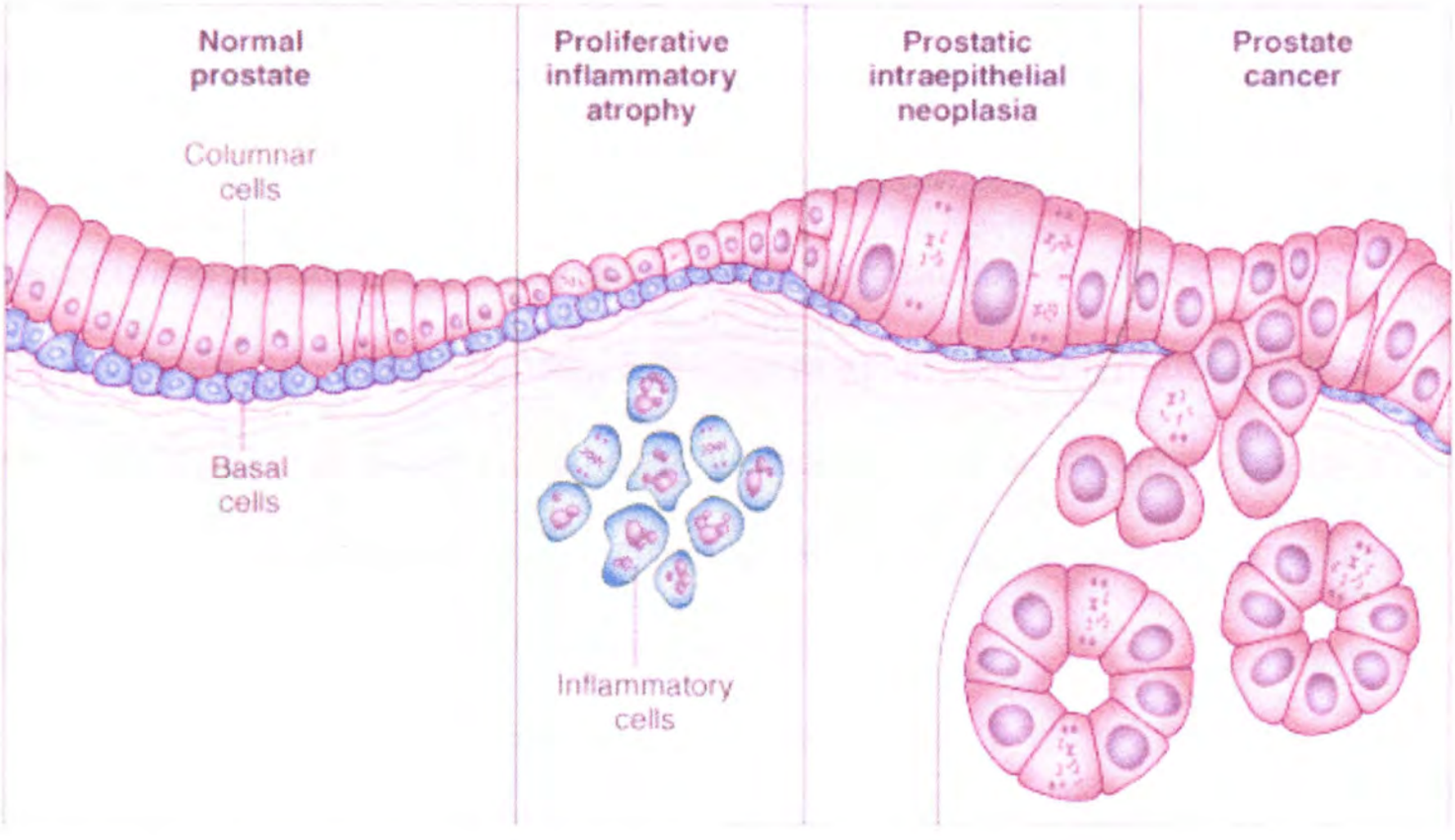


Figure 2-2: Schematic of changes from normal prostate to prostate cancer. (adapted from Nelson et al 2003[26])

disorders: prostatitis, hyperplasia or carcinoma. The first of these changes is prostatitis, or inflammation of the prostate tissue, which manifests as difficult or painful urination. It is classified as acute or chronic based upon a combination of clinical features and microscopic examination. Although the cause is often unknown, it is sometimes a result of a bacterial organism associated with other urinary tract infections[21].

Another change that can occur with age in the prostate is enlargement of cells within the transition zone, also know as benign prostatic hyperplasia (BPH)[24]. As the prostate enlarges, it begins to squeeze the urethra which may cause urinary problems by slowing or obstructing the flow of urine from the bladder to the penis. Additionally, BPH can result in retention of urine in the bladder due to the inability to completely empty it. Neither prostatitis nor BPH are known to cause cancer, however, men with one or both of these conditions may develop prostate cancer as well.

Prostate Cancer

Prostate cancer remains the most common malignancy, excluding skin cancer, and the second leading cause of cancer related deaths in men in the United States[25]. Risk factors for prostate cancer that have long been recognized include age, race, androgens and a variety of lifestyle factors, such as diet. In addition, there is a strong hereditary component in the risk of prostate cancer development. Steinberg *et al.* (1990) reported that the number of affected first-

degree relatives significantly increased the risk, whereas there was only a marginal increase in risk if distant relatives were affected[27].

More recently, molecular genetic studies of familial prostate cancer led to the identification of numerous genes and genetic alterations that has greatly enhanced our understanding of prostate cancer development and progression[4]. The most thoroughly investigated of these is hereditary prostate cancer (HPC1 gene)[28]. In addition, it has been proposed that RNASEL, MSR1, AR, CYP17, SRD5A2, and a variety of other genes are potential contributors to the risk of prostate cancer development[29-36]. Although the exact mechanism underlying prostate tumorigenesis is unknown, it is known that the clinical outcome of a prostate cancer patient is closely related to tumor grade and stage with poorly differentiated tumors having a poor prognosis[37].

Molecular Pathogenesis of Prostate Cancer

The molecular pathogenesis of prostate cancer involves a progression through a series of pre-malignant changes that ultimately lead to uncontrolled cellular division resulting in malignancy (Figure 2-3). There is an abundance of evidence suggesting a key component of this progression includes the transformation of normal epithelium to prostate inflammatory atrophy (PIA), a focal area of proliferating cells accompanied by inflammation[38]. PIA lesions occur in the peripheral zone of the prostate where most prostate carcinomas originate[38, 39] and are often located adjacent to areas of prostate intraepithelial neoplasms (PIN), a known precursor to prostate cancer[40].

Figure 2-3: Pathogenesis of prostate cancer. The effects of age and the accumulation of numerous genetic and environmental insults that contribute to the molecular pathogenesis of prostate cancer. (adapted from Nelson et al 2004[40])

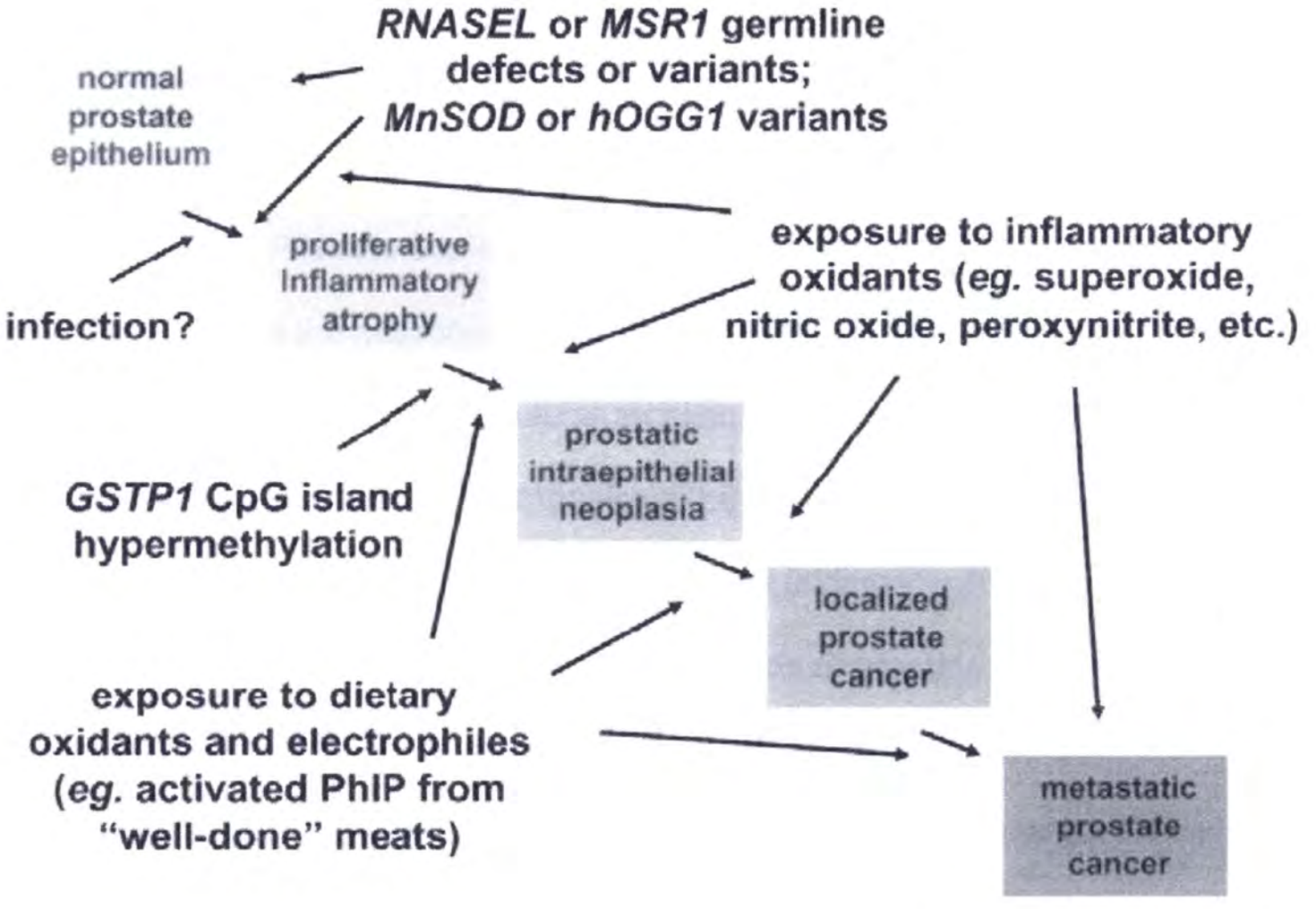


Figure 2-3: Pathogenesis of prostate cancer.

(adapted from Nelson et al 2004[40])

Several studies have identified additional changes in prostate tissue as it undergoes a transition from PIA to PIN including p53 mutations[41] and π-class glutathione S transferase (GSTP) hypermethylations[38, 42]. GSTs, long recognized to protect against cancer, are a family of enzymes that prevent DNA damage by serving as a catalyst for the scavenger glutathione to detoxify environmental carcinogens and oxidants[43, 44]. The loss of GSTP1 expression due to hypermethylation and the inflammatory state of PIA lesions have been associated with high grade PIN[26, 40, 45, 46].

The term PIN refers to established morphological criteria that simply defined describes the presence of individual, atypical cells resulting in the enhancement of the normal glandular architecture[4]. Multiple studies indicate that areas within the prostate that demonstrate the histological changes of PIN are designated as low or high grade based upon the findings[47]. It is important to note that current models of the molecular pathogenesis of prostate cancer recognize PIN as a precursor to the development of prostate cancer[48].

As in other forms of cancer, prostate neoplasms have been extensively studied for genetic alterations and candidate susceptibility genes[49]. Loss of heterozygosity (LOH) describes the loss of one allele in the tumor cells of a heterozygous individual. LOH studies in various types of cancer have identified the loss of putative tumor suppressor genes[5, 6]. Although the underlying mechanism of LOH is not completely understood, several proposed mechanisms include the loss of whole chromosomes, partial chromosome deletion, disproportionate translocation and mitotic recombination[36, 40, 50-53]. Studies have

shown that chromosome 8 possesses the most frequent cytogenetic changes observed in prostate cancer patients[54, 55].

To date, at least three host immune response genes are emerging as prostate cancer susceptibility genes: RNASEL, MSR1, and hBD-1[35, 36]. RNASEL, a ribonuclease activated in the presence of viral infections, functions to degrade cellular and viral RNA. Studies have identified three mutant alleles of RNASEL that result in a defective protein and have been associated with increased prostate cancer risk[50-52].

Another immune response gene that has emerged with a strong association to prostate cancer development is MSR1[56, 57]. Located on 8p22, the MSR1 gene encodes a macrophage scavenger protein that can bind various ligands, including bacterial lipopolysaccharides[26]. It appears the expression of MSR1 is limited to macrophages at sites of inflammation within the prostate.

Lastly, the cancer specific loss of hBD-1 has been shown in prostate and renal carcinomas[58, 59]. In addition, several studies have demonstrated that the over-expression of this peptide results in cell death in prostate, bladder, and renal cancer cell lines[60-62]. Although the exact mechanisms of these genes involvement in prostate cancer pathogenesis are unknown, the data suggest it involves host defense against inflammatory damage. Moreover, the genetic changes observed in and molecular pathogenesis of prostate cancer provide ample evidence for the role prostate inflammation may play in the development of prostate cancer.

Prostate Cancer Detection

Since the mid-1990's, the measurement of prostate specific antigen (PSA) levels has been one of the main screening methods for prostate cancer. PSA is a serine protease normally present in the acidic fluid secreted in the semen. On average, a PSA level is normal if it falls below 4ng/ml and high if it is above 10ng/ml. Elevated serum PSA levels or the palpitation of a tumor growth during a digital rectal exam (DRE) are considered to be prognostic indicators of prostate carcinoma.

Although these methods have served to increase the detection of prostate cancer, they have recognized limitations. The DRE, a quick, but rather invasive physical exam, allows for an area of detection limited to digital palpitation, thus, leaves room to miss a tumor. While the PSA screening serves to detect potential prostate cancer missed by the DRE, it has inherent problems with sensitivity and standardization. For example, an increase in PSA levels can be a result of other abnormalities of the prostate such as prostatitis or BPH[63]. Therefore, it is necessary to perform a transrectal biopsy to confirm a clinical diagnosis. Since none of the methods to date have proven to definitively diagnose prostate cancer alone, the emergence of new molecular targets may prove to identify early alterations in prostate cells accurately and efficiently with minimal invasiveness.

Prostate Cancer Grading System

Several histopathological grading systems for prostate cancer have been designed. However, the Gleason grading system, named after D.F. Gleason,

was recommended by a WHO consensus conference in 1993 and is currently the principal grading system used[64]. The Gleason grading system defines the histological patterns with decreasing differentiation in glandular architecture of the tissue[65, 66]. To obtain a Gleason score, the primary and secondary, i.e. most prevalent and second most prevalent, patterns are added[66]. Gleason pattern 1 tumors are very rare and composed of a well confined nodule of separate, tightly-associated glands that do not infiltrate into adjacent benign tissue[67]. Gleason pattern 2 describes rounded glands with smooth ends that are loosely arranged and lack the uniform size[67]. The most common is Gleason pattern 3, in which there is more glandular infiltration of adjacent benign tissue, as well as a more marked variation of distance between them[67]. In Gleason pattern 4, the glands are fused and no longer have stroma separating them, while Gleason pattern 5 describes almost a complete loss of glandular lumina[67].

The Defensin Family

Antimicrobial peptides (AMPs) are found in an extensive array of eukaryotic organisms[68] and are classified into categories based upon their structural characteristics[69]. One important category of AMPs in humans is the defensin family[70-72]. Defensins, a family of 3-4 kDa cationic peptides, are cysteine rich amphipathic peptides involved in innate immunity[73]. They can be classified into α-, β- and θ-defensins based on the pattern of six conserved cysteine residues and the precursor gene structure[74]. α-defensins are normally abundant in the microbicidal granules of polymorphonuclear leukocytes and have

been identified in the Paneth cells of the human intestine[75]. β-defensins have been identified in bovine tracheal epithelium[76] and the epithelial surfaces of respiratory, genitourinary, and gastrointestinal tracts[77, 78]. Although α-defensins exhibit broad spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses[79], β-defensins are mainly active against Gram-negative bacteria and yeast[59, 60, 80].

To be classified as a β-defensin, the peptide must consist of 36 or more amino acids with a primary structure that consists of the following identifiable disulfide bond sequence 1-5, 2-4, and 3-6[73, 81-83]. Other residues that are conserved among the β-defensins include (using hBD-1 numbering): Gly10, a cationic residue at position 13, Glu21, and hydrophobic residues at positions 24 and 36[81]. The carboxy terminal end of the four most studied hBDs, hBD-1 to hBD-4, contains a cluster of cationic residues (Lys and Arg). The aggregation of positive charges proves to be important for antimicrobial activity[73].

To date, six hBDs (hBD1-6) have been identified that are either constitutively secreted, or inducibly expressed for secretion, at the epithelial cell surface[84, 85]. The first human β-defensin, hBD-1, was purified from the plasma of patients with renal disease in 1995[86, 87]. Since then, several other β-defensins have been isolated or identified through genetic analysis. hBD-2 was purified from the lesions of psoriatic skin[88, 89], while hBD-3 was isolated from psoriatic scales[90, 91]. The remaining three β-defensins identified to date, hBDs 4-6, were originally reported based upon genome analysis[62, 75]. Searches of the human genome have recently revealed about 40 potential coding regions for β-defensins

(*DEFBs*)[91] suggesting the possibility that numerous hBDs have yet to be isolated and identified.

Currently there are two postulated mechanisms of how host defense peptides exhibit the antibacterial properties they posses (Figure 2-4)[92]: the barrel stave model and the carpet model. The models are based upon the selectivity of the peptide for its binding surface.

(1) Barrel stave model: In this model, multimeric pores form as a result of the hydrophobic bond between the peptide and the bacterial cell membrane in a non-selective manner[91]. More specifically, the hydrophobic surface of lytic peptide monomers interacts with the lipid core of the bacterial membrane causing the hydrophilic surface to point inward forming an aqueous pore. Recruitment of additional monomeric peptides to the site leads to an increase in the pore size eventually resulting in membrane depolarization ad cell death[93].

(2) Carpet model: This model describes the cell selective electrostatic interaction of the AMPs to the surface of the outer membrane. The peptides cover the membrane surface like a carpet until reaching a threshold concentration which enables the peptides to permeate the membrane. Permeation results in an intermediate formation of transient pores, known as 'toroidal' pores, that span the distance of the membrane[95, 96]. Cell death follows

Figure 2-4: Illustrations of the two mechanisms of membrane lysis by host defense peptides. Peptides that are not cell-selective bind to all types of membranes and form transmembrane pores via the 'barrel-stave' mechanism resulting in membrane depolarization followed by cell death. Peptides that are cancer cell-selective bind in the first step mainly by electrostatic interactions and align parallel to the outer membrane surface (step 1) and cover it in 'carpet-like' manner. After a threshold concentration of peptides has been reached (step 2), the peptides permeate the membrane, which, in most cases, is followed by membrane disintegration and micellization. An intermediate step is the formation of transient pores. These pores were described as 'toroidal' pores for peptides that are long enough to span the membrane. These pores might also lead directly to cell death. The figure also shows B16 melanoma cells treated with a lytic peptide labeled with rhodamine before (picture on the left showing the intact cell) and after (pictures on the right showing the remaining nucleus) a threshold concentration has been reached. (adapted from Papo and Shai 2005[94])

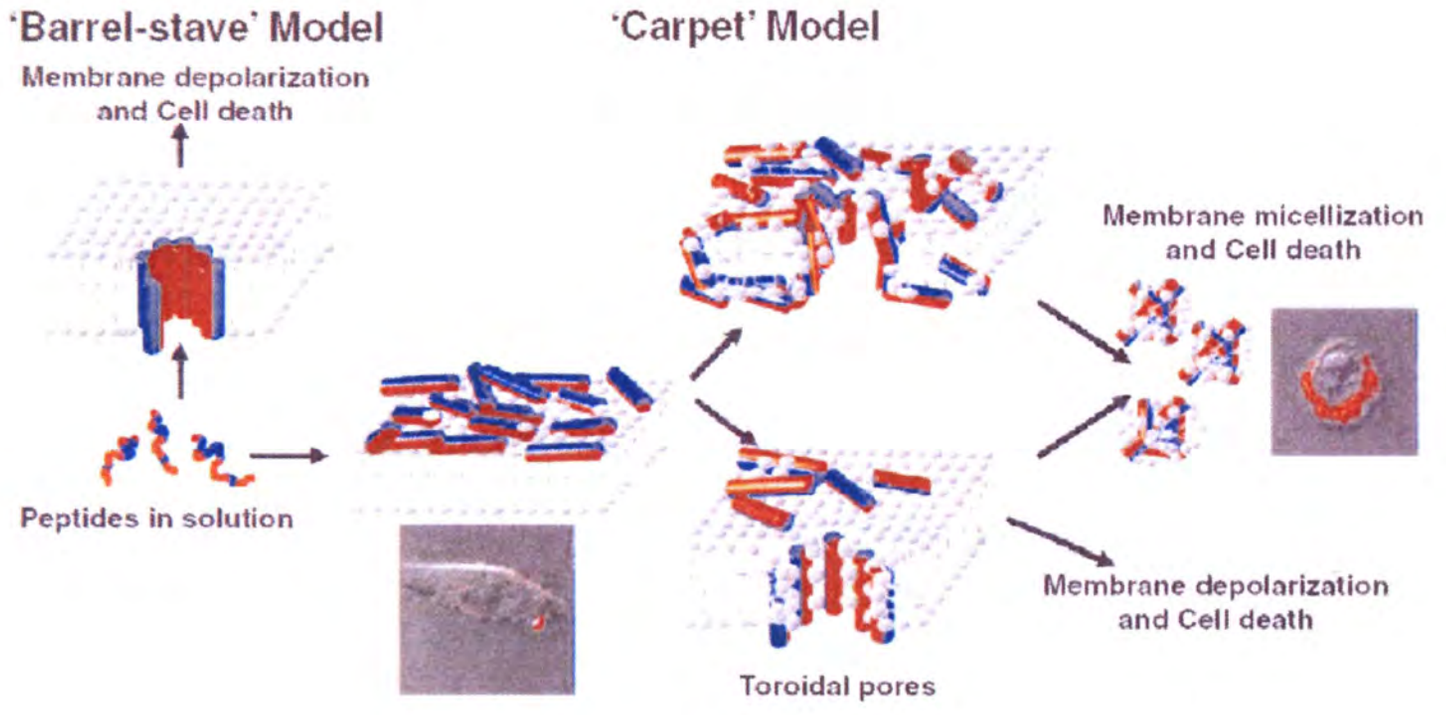


Figure 2-4: Illustrations of the two mechanisms of membrane lysis by host defense peptides. (adapted from Papo and Shai 2005[94])

pore formation due to the disintegration of the membrane and micellization[97, 98].

The proposed mechanism of β-defensin action against invading pathogens follows the carpet model with electrostatic interactions occurring between the basic defense peptides and the acidic phospholipids on the bacterial membrane[99]. Although the basic carpet model is accepted, several views exist that expound upon the mechanistic details of the process.

As previously mentioned, an AMP selectively targets based upon the composition of the cell membrane. Bacterial cell membranes consist of phospholipids with negatively charged outer leaflets, while the outer leaflet of eukaryotic cell membranes contains phospholipids consisting of mainly neutral choline[91]. It has been shown that defensins selectively target tumor cells compared to non-transformed cells[100]. There are several possible explanations for the selective targeting of tumor cells. Compared to a normal eukaryotic cell, a transformed cell has a slightly negative charge on the outer membrane due to the presence of phosphatidylserine and O-glycosylated mucine[101]. In addition, a tumor cell exhibits a partial loss of its lipid asymmetry, thus, contributing to the slight negative charge it develops[102, 103]. The transition from neutral to negatively charged allows the tumor cell to be more susceptible to the lytic effects of the peptides without harmful effects to surrounding normal cells. Another explanation for the difference in susceptibility between tumor and normal cells in response to lytic peptides may be the presence of more microvilli which provides an increased surface area on tumor cells that allows for the binding of a greater

number of peptides[94, 104, 105]. A final explanation for the difference may be that tumor cells have a higher negative potential than non-transformed cells[85].

Numerous reports indicate the importance of the antimicrobial action that defensins exhibit as part of the immune response. A demonstration of their significance centers on a correlation between defensin expression and the incidence of infection in humans[106, 107]. It was recently reported that a decrease in β-defensin expression in atopic dermatitis contributed to the presence of skin infections[106, 108]. Similarly, other reports indicate a reduction in defensin expression contributes to the chronic bacterial infections experienced by cystic fibrosis patients[6, 96, 109, 110].

Although the main function of defensins is to serve as the initial response to a pathogen as a critical part of innate defense[111], they exhibit many other properties[112](Figure 2-5). Studies have been conducted to investigate defensin mediated immune activation, IL-8 production, and chemotaxis of iDC and T-cells[113]. It has also been shown that there are inter-individual variations in expression of β-defensins modulated in response to infection and inflammation[114].

Figure 2-5: Diagram of the various biological functions of β-defensins.

Peptides of the β-defensin family mediate and participate in a variety of biological functions. These processes are both microbicidal and non-microbicidal in nature. (adapted from Klüver, et al 2006[106])

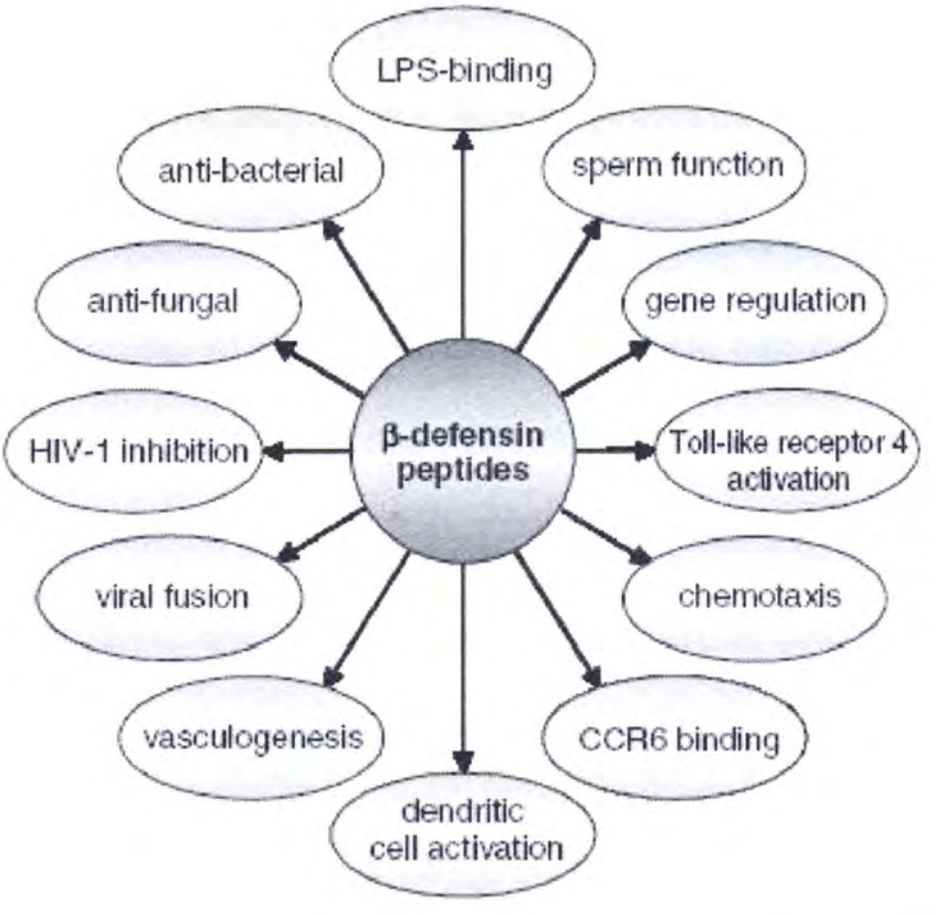


Figure 2-5: Diagram of the various biological functions of β-defensins. (adapted from Klüver et al. 2006[106])

The field of tumor immunity is gaining more attention lately as the convergence of tumorigenesis and immunity has created an exciting and promising area of research. It has been shown that in oral squamous cell carcinoma, lung cancer, basal cell carcinoma, renal and prostate carcinomas β-defensins exhibit differential gene expression profiles[115, 116]. The manipulation of the immune system to destroy tumor cells was initially described by Morales *et al.* in 1976 with the anti-tumor effect of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) in bladder cancer[116]. Recently, Ludwig *et al* published data supporting the antitumoral activity of BCG therapy is due to the response of cytokines to BCG, ultimately leading to the upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[77, 117]. TRAIL has been shown to induce apoptosis in multiple cancer cells without exhibiting cytotoxicity to normal cells[22].

In addition, growing evidence supports anti-cancer therapies using monoclonal antibodies (mAbs) to specifically target cancer cells without disrupting surrounding normal tissue[118]. Other immune-based therapies, such as cytokine and vaccine approaches, have been used in clinical trials as a treatment for a variety of cancers, including prostate cancer[78]. Fong *et al.* (2001) have shown that antigen-specific antitumor immune response can be initiated through the use of dendritic cells as a vector for presenting tumor antigens[81, 85].

The G_i-protein-coupled receptor, CCR6, mediates the recruitment of iDCs and memory T cells for β-defensins indicating their activity in adaptive immunity. Additional studies into the adaptive immune component of β-defensins have

demonstrated they are capable of facilitating the delivery of antigens to receptors on iDCs[81]. This results in an antigen-specific immune response mediated by the presentation of the tumor antigen to mature dendritic cells and T cells via MHC II molecules. However, evidence exists that demonstrates dendritic cell infiltration is decreased in patients with advanced prostate cancer, thus suggesting a possible mechanism by which immunity-mediated destruction is suppressed in prostatic tumors[106].

Human Beta Defensin-1

Human β-defensin-1 was first isolated as a 36 amino acid peptide from human plasma ultrafiltrates[119] and has been mapped to chromosome 8p23.2[75, 76]. The *DEFB1* gene has a simple structure consisting of two exons flanking an intron that spans 6962 bp[14, 78, 91, 94, 106] (Figure 2-6). The full length cDNA for hBD-1 contains only 362 bases[78, 119] (Accession No. X92744). A characteristic endoplasmic reticulum targeting signal sequence with signal peptidase cleavage sites between Gly^{21}/Gly^{22} and Ser^{16}/Glu^{17}, a short (11 or 16 amino acid) pro-piece, and a sequence for the mature hBD-1 peptide are encoded in the open reading frame[78]. Specifically, the 128 bp exon 1 encodes the prepro-piece, which is about 68 amino acids in length, and the signal sequence[78, 88, 119]. Exon 2, 234 bp in length, encodes the mature hBD-1 peptide[78, 91]. A GC box is found at position -217 bp and a TATA box sequence are located at position -34 bp relative to the first cDNA base[78].

Figure 2-6: Signature motif for β-defensin genes. The structural organization of the 3-defensin gene includes two exons surrounding a variably sized intron. Some degree of nucleotide identity is found among the defensins in exon 1, which encodes the signal sequence[14]. Exon 2 encodes the propeptide and mature peptide. (adapted from Weinberg et al. 1998[120])

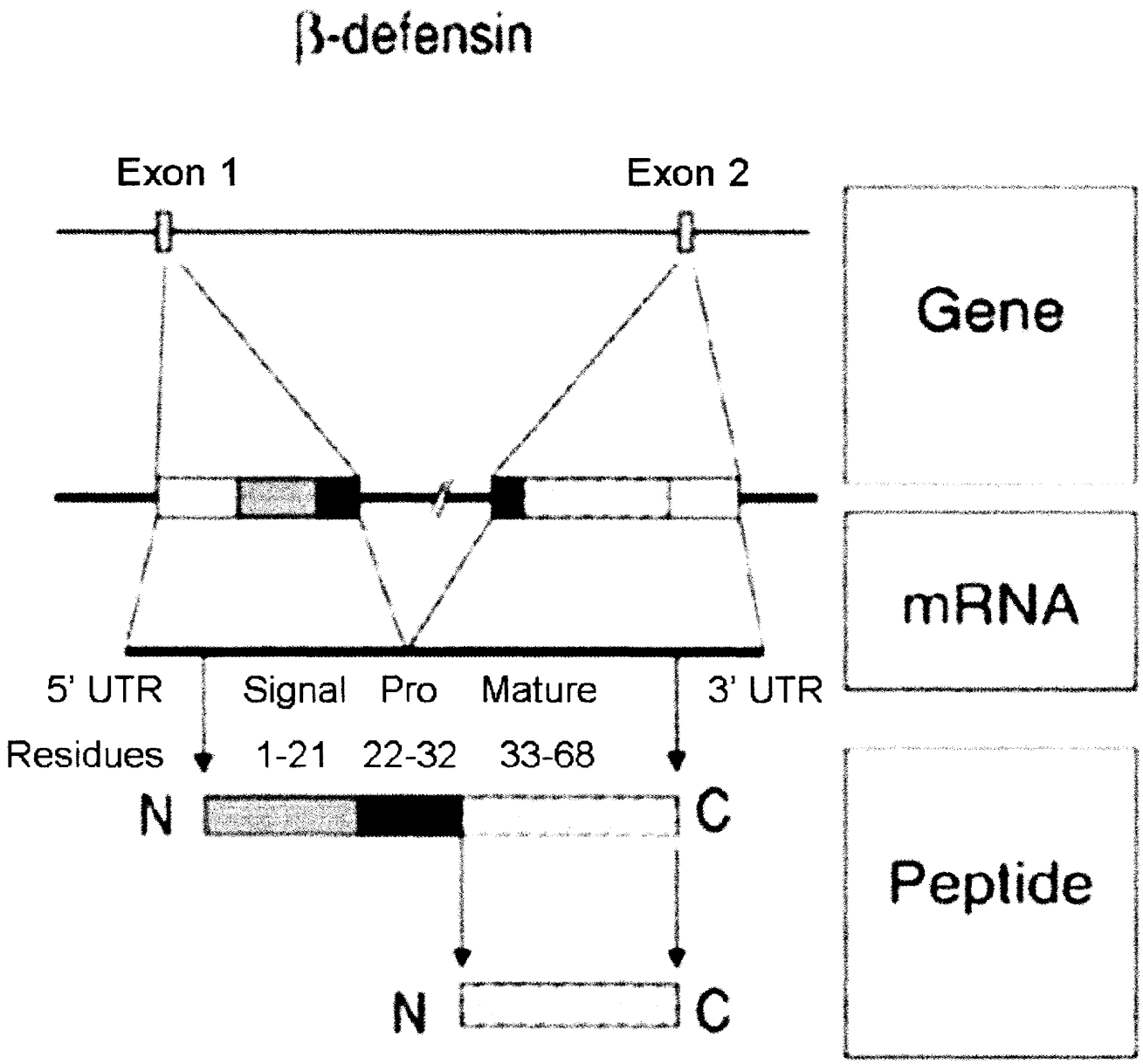


Figure 2-6: Signature motif for beta-defensin genes. (adapted from Weinberg et al. 1998[121] and Bals et al. 2000[122])

The proteolytic processing of varying functional domains of a protein generally occurs as needed during the maturation process. Several studies have identified naturally occurring N-terminally truncated molecular variants of hBD-1 from 36-47 amino acids in length in blood and urine[74, 119]. Mass spectrometry measured the molecular masses of 3928 Da for the native peptide and 4276 Da for the reduced and alkylated peptide[81].

With the initial isolation of hBD-1 from human filtrates, it was estimated that the peptide is present in nanomolar concentrations[81]. Although it has been shown that hBD-1 has cytotoxic potential toward mammalian cells[106, 119], constitutive expression at basal levels is not sufficient to independently lyse cell targets. It is known that hBD-1 is a constitutively expressed peptide in the epithelia of numerous tissues, including gingival, lung, digestive, kidney, and prostate[74, 82, 123, 124]. In addition, hBD-1 is the only human beta defensin the gene expression of which is not induced by inflammatory stimuli, such as IL-8 and LPS[78, 125, 126].

Previously Donald *et al* demonstrated the cancer specific loss of hBD-1 expression in clinical prostate and renal carcinoma samples[6]. In addition, IHC analysis indicated the loss of expression occurs as early as PIN, a precursor to prostate carcinoma, with normal glandular staining observed in adjoining benign tissue (Figure 2-7)[6]. Overall, they found benign epithelium stained positive for hBD-1 expression in both the kidney and prostate, while adjacent malignant epithelium exhibited little to no stain for hBD-1 in 90% and 82% of the samples, respectively (Figure 2-8)[6].

Figure 2-7: IHC analysis of hBD-1 in the prostate. A, Normal prostate expresses hBD-1 (*black arrow*), while adjacent malignant tissue does not (*red arrow*). B, PIN exhibits loss of hBD-1 expression (*red arrow*), while expression is maintained in a normal gland (*black arrow*). C, Malignant prostate tissue shows the absence of hBD-1 stain (*red arrow*), while normal glands are positive for hBD-1 (*black arrow*). (adapted from Donald *et al* 2003[6])

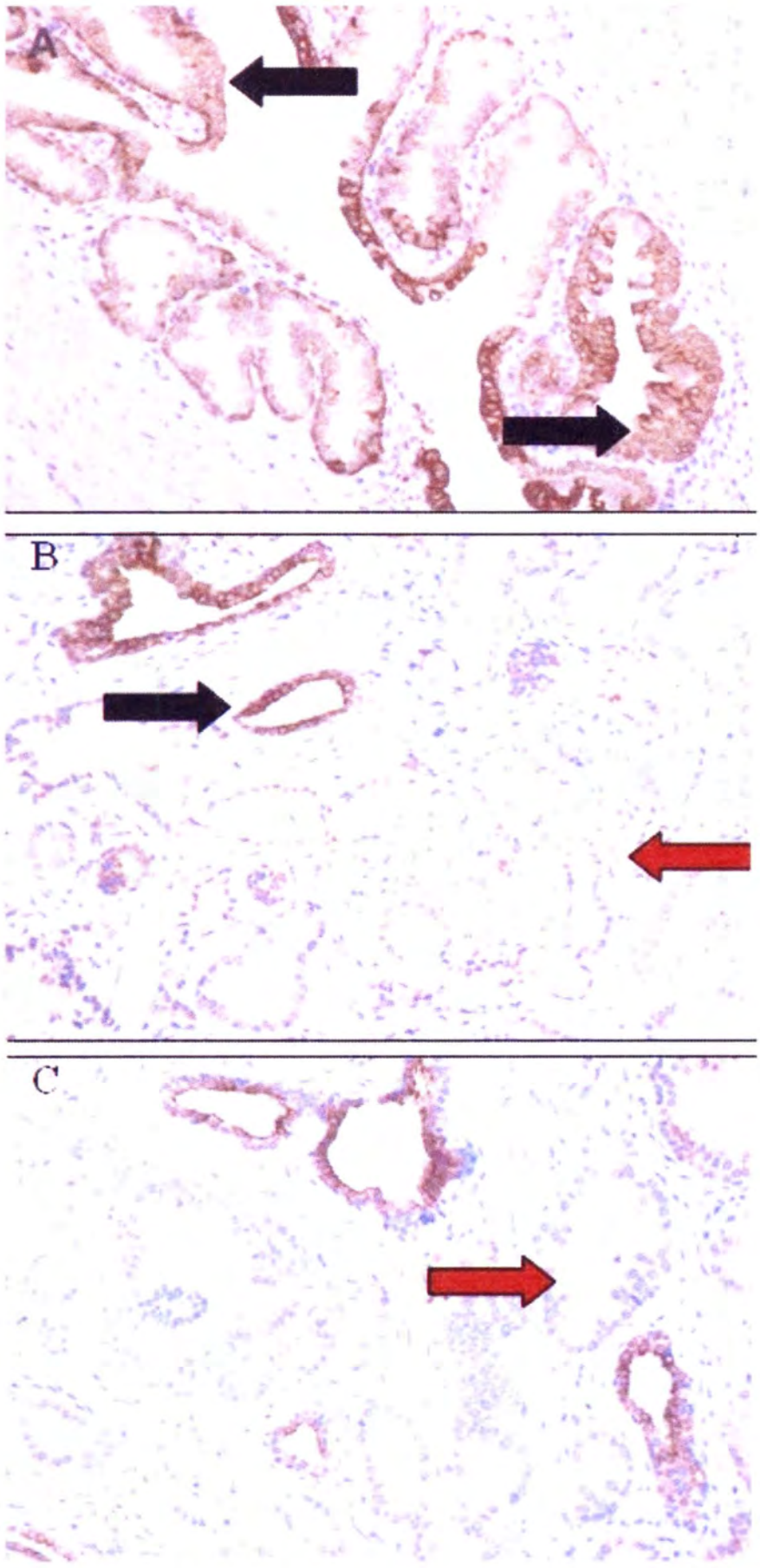


Figure 2-7: IHC analysis of hBD-1 in the prostate.

(adapted from Donald *et al* 2003[6])

Figure 2-8: Staining intensities of hBD-1 protein in kidney and prostate cancers clinical samples. A, Prostate cancer (n=100); B, renal cancer (n=48). Each graph summarizes the results of IHC staining intensity of two pathologists. The staining intensities were determined on an individual basis according to the percentage of malignant cells positive for hBD-1 expression as follows: Negative= no positive cells; Weak= 1% to 25%; Moderate= 26% to 75%; Strong= 76% to 100%. (adapted from Donald *et al* 2003[6])

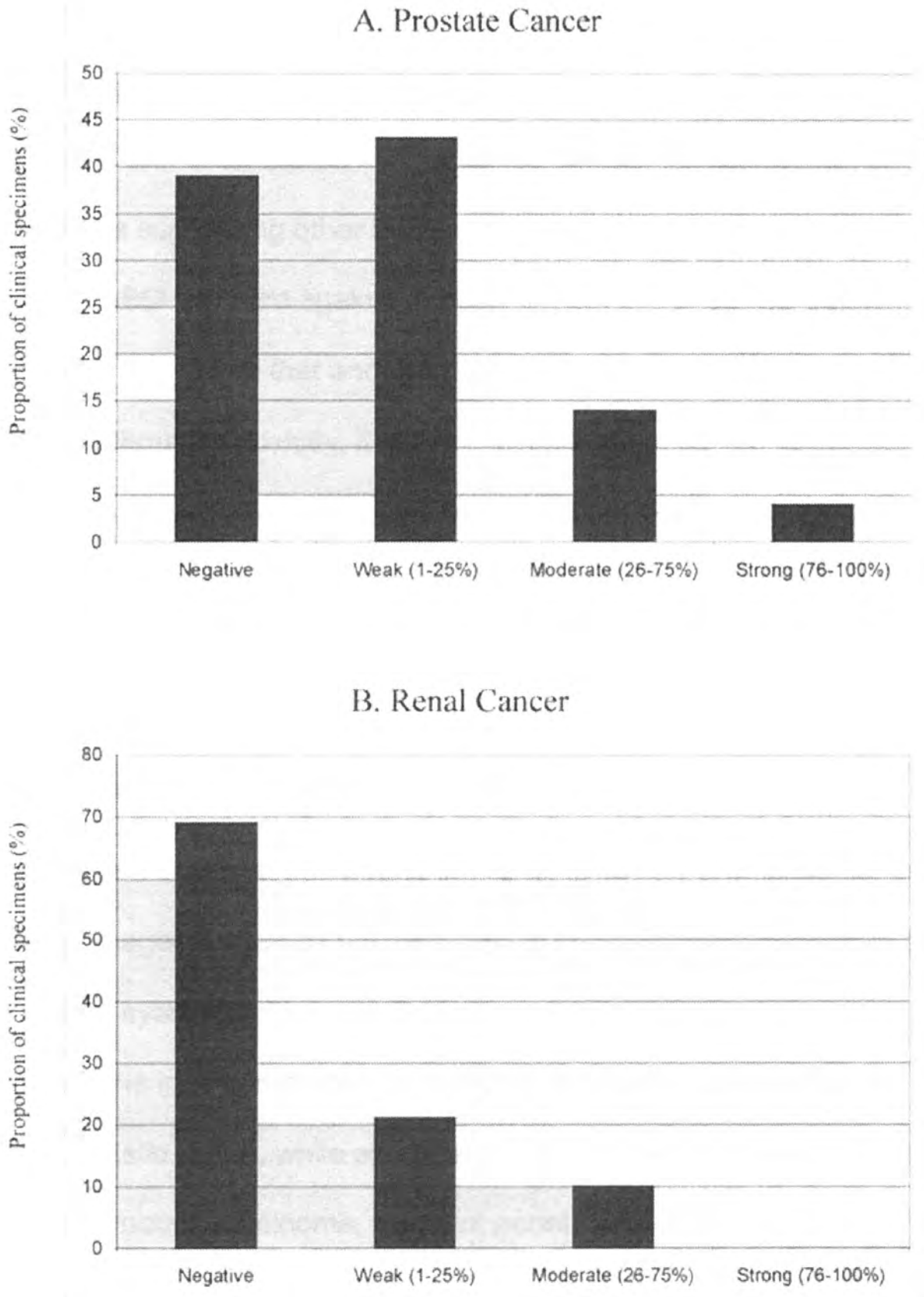


Figure 2-8: Staining intensities of hBD-1 protein in kidney and prostate cancers clinical samples. (adapted from Donald *et al* 2003[6])

As mentioned earlier β-defensins function as antimicrobial or chemotactic agents. However, numerous studies have demonstrated the rather weak antimicrobial[119] and chemotactic[77, 127] activities of hBD-1 as compared to other family members suggesting other biological functions. Recently, the strong cytotoxicity of hBD-1 *in vitro* against multiple cancer cell lines has been demonstrated[56, 57]. Given that endogenous hBD-1 is constitutively expressed by normal cells without cytotoxicity, it may be distinct phenotypic characteristics that render tumor cells sensitive to the cytotoxic effects of hBD-1. Thus, it is a plausible suggestion that hBD-1 plays a critical role in tumor immunity.

Hypothesis, Specific Aims and Significance

Although it is known that innate immunity is key for protecting the body against foreign agents such as bacteria, little is known about elements of the innate immune system that have anti-tumor activity. hBD-1, an important component of the innate immune response, is lost at high frequencies in malignant prostatic tissue, while expression is maintained in adjacent benign regions[14]. In prostate carcinoma, frequent genetic alterations occur in the 8p22-23 region and several studies indicate there may be multiple tumor suppressor genes present within this region[21, 128, 129]. The high incidence of loss of hBD-1 expression in prostate cancer, along with its chromosomal location of 8p23.2, raised the possibility that it may play a role in tumor suppression. Therefore, **we**

hypothesize that hBD-1 may be a tumor suppressor gene involved in tumor immunity in prostate cancer.

To test this hypothesis, we propose the following specific aims:

Specific Aim 1: Characterize the effects of ectopic expression of hBD-1 on prostate cancer cell lines.

The hBD-1 gene has been cloned into an inducible expression system that will be utilized to characterize its function in prostate cancer cells. This will be determined by examining the effect hBD-1 has on cell viability, as well as morphological changes to cell membrane and annexin V and propidium iodide uptake in cells induced for hBD-1 expression.

Specific Aim 2: Determine the cellular mechanisms associated with the effects of hBD-1 on prostate cancer cells.

The inducible hBD-1 expression system will be utilized to determine if hBD-1 induces apoptosis in prostate cancer cells. This will be determined by analysis of caspase activation and changes in the expression of various regulators of apoptosis, such as BCL-2 family members and AKT.

Specific Aim 3: Determine the role of hBD-1 in the progression of prostate cancer.

Here, we will determine if hBD-1 functions as a component of a tumor surveillance system by acting on neighboring cells via a 'bystander effect.' The cell culture supernatant exposed to normal prostate epithelial cells will be harvested and analyzed for secretion of hBD-1 peptide. Additionally, the expression of hBD-1 will be profiled in prostate tissue samples from benign, tumor and areas of PIN.

Significance:

Numerous studies have indicated the importance of chromosome 8 instability in prostate tumorigenesis[22]. The goal of this proposal is to characterize the function of hBD-1, an important component of the innate immune response that is lost in prostate cancer. Since the gene encoding hBD-1 is located within a region of chromosome 8p thought to contain three separate tumor suppressor genes[130], the studies will focus on hBD-1 as a potential tumor suppressor in prostate cancer. The proposed aims will allow further understanding how hBD-1 functions in prostate cancer and may eventually aid in the development of strategies to prevent prostate cancer incidence and mortality.

CHAPTER 3

CHARACTERIZATION OF THE EFFECTS OF HBD-1 ON PROSTATE CANCER CELLS

INTRODUCTION

Prostate cancer is the most common non-cutaneous malignancy in American men and is the second leading cause of cancer-related death in males in the United States[131]. The clinical outcome of prostate cancer is closely related to tumor grade and stage, with poorly differentiated tumors having a poor prognosis[131]. Prostate tumor progression has been linked to an increase in immune suppression in prostate cancer patients[6]. It has also been suggested that an imbalance between immune activation and suppression during tumor growth allows a tumor to escape immune destruction[132]. However, little is known about specific components of the innate immune system that play a role in prostate tumor suppression.

The defensin genes, a family of 3-4 kDa polycationic peptides clustered on the short arm of chromosome 8 at segment 8p22-8p23, have been shown to act as antimicrobial agents by disrupting membrane integrity resulting in cell lysis and death[6]. The involvement of defensin proteins in cytotoxicity and cytolysis, with respect to tumor immunity, is gaining more attention. Human beta defensin-1 (hBD-1) is a potent "microchemokine" that attracts immature dendritic cells and memory T cells via a specific interaction with the chemokine receptor CCR6[91]. It is through this action that β-defensins are thought to play an important role in both innate and adaptive immune responses, but their role in prostate tumor progression remains unclear[133]. We have previously shown that the expression of hBD-1 is lost or suppressed in 82% of malignant prostate tissue, whereas benign tissue generally expressed moderate to high levels of this peptide[134].

Although hBD-1 expression is lost at high frequency in prostate cancer, little is known about its function or whether this phenomenon contributes to prostate tumor progression.

These observations led to the hypothesis that hBD-1 may have anti-tumor activities and that its loss may contribute to prostate tumorigenesis. In this study, we test this hypothesis by assessing the function of hBD-1 in prostate cancer. Initial screening for hBD-1 expression revealed high levels of hBD-1 expression in benign prostatic tissue and the prostate primary culture hPrEC. However, we observed a loss of hBD-1 expression in adjacent malignant prostatic tissue, as well as in DU145, PC3 and LNCaP prostate cancer cell lines. Our functional data show that the induction of hBD-1 expression results in rapid cell death in the late-stage prostate cancer cell lines, PC3 and DU145, but had no effect on the early-stage prostate cancer cell line, LNCaP, or normal prostate epithelial cell line, hPrEC, cell viability. With this, we provide the first experimental evidence that hBD-1 may play a specific role in tumor suppression of advanced prostate cancer. Furthermore, these data support the potential utility of hBD-1 as a therapeutic agent for late-stage prostate cancer without cytotoxic effects to the host.

MATERIALS AND METHODS

Cell culture

The prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) (Table 1). DU145 cells were cultured in DMEM medium, PC3 and PC3/AR+ were grown in F12 medium, LNCaP were

grown in RPMI medium (Life Technologies, Inc., Grand Island, NY). Growth media for all three lines was supplemented with 10% (v/v) fetal bovine serum (Life Technologies). The hPrEC primary culture was obtained from Cambrex Bio Science, Inc. (Walkersville, MD) and cells were grown in prostate epithelium basal media (Table 1). All cell lines were maintained at 37° C and 5% CO_2.

Tissue Samples and Laser Capture Microdissection

Prostate tissues were obtained from patients who provided informed consent prior to undergoing radical prostatectomy. Samples were acquired through the Hollings Cancer Center tumor bank in accordance with an Institutional Review Board-approved protocol. This included guidelines for the processing, sectioning, histological characterization, RNA purification and PCR amplification of samples. Prostate specimens received from the surgeons and pathologists were immediately frozen in OCT compound. Each OCT block was cut to produce serial sections which were stained and examined. Areas containing benign cells, Prostatic Intraepithelial Neoplasia (PIN), and cancer were identified and used to guide our selection of regions from unstained slides using the Arcturus PixCell II System (Sunnyvale, CA). Caps containing captured material were exposed to 20 µl of lysate from the Arcturus Pico Pure RNA Isolation Kit and processed immediately. RNA quantity and quality was evaluated using sets of primers that produce 5' amplicons. The sets include those for the ribosomal protein L32 (the 3' amplicon and the 5' amplicon are 298 bases apart), for the glucose phosphate

Table 1: Prostate cell lines.

Normal	Cancer	Site of Metastasis
hPrEC	DU145	Brain carcinoma
	PC3	Bone adenocarcinoma
	LNCaP	Left supraclavicular lymph node carcinoma

isomerase (391 bases apart), and for the glucose phosphate isomerase (842 bases apart). Ratios of 0.95 to 0.80 were routinely obtained for these primer sets using samples from a variety of prepared tissues. Additional tumor and normal samples were grossly dissected by pathologists, snap frozen in liquid nitrogen and evaluated for hBD-1 expression.

Cloning of hBD-1 Gene

hBD-1 cDNA was generated from RNA by reverse transcription-PCR using primers generated from the published hBD-1 sequence (Accession No. U50930)[62]. The PCR primers were designed to contain *ClaI* and *KpnI* restriction sites. hBD-1 PCR products were restriction digested with *ClaI* and *KpnI* and ligated into a TA cloning vector. The TA/hBD-1 vector was then transfected into the XL-1 Blue strain of E. coli by heat shock and individual clones were selected and expanded. Plasmids were isolated by Cell Culture DNA Midiprep (Qiagen, Valencia, CA) and sequence integrity verified by automated sequencing. The hBD-1 gene fragment was then ligated into the pTRE2 digested with *ClaI* and *KpnI*, which served as an intermediate vector for orientation purposes. The pTRE2/hBD-1 construct was digested with *ApaI* and *KpnI* to excise the hBD-1 insert. The insert was ligated into pIND vector of the Ecdysone Inducible Expression System (Invitrogen, Carlsbad, CA) also double digested with *ApaI* and *KpnI*. The construct was again transfected into E. coli and individual clones

were selected and expanded. Plasmids were isolated and sequence integrity of pIND/ hBD-1 was again verified by automated sequencing.

Transfection

Cells were seeded into appropriate dishes at least 5 hours prior to transfection. Next, the cells were co-transfected using Lipofectamine 2000 (Invitrogen) with 2 pg/cell of pvgRXR plasmid, which expresses the heterodimeric ecdysone receptor, and 2 pg/cell of the pIND/hBD-1 vector construct in Opti-MEM media (Life Technologies, Inc.). As a control, cells were transfected with 4 pg/cell of the pvgRXR plasmid. Transfection efficiency was determined by transfecting cells with 4 pg/cell of a pcMV-GFP vector (gift from Christina Voelkel-Johnson laboratory, MUSC, Charleston, SC) and expressed as percent gated following FACS analysis.

Immunocytochemistry

In order to verify hBD-1 protein expression, DU145 and hPrEC cells were seeded onto 2-chamber culture slides (BD Falcon, USA) at 1.5-2 x 10^4 cells per chamber. DU145 cells transfected with pvgRXR alone (control) or with the hBD-1 plasmid were induced for 18 hours with media containing 10 μM Pon A, while untransfected cells received fresh growth media. Following induction, cells were washed in 1x PBS and fixed for 1 hour at room temperature with 4% paraformaldehyde. Cells were then washed 6 times with 1x PBS and blocked in 1x PBS supplemented with 2% BSA, 0.8% normal goat serum (Vector

Laboratories, Inc., Burlingame, CA) and 0.4% Triton-X 100 for 1 hour at room temperature. Next, cells were incubated overnight in primary rabbit anti-human BD-1 polyclonal antibody (PeproTech Inc., Rocky Hill, NJ) diluted 1:1000 in blocking solution. Following this, cells were washed 6 times with blocking solution and incubated 1 hour at room temperature in Alexa Fluor 488 goat anti-rabbit IgG (H+L) secondary antibody at a dilution of 1:1000 in blocking solution. After washing cells with blocking solution 6 times, coverslips were mounted with Gel Mount (Biomeda, Foster City, CA). Finally, cells were viewed under differential interference contrast (DIC) and under laser excitation at 488 nm. The fluorescent signal was analyzed using a confocal microscope (Zeiss LSM 5 Pascal) and a 63x DIC oil lens with a Vario 2 RGB Laser Scanning Module. The digital images were exported into Photoshop CS Software (Adobe Systems) for image processing and hard copy presentation.

RNA Isolation and Quantitative RT-PCR

QRT-PCR was performed as previously described[135]. Briefly, total RNA (0.5 µg per reaction) from both sources was reverse transcribed into cDNA utilizing random primers (Promega). Two-step QRT-PCR was performed on cDNA generated using the MultiScribe Reverse Transcriptase from the TaqMan Reverse Transcription System and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primer pairs for hBD-1 and c-MYC were generated from the published sequences (Table 2). Forty cycles of PCR were performed under standard conditions using an annealing temperature of 56.4° C

for hBD-1 and c-MYC. In addition, β-Actin (Table 2) was amplified as a housekeeping gene to normalize the initial content of total cDNA. Gene expression in benign prostate tissue samples was calculated as the expression ratio compared to β-Actin. Relative hBD-1 expression was calculated by comparing hBD-1 expression in malignant prostate tissue, hPREC prostate primary culture, and prostate cancer cell lines before and after being induced for hBD-1 expression to the average level of hBD-1 expression in benign prostate tissue. In all cases, unless specified in the figure legend, the dissociation curves were analyzed and matched for samples used to generate data. Specifically, the Tm for each primer set was 77° for hBD-1 and 79° for β-Actin and cMYC. As a negative control, QRT-PCR reactions without cDNA template were also performed. All reactions were run a minimum of three times.

Phase Contrast Microscopic Analysis

Phase contrast microscopy was utilized to evaluate the effect of hBD-1 on prostate cancer cells. DU145, PC3 and LNCaP cells containing no vector, empty plasmid or hBD-1 plasmid were seeded onto 6-well culture plates (BD Falcon). The following day plasmid-containing cells were induced for a period of 48 hours with media containing 10 μM Pon A, while control cells received fresh media. The cells were then viewed under an inverted Zeiss IM 35 microscope (Carl Zeiss, Germany). Phase contrast pictures of a field of cells were obtained using the SPOT Insight Mosaic 4.2 camera (Diagnostic Instruments, USA). Cells were examined by phase contrast microscopy under 32X magnification. The digital

Table 2: Sequences of QRT-PCR primers.

	Sense (5'–3')	Antisense (5'-3')
β-actin	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'
hBD-1	5'-TCAGCAGTGGAGGGCAATG-3'	5'-CCTCTGTAACAGGTGCCTTGAAT-3'
c-MYC	5'-ACAGCAAACCTCCTCACAGCC-3'	5'-TGGAGACGTGGCACCTCTTG-3'
GAPDH	5'-CCACCCATGGCAAATTCCATGGCA-3'	5'-TCTAGACGGCAGGTCAGGTCAACC-3'
AKT	5'-TCAGCCCTGGACTACCTGCA-3'	5'-GAGGTCCCGGTACACCACGT-3'
BAD	5'-CTCAGGCCTATGCAAAAAGAGGA-3'	5'-GCCCTCCCTCCAAAGGAGAC-3'
BID	5'-AACCTACGCACCTACGTGAGGAG-3'	5'-CGTTCAGTCCATCCCATTTCTG-3'

images collected were stored as uncompressed TIFF files and exported into Photoshop CS software (Adobe Systems) for image processing and hard copy presentation.

MTT Cell Viability Assay

To examine the effects of hBD-1 on cell growth, we performed metabolic 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. DU145, LNCaP, PC3 and PC3/AR+ cells co-transfected with pvgRXR plasmid and pIND/hBD-1 construct or control pvgRXR plasmid were seeded onto a 96-well plate at 1-5 $x10^3$ cells per well. Twenty-four hours after seeding, fresh growth medium was added containing 10 μM Pon A daily to induce hBD-1 expression for 24, 48 and 72 hours after which the MTT assay was performed according to the manufacturer's instructions (Promega). Reactions were performed three times in triplicate.

Analysis of Membrane Integrity

Acridine orange (AO)/ethidium bromide (EtBr) dual staining was performed to identify changes in cell membrane integrity, as well as apoptotic cells by staining the condensed chromatin. AO stains viable cells and early apoptotic cells, whereas EtBr stains late stage apoptotic cells that have compromised membranes. Briefly, PC3, DU145 and LNCaP cells were seeded into 2-chamber culture slides (BD Falcon). Cells transfected with empty plasmid or hBD-1 plasmid were induced for 24 or 48 hours with media containing 10 μM

Pon A, while control cells received fresh growth media at each time point. After induction, cells were washed once with PBS and stained with 2 ml of a mixture (1:1) of AO (Sigma, St. Louis, MO) and EtBr (Promega) (5 µg/ml) solution for 5 min and were again washed with PBS.

Fluorescence was viewed by a Zeiss LSM 5 Pascal Vario 2 Laser Scanning Confocal Microscope (Carl Zeiss). The excitation color wheel contains BS505-530 (green) and LP560 (red) filter blocks which allowed for the separation of emitted green light from AO into the green channel and red light from EtBr into the red channel. The laser power output and gain control settings within each individual experiment were identical between control and hBD-1 induced cells. The excitation was provided by a Kr/Ar mixed gas laser at wavelengths of 543 nm for AO and 488 nm for EtBr. Slides were analyzed under 40x magnification and the images were collected and processed as previously mentioned.

Flow Cytometry

PC3 and DU145 cells transfected with the hBD-1 expression system were grown in 60-mm dishes and induced for 12, 24, and 48 hours with 10 µM Pon A. Following each incubation period, the medium was collected from the plates (to retain any detached cells) and combined with PBS used to wash the plates. The remaining attached cells were harvested by trypsinization and combined with the detached cells and PBS. The cells were then pelleted at 4° C (500 x g) for 5 min, washed twice in PBS, and resuspended in 100 µl of 1x Annexin binding buffer (0.1 M Hepes/NaOH at pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) containing 5 µl of

Annexin V-FITC and 5 µl of PI. The cells were incubated at RT for 15 min in the dark, then diluted with 400 µl of 1x Annexin binding buffer and analyzed by FACscan (Becton Dickinson, San Jose, CA). All reactions were performed three times.

Caspase Detection

Detection of caspase activity in the prostate cancer cell lines was performed using APO LOGIX^{TM} Carboxyfluorescin Caspase detection kit (Cell Technology, Mountain View, CA). Active caspases were detected through the use of carboxyfluorescein labeled peptide fluoromethyl ketone (FAM-VAD-FMK) that irreversibly binds to active caspases. Briefly, DU145 and LNCaP cells (1.5-3 X10^5) containing the hBD-1 expression system were plated in 35 mm glass bottom dishes (Matek, Ashland, MA) and treated for 24 hours with media only or with media containing 10 µM Pon A as previously described. Next, 10 µl of a 30X working dilution of FAM-VAD-FMK was added to 300 µl of media and added to each 35 mm dish. Cells were then incubated for 1 hour at 37° C under 5% CO_2. The medium was aspirated and the cells were washed twice with 2 ml of a 1x working dilution Wash Buffer. Cells were viewed under differential interference contrast (DIC) or under laser excitation at 488 nm. The fluorescent signal was analyzed using a confocal microscope (Zeiss LSM 5 Pascal) and a 63x DIC oil lens with a Vario 2 RGB Laser Scanning Module.

Statistical Analysis

Statistical analysis was performed by using the Student's t-test for unpaired values. P values were determined by a two-sided calculation, and a P value of less than 0.05 was considered statistically significant. Statistical differences are indicated by asterisks.

RESULTS

hBD-1 Expression in Prostate Tissue

We previously demonstrated that 82% of prostate cancer frozen tissue sections analyzed exhibited little or no expression of hBD-1[136]. In addition, in that study we observed a potential correlation between clinical prostate samples with little or no expression of hBD-1 and higher rates of cancer recurrence (unpublished data). To compare hBD-1 expression levels, QRT-PCR analysis was performed on normal prostate tissue obtained by gross dissection or LCM of normal prostate tissue adjacent to malignant regions which were randomly chosen. Here, hBD-1 was detected in all of the gross dissected normal clinical samples with range of expression that represents approximately a 6.6-fold difference in expression level (Figure 3-1A). LCM captured normal samples expressed hBD-1 at levels in a range that represents a 32-fold difference in expression (Figure 3-1B). Matching sample numbers to corresponding patient profiles revealed that in most cases, the hBD-1 expression levels was higher in patient samples with a Gleason score of 6 than in patient samples that had a

Figure 3-1: Analysis of hBD-1 expression in human prostate tissue. hBD-1 relative expression levels were compared in normal clinical samples from patients that underwent radical prostatectomies. The dashed line serves as a point of reference to compare values obtained between gross and LCM-derived specimen, and corresponding Gleason scores are indicated above each bar. (A) hBD-1 expression levels were compared in tissues obtained by gross dissection. (B) hBD-1 expression levels were compared in tissue obtained by Laser Capture Microdissection.

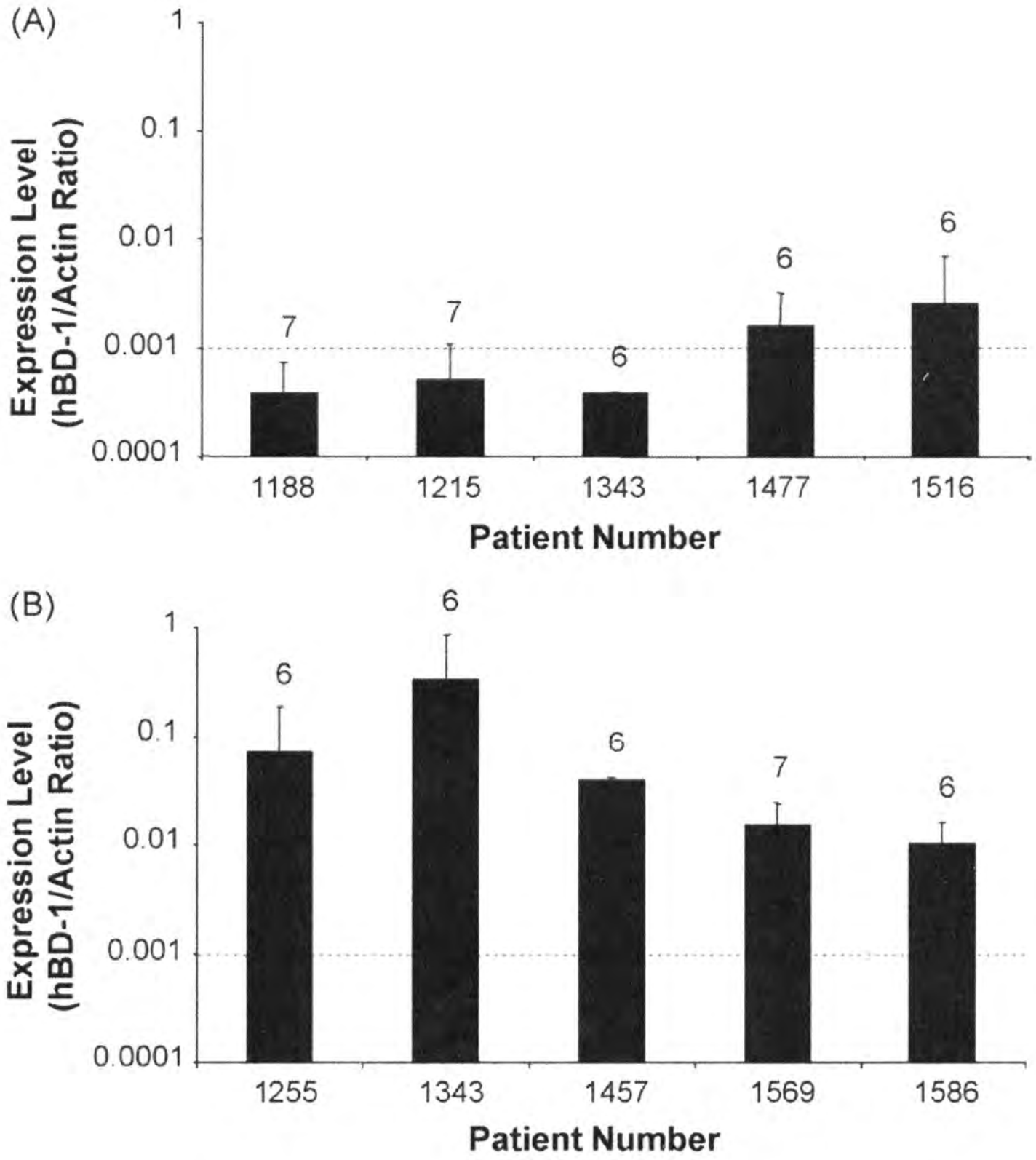


Figure 3-1: Analysis of hBD-1 expression in human prostate tissue.

Gleason score of 7. In addition, a comparison of hBD-1 expression levels in tissue obtained by gross dissection and LCM for the same patient, #1343, demonstrated an 854-fold difference in expression between the two isolation techniques. Therefore, our results indicate LCM is a more sensitive technique to assess hBD-1 expression in prostate tissue.

Transfection Efficiency in Prosate Cancer Cell Lines

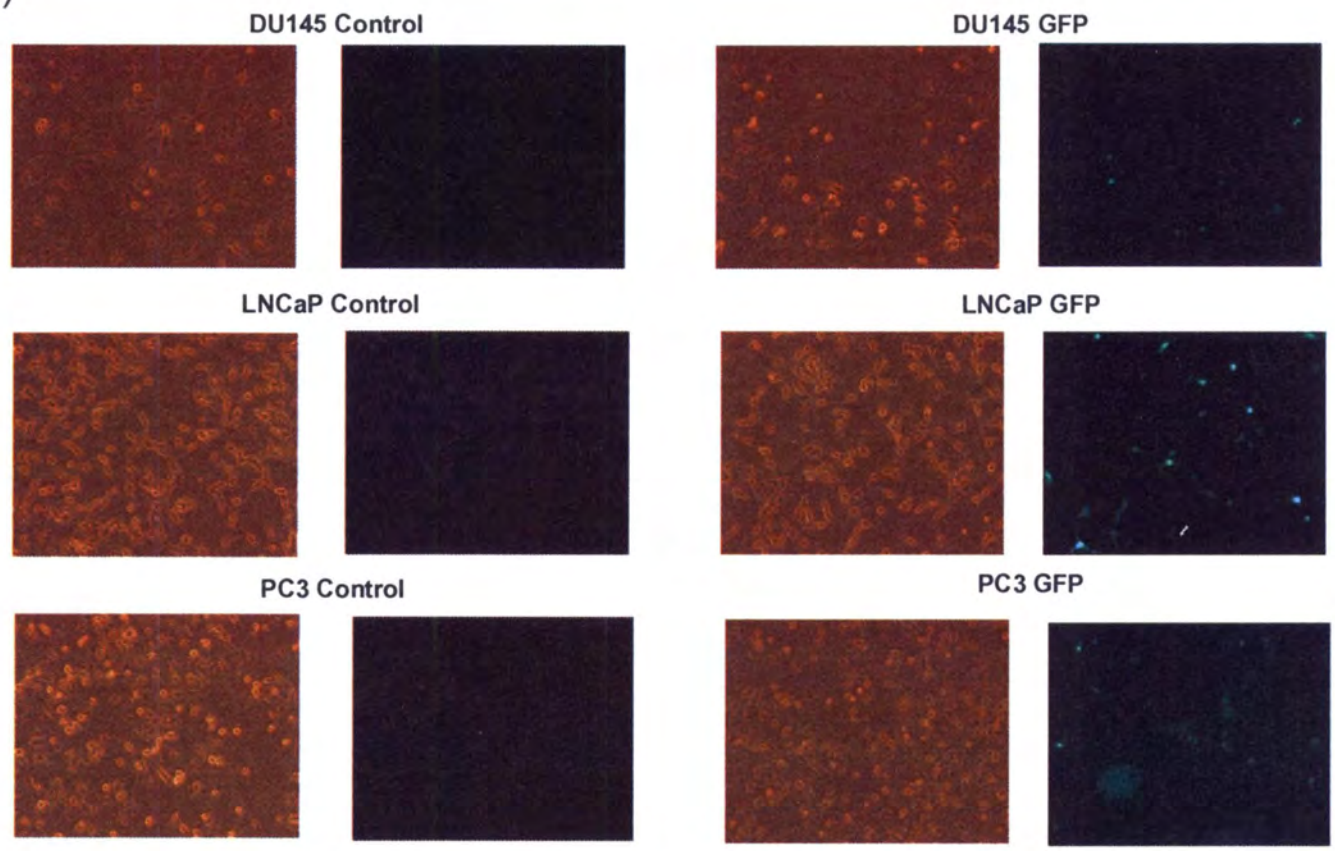
To determine the transfection efficiency, the prostate cancer cell lines were transfected with a GFP vector and fluorescence was monitored. Microscopic analysis revealed green fluorescence in only the prostate cancer cell lines transfected with GFP (Figure 3-2). In addition, the flow analysis of GFP positive cells demonstrated an approximate 50% transfection efficiency in all three prostate cancer cell lines.

hBD-1 Expression in Prostate Cell Lines

To verify upregulation of hBD-1 in the prostate cancer cell lines after transfection with the hBD-1 expression system, QRT-PCR was performed. In addition, no template negative controls were also performed, and amplification products were verified by gel electrophoresis (Figure 3-3). Here, hBD-1 expression was significantly lower in the prostate cancer cell lines compared to hPrEC cells. Following a 24 hour induction period, relative expression levels of

Figure 3-2: Analysis of Transfection Efficiency with GFP Plasmid. (A) Microscopic analysis of GFP transfection efficiency. (B) Graph representing the percent gated during flow analysis for GFP.

(A)



(B)

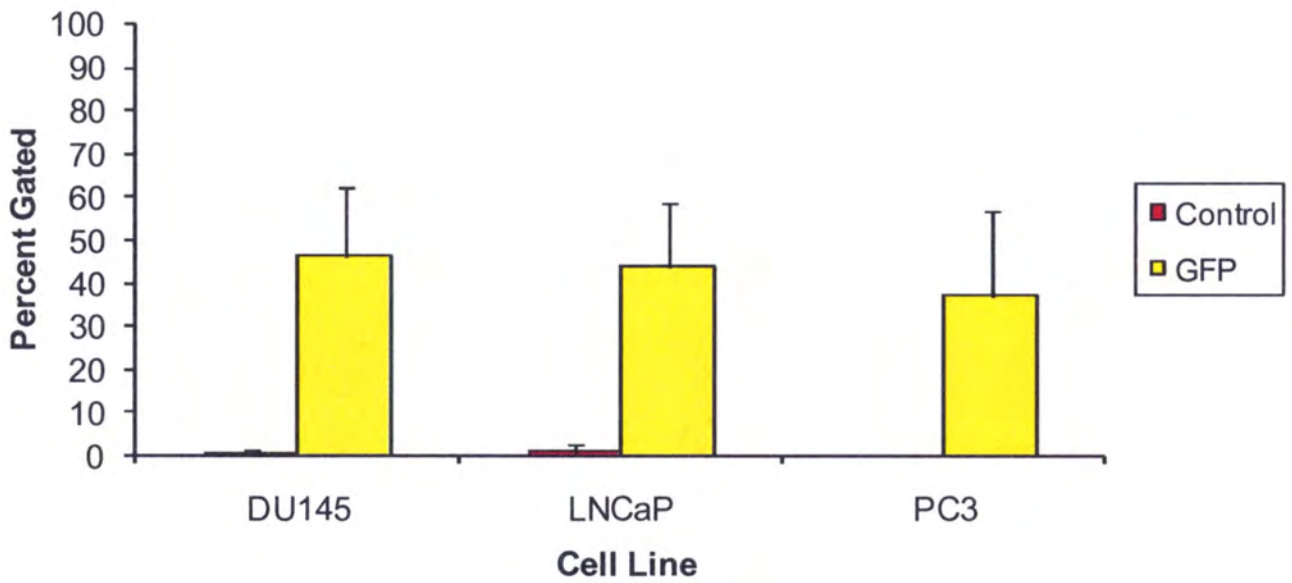
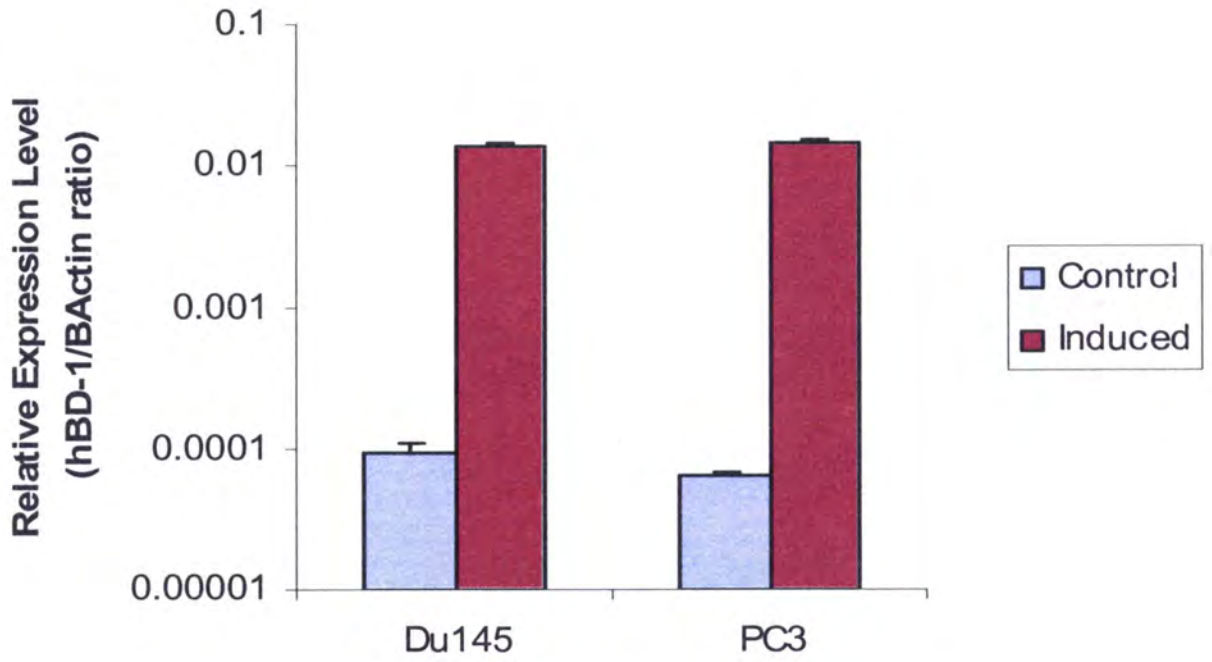


Figure 3-2: Analysis of Transfection Efficiency with GFP Plasmid.

Figure 3-3: Analysis of PCR products. (A) RT-PCR was performed in DU145 and PC3 after induction for hBD-1 expression. (B) Products were verified by gel electrophoresis.

(A)



(B)

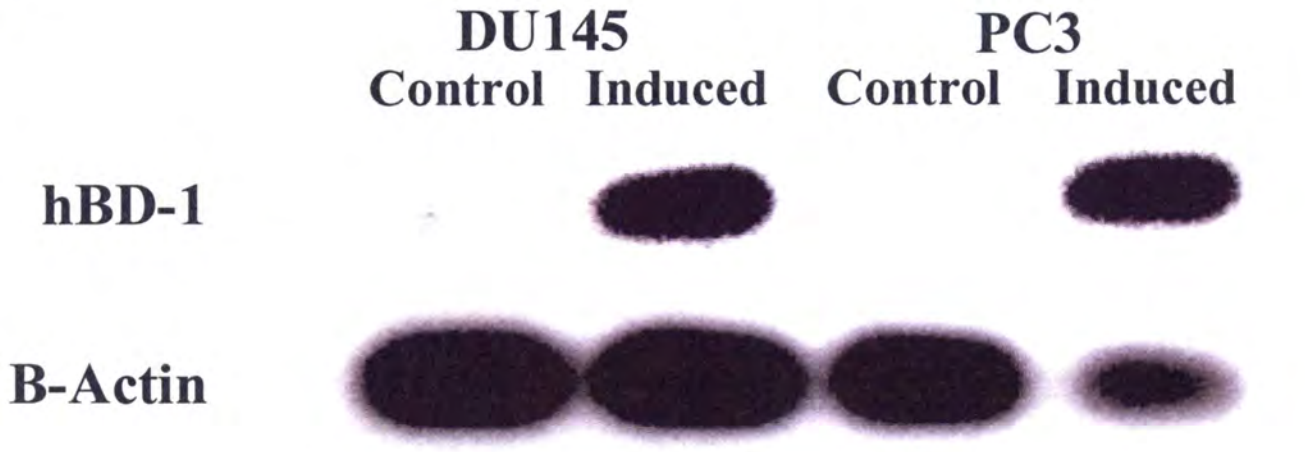


Figure 3-3: Analysis of PCR products.

Figure 3-4: Analysis of hBD-1 expression in prostate cell lines. hBD-1 expression levels were compared relative to hPrEC cells in prostate cancer cell lines before and after hBD-1 induction. An asterisk represents statistically higher expression levels compared to hPrEC. Double asterisks represent statistically significant levels of expression compared to the cell line before hBD-1 induction (Student's *t*-test, $p < 0.05$).

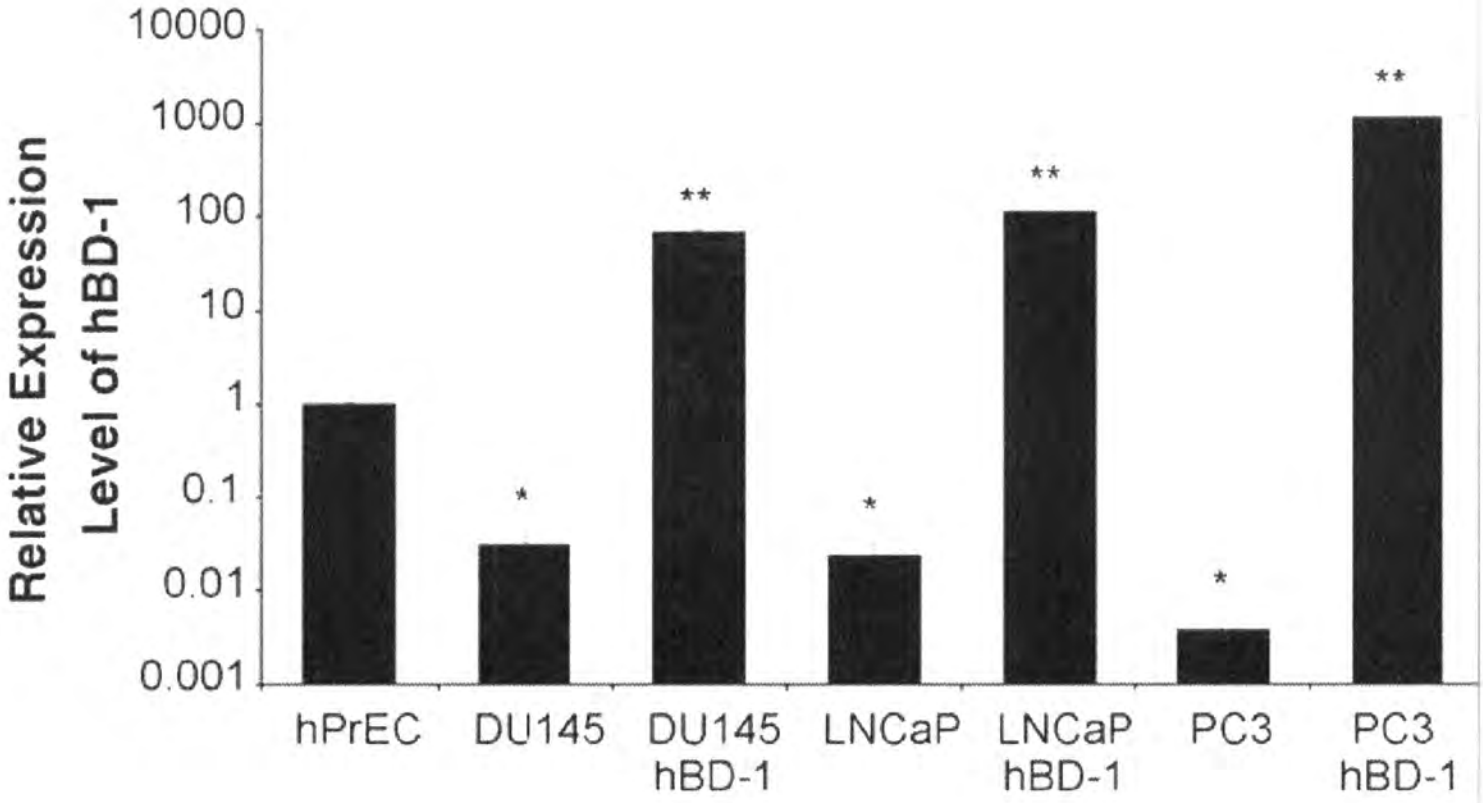


Figure 3-4: Analysis of hBD-1 expression in prostate cell lines.

hBD-1 significantly increased in DU145, PC3 and LNCaP as compared to the cell line prior to hBD-1 induction (Figure 3-4). Analysis of hBD-1 relative expression in the prostate cancer cell lines revealed that we can induce hBD-1 in prostate cancer cells as compared to endogenous hBD-1 expression in normal prostate cells.

Next, we verified protein expression of hBD-1 in DU145 cells transfected with the hBD-1 expression system after induction with Pon A utilizing immunocytochemistry. As a positive control, hBD-1 expressing hPrEC prostate epithelial cells were also examined. Cells were stained with primary antibody against hBD-1 and protein expression was monitored based on the green fluorescence of the secondary antibody (Figure 3-5). Analysis of cells under DIC verifies the presence of hPrEC cells and DU145 cells at 18 hours. Excitation by the confocal laser at 488 nm revealed green fluorescence indicating the presence of hBD-1 protein in hPrEC. However, there was no detectable green fluorescence in control DU145 and pvgRXR induced DU145 cells demonstrating no hBD-1 expression (d and f, respectively). Confocal analysis of DU145 (h) cells induced for hBD-1 expression revealed green fluorescence indicating the presence of hBD-1 protein following induction with Pon A.

Expression of hBD-1 Results in Decreased Cell Number and Viability

To test the effect of hBD-1 on prostate cancer cell lines, we performed phase contrast microscopic analysis of prostate cancer cell lines. Induction of

Figure 3-5: Analysis of hBD-1 protein expression. Ectopic hBD-1 expression was verified in the prostate cancer cell line DU145 by immunocytochemistry. hPrEC cells were stained for hBD-1 as a positive control (a: DIC and b: fluorescence). DU145 control cells (c: DIC and d: fluorescence) or DU145 cells transfected with pvgRXR alone (e: DIC and f: fluorescence) or hBD-1 and induced for 18 h (g: DIC and h: fluorescence). Size bar = 20μM.

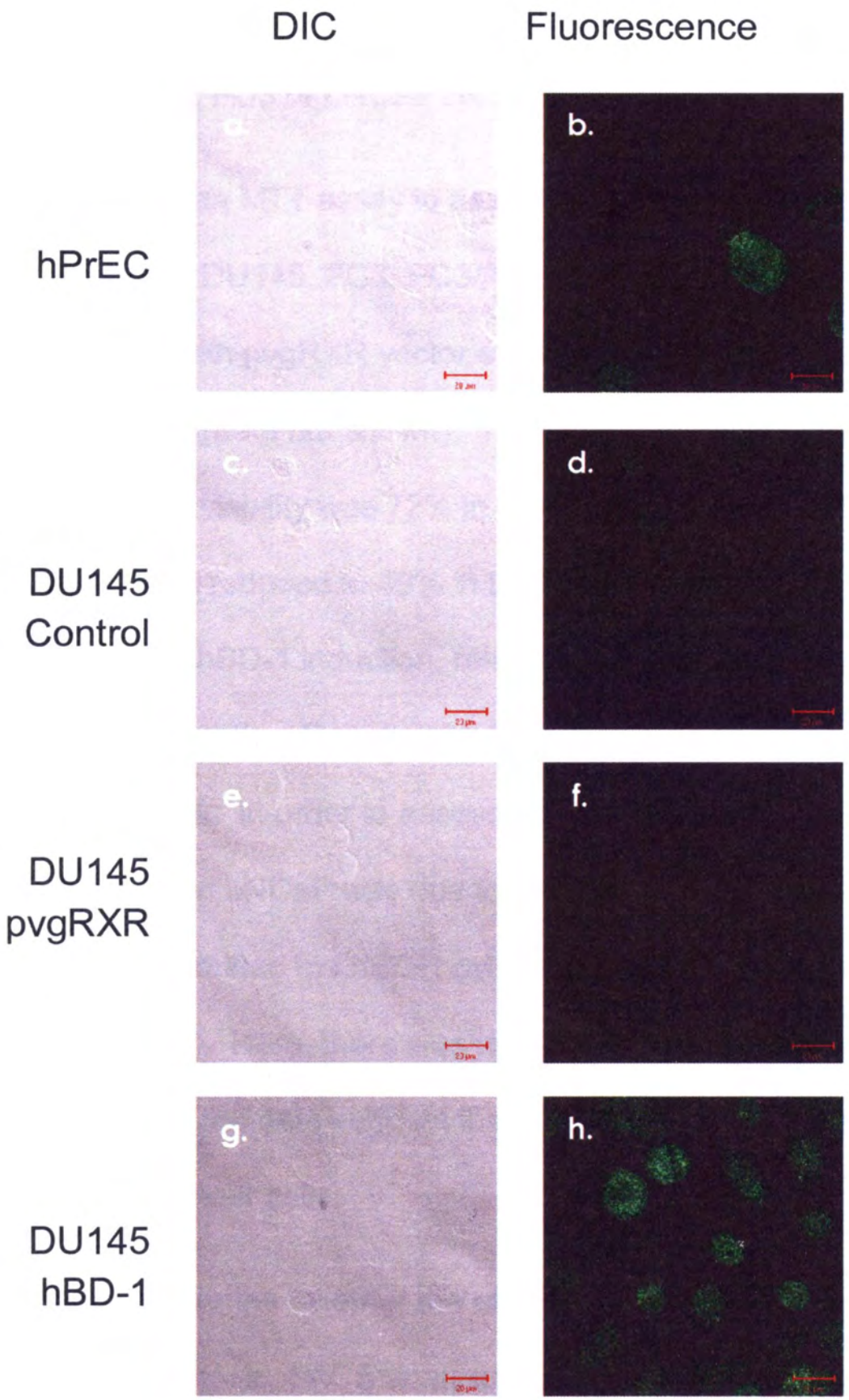


Figure 3-5: Analysis of hBD-1 protein expression.

hBD-1 in the prostate cancer cell lines resulted in a significant reduction in cell number in DU145 and PC3, but had no effect on cell proliferation in LNCaP (Figure 3-5). As a negative control, cell growth was monitored in all three lines after the induction of the pvgRXR vector. There were no observable changes in cell number in DU145, PC3 or LNCaP cells.

We performed an MTT assay to assess the effect of hBD-1 expression on relative cell viability in DU145, PC3, PC3/AR+ and LNCaP prostate cancer cell lines. MTT analysis with pvgRXR vector exhibited no statistically significant change in cell viability (data not shown). Twenty-four hours following hBD-1 induction, relative cell viability was 72% in DU145 and 56% in PC3, and after 48 hours cell viability was reduced to 49% in DU145 and 37% in PC3 (Figure 3-7). Following 72 hours of hBD-1 induction, relative cell viability decreased to 44% in DU145 and 29% PC3 cells. Conversely, there was no significant effect on the viability of LNCaP cells. In order to assess whether the resistance to hBD-1 cytotoxicity observed in LNCaP was due to the presence of the androgen receptor (AR), we examined the hBD-1 cytotoxicity in PC3 cells with ecpotic AR expression (PC3/AR+). Here, there was no difference between PC3/AR+ and PC3 cells. Therefore, the data suggest that hBD-1 is cytotoxic specifically to late-stage prostate cancer cells.

In order to determine whether the effects of hBD-1 on PC3 and DU145 were cytostatic or cytotoxic, FACS analysis was performed to measure cell death. Under normal growth conditions, more than 90% of PC3 and DU145 cultures were viable and non-apoptotic (lower left quadrant) that did not stain with

Figure 3-6: Analysis of hBD-1 on prostate cancer cells. Cell number in DU145, PC3 and LNCaP was monitored by phase contrast microscopy after 48 hours of hBD-1 induction. Size bar= 20 μM.

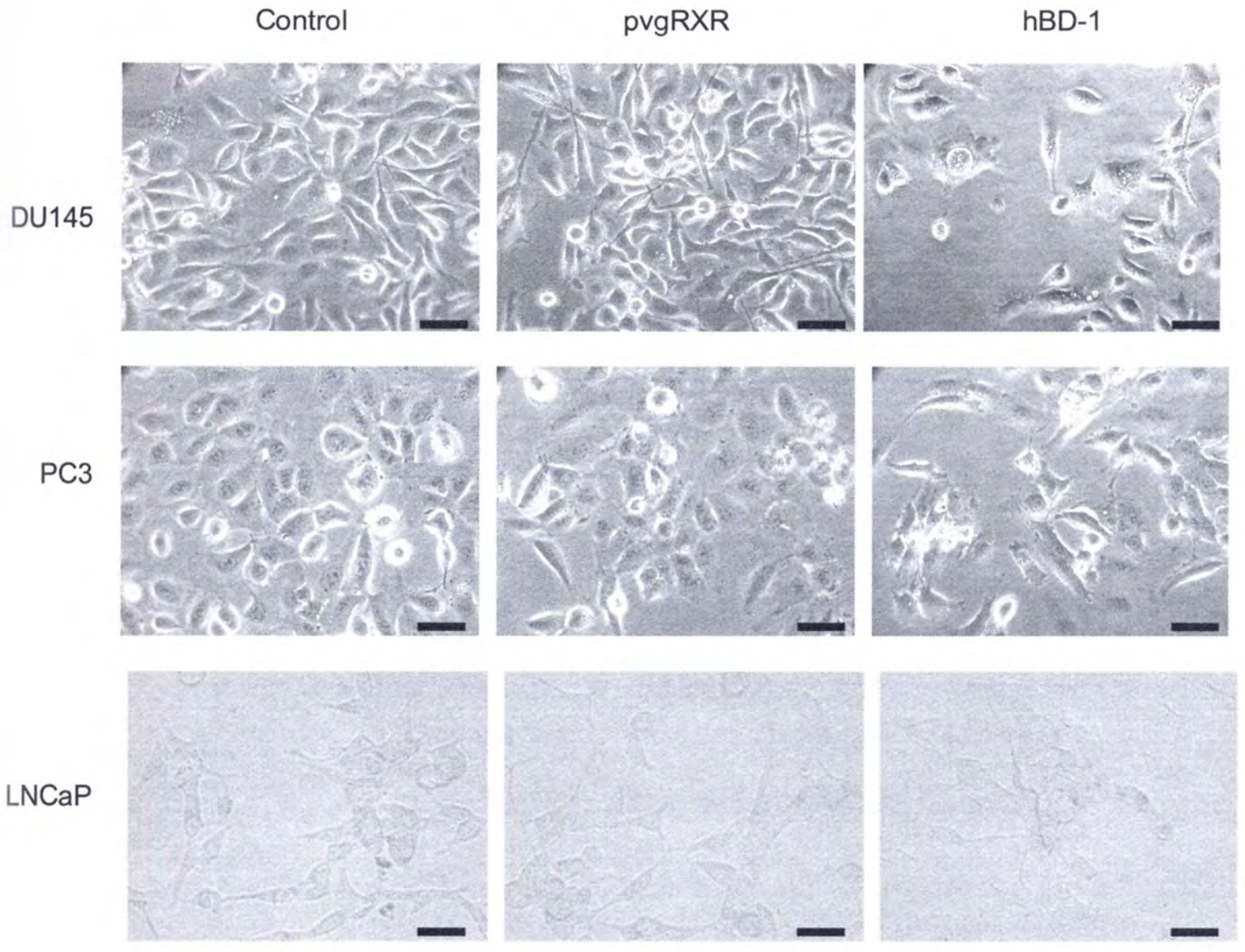


Figure 3-6: Analysis of hBD-1 on prostate cancer cells.

Figure 3-7: Analysis of hBD-1 effect on cell viability. The prostate cell lines DU145, PC3, PC3/AR+ and LNCaP were treated with Pon A to induce hBD-1 expression for 1–3 days after which MTT assay was performed to determine cell viability. Each bar represents the mean ± S.E.M. of three independent experiments performed in triplicate.

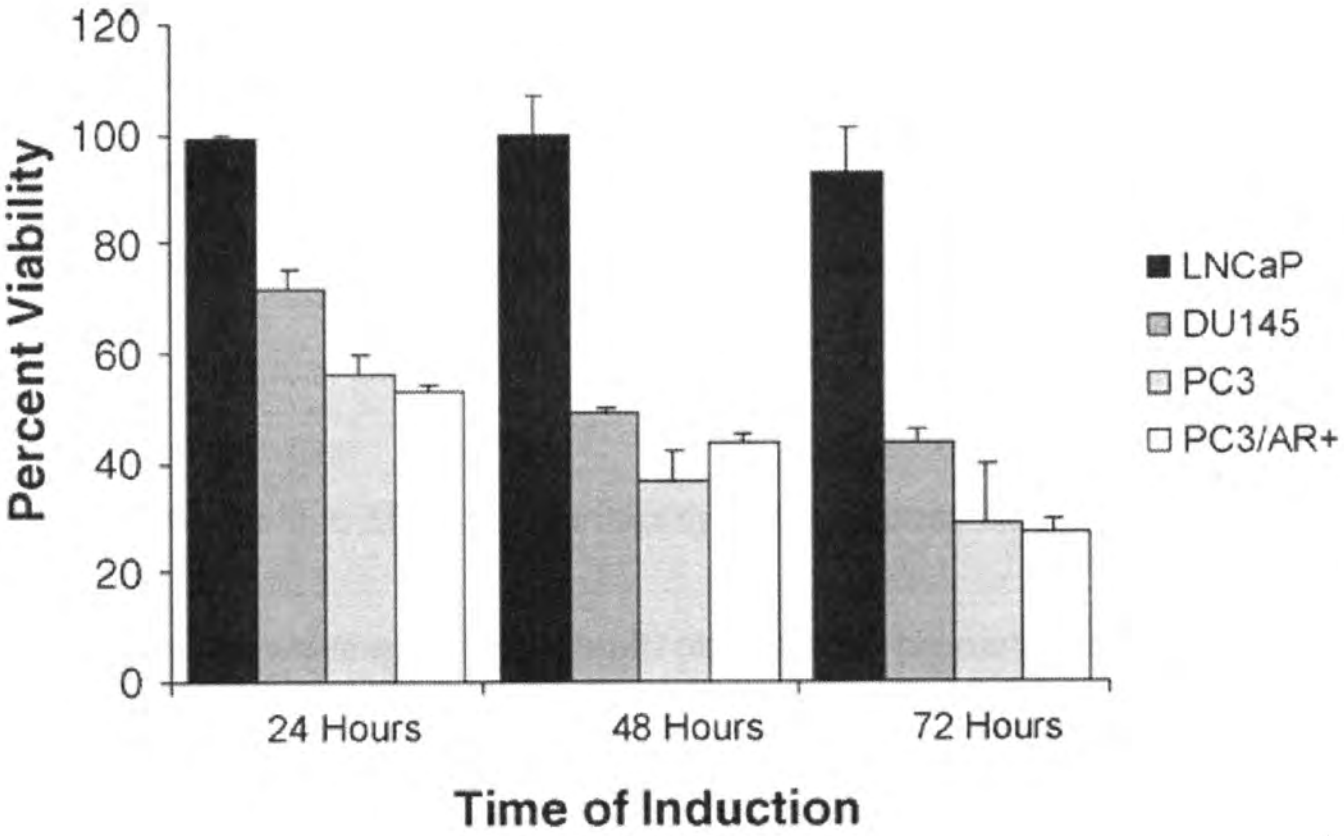


Figure 3-7: Analysis of hBD-1 effect on cell viability.

annexin V or PI (Figure 3-8). After inducing hBD-1 expression in PC3 cells, the number of cells undergoing early apoptosis and late apoptosis/necrosis (lower and upper right quadrants, respectively) totaled 10% at 12 hours, 20% at 24 hours, and 44% at 48 hours. For DU145 cells, the number of cells undergoing early apoptosis and late apoptosis/necrosis totaled 12% after 12 hours, 34% at 24 hours, and 59% after 48 hours of induction. No increase in apoptosis was observed in cells containing empty plasmid following induction with Pon A (data not shown). Annexin V and propidium iodide uptake studies have demonstrated that hBD-1 has cytotoxic activity against DU145 and PC3 prostate cancer cells and results indicate apoptosis as a mechanism of cell death.

hBD-1 Causes Alterations in Membrane Integrity and Caspase Activation

We investigated whether the cell death observed in prostate cancer cells after hBD-1 induction is caspase-mediated apoptosis. To better understand the cellular mechanisms involved in hBD-1 expression, confocal laser microscopic analysis was performed (Figure 3-9) on DU145, PC3, and LNCaP cells induced for hBD-1 expression. Pan-caspase activation was monitored based on the binding and cleavage of green fluorescing FAM-VAD-FMK to caspases in cells actively undergoing apoptosis. Analysis of cells under DIC showed the presence of viable control DU145 (a), PC3 (e), and LNCaP (i) cells at 0 hours. Excitation

Figure 3-8: Analysis of hBD-1 cytotoxicity in prostate cancer cells. Cells positive for propidium iodide and annexin V were considered apoptotic. Times of induction are shown under each panel. Numbers next to the boxes for each time point represent the percentages of propidium iodide (PI)- annexin V+ cells (lower right quadrant), and PI+ annexin V+ cells (upper right quadrant).

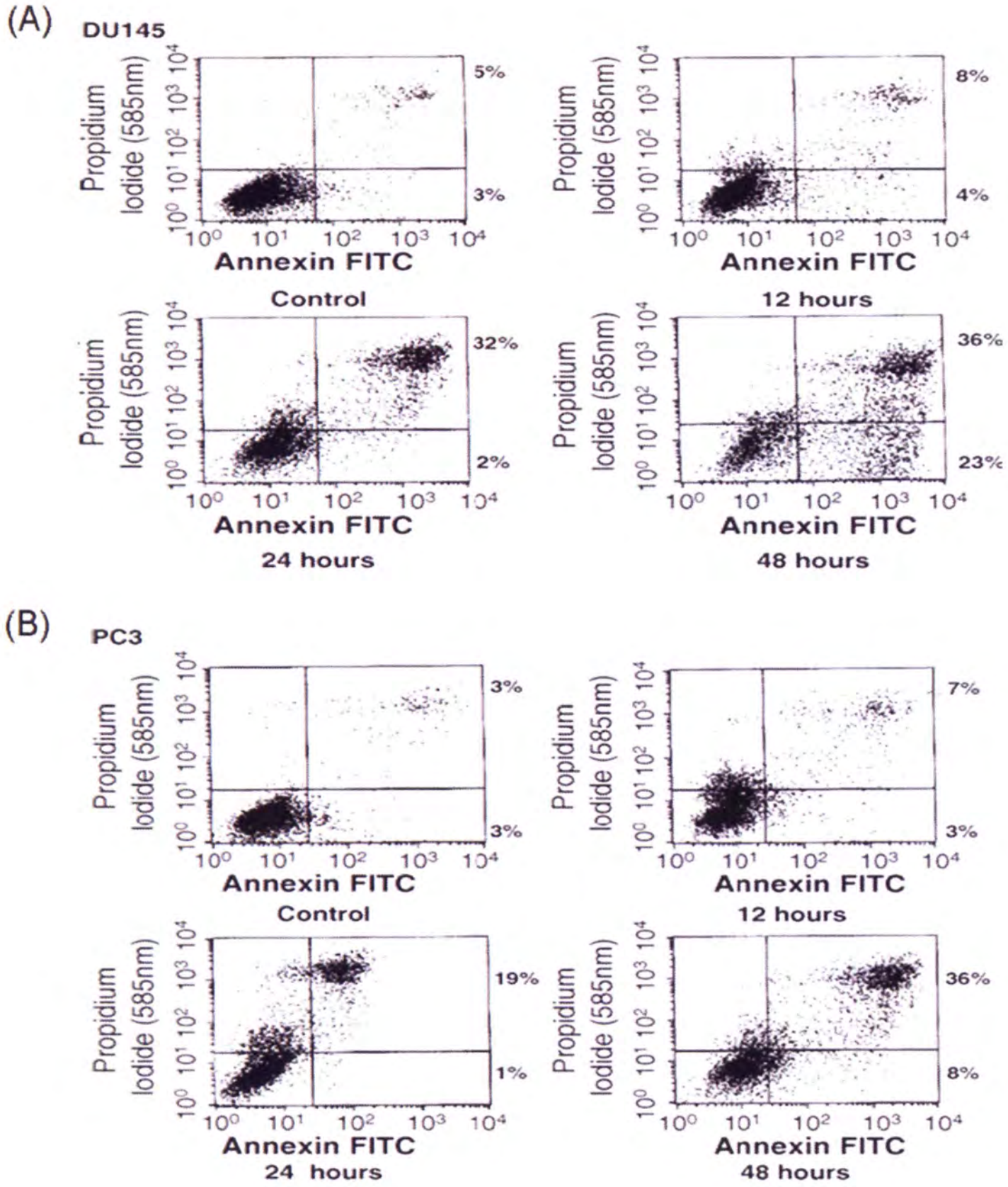


Figure 3-8: Analysis of hBD-1 cytotoxicity in prostate cancer cells.

Figure 3-9: Analysis of pan-caspase activation. Analysis of hBD-1 mediated cell death in DU145, PC3, and LNCaP prostate cancer cells. DIC visualization of cells before (a, e, and I, respectively) and after (c, g, and k, respectively) hBD-1 induction. Fluorescence analysis revealed no caspase activity in control cells (b, f, and j, respectively), however, cells treated with Pon A for 24 h to induce hBD-1 revealed caspase activity in DU145 and PC3 (d and h respectively), but not in LNCaP (l).

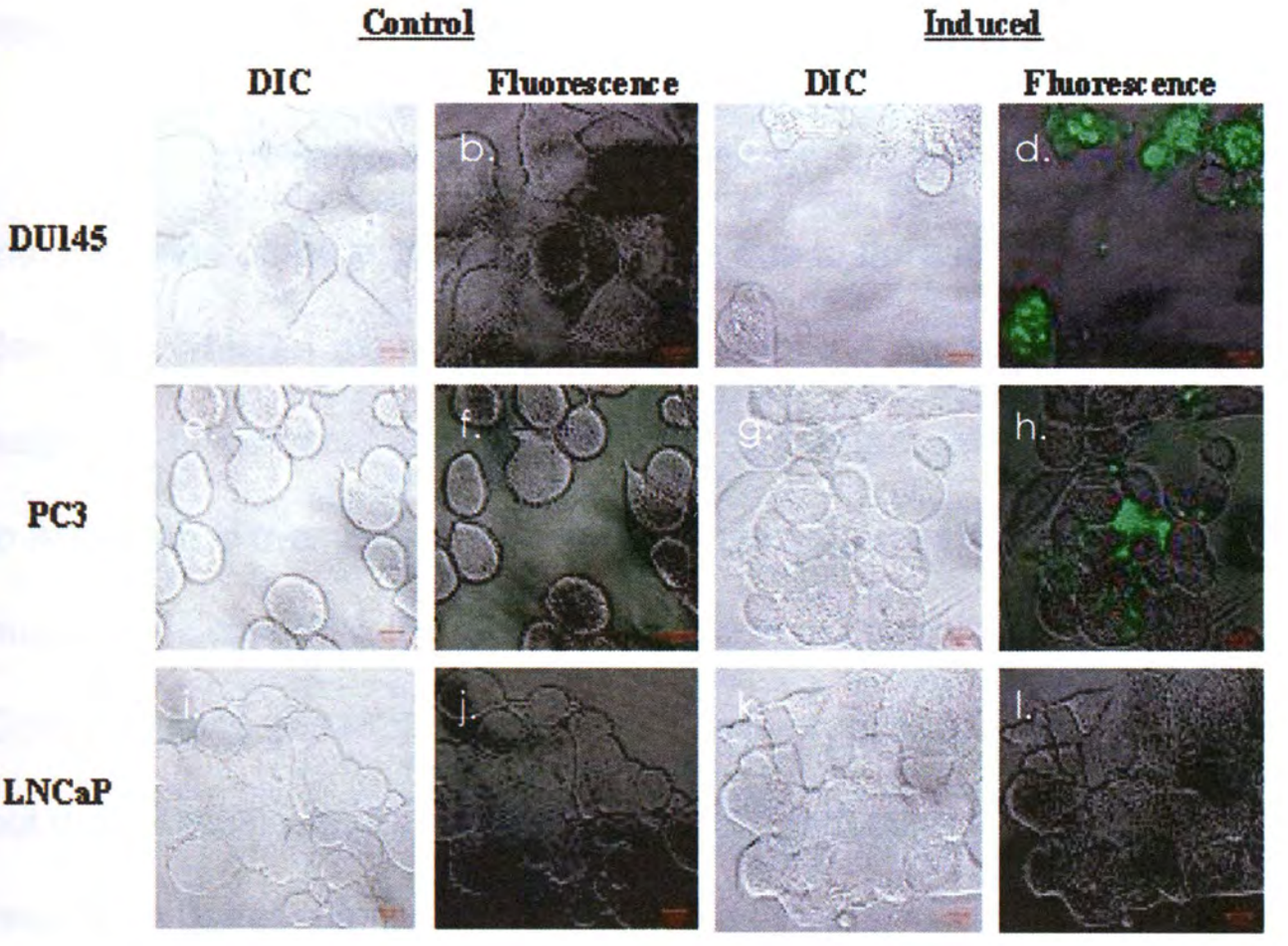


Figure 3-9: Analysis of pan-caspase activation.

by the confocal laser at 488 nm produced no detectable green staining which suggests no caspase activity in DU145 (b), PC3 (f), or LNCaP (j) control cells.

Following induction for 24 hours, DU145 (c.), PC3 (g.), and LNCaP (h.) cells were again visible under DIC. Confocal analysis under fluorescence revealed green staining in DU145 (d.) and PC3 (h.) cells indicating pan-caspase activity after the induction of hBD-1 expression. However, there was no green staining in LNCaP (l.) cells induced for hBD-1 expression. Therefore, cell death observed following induction of hBD-1 is caspase-mediated apoptosis.

The proposed mechanism of antimicrobial activity of defensin peptides is the disruption of the microbial membrane due to pore formation [33, 64]. In order to determine if hBD-1 expression altered membrane integrity EtBr uptake was examined by confocal analysis (Figure 3-10). Intact cells were stained green due to AO which is membrane permeable, while only cells with compromised plasma membranes stained red due to incorporation of membrane impermeable EtBr. Control DU145 and PC3 cells stained positively with AO and emitted green color, but did not stain with EtBr. However, hBD-1 induction in both DU145 and PC3 resulted in the accumulation of EtBr in the cytoplasm at 24 hours as indicated by the red staining. By 48 hours, DU145 and PC3 possessed condensed nuclei and appeared yellow due to the co-localization of green and red staining from AO and EtBr, respectively. Conversely, there were no observable alterations to membrane integrity in LNCaP cells after 48 hours of induction as indicated by positive green fluorescence with AO, but lack of red EtBr fluorescence. This finding suggests that alterations to membrane integrity and permeabilization in

Figure 3-10: Analysis of membrane integrity. Membrane integrity of DU145, PC3 and LNCaP was analyzed by confocal microscopy 24 and 48 h after hBD-1 induction. Green staining indicates the localization of AO, red staining represents EtBr and yellow staining represents the co-localization of both AO and EtBr. Size bars = 10 μM.

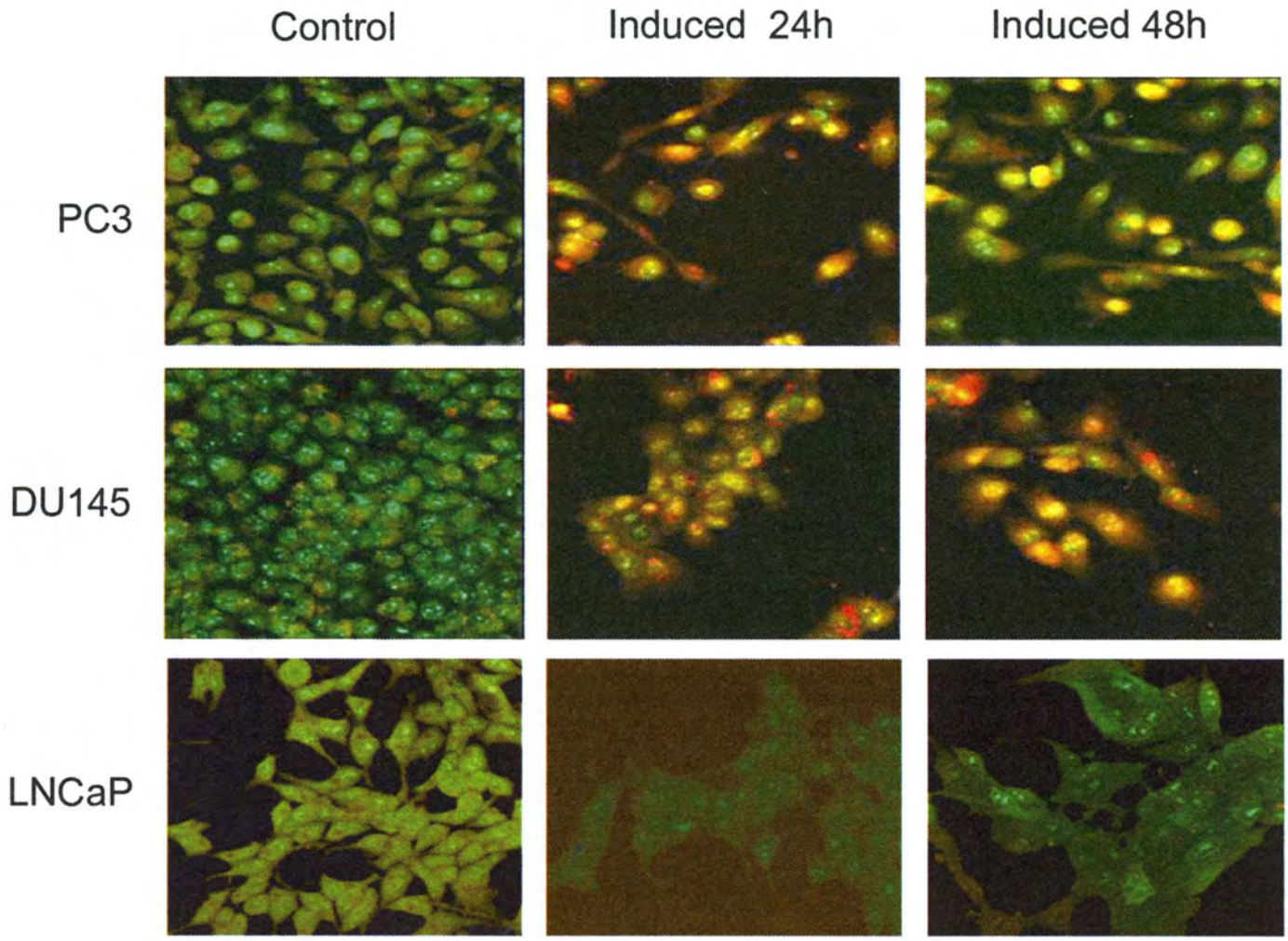


Figure 3-10: Analysis of membrane integrity.

response to hBD-1 expression differ between early- and late-stage prostate cancer cells.

hBD-1 Expression Correlates with cMYC and PAX2

QRT-PCR analysis was performed on LCM prostate tissue sections from three patients. Here, hBD-1 expression exhibited a 2.7-fold decrease from normal to PIN, a 3.5-fold decrease from PIN to tumor and a 9.3-fold decrease from normal to tumor in patient # 1457 (Figure 3-11). Likewise, we observed

cMYC expression followed a similar expression pattern in patient # 1457 where expression decreased by 1.7-fold from normal to PIN, 1.7-fold from PIN to tumor and 2.8-fold from normal to tumor. In addition, we saw a statistically significant decrease in cMYC expression in the other two patients. Patient # 1569 had a 2.3-fold decrease from normal to PIN, while in patient # 1586 we saw a 1.8-fold decrease from normal to PIN, a 4.3-fold decrease from PIN to tumor and a 7.9-fold decrease from normal to tumor. Conversely, PAX2 expression increased from normal to malignant tissue by 6.6-fold in patient # 1586. Although we did not observe a direct correlation of hBD-1 and PAX2 expression, our results indicate that both PAX2 and cMYC expression may influence hBD-1 levels.

Figure 3-11: QRT-PCR analysis of hBD-1 and cMYC expression in LCM human prostate tissue sections of normal, PIN, and tumor. Expression for each gene is presented as expression ratios compared to β-actin. (A) Comparison of hBD-1 expression levels in normal, PIN and tumor sections. (B) Comparison of cMYC expression level in normal, PIN and tumor sections.

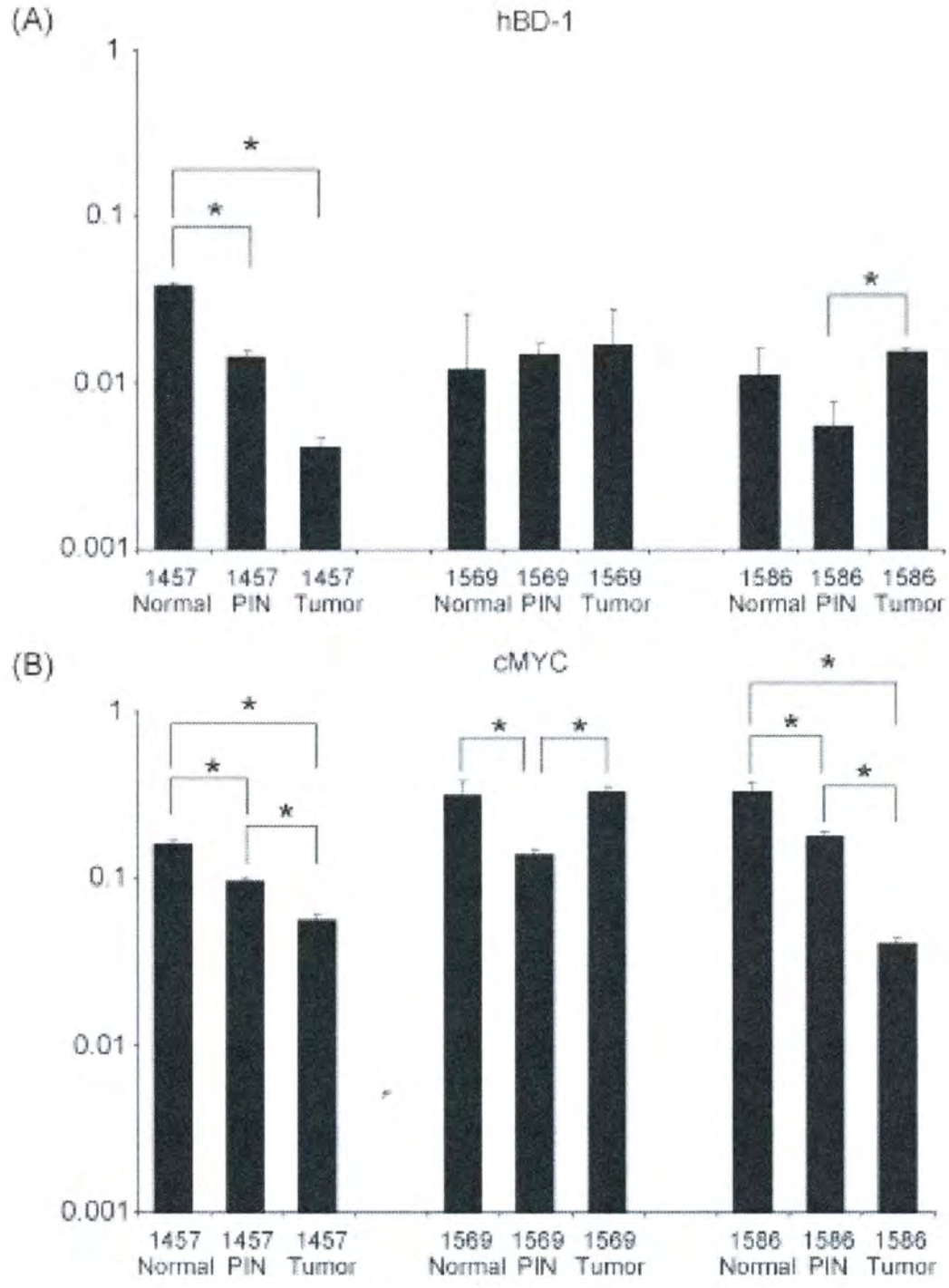


Figure 3-11: QRT-PCR analysis of hBD-1 and cMYC expression in LCM human prostate tissue sections of normal, PIN, and tumor.

Induction of hBD-1 Expression Following PAX2 Inhibition

To further examine the role of PAX2 in regulating hBD-1 expression, we utilized siRNA to knockdown PAX2 expression and performed QRT-PCR to monitor hBD-1 expression. Treatment of hPrEC cells with PAX2 siRNA exhibited no effect on hBD-1 expression (Figure 3-12). However, PAX2 knockdown resulted in a 42-fold increase in LNCaP, a 37-fold increase in PC3 and a 1026-fold increase in DU145 expression of hBD-1. As a negative control, cells were treated with non-specific siRNA which had no significant effect on hBD-1 expression (data not shown).

DISCUSSION

The identification of susceptibility genes and genetic alterations associated with prostate cancer has greatly enhanced our understanding of the disease [65]. However, the basic mechanism underlying prostate carcinogenesis is still poorly understood. Recent interest has focused on the role inflammation or infection in the progression of prostate cancer [65-67]. Members of the defensin family of peptides have an important role in host defense against bacterial, fungal and viral infections [68] and are considered a critical part of the initial response to a pathogen [35]. In contrast to other members of the defensin family, hBD-1 exhibits no activity against fungal and viral infections and only very weak antimicrobial activity. Given this, the exact role of hBD-1 expression in the epithelial cell remains unclear [69-71].

Figure 3-12: QRT-PCR analysis of hBD1 expression following PAX2 knockdown with siRNA. hBD-1 expression levels are presented as expression ratios compared to β-actin. An asterisk represents statistically higher expression levels compared to the cell line before PAX2 siRNA treatment (Student's *t*-test, $p < 0.05$).

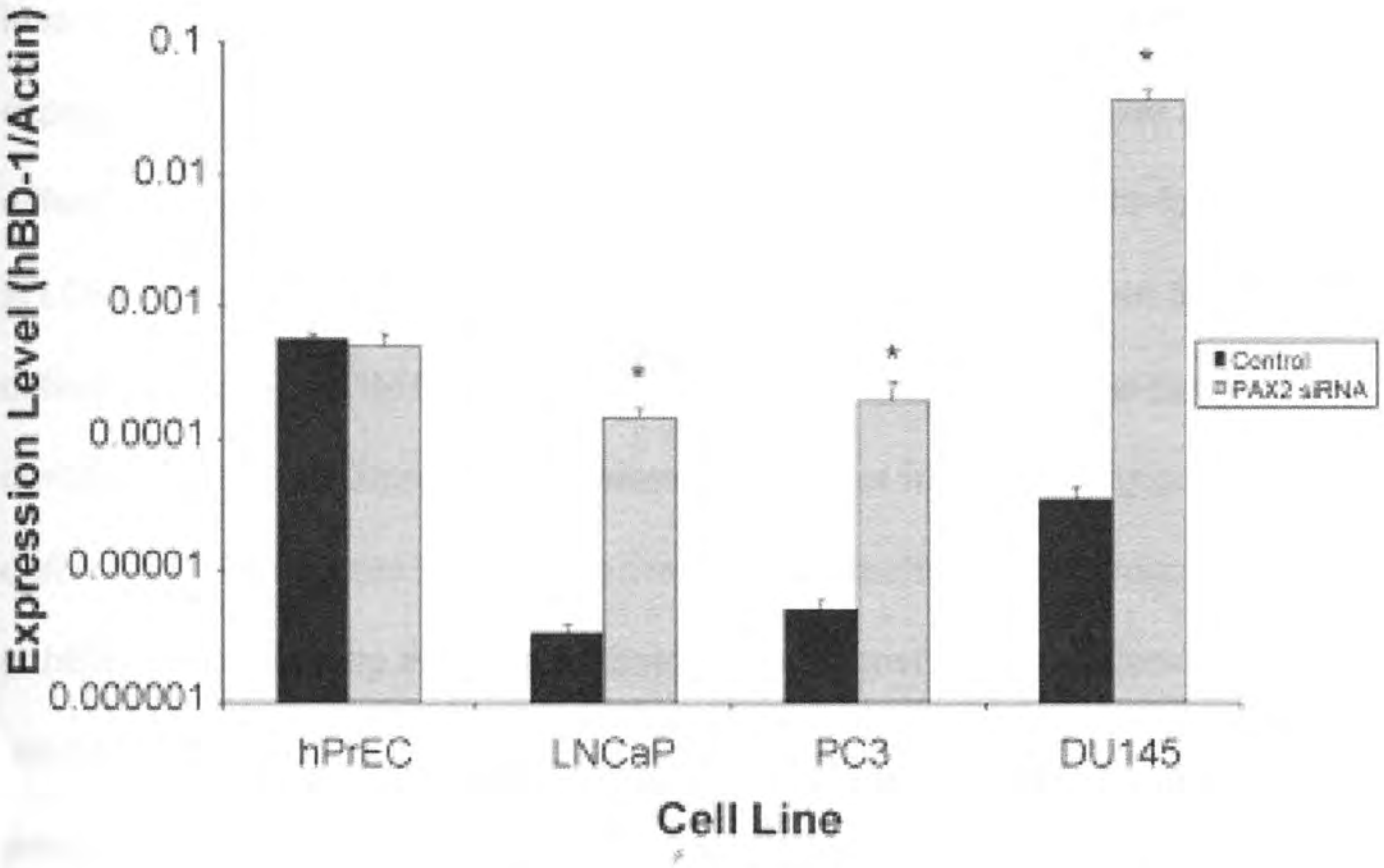


Figure 3-12: QRT-PCR analysis of hBD1 expression following PAX2 knockdown with siRNA.

Our previous studies have shown that hBD-1 expression is lost at a high frequency in prostate cancer[20]. However, little is known about its function in cancer or whether the loss of expression contributes to prostate tumor progression. There has been increasing interest in whether host defense peptides possess anti-cancer activity[137]. Here we show that hBD-1 is cytotoxic to prostate cancer cells. Overall, hBD-1 expression was 10- to almost 1000-fold less in grossly dissected tissues compared to LCM tissue samples. Furthermore, expression in benign prostate tissue adjacent to cancer varied over a range differing by 6.6-fold difference in gross dissected tissues and a 32-fold difference in LCM derived tissue. In this, we found that patient #1343 had an 854-fold difference in detectable hBD-1 expression when comparing gross and LCM derived tissue. The increase in detectable levels of hBD-1 from gross to LCM derived tissue indicates LCM is a more sensitive technique to analyze expression of hBD-1. Additionally analysis indicated that in most cases, patient samples with higher hBD-1 expression levels had a Gleason score of 6, while those with less expression were diagnosed with cancers having Gleason score of 7. Although LCM derived tissue appeared to display the trend of higher hBD-1 expression and lower Gleason score, other factors such as degree of inflammation within the tissue were not evaluated in this study. In addition, we have preliminary data from a previous long-term patient follow-up which suggests that those with little to no hBD1 expression experienced higher cancer recurrence rates compared to those with higher expression levels. Taken

together, these results suggest that decreased or loss of hBD-1 expression and its subsequent anti-cancer activity may contribute to prostate tumor progression.

In 2006, Sherman et al. published data indicating that hBD-1 is upregulated in response to BSA treatment and the pathway involved may be regulated by the c-Myc proto-oncogene[137, 138]. C-myc has also been shown to sensitize tumor cells to various apoptotic stimuli[139]. One of these pathways involves the permeabilization of the cellular and mitochondrial membranes, and the release of cytochrome C. Therefore, c-Myc must act to potentiate the ability of an additional factor to induce permeabilization and cytochrome C release. However, in cancer cells c-Myc-induced release of cytochrome C is suppressed by cell survival factors, which subsequently exacerbates c-Myc oncogenicity.

Given that all potential pro-apoptotic actions of c-Myc involve its functional integrity as a transcription factor, it is possible that c-Myc modulates target genes that encode proteins which can permeabilize the mitochondrial membrane. Here we show that hBD-1 causes rapid cell death in late-stage prostate cancer cells via the disruption of cell membrane potential and caspase activation. Furthermore, we found a positive correlation between cMyc and hBD-1 in one of three prostate tissue samples, where tissue exhibiting decreased cMyc expression also possessed a decreased hBD-1 level. This suggests that the loss of hBD1 observed in prostate cancer may be due to the down-regulation of cMyc expression or by the up-regulation of transcription factors acting as pro-survival factors, therefore preventing the triggering of apoptosis, and ultimately promoting tumor progression.

Paired box 2, PAX2, is a member of the PAX gene family of transcriptional regulators that functions early in the development of the urogenital system[140]. Although PAX2 is typically repressed upon terminal differentiation, in urogenital cancers including prostate cancer it has been shown to be overexpressed[141, 142]. In addition, studies have demonstrated that PAX2 expression can stimulate proliferation stimulus, thus serving as a critical component of a multi-step oncogenic transformation process[143]. Therefore, PAX2 may offer an advantage for the survival and growth of cancer cells.

PAX genes function to activate or repress transcription by transactivating the promoters of target genes that regulate cell growth and apoptosis[144]. Unique recognition sequences are contained within the paired domain and activating domains in PAX2 that aid to activate or repress the transcription of specific genes[145, 146]. Examination of the hBD-1 promoter revealed a PAX2 recognition sequence between bases-172 and -157 upstream of the TATA box (data not shown). Here we demonstrate a 6.6-fold increase from normal to tumor in PAX2 expression in patient # 1586. Although we did not observe a direct correlation with hBD-1 and PAX2 expression in the patient samples tested, we did demonstrate that suppression of PAX2 protein expression by siRNA results in the re-expression of hBD1. These findings suggest that PAX2 is a transcriptional repressor of hBD-1 expression although this was not examined in this study. Taken together, it is plausible that the delicate balance of the regulation of hBD-1 expression may play a key role in determining the fate of cancer cells and tumor progression.

In summary, data presented here demonstrate that hBD-1 expression is highly cytotoxic to DU145 and PC3 which represent AR negative, hormone refractory late-stage prostate cancer. These findings suggest that hBD-1 has anti-cancer and/or tumor suppressive activities and that its suppression or loss contributes to cancer cell survival and tumor progression. Cytotoxic effects of hBD-1 observed in DU145 and PC3, but not in LNCaP suggest that hBD-1 specifically affects late-stage prostate cancer cells. This reasoning is further supported by the high frequency of cancer-specific loss of hBD-1 expression, and that hBD-1 loss of expression may influence Gleason grading and cancer recurrence. Therefore, hBD-1 may be an integral component of the innate immune system involved in the recognition and destruction of cancer cells. In conclusion, our studies are the first to demonstrate hBD-1 functionality in prostate cancer progression, thus offering hBD-1 as a viable therapeutic agent for the treatment of late-stage prostate cancer.

CHAPTER 4

CELLULAR MECHANISMS ASSOCIATED WITH THE EFFECTS OF HBD-1 ON PROSTATE CANCER CELLS

Apoptosis is a mechanistic process whereby the activation of cysteine-aspartate proteases (caspases) results in programmed cell death that allows multicellular organisms to regulate cell number within tissues. It is characterized by stereotypical morphological changes, such as chromatin condensation and fragmentation, cell shrinkage, and alterations of the plasma membrane. In part, apoptosis is regulated by the Bcl-2 family of proteins[147]. There are two pathways of apoptosis, an extrinsic pathway involving ligand activated cell death receptors and an intrinsic pathway involving mitochondrial alterations initiated by a variety of stress signals[141]. The extrinsic pathway involves the recruitment of adapter proteins that lead to the activation of caspase 8 and the initiation of the caspase cascade. Activated caspase 8 catalyzes the cleavage of BID leading to the translocation of tBID to the mitochondria and subsequent cell death. In the intrinsic pathway, the release of cytochrome c from the mitochondria, in conjunction with APAF-1, results in the formation of the apoptosome. This aids in the cleavage of procaspase-9 in the presence of ATP generating an active caspase 9, which, in turn, activates the effector caspases. The extrinsic and intrinsic pathways merge at caspase 3 activation to complete the apoptotic process. Ultimately, the process results in the morphological and biochemical changes characteristic of apoptotic death.

Apoptosis is an important and physiologically normal course of action for multicellular organisms[148]. It is accepted that the growth and progression of a tumor is regulated by deregulation of the balance between proliferation and

apoptosis[149, 150]. The ability of a cell to evade apoptosis is an important characteristic that defines the transition from a normal cell to malignant[151]. Further study into the molecular mechanisms underlying the process could lead to the development of possible preventative strategies.

In prostate cancer, mortality is a consequence of metastasis to the lymph nodes and bone, as well as a progression from androgen-dependent to androgen-independent growth[152]. There are limited treatment options beyond localized prostate cancer due to the ineffective nature of chemotherapy to increase patient survival in metastatic prostate cancer[153]. Although the accumulation of genetic alterations and environmental insults contributes to the development and progression of prostate cancer, the exact molecular mechanisms underlying these processes are unknown. An important pathophysiological response that appears to contribute to prostate cancer involves the inhibition of apoptosis. Therefore, identification of potential therapeutic targets that manipulate the apoptotic process may serve as alternative treatment methods leading to a decrease in prostate cancer mortality rates.

MATERIALS AND METHODS

Specific Caspase Activity

Specific caspase activity in the prostate cancer cell lines was detected using the Carboxyfluorescein or Sulforhodamine FLICA Apoptosis Detection Kit Caspase Assay (Immunochemistry Technologies, LLC, Bloomington, MN).

Caspase 8 was detected with a FAM-LETD-FMK peptide, caspase 9 with a FAM-LEHD-FMK peptide, and caspases 3 & 7 with a SR-DEVD-FMK peptide that irreversibly binds to active caspases. Briefly, 1.5-3x10^5 DU145, LNCaP and PC3 cells were plated on 35-mm glass bottom dishes (MATEK, Ashland, MA) and were transfected with the hBD-1 expression system. As previously described, cells were exposed to media only or media containing Pon A for 24-48 hours. Next, 10 ul of a 30X working dilution of the appropriate fluoromethyl ketone labeled peptide was added to 300 ul of media and incubated with each 35-mm dish for 1 hour at 37°C under 5% CO_2. Then, the medium was aspirated and the cells were washed twice with 2 ml of 1X working dilution Wash buffer. Cells were viewed under differential interference contrast (DIC) or laser excitation at 488 nm. The fluorescent signal and DIC images were analyzed using a confocal microscope (Zeiss LSM 5 Pascal) and 63X oil lens with a Vario 2 RGB Laser Scanning Module. Images were collected and processed as previously mentioned.

RNA Isolation and Quantitative RT-PCR

QRT-PCR was performed as previously described[135]. Briefly, total RNA (0.5 µg per reaction) from both sources was reverse transcribed into cDNA utilizing random primers (Promega). Two-step QRT-PCR was performed on cDNA generated using the MultiScribe Reverse Transcriptase from the TaqMan Reverse Transcription System and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primer pairs for AKT, BAD, and BID were

designed from the published sequences (Table 2). Forty cycles of PCR were performed under standard conditions using an annealing temperature of 58° C. In addition, GAPDH (Table 2) was amplified as a housekeeping gene to normalize the initial content of total cDNA. Gene expression in benign prostate tissue samples was calculated as the expression ratio compared to GAPDH. Relative expression was calculated by comparing mRNA levels in prostate cancer cell lines after being induced for hBD-1 expression to the level of mRNA expression in control cells. In all cases, the dissociation curves were analyzed and matched for samples used to generate data. As a negative control, QRT-PCR reactions without cDNA template were also performed. All reactions were run a minimum of three times.

Western Blot Analysis

Protein levels were analyzed by western blot analysis. Briefly, 5-7.5 $x10^5$ cells were plated on 100mm dishes and transfected with the appropriate amount of pvgRXR vector or pIND/hBD-1 plasmid. Lysates were collected following 20h of induction with Pon A. Samples were prepared for a triple load: 75 μl sample, 15 μl sample buffer, and 1.5 μl 1M DTT. After incubation at 70°C for 10 min, the samples were loaded onto a 4-12% NuPAGE gel and run for 50 min at 200V with MES running buffer containing anti-oxidant (Invitrogen). Transfer was performed at 30V for 1 hr (one gel/power supply) or 1.5 hrs (two gels/power supply). The nitrocellulose was blocked in 5% milk in TBS-T for a minimum of 30 min at RT. Incubation O/N at 4°C in 5% milk/TBS-T with a primary antibody concentration of

1:1000 for anti-caspase 3, anti-caspase 8, anti-caspase 9, Bid, Bad, AKT, pAKT, and β-actin (Cell Signaling, Beverly, MA) was followed with 3x10 min washes with TBS-T. Then, the blots were incubated for 1-2 hrs at RT in 5% milk-TBS-T with secondary antibody (1:5000 mouse, 1:50000 rabbit), washed 3x10 min TBS-T and incubated with chemiluminescent reagent prior to development.

RESULTS

hBD-1 Causes Caspase Activation

We have shown that the induction of hBD-1 expression in DU145 and PC3 prostate cancer cell lines results in the activation of caspases, while there are no active caspases observed in LNCaP. To better understand the molecular mechanisms involved in caspase-mediated cell death, confocal microscopic analysis was performed. In order to delineate the apoptotic response in cells induced for hBD-1 expression, caspase 8 from the extrinsic pathway, caspase 9 for the intrinsic pathway and caspase 3 where the two pathways converge were monitored with a FAM-FLICA or SR-FLICA kit that reacts with the active caspase within minutes.

Analysis of caspase 8 activation with FAM-LETD-FMK, a fluorescent green probe, demonstrated no detectable staining in DU145, PC3 or LNCaP control cells (Figure4-1). However, confocal analysis of DU145 and PC3 cells induced for hBD-1 expression revealed green staining indicative of caspase 8 activation. Similarly, specific activation of caspase 9 was monitored with FAM-

Figure 4-1: Analysis of caspase 8 activation. Fluorescence analysis revealed no caspase activation in DU145, PC3, or LNCaP control cells. However, caspase 8 activation was observed in DU145 and PC3 induced for hBD-1 expression.

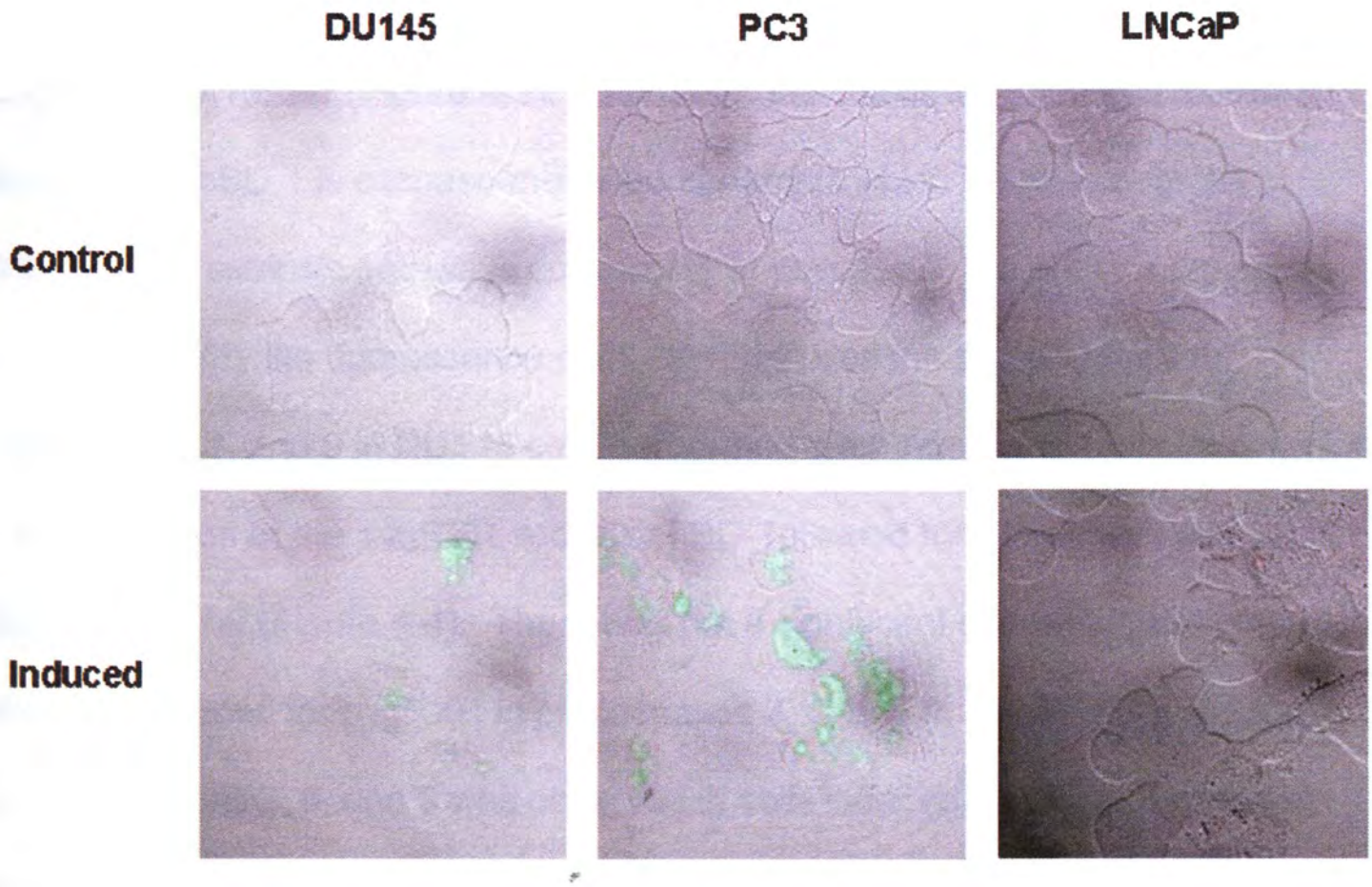


Figure 4-1: Analysis of caspase 8 activation.

LEHD-FMK, a green fluorescent probe. Again, the analysis of control cells produced no detectable green fluorescence, while DU145 and PC3 cells induced for hBD-1 expression revealed the green fluorescence indicating the presence of active caspase 9 (Figure 4-2). The activation of caspase 3 was monitored with SR-DEVD-FMK, a fluorescent red probe, in DU145, PC3 and LNCaP prostate cancer cell lines. There was no detectable staining in control cells, however, red fluorescence was observed in DU145 and PC3 cells following induction for the expression of hBD-1 (Figure 4-3). Therefore, the cell death observed following induction of hBD-1 is caspase-mediated apoptosis which involves both the intrinsic and extrinsic pathways converging at caspase 3, the effector caspase.

To verify the fluorescence data, we monitored the protein levels of caspases 3, 8, and 9 in DU145 cells before and after induction. Cells induced for the expression of the pvgRXR vector or hBD-1plasmid for 24hrs were analyzed by western blot (Figure 4-4). There was not a significant decrease from control in the cells induced for pvgRXR in procaspases 3, 8, and 9. However, a reduction in procaspases 3, 8, and 9 was observed in cells induced for hBD-1 expression. Additionally, the fragments that result from the cleavage of procaspase 3 and 9 into the active forms were shown. ImageJ analysis verified the decrease in procaspase 3, 8, and 9 levels observed by western blot (data not shown).

Figure 4-2: Analysis of caspase 9 activation. Fluorescence analysis revealed no caspase activation in DU145, PC3, or LNCaP control cells. However, caspase 9 activation was observed in DU145 and PC3 induced for hBD-1 expression.

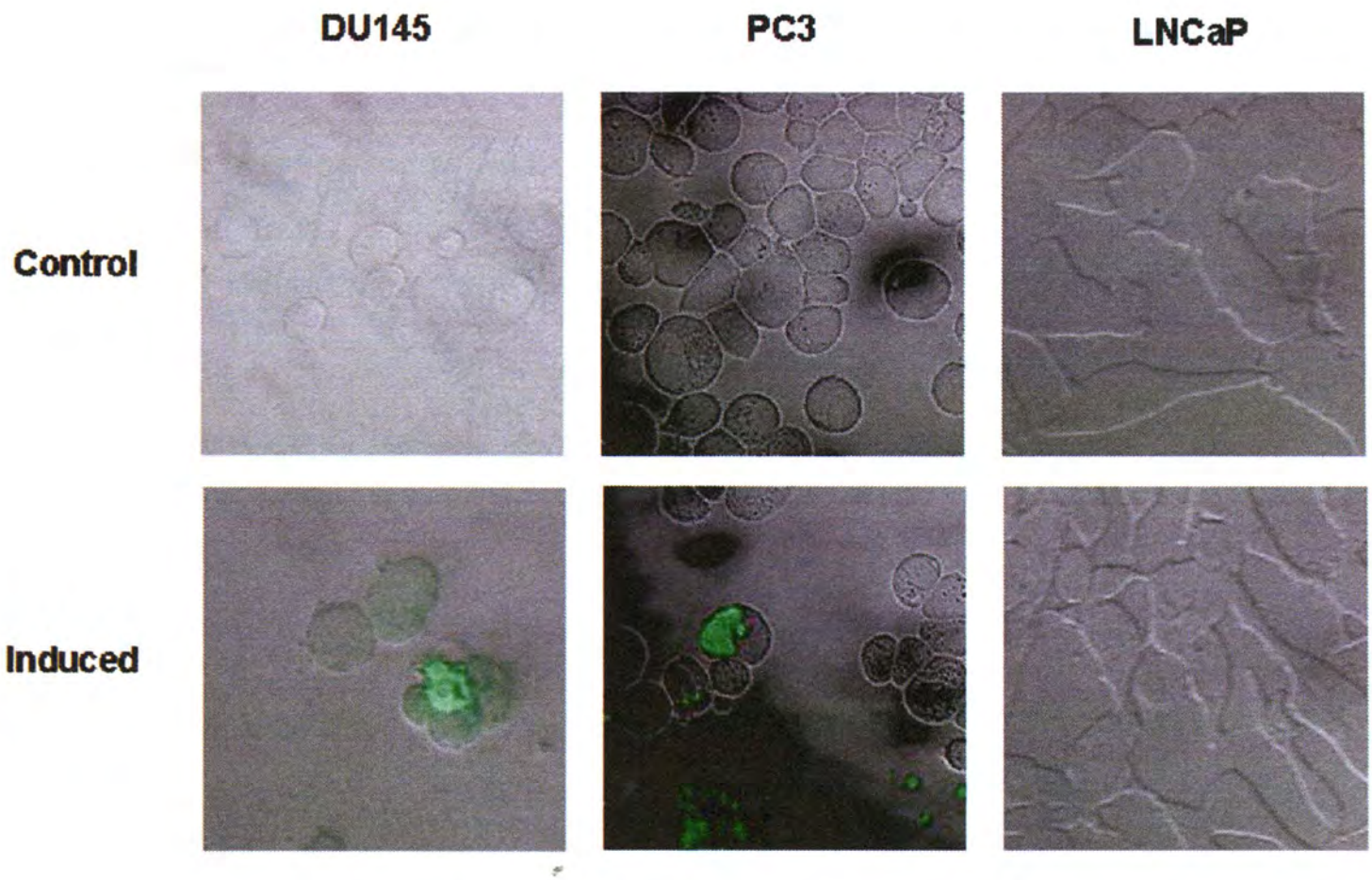


Figure 4-2: Analysis of caspase 9 activation.

Figure 4-3: Analysis of caspase 3 activation. Fluorescence analysis revealed no caspase activation in DU145, PC3, or LNCaP control cells. However, caspase 3 activation was observed in DU145 and PC3 induced for hBD-1 expression.

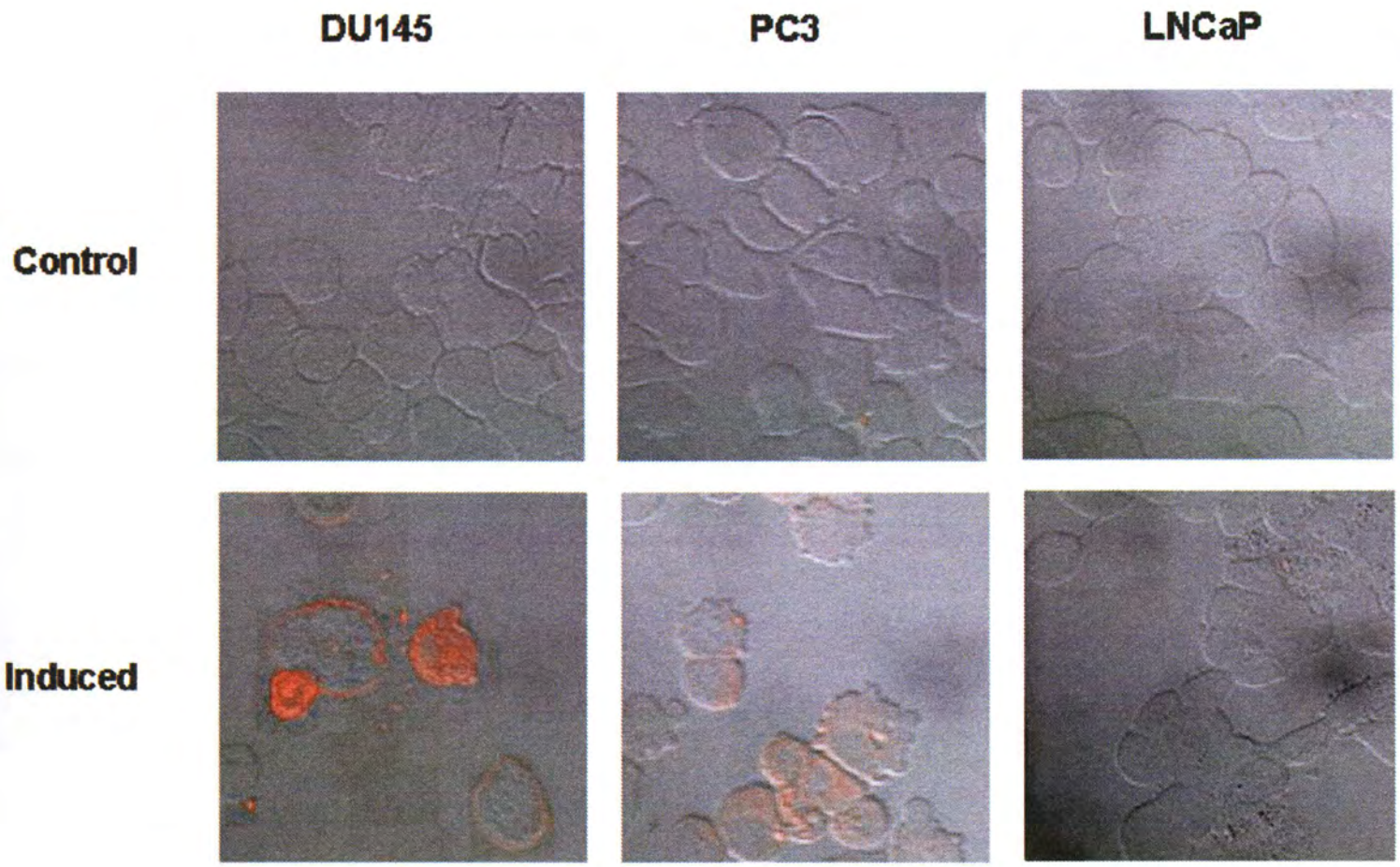


Figure 4-3: Analysis of caspase 3 activation.

Figure 4-4: Western blot analysis of caspase activation. Protein from DU145 cells was collected to perform a western blot analysis following induction for hBD-1 expression.

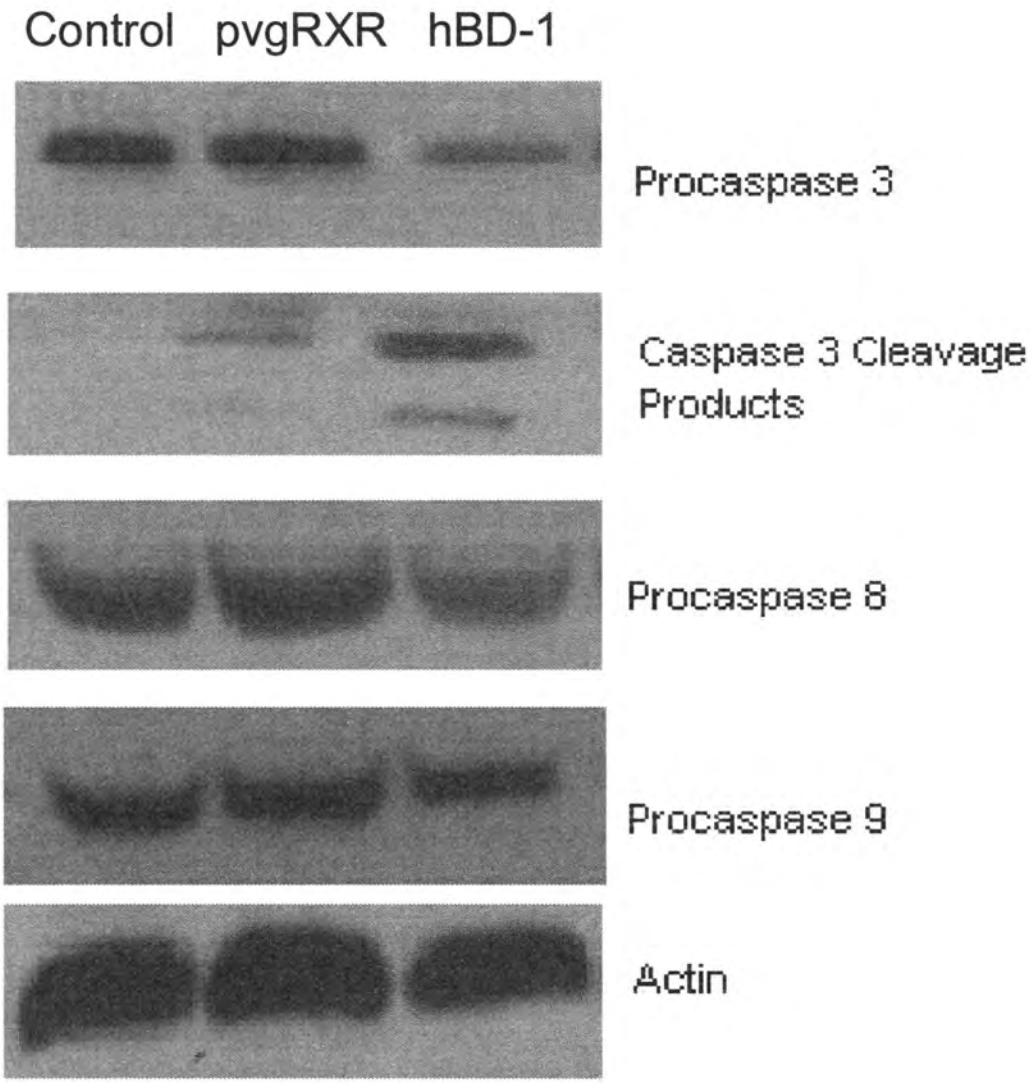


Figure 4-4: Western blot analysis of caspase activation.

Regulators of Apoptosis following hBD-1 expression

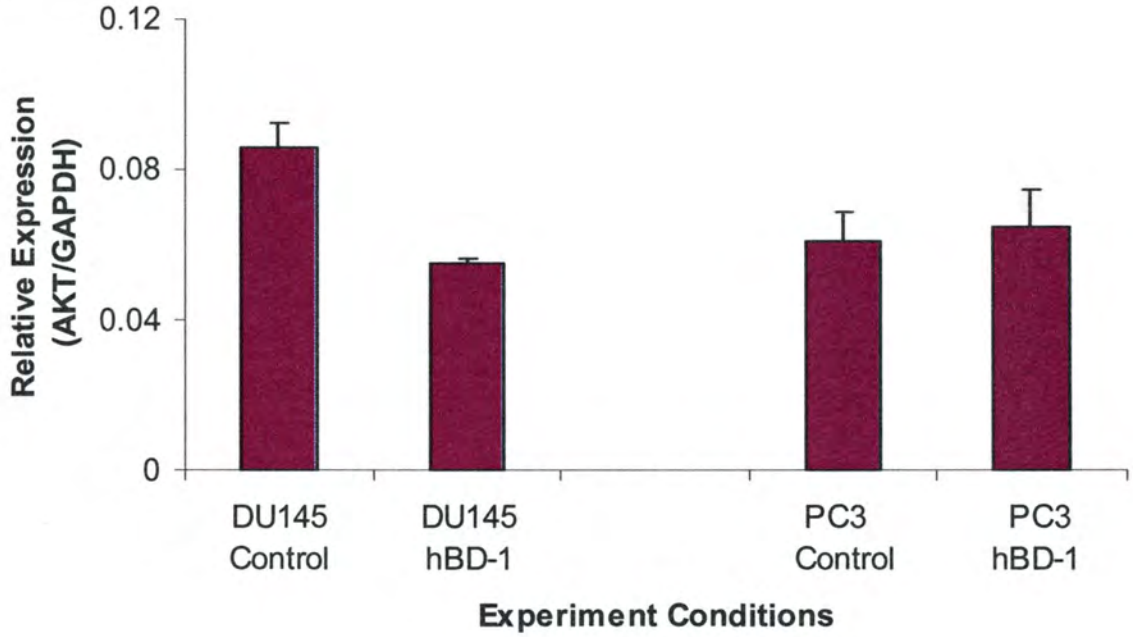
Several regulators of apoptosis are important to the activation or suppression of cell death. Akt, an important pro-survival kinase, is involved in protecting cells from apoptosis. Stimulation with growth factors leads to Akt recruitment to the plasma membrane and phosphorylation at two key regulatory sites, Thr^{308} and Ser^{473}, by 3-phosphoinositide-dependent protein kinase-1 (PDK1)[154]. Analysis of the effect of hBD-1 expression on Akt mRNA levels revealed a decrease in DU145 and no change in PC3, while protein analysis in DU145 demonstrated no change in Akt levels and a slight decrease in pAkt (Figure 4-5).

One important substrate of Akt is Bad, a BH3-domain protein which if phosphorylated becomes sequestered to the cytoplasm and prevents it from translocating to the mitochondrion where it exerts its pro-apoptotic action[155]. An increase in Bad mRNA levels was observed in both DU145 and PC3 following the induction of hBD-1 (Figure 4-6). Subsequent analysis of Bad protein levels demonstrated a decrease in hBD-1 induced cells from those of control DU145. At first, the observed decrease in Bad protein levels suggests that there is less apoptosis. However, it is important to note that the RNA and protein isolates were collected at the 24h time point. Since an increase in transcript levels was observed, additional later time points may have demonstrated the expected increase in Bad protein levels.

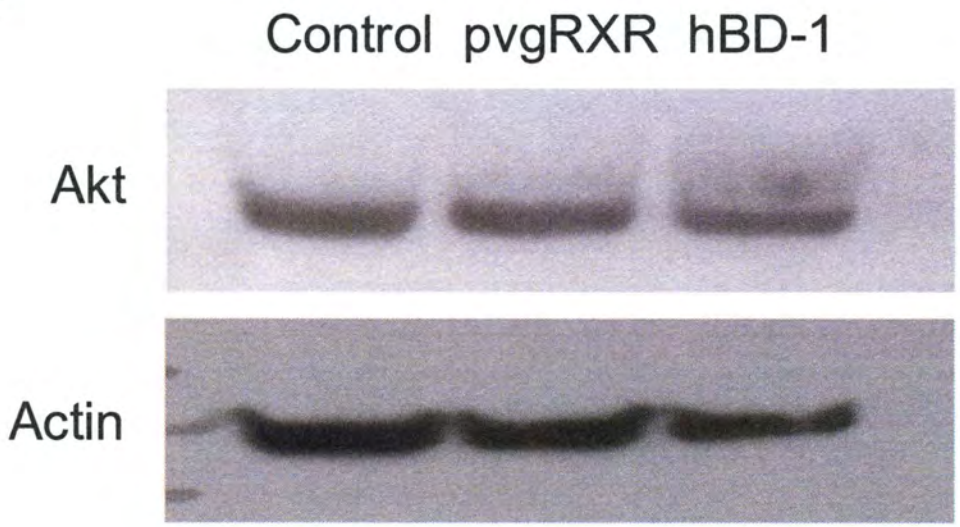
The final pro-apoptotic factor that was analyzed, Bid, is cleaved by activated caspase 8 into a truncated BH-3 only domain form known as tBid. It

Figure 4-5: Effect of hBD-1 on Akt and pAkt expression. (A) Akt mRNA expression in DU145 and PC3 before and after induction for hBD-1 expression. (B) Akt protein expression in DU145. (C) pAkt protein expression in DU145.

(A)



(B)



(C)

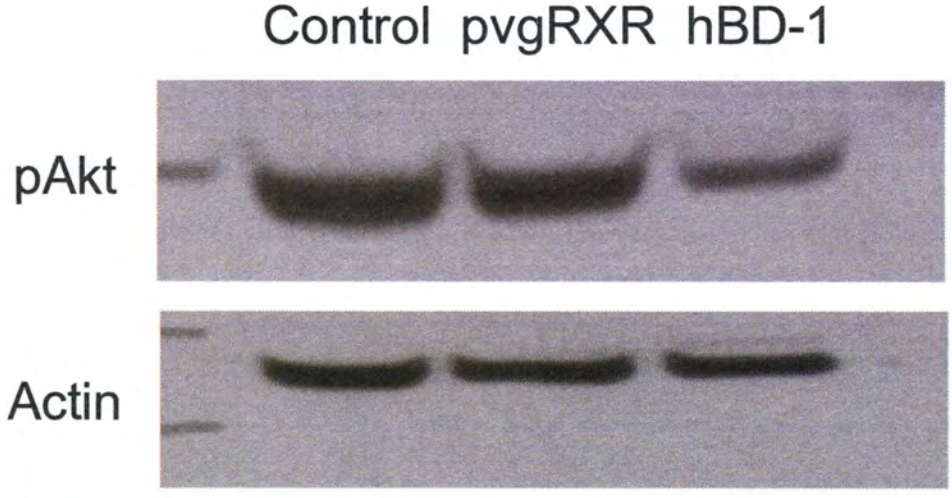
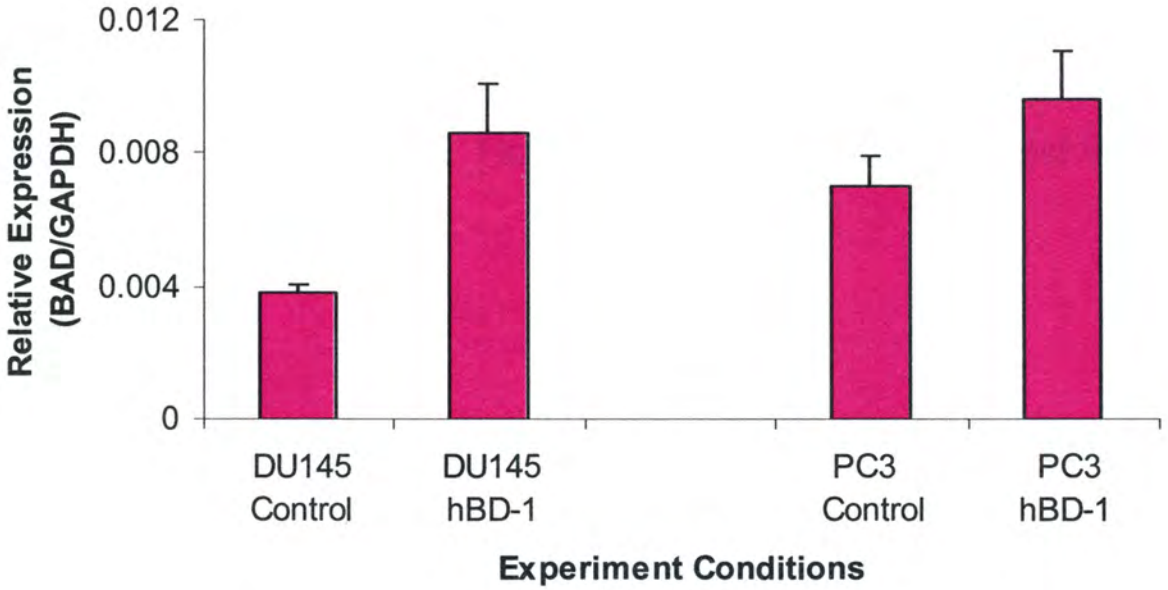


Figure 4-5: Effect of hBD-1 on AKT and pAKT expression.

Figure 4-6: Effect of hBD-1 on Bad expression. (A) mRNA expression in DU145 and PC3 before and after induction for hBD-1 expression. (B) Protein expression in DU145: lane 1-control cells, lane 2- cells induced for pvgRXR vector expression, lane 3- cells induced for hBD-1 expression.

(A)



(B)

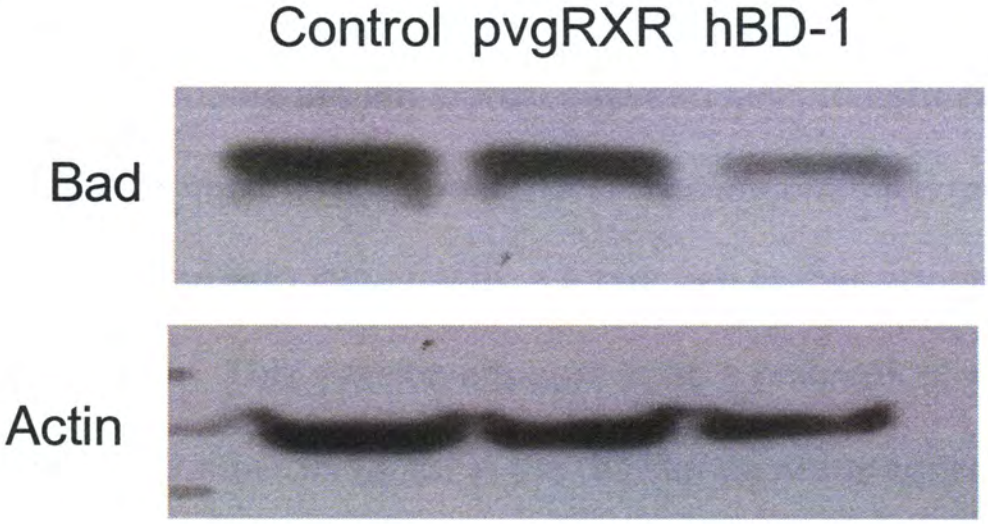


Figure 4-6: Effect of hBD-1 on Bad expression.

then translocates to the mitochondria where it contributes to the caspase cascade by causing the release of cytochrome c[156]. Analysis of Bid mRNA levels showed a decrease in both DU145 and PC3 cells following induction of hBD-1 (Figure 4-7). In addition, a decrease in Bid protein level was observed in DU145 cells induced for hBD-1 expression as compared to control cells. The observed decrease in protein levels of Bid correlates with the truncation of Bid to tBid prior to translocation to the mitochondria. However, we did not monitor tBid in this study.

DISCUSSION

Several lines of evidence indicate the transformation of normal cells into malignant derivatives in humans is a multi-step process. This creates a challenge in the search for the origin, prevention, and treatment of cancer due to the complexity. It appears the imbalance between cell proliferation and attrition in a tumor creates a microenvironment that favors uncontrolled growth within a tissue. In combination with this growth, a tumor cell eludes apoptotic destruction in an effort to survive. This evasion appears to be a hallmark of many malignant neoplasms and a contributory factor in the resistance many aggressive cancers demonstrate[149, 157, 158].

Recently there has been an emergence of data implicating β-defensins in mammalian cell apoptosis. In 2006, Sun et al. published data indicating that the addition of exogenous synthetic hBD-1 diminutively inhibited the proliferation of tumor cells, while the endogenous overexpression induced apoptosis[57]. The

Figure 4-7: Effect of hBD-1 on Bid expression. (A) mRNA expression in DU145 and PC3 before and after induction for hBD-1 expression. (B) Protein expression in DU145: lane 1-control cells, lane 2- cells induced for pvgRXR vector expression, lane 3- cells induced for hBD-1 expression.

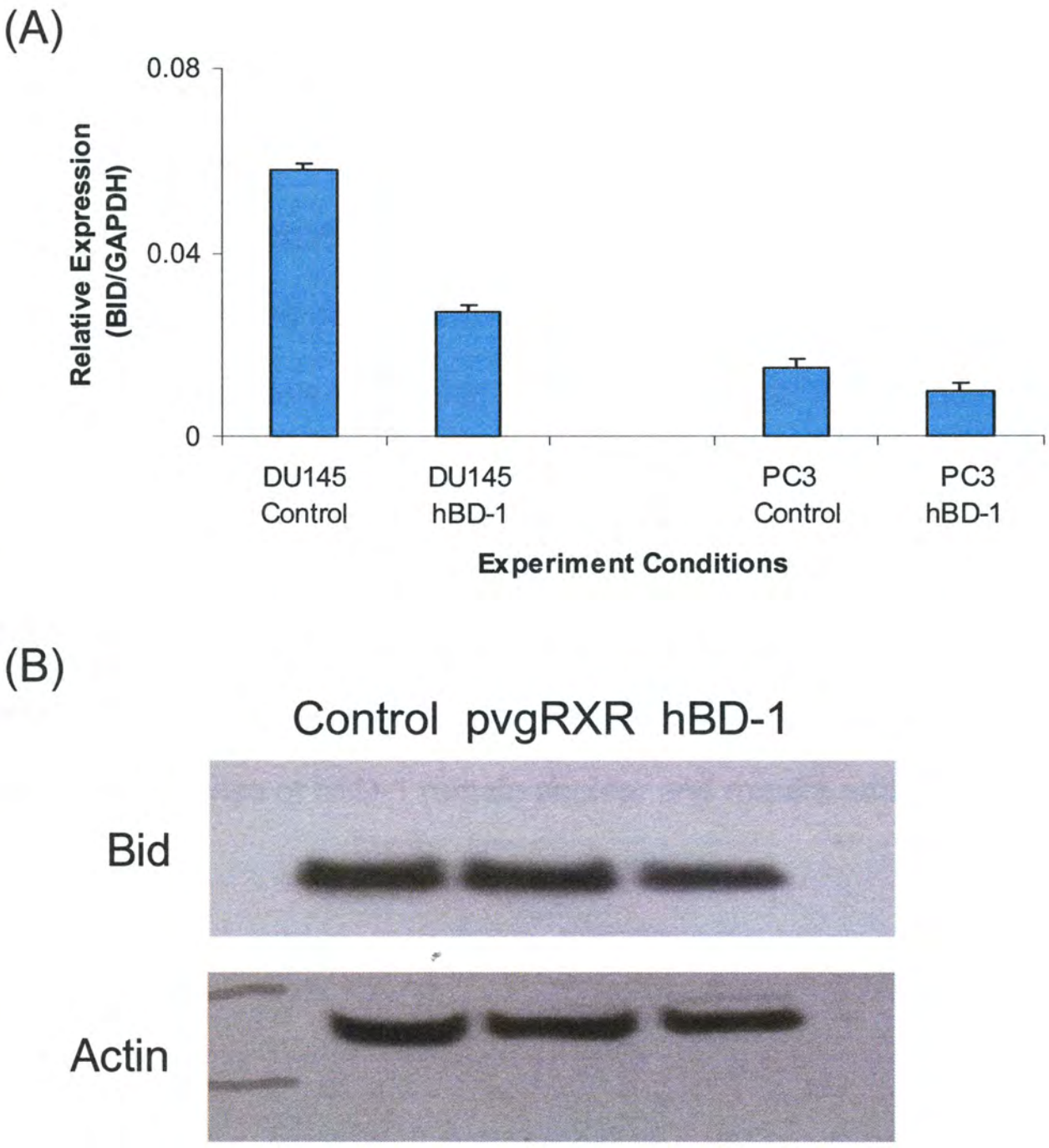


Figure 4-7: Effect of hBD-1 on Bid expression.

results were evidenced by a decrease in cell number and the appearance of cleaved caspase 8 and 9 fragments[57]. Furthermore, in mice it has been shown the over-expression of mouse β-defensin-6 results in myofiber apoptosis[159].

In summary, data presented here support the recent findings that demonstrate hBD-1 expression is highly cytotoxic in human prostate cancer cells. Specifically, we have shown that the death observed in DU145 and PC3 prostate cancer cells involves the activation of caspases 3, 8 and 9. In addition, there were alterations in the expression pattern of various regulators of apoptosis. We are confidant the induced cell death is due to hBD-1 expression because appropriate vector controls were used in each experiment. These findings suggest that hBD-1 has anti-cancer activities via an apoptotic cell death. Taken together, it is plausible that hBD-1 expression within the tumor microenvironment may play a key role in determining the fate of cancer cells and tumor progression. However, the mechanisms and receptors, if any, involved in the pro-apoptotic response of hBD-1 remain unclear and require additional studies.

CHAPTER 5

ROLE OF HBD-1 IN PROSTATE CANCER PROGRESSION

INTRODUCTION

One philosophy for the successful treatment of cancer involves eradicating all of the tumor cells from the body. Conventional modalities to eliminate cancer cells include surgical removal of the tumor mass, radiotherapy and chemotherapy which use exposure to toxic ionizing radiation and chemicals, respectively, to destroy tumor cells. Although these methods are regularly used to treat cancer, they have associated limitations and side effects.

There are two significant limitations connected with the use of radio- or chemo-therapy. First, there is an observed toxicity to both malignant cells and their normal counterparts rapidly dividing within the treatment field. Second, there is significant data that shows the development of drug resistance within a subset of malignant cells following treatment. In an effort to circumvent these limitations, research has been conducted to identify new modalities without the side effects to the host.

One particular area of interest involves the phenomena termed bystander effect. Typically, the term bystander effect is used in radiobiology to describe the effects of radiation on cells adjacent to those directly affected by radiation. However, it has recently been adapted more generally to refer to the effect a particular treatment of cells has on those that have not received any treatment.

The work presented here was designed to test our hypothesis that hBD-1 exhibits a bystander effect. In this study, we test this hypothesis by assessing the effect of hBD-1 containing media on prostate cancer cell lines. Although we

do not observe a bystander effect in our studies, our results indicate a possible upward trend. This suggests that under conditions other than those used in this study, hBD-1 may exhibit a bystander effect. Due to the role hBD-1 has in innate and adaptive immunity, the restoration of expression in cancer cells where it has been lost may prove to be a useful antitumor therapy.

MATERIALS AND METHODS

MTT Cell Viability Assay

To examine the bystander effects of hBD-1 on prostate cancer cells, we performed metabolic 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. DU145 and PC3 cells were seeded onto a 96-well plate at 5 $x10^3$ cells per well. Twenty-four hours after seeding, fresh growth medium was added to control cells, while test conditions were treated with medium exposed to hPrEC cells for 24-48 hours. After incubation with the conditioned media for 24, 48, and 72 hours a MTT assay was performed according to the manufacturer's instructions (Promega). Reactions were performed three times in triplicate.

AO/EtBr Confocal Analysis

AO/EtBr staining was performed to identify changes in cell membrane integrity as described previously. Briefly, DU145 and LNCaP cells were seeded onto 35mm dishes. Cells transfected with pvgRXR vector or hBD-1 plasmid were induced for 24 or 48 hours with media containing 10 μM Pon A,

while control cells received fresh growth media at each time point. At the appropriate time point, the media was collected and placed onto DU145 and LNCaP cells seeded onto 2-chamber slides. The cells were incubated with the conditioned medium for 24 or 48 hours prior to staining. Then, cells were washed once with PBS and stained with 2 ml of a mixture (1:1) of AO (Sigma, St. Louis, MO) and EtBr (Promega) (5 µg/ml) solution for 5 min and were again washed with PBS. Fluorescence was viewed by a Zeiss LSM 5 Pascal Vario 2 Laser Scanning Confocal Microscope (Carl Zeiss) under 40x magnification and the images were collected and processed as previously mentioned.

Trypan Blue Exclusion

DU145 and LNCaP cells were plated on 60mm dishes and transfected with pvgRXR vector or hBD-1 plasmid. Following induction for 24 hours with media containing 10 µM Pon A or fresh growth media for control cells, the media was collected and place onto DU145 and LNCaP cells seeded into 6-well plates. After incubation with the conditioned medium for 24 hours, the cells were washed with PBS and collected. The cells were stained with a mixture (1:1) of cells and PBS containing 0.4% Trypan Blue (Invitrogen). Cells were counted after a 5 min incubation at RT as viable (no blue stain) or non-viable (blue stain) in four quadrants of a hemocytometer. Data are presented as percent non-viable as follows: % non-viable= # non-viable/total (viable+non-viable) x 100. Phase contrast pictures of the field of cells on the hemocytometer were obtained and the images processed as previously described.

Hoechst Stain

DU145, PC3 and LNCaP control cells or transfected with pvgRXR vector or hBD-1 plasmid were induced for 24 and 48 hours with 10 μM Pon A or fresh growth media (controls). Following each incubation period the medium was collected from the plates, spun briefly and placed on DU145, PC3 or LNCaP recipient cells. Recipient cells were exposed to the conditioned donor medium for 24, 48, or 72 hours prior to analysis, while donor cells received new media containing 30μl of 1x Hoechst 33342 dye. After 5 min of incubation, cells were analyzed under UV light on a Zeiss fluorescent microscope under 20x magnification. Images were captured and processed as previously described. The protocol was performed on recipient cells following the appropriate exposure to conditioned medium with the addition of 15μl of 1x Hoechst 33342 dye.

Flow Cytometry

DU145 cells transfected with the hBD-1 expression system and control cells were grown in 60-mm dishes were induced for 24 and 48 hours with 10 μM Pon A or fresh growth media, respectively. The donor cells were used to generate conditioned media for recipient cells. Following each incubation period the medium was collected from the donor plates, spun briefly and placed on DU145 and LNCaP recipient cells. Recipient cells were exposed to the conditioned donor medium for 24, 48, or 72 hours prior to a wash with PBS. The attached donor or recipient cells were harvested by trypsinization and pelleted at

4° C (1500 rpm) for 5 min, washed in PBS, and resuspended in 100 µl PBS. While vortexing or bubbling with a pipette, 2 ml of 70% EtOH was added drop-wise. Samples were stored 1h to 3 days at 4° C. Then, the suspension was washed with PBS and pelleted at 4° C (3000 rpm) for 5 min. The pellet was resuspended in 100μl of PBS prior to the addition of 100μl RNase (Sigma, 1mg/ml) and 200μl of propidium iodide solution (1:100 dilution of a 1mg/ml stock). Samples were vortexed and stored at 4° C for at least 30 min wrapped in foil prior to flow analysis by FACscan (Becton Dickinson). All reactions were performed two times.

RESULTS

Effect of conditioned media on cell viability

To better understand the mechanism of action of hBD-1, it is important to assay whether the peptide acts in an autocrine or paracrine manner. It is widely accepted that hBD-1 is constitutively expressed by epithelial cells[90]. Several groups have reported the secretion of hBD-1 peptide into the media of human epithelial cell lines including gastric (AGS)[160], colon (HT-29 and Caco-2)[161], and gingival (HGEC)[162]. Therefore, normal prostate epithelial cells, hPrEC, were used to determine if hBD-1 exhibits a bystander effect on neighboring cells (Figure 5-1A).

Here, media exposed to hPrEC for 48 hours was collected and used to treat DU145 and PC3 cells for 24, 48, and 72 hours. MTT analysis of DU145

Figure 5-1: Basic diagram of bystander studies. (A) A diagram that demonstrates the bystander assay performed using conditioned media from hPrEC. (B) A diagram illustrating the use of conditioned media from prostate cancer cell lines induced for hBD-1 expression.

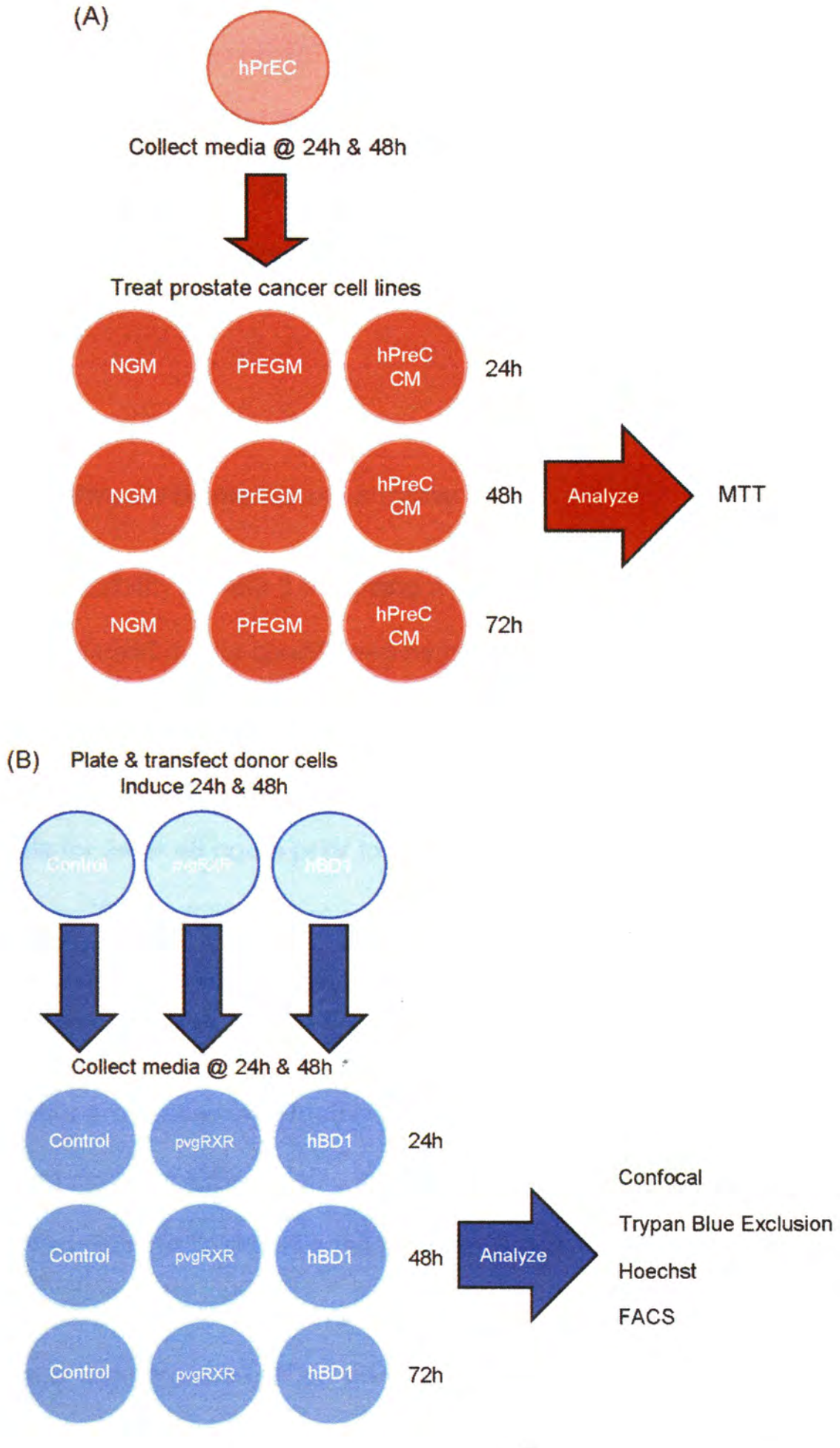


Figure 5-1: Basic diagram of bystander assays.

cells following a 48h exposure to hPrEC conditioned media revealed mixed results (Figure 5-2). It appears that the difference between control media and conditioned media ranged from no to moderately significant. Therefore, alternate methods were used to investigate whether hBD-1 exhibits a bystander effect on adjacent cells.

Effect of conditioned media on membrane integrity

To test the possibility of hBD-1 exhibiting a bystander effect with an alternate method, we used media conditioned by prostate cancer cells induced to express hBD-1 (Figure 5-1B). Since the proposed mechanism of action of defensin peptides involves pore formation, recipient cells were exposed to the conditioned media for 24 or 48 hours prior to analysis of membrane integrity by confocal microscopy (Figure 5-3). Intact cells stained green due to the fluorescence of membrane permeable AO, while cells with compromised membranes stained red due to the fluorescence of membrane impermeable EtBr. When DU145 cells were treated with media conditioned by DU145 cells, there was a slight accumulation of EtBr staining in hBD-1 exposed (A). However, LNCaP cells treated with media conditioned by DU145 cells did not demonstrate a difference in staining from control or pvgRXR exposed (B). Media conditioned by LNCaP was also used to treat LNCaP and did not demonstrate a difference in EtBr staining from pvgRXR exposed (C). This finding suggests that alterations to

Figure 5-2: Effect of conditioned media on cell viability. An MTT assay was performed to determine cell viability after 48h exposure to conditioned media from hPrEC.

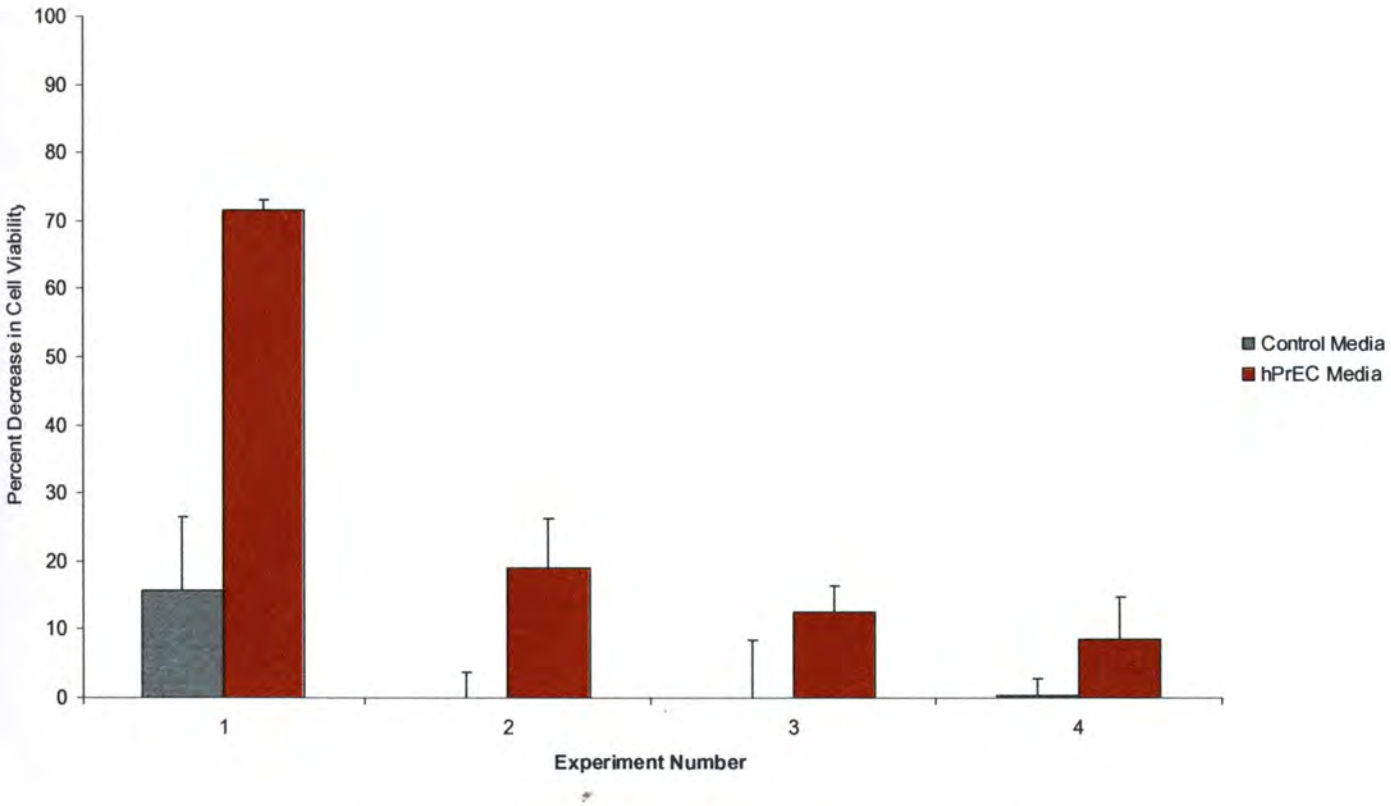


Figure 5-2: Effect of conditioned media on cell viability.

Figure 5-3: Effect of conditioned media on membrane integrity. Membrane integrity of recipient cells was analyzed by confocal microscopy 24 h after hBD-1 induction. (A) DU145 conditioned media on DU145 recipient cells. (B) DU145 conditioned media on LNCaP recipient cells. (C) LNCaP conditioned media on LNCaP recipient cells. Green staining indicates the localization of AO, red staining represents EtBr and yellow staining represents the co-localization of both AO and EtBr. Size bars = 10 μM.

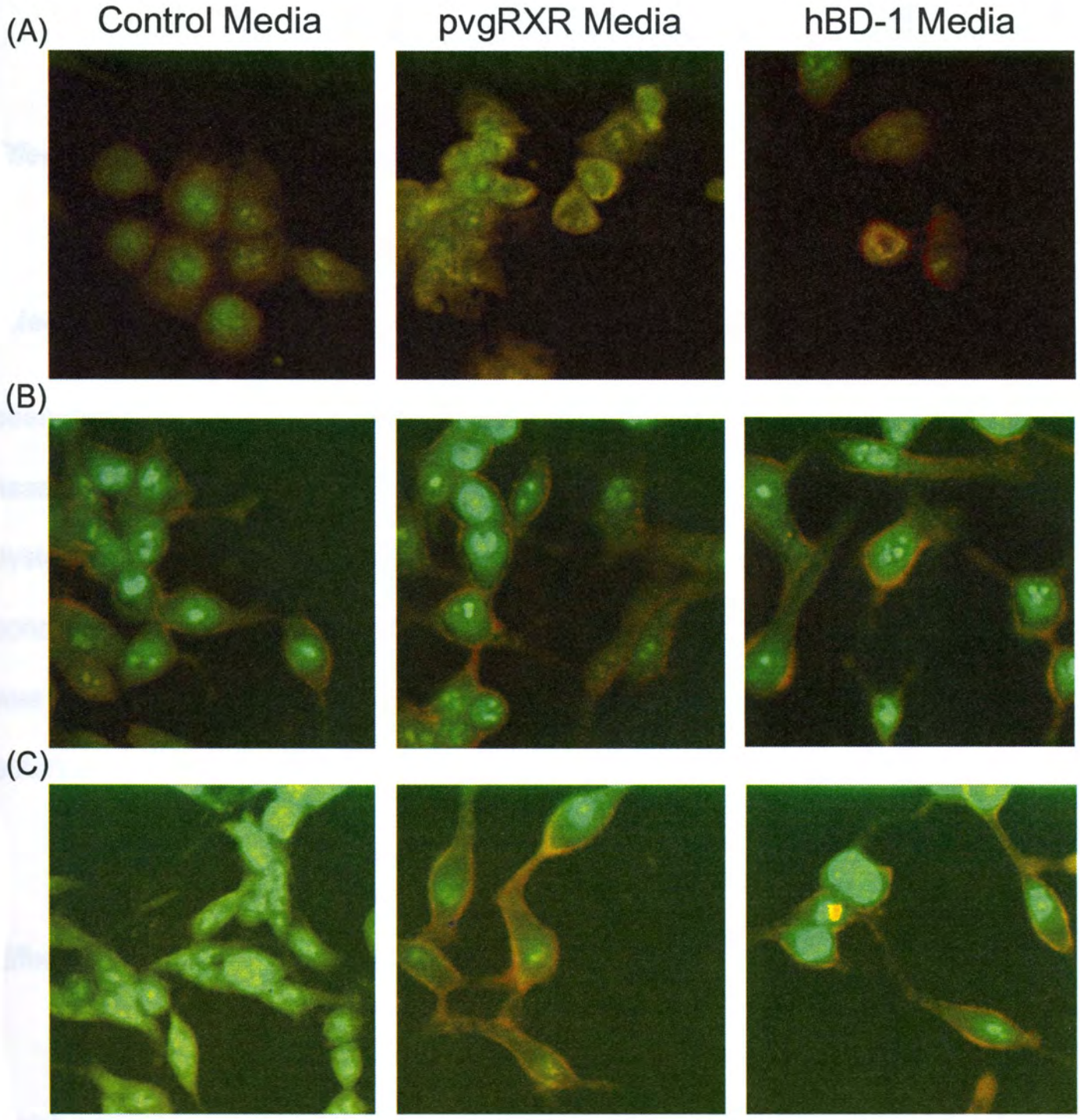


Figure 5-3: Effect of conditioned media on membrane integrity.

membrane integrity may occur in response to exposure of hBD-1 containing media in some cases.

Effect of conditioned medium on trypan blue exclusion

Similar to the previous experiment, trypan blue is a vital stain used to selectively color dead cells blue, while viable cells exclude the dye. Since it has been demonstrated that hBD-1 is cytotoxic to cells, dye exclusion was used to assess the effect conditioned media had on other cells to test the possibility of a bystander effect. As indicated in Figure 5-4, there was not a significant effect of conditioned media from DU145 or LNCaP on recipient cell lines. However, there was a minimal increase in hBD-1 exposed cells indicating a slight trend toward a potential bystander effect.

Effect of conditioned media on the cell cycle

The cell cycle is comprised of a series of checkpoints and phases through which proliferating cells progress. The cell cycle consists of the following: G_1, presynthetic growth phase 1; S, DNA systhesis phase; G_2; and M, mitotic phase. Quiescent cells are in a state called G_0, however, most tissues consist of cells at various phases of the cell cycle.

Figure 5-4: Analysis of dye exclusion after exposure to conditioned media. The effect of hBD-1 conditioned media on DU145 and LNCaP prostate cancer cells was quantified as percent non-viable under the various conditions.

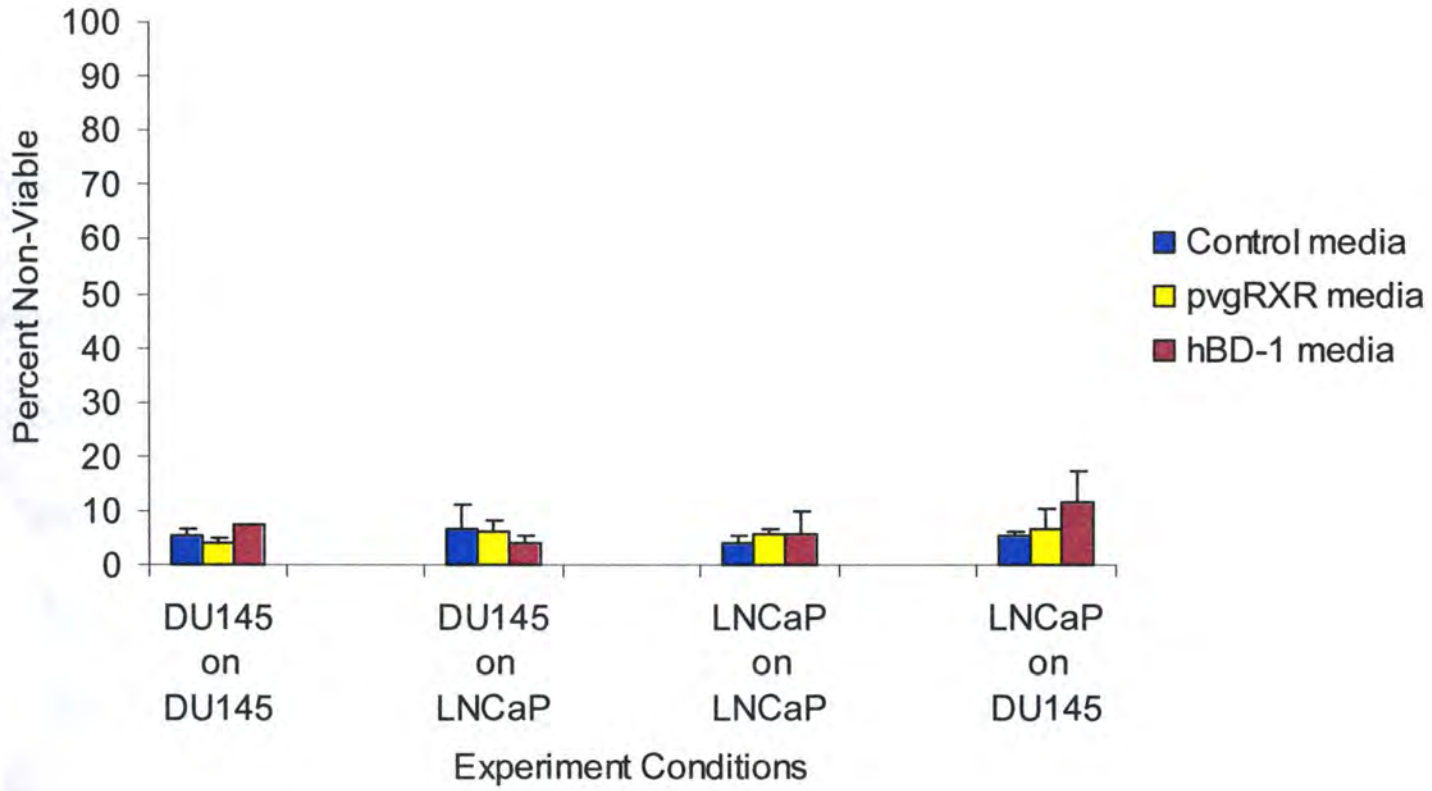


Figure 5-4: Analysis of dye exclusion after exposure to conditioned media.

The most commonly utilized dye for DNA content and cell cycle analysis is PI, which intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct. Here, FACS analysis was performed to assess whether hBD-1 conditioned media had any effect on donor or recipient cells. Neither the results at 24 hours (Figure 5-5) nor 48 hours (Figure 5-6) indicated a difference from the negative control, pvgRXR.

Effect of conditioned media on cells

Since we have shown hBD-1 induces apoptotic cell death with expression, Hoechst 33342 was used to assess the effect of hBD-1 on donors and recipients. Hoechst dyes bind to AT-rich regions of DNA that can be excited with a UV source. It has been observed that short exposures of cells to low concentrations of Hoechst dye strongly labels apoptotic cells[163, 164]. Therefore, we utilized this method as a method to assess the end-point of hBD-1 expression observed in prostate cancer cell lines. Donor cells induced for hBD-1 expression demonstrated a significant increase in condensed nuclei from pvgRXR and controls (Figure 5-7). Media from the 24 hour donors was exposed to DU145 recipient cells for 24, 48, and 72 hours. We found that although there was not a significant difference from pvgRXR and controls, there appears to be a trend consistent with a potential effect (Figure 5-8). DU145 recipient cells exposed to media from 48 hour donors demonstrated a slight, but insignificant increase in

Figure 5-5: Cell cycle analysis after treatment with 24h conditioned media. The effect of 24 hour conditioned media on the cell cycle was analyzed by flow cytometry. Data are presented as percentages of modeled events measured at each phase.

Cell Type	Cell Cycle Phase			% CV	Apoptosis	Modeled Events
	G0/1	G2/M	S			
DU145 NGM	78.22%	10.21%	11.57%	8.24%	0.11%	9548
DU145 Donors:						
24h DU145 Control	56.75%	22.18%	21.06%	7.05%	ND	9339
24h DU145 pvgRXR	69.57%	21.22%	9.21%	9.58%	0.02%	9567
24h DU145 hBD1	66.83%	23.41%	9.75%	10.29%	0.00%	9565
DU145 Recipients:						
24h DU145 NGM	78.87%	4.53%	16.60%	11.51%	ND	584
24h DU145 10 uM PonA	3.24%	0.00%	96.76%	2.64%	ND	58
24h DU145 H202	7.55%	0.00%	92.45%	2.58%	ND	61
24/24 DU:DU Control	5.35%	0.29%	94.37%	0.24%	ND	53
24/24 DU:DU pvgRXR	ND	ND	ND	ND	ND	ND
24/24 DU:DU hBD1	16.57%	0.00%	83.43%	3.78%	3.62%	67
24/48 DU:DU Control	63.66%	18.63%	17.71%	10.79%	ND	1652
24/48 DU:DU pvgRXR	ND	ND	ND	ND	ND	ND
24/48 DU:DU hBD1	53.34%	43.43%	3.23%	12.19%	ND	42
24/72 DU:DU Control	ND	ND	ND	ND	ND	ND
24/72 DU:DU pvgRXR	ND	ND	ND	ND	ND	ND
24/72 DU:DU hBD1	ND	ND	ND	ND	ND	ND
LNCaP Recipients:						
24h LNCaP NGM	50.70%	25.07%	24.23%	9.20%	ND	9278
24h LNCaP 10 uM PonA	66.53%	16.73%	16.74%	8.99%	ND	1501
24h LNCaP H202	62.43%	19.36%	18.20%	9.55%	0.03%	9396
24/24 DU:LN Control	60.70%	13.96%	25.35%	8.15%	ND	841
24/24 DU:LN pvgRXR	17.90%	1.21%	80.88%	1.36%	ND	39
24/24 DU:LN hBD1	52.06%	21.89%	26.05%	10.13%	ND	16
24/48 DU:LN Control	38.36%	0.00%	61.64%	8.96%	ND	41
24/48 DU:LN pvgRXR	21.11%	0.00%	78.89%	11.26%	ND	17
24/48 DU:LN hBD1	ND	ND	ND	ND	ND	ND
24/72 DU:LN Control	ND	ND	ND	ND	ND	ND
24/72 DU:LN pvgRXR	ND	ND	ND	ND	ND	ND
24/72 DU:LN hBD1	ND	ND	ND	ND	ND	ND

Figure 5-5: Cell cycle analysis after treatment with 24h conditioned media.

Figure 5-6: Cell cycle analysis after treatment with 48h conditioned media. The effect of 48 hour conditioned media on the cell cycle was analyzed by flow cytometry. Data are presented as percentages of modeled events measured at each phase.

Cell Type	Cell Cycle Phase			% CV	Apoptosis	Modeled Events
	G0/1	G2/M	S			
DU145 NGM	78.22%	10.21%	11.57%	8.24%	0.11%	9548
DU145 Donors:						
48h DU145 Control	58.63%	20.56%	20.80%	9.24%	ND	9469
48h DU145 pvgRXR	69.81%	23.51%	6.67%	10.73%	ND	1957
48h DU145 hBD1	ND	ND	ND	ND	ND	ND
DU145 Recipients:						
48h DU145 NGM	61.67%	22.35%	15.97%	8.66%	ND	9046
48h DU145 H202	66.55%	14.88%	18.57%	8.69%	ND	9142
48/24 DU:DU Control	65.46%	19.46%	15.08%	9.20%	ND	4089
48/24 DU:DU pvgRXR	100.00%	0.00%	0.00%	7.00%	ND	5
48/24 DU:DU hBD1	12.99%	0.00%	87.01%	0.30%	4.37%	50
48/48 DU:DU Control	ND	ND	0.00%	ND	ND	0
48/48 DU:DU pvgRXR	22.94%	5.74%	71.33%	0.66%	ND	39
48/48 DU:DU hBD1	51.95%	4.24%	43.81%	6.21%	0.70%	326
48/72 DU:DU Control	12.03%	2.59%	85.38%	0.72%	ND	181
48/72 DU:DU pvgRXR	56.38%	19.76%	23.86%	10.61%	2.73%	868
48/72 DU:DU hBD1	7.84%	0.00%	92.16%	0.22%	3.82%	160
LNCaP Recipients:						
48h LNCaP NGM	83.56%	11.38%	5.07%	9.20%	ND	841
48h LNCaP H202	63.25%	0.25%	36.50%	5.04%	ND	33
48/24 DU:LN Control	ND	ND	ND	ND	ND	ND
48/24 DU:LN pvgRXR	81.12%	18.88%	0.00%	13.29%	ND	23
48/24 DU:LN hBD1	ND	ND	ND	ND	ND	ND
48/48 DU:LN Control	0.00%	ND	ND	0.00%	100.00%	3
48/48 DU:LN pvgRXR	ND	ND	0.00%	ND	ND	0
48/48 DU:LN hBD1	ND	ND	ND	ND	ND	ND
48/72 DU:LN Control	89.72%	6.27%	4.00%	16.42%	3.47%	215
48/72 DU:LN pvgRXR	83.48%	5.72%	10.80%	10.23%	0.77%	731
48/72 DU:LN hBD1	82.17%	8.22%	9.61%	11.01%	1.57%	923

Figure 5-6: Cell cycle analysis after treatment with 48h conditioned media.

Figure 5-7: Analysis of apoptosis in donor cells. (A) Microscopic analysis of condensed nuclei in DU145 cells induced for hBD-1 expression for 24 hours. (B) Graphical representation demonstrating the percentage of condensed nuclei in DU145 cells induced for hBD-1 expression for 24 hours.

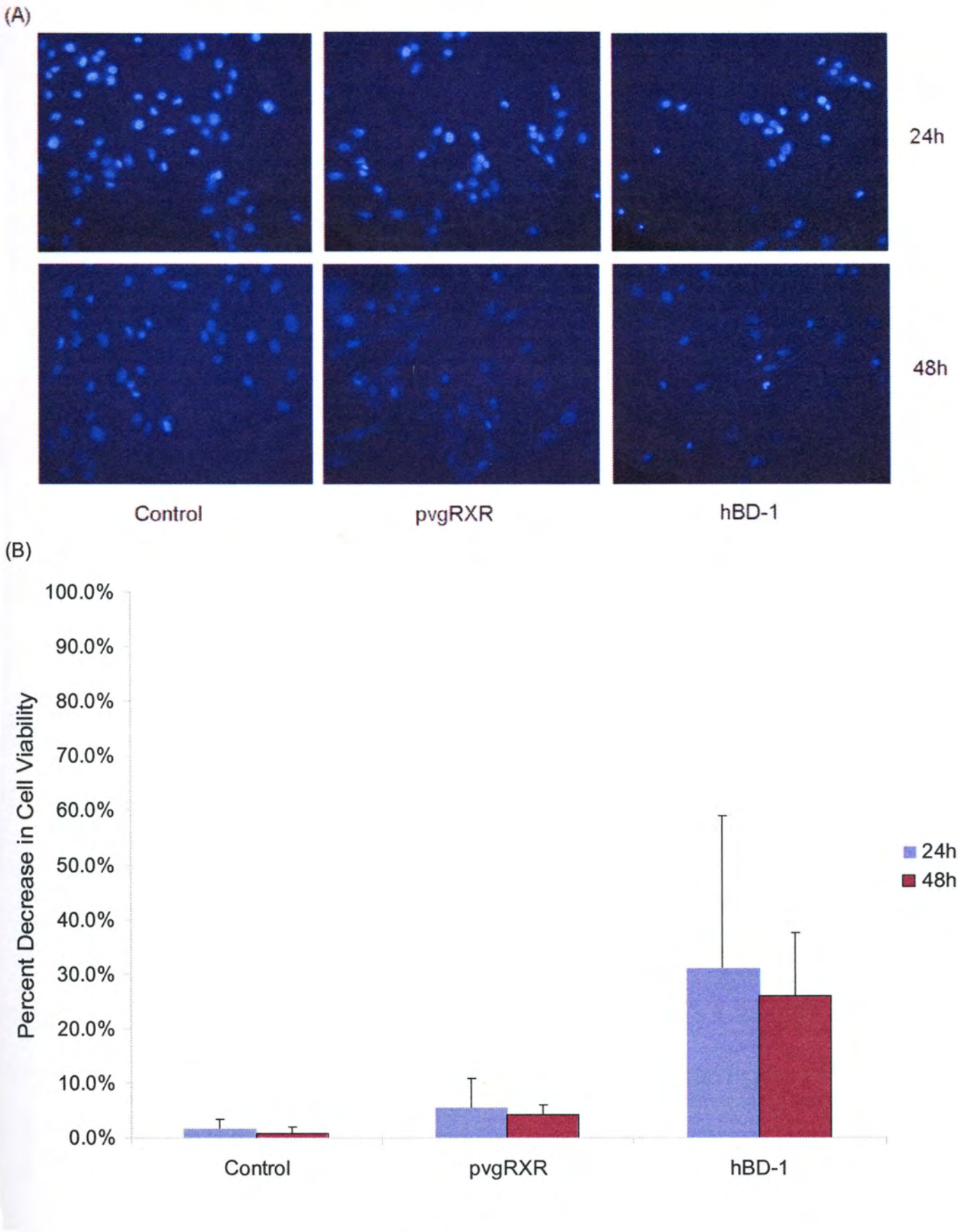


Figure 5-7: Analysis of apoptosis in donor cells.

Figure 5-8: Analysis of apoptosis after exposure to 24h conditioned media. (A) Microscopic analysis of condensed nuclei in DU145 cells exposed to 24h conditioned media at 24, 48, and 72 hours. (B) Graphical representation demonstrating the percentage of condensed nuclei in DU145 cells exposed to 24h conditioned media at 24, 48, and 72 hours.

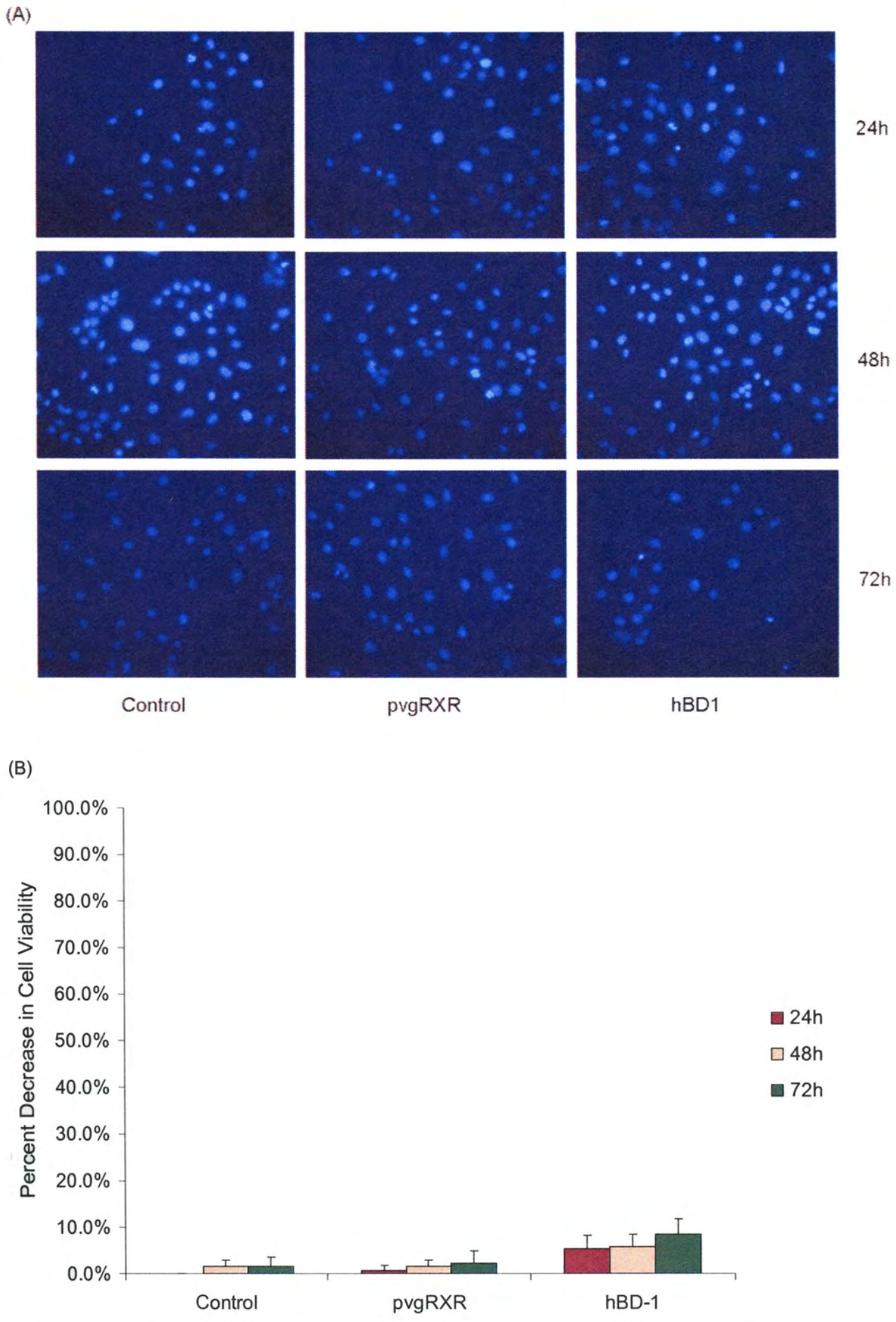


Figure 5-8: Analysis of apoptosis after exposure to 24h conditioned media.

apoptotic nuclei (Figure 5-9). Additionally, similar results were found for PC3 cells under the same conditions (data not shown).

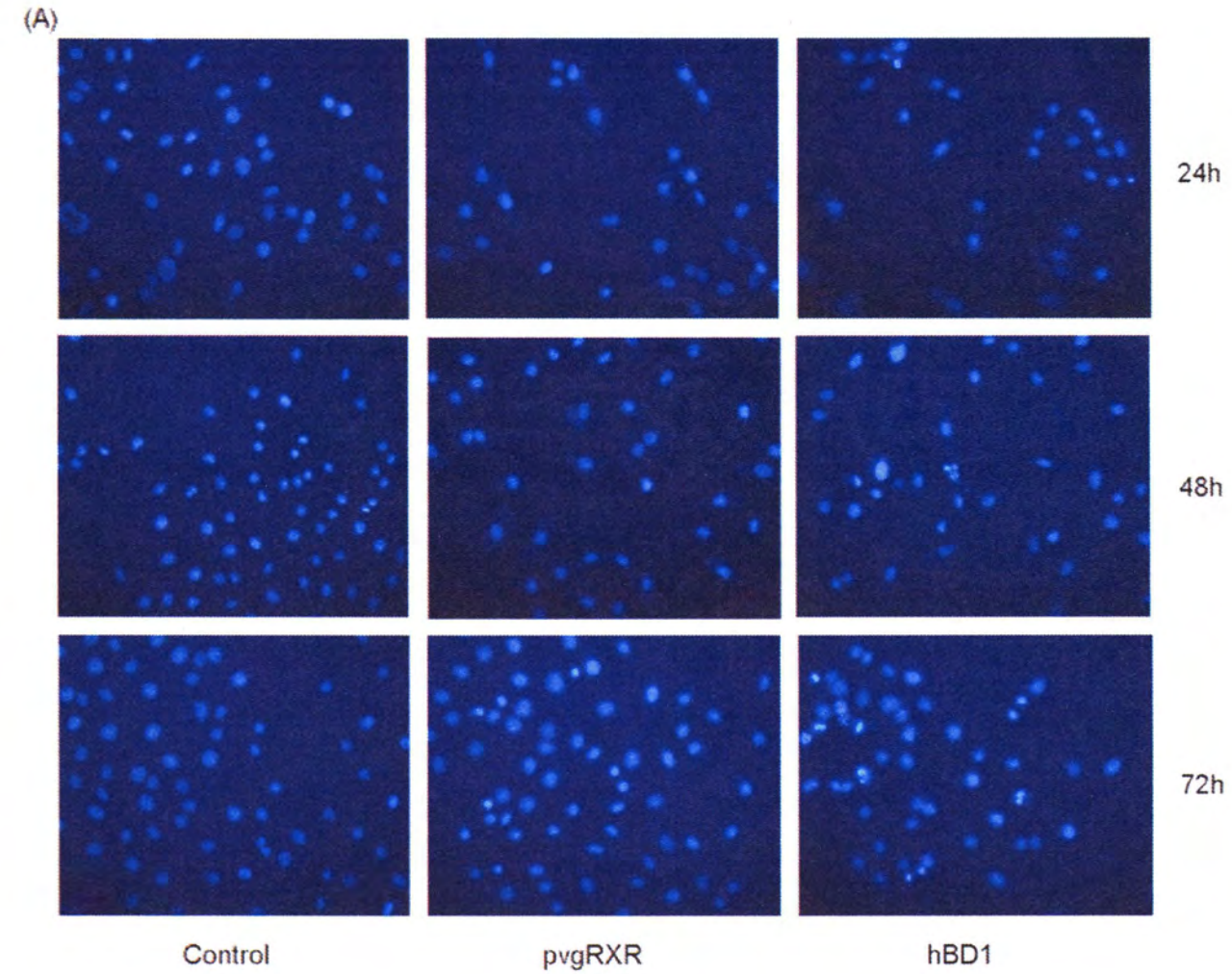
DISCUSSION

The field of tumor immunity has been rapidly expanding in recent years. There is increasing evidence that altered immune function may not only accompany, but even facilitate the development and progression of cancer. To date, numerous AMPs and other host-defense molecules have been shown to have altered expression profiles in a variety of cancers[6, 56, 165].

Previously we demonstrated the high frequency of hBD-1 expression in prostate and renal carcinomas[6]. The cancer-specific cytotoxicity of hBD-1 was recently shown in a renal cancer cell line[57]. In addition, our studies have confirmed that late-stage prostate cancer cells induced to express hBD-1 undergo a caspase-mediated cell death, while normal prostate cells and early-stage prostate cancer cells do not[56].

It is known that hBD-1 exhibits antimicrobial properties by binding to the bacterial membrane and forming pores that ultimately result in death[166]. However, the exact mechanism by which hBD-1 exhibits cytotoxicity toward cancer cells is unknown. To better understand the mechanism of cytotoxic action of hBD-1 in human cancer cell lines, we designed experiments to elucidate whether the secreted peptide acts in an autocrine or paracrine fashion.

Figure 5-9: Analysis of apoptosis after exposure to 48h conditioned media. (A) Microscopic analysis of condensed nuclei in DU145 cells exposed to 48h conditioned media at 24, 48, and 72 hours. (B) Graphical representation demonstrating the percentage of condensed nuclei in DU145 cells exposed to 48h conditioned media at 24, 48, and 72 hours.



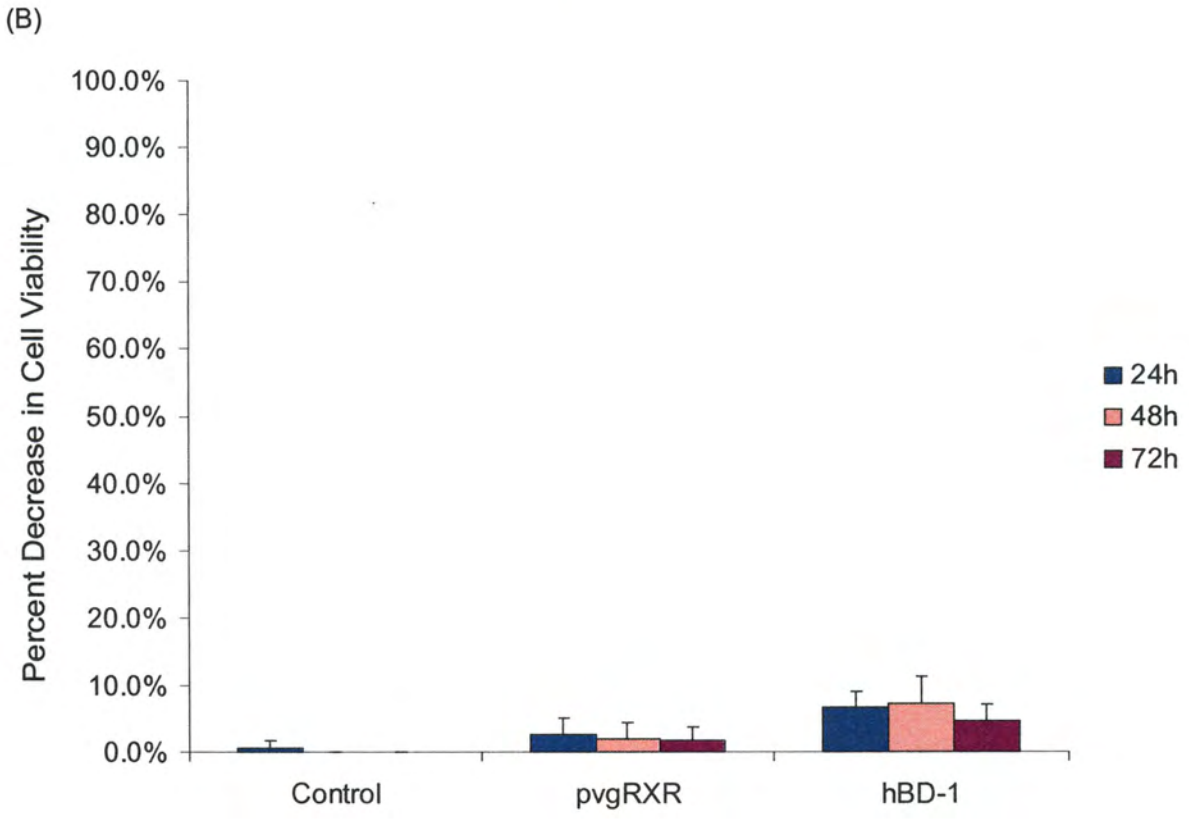


Figure 5-9: Analysis of apoptosis after exposure to 48h conditioned media.

Specifically, we performed an array of assays in an effort to determine if hBD-1 exhibited a bystander effect on adjacent cells. In our system we were unable to demonstrate a bystander effect attributed to the expression of hBD-1. However, several assays resulted in data that indicates a slight trend toward a potential bystander effect.

There are several reasons why a bystander effect was not observed within our test parameters. The first possibility involves the proteolytic processing of the peptide. It is known the functionally active protein in host defense is the 36 amino acid peptide[81, 118]. Additionally, it has been shown that significant amounts of hBD-1 peptide are released into the lumen of the kidneys as a 47 amino acid pro-peptide that undergoes proteolytic cleavage to a variety of truncated forms[118]. Although the microbicidal activity of other forms of hBD-1 was reduced in some conditions, it has been shown that these effects were not observed with the biologically active 36 amino acid peptide[74]. Given that the exact size of hBD-1 peptide generated in our expression system is unknown, it is a possibility that there is a decrease in biological activity due to proteolytic processing.

The observed trend toward a potential bystander effect may be due to the expression system used to induce hBD-1 in the cell lines. hBD-1 was cloned into the Ecdysone Inducible Mammalian Expression System (Invitrogen), a dual vector system that permits the regulation of gene expression. In this system, both subunits of a functional ecdysone receptor from Drosophila are constitutively expressed from the regulator vector, pvgRXR. The ecdysone-responsive promoter (p-Δ-HSP) that ultimately drives the expression of hBD-1 is

located on the second inducible vector. In the presence of the inducer, Pon A, the functional ecdysone receptor binds upstream of the ecdysone responsive promoter and activates gene expression. Although expression is supposed to be ligand-induced, it has been shown that this system is leaky and allows for gene expression in the absence of the inducing agent[167].

Due to the leaky nature of the expression system, it was difficult to generate stable clones in the prostate cancer cell lines since hBD-1 is cytotoxic to them. Therefore, transient transfections were performed to evaluate hBD-1 effect on the cultured cells. Although an estimation of transfection efficiency was completed to derive an average, there was variation between the experiments. Taken together, it is possible that the optimal transfection efficiency in this transient system was not achieved to observe a bystander effect.

A final reason for the lack of bystander effect in the methods tested may be due to the concentration of hBD-1 present. It is believed that when a peptide is selective for a cancer cell a threshold concentration must be reached prior to membrane disruption[91]. In the methods used there was not a quantitative analysis of hBD-1 in the conditioned media, thus, making it possible that the difference in responses between donor and recipient cells may be due to altered media concentrations of the peptide. Additionally, the experiments did not assess the degradation of the peptide to see if this potentially contributed to a decrease concentration of hBD-1 in the conditioned media exposed to recipient cells.

In summary, we utilized hPrEC which are believed to constitutively express hBD-1 and prostate cancer cell lines induced to express hBD-1 to test the possibility of a bystander effect. In donor DU145 and PC3 prostate cancer cells induced for hBD-1 expression, a clear cytotoxic effect was observed. However, the treatment of recipient cells with conditioned media did not generate similar results. Data indicate that although there is not a bystander effect under the conditions tested, there is a trend upward indicating the possibility of a bystander effect. Therefore, it may be interesting to study the potential bystander effect of hBD-1 under different conditions.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Prostate cancer continues to be the most common non-cutaneous cancer and a leading cause of death in American men[25]. A variety of factors increase the risk for development of prostate cancer, including diet, environment and genetics. Although the exact mechanism of prostate tumorigenesis is unknown, it is known that the molecular pathogenesis involves a series of pre-malignant changes that lead to the uncontrolled cell growth indicative of prostate cancer.

Currently there are limited treatment methods that improve survival beyond localized prostate cancer[153]. Additionally, the clinical outcome of prostate cancer is closely related to the tumor grade and stage, with poorly differentiated tumors having a poor prognosis[131]. In prostate cancer patients, tumor progression has been linked to an increase in immune suppression[6]. It has also been suggested that tumors escape destruction due to an imbalance between immune activation and suppression during tumor growth[132]. However, little is known about specific components of the innate immune system that play a role in prostate tumor suppression. Therefore, the emergence of new molecular targets that have strong anti-tumor effects without corresponding host toxicity may prove to be an essential treatment modality in prostate cancer.

The involvement of defensin proteins in cytotoxicity and cytolysis, with respect to tumor immunity, is gaining more attention. Defensins are a family of 3-4 kDa polycationic peptides involved in host immunity[80]. They are classified into subfamilies, α-, β-, and θ-defensins, based on the connectivity of cysteine residues in the precursor gene structure[74]. The α-defensins are predominantly

abundant in the granules of polymorphonuclear leukocytes and exhibit broad spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses[75, 79]. On the other hand, β-defensins have been identified in epithelial cells and are mainly active against Gram-negative bacteria and yeast[60, 77].

The core motif for β-defensins includes 9 highly conserved amino acid residues that probably serve as essential components of the primary structural motif[81, 168]. To date, six hBDs have been identified that are constitutively secreted, hBD-1, or induced for secretion, hBDs 2-6, at the epithelial cell surface[84, 85]. Of particular interest in our lab was hBD-1, which was first isolated from the plasma of patients with renal disease in 1995[81]. Several N-terminally truncated molecular variants of hBD-1 have been identified from 36-47 amino acids in length. However, the 36 amino acid peptide is considered the biologically active form[74]

Previously Donald et *al* have shown the cancer specific loss of hBD-1 expression in prostate and renal carcinoma tissue[6]. Specifically, 82% of the malignant prostate tissue demonstrated little to no hBD-1 expression compared to adjacent benign tissue[6]. In addition, the observed loss of expression occurred as early as PIN with adjacent benign tissue displaying normal glandular staining for hBD-1[6].

These observations led to the hypothesis that hBD-1 may have anti-tumor activities and that its loss may contribute to prostate tumorigenesis. In this study,

we test the hypothesis that hBD-1 may have anti-tumor activities by characterizing its function in prostate cancer. Initial screening of benign prostate tissue and the primary prostate cell culture hPrEC revealed high levels of hBD-1 expression. However, we observed a decrease or loss of hBD-1 expression in adjacent malignant prostate tissue, as well as in DU145, PC3 and LNCaP prostate cancer cell lines.

Induction of hBD-1 expression in our prostate cancer cell lines caused a significant reduction in cell viability and proliferation in the late-stage prostate cancer cell lines, DU145 and PC3. To assess whether the effect was cytostatic or cytotoxic, we performed FACS analysis to measure cell death. After inducing hBD-1 in DU145 and PC3 cell lines, the number of cells undergoing early apoptosis and late apoptosis/necrosis totaled 59% and 44%, respectively, by 48 hours. Our results demonstrated the cytotoxic activity of hBD-1 on DU145 and PC3 prostate cancer cells.

The mechanism of cell death indicated in our cytotoxicity studies was apoptosis. To better understand the actions of hBD-1 in prostate cancer cells, we investigated whether hBD-1 induction resulted in caspase activation. Initial studies indicated the general activation of caspases in DU145 and PC3 cells. Analysis of specific caspases demonstrated that hBD-1 expression results in the activation of caspases 3, 8, and 9. In addition, there were alterations in the expression profiles of several pro- and anti-apoptotic factors. These findings suggest that hBD-1 expression may play a key role in determining the fate of cancer cells and, hence, tumor progression.

The proposed mechanism of antimicrobial action of defensin peptides involves the disruption of the microbial membrane due to the formation of pores[80]. To determine if hBD-1 expression altered the membrane integrity of the prostate cancer cells, EtBr uptake was examined by confocal analysis. Our results demonstrated permeable membranes in DU145 and PC3 cell induced for hBD-1 expression, while LNCaP did not have observable membrane alterations. This finding suggests that alterations to membrane integrity in response to hBD-1 expression differ between early- and late-stage prostate cancer cells. Additionally, it provides the first line of evidence into the mechanism of hBD-1 induced cell death.

To further delineate the mechanism of hBD-1 cytotoxicity, we examined if the death observed was due to a bystander effect. Several groups have demonstrated the secretion of hBD-1 into the media of a variety of epithelial cell lines[160, 161]. Therefore, hPrEC generated conditioned media was used to determine if hBD-1 exhibits a bystander effect. The results of the initial study revealed a range from insignificant to significant reduction in cell viability after exposure to the conditioned media. Due to the variation in the results and the lack of concrete evidence supporting the secretion of hBD-1 by hPrEC, alternate methods to generate conditioned media were employed.

As an alternate method to generate conditioned media, prostate cancer cell lines were induced for hBD-1 expression and the media was collected at various time points to treat non-hBD-1 expressing prostate cancer cells. We have previously demonstrated that hBD-1 expression in DU145 and PC3 results

in a decrease in cell viability, an increase in membrane permeability and apoptosis. Therefore, we assessed recipient cells following treatment with conditioned media by dye exclusion, AO/EtBr staining, and Hoechst staining to observe the effects, if any, to cell vitality, membrane integrity and nuclear condensation. Although the results did not indicate a bystander effect within the experiment parameters, there was a minimal increase in staining in each of the assays indicating a slight trend toward a potential bystander effect. In addition, cell cycle analysis following treatment with hBD-1 conditioned media was performed. However, the results indicated no difference from the negative control pvgRXR conditioned media.

There are several reasons why a bystander effect was not attributed to the expression of hBD-1 in our system. Since it is unknown what form of the peptide is expressed in these experiments, it is possible that differences in the proteolytic processing of the peptide have reduced the activity. Also, the concentration of peptide in the media is unknown. It has been shown that synthetic hBD-1 peptide caused a decrease in cell viability in bladder cancer cells at 50μg/ml[57]. The trend toward, but lack of, a bystander effect may be due to the fact that the threshold concentration of hBD-1 peptides was never reached to see optimal results. In addition, a significant limitation to concentration of hBD-1 in our system is the transient nature of our transfections.

Taken together, our data support the hypothesis that hBD-1 is a tumor suppressor gene. Also, the data indicate the loss of hBD-1 in the surrounding tissue in may create an environment that promotes the progression of prostate

cancer. Due to the decreased expression of a host defense peptide in cancer, there is a suggestion that defensins may contribute to tumor immunity.

The anti-tumor effect of defensins is an emerging field. Preliminary experiments by Huang et al. demonstrated the slower growth of tumors injected with hBD-2 vectors compared to control possibly due to an antitumor effect of hBD-2[169]. Also, others have shown the differential gene expression profiles of defensins in a variety of cancers[115, 116]. Therefore, additional studies of the anti-tumor effect of defensins are necessary to delineate the specific contribution of defensins to tumor immunity.

The scope of this study was limited to characterizing the effects of hBD-1 in prostate cancer cells. However, there are several key experiments that would significantly contribute this particular area of research. First, the generation of a better expression system is essential. The ability to perform quality experiments in the future will require stable clones that can be induced without accompanied leakiness of gene expression. Also, a triple stain experiment to verify which cells are undergoing apoptosis after exposure to hBD-1 containing media would provide further insight into the cytotoxic mechanism of this defense peptide. Ultimately, *in vivo* experiments that investigate the anti-timor effects of hBD-1 will aid in determining the clinical relevance of our findings.

In summary, this data demonstrates the induction of hBD-1 expression resulted in rapid cell death in the late-stage prostate cancer cell lines, PC3 and DU145, but had no effect on the early-stage prostate cancer cell line, LNCaP.

With this, we provide the experimental evidence that hBD-1 may play a specific role in tumor suppression of advanced prostate cancer. Furthermore, these data support the potential utility of hBD-1 as a therapeutic agent for late-stage prostate cancer without cytotoxic effects to the host.

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Rebecca Suzanne Bullard

Office: 165 Ashley Avenue, Suite 309
PO Box 250908
Charleston, SC 29425
(843) 792-9942
bullard@musc.edu

Home: 510 Amy Drive
Goose Creek, SC 29445
(843) 812-7731

ACADEMIC PREPARATION

Ph.D. Candidate, Biomedical Sciences May 2008
Department of Pathology and Laboratory Medicine
Medical University of South Carolina, MUSC
Charleston, SC
Dissertation: *Functional Analysis of Human Beta Defensin-1: novel insight into its role in prostate cancer*
Advisor: Dr. Carlton D. Donald

B.S., Biology December 2001
Concentration: Molecular and Cellular Biology
Department of Biology
University of South Carolina Aiken, USCA
Aiken, SC
Dissertation: *Transfection and Infection of AML-1B in Mammalian Tissue Culture Cell Lines*
Advisor: Dr. David K. Strom

LANGUAGE COMPETENCY

Spanish

HONORS AND AWARDS

Recipient of the 2007-2008 Provost Scholarship
Recipient of the Service and Leadership Award, Department of Pathology and Laboratory Medicine, 2007
Recipient of the 2006-2007 Abney Foundation Scholarship Award
Selected for Oral Presentation at 5th Annual Hollings Cancer Center Retreat, November 18, 2005
3rd place award for Outstanding Performance in Presenting Research, Pathology and Laboratory Medicine Seminar Series, MUSC, 2005
Honors Credit for Research Laboratory Rotation, MUSC, Charleston, SC, 2003
Volunteer Service Award, Circle K International, USCA, 1999-2002

Honors Biology, USCA, Department of Biology and Geology, Aiken, SC, 1997
Who's Who in American Colleges and Universities, USCA, 1998-2001

PROFESSIONAL EXPERIENCE

Research Assistant

Department of Biology and Geology
USCA, Aiken, SC, Summer 2002

- Assisted Dr. David K. Strom on the AML-1B Project
- Directed maintenance of tissue culture laboratory; organized equipment and materials for tissue culture
- Performed transfection and infection experiments in tissue culture
- Trained undergraduate students in tissue culture procedures

PUBLICATIONS

Carlton R. Cooper, Hassan Chaib, Bianca Graves, Linda Sequeira, Freddie Pruitt, Angelo Evans, Jill Lynch, Kirk Cymzek, **Rebecca S. Bullard**, Carlton D. Donald, Katia Sol-Church, Christopher H. Chay, James D. Gendernalik, Mary C Farach-Carson, Robert A Sikes, Jill A. Macoska, and Kenneth J. Pienta: Reticulocalbin surface expression in bone endothelial cells and its role in prostate cancer cell adhesion to bone endothelium. *J Cell* Biochem, 2008. (in press)

Sudeep K. Bose, **Rebecca S. Bullard**, and Carlton D. Donald. Oncogenic Role of Engrailed-2 (*En-2*) in Prostate Cancer Cell Growth and Survival. *Translational Oncogenomics*, 2008. (in press)

Rebecca S. Bullard, Willietta Gibson, Sudeep K. Bose, Jamila K. Belgrave, Andre C. Eaddy, Corey J. Wright, Debra J. Hazen-Martin, Janice M. Lage, Thomas E. Keane, Tomas A. Ganz and Carlton D. Donald: Functional Analysis of the Host Defense Peptide Human Beta Defensin-1: New Insight into its Potential Role in Cancer. *Molecular Immunology*, 2008 Feb; 45(3):839-48. Epub ahead of print: 2007 Sep 14.

Willietta Gibson, Ashley Green, **Rebecca S. Bullard**, Andre C. Eaddy and Carlton D. Donald: Inhibition of PAX2 expression results in alternate cell death pathways in prostate cancer cells differing in p53 status. *Cancer Letters.* 2007 April 18; 248(2):251-61.

MANUSCRIPTS IN PREPERATION

Willietta Gibson, Sudeep K. Bose, **Rebecca S. Bullard**, Carlton D. Donald. PAX2 Oncogene Negatively Regulates the Expression of the Putative Tumor Suppressor Human Beta Defensin-1 in Prostate Cancer.

PROFESSIONAL PRESENTATIONS

Functional Analysis of Human Beta-Defensin 1 (hBD1): insight into its role in prostate cancer. Presented a seminar to the Department of Pathology and Laboratory Medicine, MUSC, SC, November 26, 2007.

Defense peptide human Beta Defensin-1: induction of apoptosis and its potential role in the suppression of prostate cancer development. Presented a poster at the 2nd Annual Pathology Research Day, MUSC, SC, August 24, 2007.

Human Beta Defensin-1: novel insight into its potential role in prostate tumor immunity. Presented a seminar at Student Research Day, MUSC, SC, November 3, 2006.

Defense peptide human Beta Defensin-1: induction of apoptosis and its potential role in the suppression of prostate cancer development. Presented a poster at the 1st Annual Pathology Research Day, MUSC, SC, August 25, 2006.

Functional Analysis of Human Beta-Defensin 1 and its Potential Role in Prostate Cancer Tumor Immunity. Presented a seminar at Student Research Day, MUSC, SC, November 4, 2005.

Functional Analysis of Human Beta-Defensin 1 as a Putative Tumor Suppressor Gene in Prostate Cancer. Presented a seminar to the Department of Pathology and Laboratory Medicine, MUSC, SC, November 15, 2004.

Functional Analysis of a Putative Tumor Suppressor Gene Human Beta-Defensin 1 in Prostate Cancer. Presented a seminar at Student Research Day, MUSC, SC, November 5, 2004.

Cancer-specific Loss of Human Beta Defensin-1 in Prostate Carcinoma. Presented a poster at the Departmen of Pathology and Laboratory Medicine Program Exposure Day for First Year Graduate Students, MUSC, SC, September 2004.

Functional Analysis of Human Beta Defensin-1 in Prostate Cancer. Presented a seminar to the Department of Pathology and Laboratory Medicine, MUSC, SC, April 5, 2004.

Cancer-specific Loss of Human Beta Defensin-1 in Prostate Carcinoma. Presented a poster at the Hollings Cancer Center 3rd Annual Cancer Research Retreat, MUSC, SC, December 5, 2003.

Cancer-specific Loss of Human Beta Defensin-1 in Carcinoma. Presented a poster at Student Research Day, MUSC, SC, November 7, 2003.

Cancer-specific Loss of Human Beta Defensin-1 in Carcinoma. Presented a poster at the Program Exposure Day for First Year Graduate Students, MUSC, SC, September 2003.

Transfection and Infection in Mammalian Tissue Culture Cells. Presented a seminar at the Senior Seminar for the Department of Biology and Geology, USCA, Aiken, SC, November 13, 2001.

INVITED PRESENTATIONS

Functional analysis of human beta-defensin 1 and its potential role in prostate cancer tumor immunity. 5th Annual Hollings Cancer Center Research Retreat, MUSC, Charleston, SC, Novemeber 18, 2005.

Fumctional analysis of human beta-defensin 1 as a putative tumor suppressor gene. US TOO Prostate Cancer Support Group, Charleston, SC, August 2, 2005.

Expression of AML-1B by a Retroviral Vector. 75th Annual Meeting of the South Carolina Academy of Science, USCA, Aiken, SC, March 2002.

ABSTRACTS

Rebecca S. Bullard, Willietta Gibson, Sudeep K. Bose and Carlton D. Donald: Defense peptide human Beta Defensin-1: induction of apoptosis and its potential role in the suppression of prostate cancer development. *American Association for Cancer Research 98th Annual Meeting 2007.*

Rebecca S. Bullard, Debra Hazen-Martin, Gian Re, Janice M. Lage, Andrew S. Kraft, Thomas E. Keane, and Carlton D. Donald: Analysis of human beta defensin-1 cytotoxicity to prostate cancer via bystander effect. *American Association for Cancer Research 97th Annual Meeting 2006.*

Rebecca S. Bullard, Jamila K. Belgrave, Willietta Gibson, Andre C. Eaddy, Corey J. Wright, Tomas A. Ganz, Janice M. Lage, Thomas E. Keane and Carlton D. Donald: Novel role of Human Beta Defensin-1 as a tumor suppressor that may be part of the innate immune system involved in tumor immunity. *American Association for Cancer Research 96th Annual Meeting 2005.*

Jamila K. Belgrave, **Rebecca S. Bullard**, Willietta Gibson, Carlton D. Donald: Functional analysis of human beta defensin-1 in prostate cancer. *American Association for Cancer Research 95th Annual Meeting 2004.*

CURRENT RESEARCH INTERESTS

Cancer-specific loss of human beta-defensin 1 in prostate carcinoma
Tumor immunity
Cancer and nutrition
Complimentary alternative medicine

GRANT

Senior Research Grant, Department of Biology and Geology, USCA, Aiken, SC, Awarded Fall 2001

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science
American Association for Cancer Research
Gamma Beta Phi Society
Omicron Delta Kappa National Leadership Honor Society
Society for Basic Urological Research

TEACHING EXPERIENCE

Teaching Techniques, MUSC, Charleston, SC, 2005

- Learned theories and methodologies of teaching.
- Developed and taught a 15 minute lesson plan to demonstrate learned techniques.

Tutor, Spanish, USCA, Aiken, SC, 1998-2001

Lecturer, Biology Department, USCA, Aiken, SC, 2001

- Shared lecture responsibilities of ABIO 502, Advanced Cell/Molecular Biology, with other course members under the supervision of Dr. James Yates.

Teacher Cadet, Beaufort High School, Beaufort, SC, 1996-1997

- Received insight into the nature of the teaching profession.
- Developed and taught lesson plans in Math for a 3rd grade class at Mossy Oaks Elementary School.

STUDENT ACTIVITIES

Student Government Association Representative, MUSC, 2004-2007

- Pitts Memorial (Ethics) Lectureship Committee, University-wide, 2004-2006
- Communications Committee, 2005-2006
- Student Welfare Committee, 2004-2007

Graduate Student Association Representative, MUSC, 2002-2007

- President 2004-2006
- Vice President, 2004-2005
- Alcohol Awareness Committee, 2004-2005

Multicultural Graduate Student Association, MUSC 2003-2007

- Vice President, 2006-2007

Student Facilitator at the 1st Annual Interprofessional Day, MUSC, 2006
Honorary Degree Committee, University-wide, MUSC, 2005-2006

Distinguished Graduate of the Year Award Committee, College of Graduate Studies, MUSC, 2005 and 2006
Health Sciences Foundation Teaching Excellence Awards Selection Committee, University-wide, MUSC, 2005 and 2006
Circle K International, USCA, 1997-2002

- Piedmont Division Lieutenant Governor, 2000-2001
- USCA Chapter Vice President, 1999-2000
- Committee Chair, 1998-1999

Emerging Leaders, USCA, 1998

VOLUNTEER EXPERIENCES

Mentor for teen girls weekly, October 2006-present
Blood donor for American Red Cross, 2001-present
Participated in and raised over $100 for the American Heart Association Heart Walk, October 2, 2004
Volunteered at the Charleston County Public Library to assist students with Science projects, December 2002-February 2003

REFERENCES

Dr. Lisa Cunningham
Coordinator, Graduate Studies
165 Ashley Avenue, Suite 309
PO Box 250908
Charleston, SC 29425
Department of Pathology and Laboratory Medicine
(843) 792-8324
cunninll@musc.edu

Dr. Perry V. Halushka
Dean, College of Graduate Studies
171 Ashley Avenue
Charleston, SC 29425
Department of Pharmacology
(843) 792-3012
halushpv@musc.edu

Dr. Debra Hazen-Martin
165 Ashley Avenue, Suite 309
PO Box 250908
Charleston, SC 29425
Department of Pathology and Laboratory Medicine
(843) 792-2906
hazenmad@musc.edu