TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Expression, Localization, and Function of the Nucleolar Protein BOP1 in Prostate Cancer Progression

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Differentiating between indolent and aggressive prostate cancers (CaP) is important to decrease overtreatment and increase survival for men with the aggressive disease. Nucleolar prominence is a histologic hallmark of CaP; however, the expression, localization, and functional significance of specific nucleolar proteins have not been investigated thoroughly. The nucleolar protein block of proliferation 1 (BOP1) is associated with multiple cancers but has not been implicated in CaP thus far. Meta-analysis of publicly available data showed increased BOP1 expression in metastatic CaP and recurrent CaP, and was inversely associated with overall survival. Multiplexed immunohistochemistry was used to analyze expression and localization of BOP1 and nucleolar protein 56 in human tissue samples from various stages of CaP progression. Here, increased BOP1 expression was observed at later stages of CaP progression, coinciding with a localization change from nuclear to cytoplasmic. In patient samples, cytoplasmic BOP1 was also inversely associated with overall survival. In models of prostate cancer progression, BOP1 expression showed expression and localization similar to that in human patient samples. The functional significance of BOP1 in metastatic CaP was assessed by genetic knockdown, where BOP1 knockdown resulted in decreased proliferation and motility compared with control. Taken together, these data suggest prognostic significance of BOP1 expression and localization in CaP progression and provide a foundation for further investigation into the functional role of nucleolar proteins in advanced CaP. (*Am J Pathol* 2021, 191: 168–179; <https://doi.org/10.1016/j.ajpath.2020.09.010>)

Prostate cancer (CaP) is a widespread disease with approximately 12.1% of men in the United States diagnosed with CaP at some point during their lifetime, according to the Surveillance, Epidemiology, and End Results Program.^{1,2} Fortunately, accessible screening for CaP has led to a high 5-year survival rate of 97.8%.^{1,2} However, when CaP diagnoses are stratified by stage, the 5-year survival rate for men with localized and regional CaP is 100%, whereas the 5-year survival rate decreases to only 30.2% for metastatic prostate cancer.^{1,2} Because of this wide range of CaP prognoses, many cases of indolent (localized) CaP are overtreated clinically, whereas aggressive CaPs that will metastasize are not identified, resulting in undertreatment and/or disease recurrence and decreased survival.^{3–8} This dichotomy between survival among CaP stages shows a

need for biomarkers that can differentiate indolent versus aggressive CaP to better inform treatment strategies, decrease the financial and psychologic burden of overtreatment, and improve survival rates for metastatic/recurrent CaP.

Histologically, cellular changes in CaP progression can inform the severity of disease. One such hallmark of CaP is the development of prominent nucleoli, which has been shown to occur in CaP stages as early as high-grade intraepithelial neoplasia, and persist through later stages,

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including metastasis.^{9,10} In one study, nucleolar size was measured in localized versus metastatic CaP, and indicated a significantly increased nucleolar surface area in metastatic CaP versus localized CaP.¹⁰ Because the nucleolus is the site of ribosomal biogenesis, prominent nucleoli have been associated with several cellular functions including cell-cycle progression and increased proliferation.^{11–14} Although a multitude of proteins and RNAs have been identified within these nucleolar complexes,¹⁵ expression/localization changes of specific nucleolar proteins in the progression of CaP have not been studied as potential biomarkers.

Block of proliferation 1 (BOP1) may be a potential biomarker for indolent versus aggressive CaP. As a component of the pescadillo ribosomal biogenesis factor 1 (PES1)/BOP1/WD Repeat Domain 12 (PeBoW) complex, BOP1 is required for the maturation of the 28S and 5.8S ribosomal RNAs (rRNA), with processing of these rRNAs resulting in the formation of the 60S ribosomal subunit.^{16,17} Functionally, BOP1 is involved in several stages of rRNA processing, and its inactivation in mice results in decreased synthesis of the 60S ribosomal subunit and subsequent cell-cycle arrest.^{12,18} BOP1 also has been implicated in several cancer types including colorectal carcinoma, hepatocellular carcinoma, and melanoma.^{19–21} Consistent with its role in cell-cycle progression, BOP1 overexpression in colorectal cancer results in increased multipolar spindles and chromosomal instability.¹⁹ Interestingly, the role of BOP1 in hepatocellular carcinoma is not limited to its functions in ribosomal biogenesis; in this context, BOP1 promotes invasion and motility, and induces epithelial-to-mesenchymal transition.²⁰ Recently, BOP1 also was implicated in a therapy-resistance mechanism in melanoma, suggesting its function may change at later stages of disease progression.²¹ Importantly, despite well-established nucleolar histologic changes associated with prostatic disease, BOP1 expression, localization, and function in CaP progression is not known.

Materials and Methods

Meta-Analysis

Oncomine was accessed online (<https://www.oncomine.org>, last accessed June 14, 2020) and data sets were analyzed for prostate cancer using cancer versus normal analysis for *BOP1*.²² cBioPortal was accessed online (<https://www.cbioportal.org>, last accessed June 6, 2020), with data sets analyzed for prostate cancer by filtering for copy number alterations and *BOP1*.^{23,24} Gene Expression Omnibus (GEO) data sets (<https://www.ncbi.nlm.nih.gov/geo>, accession numbers GSE6919, public on January 30, 2007,²⁵ GSE25136, public on November 5, 2010,²⁶ and GSE16560, public on March 5, 2010)²⁷ were analyzed with the GEO2R analysis tool for *BOP1* (ID: 35615_at, 212563_at, and DAP4_5968, respectively).

Patient Samples

The prostate cancer progression tissue microarray (TMA) was generated by a board-certified pathologist (W.H.) and contains approximately 340 cores from approximately 170 patients (2 cores/patient). Histologically determined disease stage was used to stratify patients, resulting in 120 cores (60 patients) of benign tissue, 44 cores (22 patients) of high-grade prostatic intraepithelial neoplasia, 130 cores (65 patients) of CaP, and 32 cores (16 patients) of metastatic tissue (mets).²⁸ Diagnosis of cores was confirmed by a pathologist (W.H.) at 10-section intervals. Cores that contained more than 5% intermixed glands, fewer than 100 total cells, or were damaged significantly during processing were excluded from the analysis. The outcomes TMA contains duplicate CaP cores with associated clinical data, including survival.²⁸ Stratification by survival time (in years) resulted in the following sample sizes: 8+ years, $n = 35$; 7 to 8 years, $n = 23$; 6 to 7 years, $n = 44$; and fewer than 6 years, $n = 81$. Both TMAs were constructed in a single institution over the years 1998 to 2006, and the age range for specimens on the TMAs were 37 to 86 years.

Multiplexed Immunohistochemistry and Analysis

Multiplexed immunohistochemistry was performed using commercially available antibodies against BOP1 (A302-149A, 1:125 dilution; Bethyl Laboratories, Inc., Montgomery, TX), nucleolar protein 56 (NOP56; AMAb91013, 1:125 dilution; Sigma Life Science, St. Louis, MO), and E-cadherin (790-4497, undiluted; Ventana, Oro Valley, AZ). All antibodies used in this study have been validated for immunohistochemistry by the vendor. Quantification was performed as previously described.^{28,29} Briefly, the TMA slides were scanned and imaged at $\times 20$ magnification using the Vectra2 quantitative pathology imaging system. inForm Cell Analysis software version 2.1 (PerkinElmer, Waltham, MA) was used to generate spectral libraries of diaminobenzidine, Renoir Red, Vina Green, and hematoxylin, allowing for optical separation of the four stains. Using the Tissue Finder function of inForm, specimens were segmented digitally by tissue type (stroma, epithelia) and cell compartment (nuclear, cytoplasmic).²⁸ The mean OD, measured in intensity per pixel, was calculated for each, and the two cores from the same patient were averaged to generate a single mean OD value for each patient.

Cell Culture

Standard prostate cell lines prostate epithelial transformed by HPV (RWPE-1), prostate cancer bone metastasis (PC3), and prostate cancer brain metastasis (DU145) were obtained from ATCC (Manassas, VA). Benign Prostatic Hyperplasia cell line 1 (BPH1) was generously provided by Dr. Simon Hayward at NorthShore University Health Systems (Evanston, IL), and lymph node carcinoma for the prostate (LNCaP) cells were obtained from collaborators at the University of

Wisconsin-Madison. BPH1 to Prostate Cancer (BCaP) cell lines were generated in Dr. William A. Ricke's laboratory (Madison, WI) as described previously.^{30–32} All cell lines were maintained in RPMI1640 + 2.05 mmol/L L-glutamine media (Hyclone, Chicago, IL) supplemented with 5% fetal bovine serum (Hyclone), 2.5% HEPES (Hyclone), 1% penicillin/streptomycin (Hyclone), 0.2% normacin (Invivogen, San Diego, CA), and routinely passaged.

Quantitative PCR

RNA was isolated using the Maxwell 16 LEV simplyRNA Purification kit (AS1270; Promega, Madison, WI), and cDNA was made using iScript Reverse Transcription Supermix (1708841; BioRad, Hercules, CA). Quantitative PCR for BOP1 (forward: 5'-GTGGGCTTCAACCCCTATGAG-3', reverse: 5'-CCATGCGAGAGACCTTCTCC-3') was performed using SSO Universal SYBR (1725271; BioRad), and normalization was calculated in reference to TATA-box binding protein (forward: 5'-CCACTCACA-GACTCTCACAAC-3', reverse: 5'-CTGCGGTA-CAATCCCAGAACT-3') and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (forward: 5'-TGATCCCCAATGCTTCACAAG-3', reverse: 5'-GCCAAGGTAACGGTAGTAATCT-3').

Western Blot

Western blot protocol using BioRad precast gels and nitrocellulose membrane transfer packages was performed as previously described.³³ For subcellular fractionation, the nuclear and cytoplasmic fractions were separated by centrifugation following the Abcam (Cambridge, UK) protocol. Primary antibodies specific to BOP1, histone 3 (H3; 17168-1-AP; Proteintech, Rosemont, IL), and α -tubulin (Cell Signaling Technology, Danvers, MA) were diluted in 5% bovine serum albumin/Tris-buffered saline/Tween-20 (1:5000 BOP1, 1:1000 H3, and 1:1000 α -tubulin) and incubated overnight at 4°C. Species-specific, horseradish-peroxidase-conjugated secondary antibodies (Bethyl Laboratories, Inc.) were incubated at room temperature for 1 hour and visualized using chemiluminescent substrate DURA (Thermo Fisher Scientific, Waltham, MA). Band intensity was quantified with ImageJ software version 1.49³⁴ (NIH, Bethesda, MD; <https://imagej.nih.gov/ij/>) after it was normalized to α -tubulin (cytoplasmic fraction, whole cell) or H3 (nuclear fraction). To show antibody specificity, an uncropped BOP1 Western blot is included showing a single band around 83 kDa, as expected (Supplemental Figure S1A).

Immunofluorescence

Immunofluorescence was performed according to Abcam's protocol. Briefly, cells were fixed, permeabilized, and incubated with primary antibodies BOP1 (diluted 1:500) and NOP56 (diluted 1:200) for 1 hour at room temperature. Secondary

antibodies [anti-rabbit conjugated to AlexaFluor 488 (A-21206; Thermo Fisher Scientific) and anti-mouse conjugated to AlexaFluor 555 (A-32773; Thermo Fisher Scientific)] were incubated for 1 hour at room temperature. DAPI was used for the nuclear stain, and images were taken at $\times 40$ magnification.

Proliferation and Motility Assays

SMARTpool ON-TARGETplus Human *BOP1* siRNA (siBOP1) was acquired from Dharmacon (Lafayette, CO; L-014065-01-0005), with the associated siRNA nontargeting scramble control (D-001810-10-05). BCaP metastatic 1 (BCaP^{M1}) cells were transfected transiently with either 37.5 nmol/L siBOP1 or siRNA nontargeting scramble control using the Mirus TransIT-X2 Dynamic Delivery System (MIR 6005; Mirus, Madison, WI), according to the Mirus protocol. Proliferation was assessed by staining with MTT (M5655; Sigma-Aldrich, St. Louis, MO). To assess motility, BCaP^{M1} cells were plated at a seeding density of 0.25×10^6 cells/well and allowed to adhere overnight. The adherent cells were treated with siBOP1 or control, and a scratch was made through the cell monolayer in the center of each well as previously described.³¹ Images were taken at 0, 6, 12, and 24 hours, and the area of the scratch was quantified with ImageJ. Statistics reflect $n = 3$ biological replicates.

Statistics

Most bar graphs reflect the mean of the samples within each category with error bars showing the SEM, cBioPortal data show error bars of SD. GraphPad Prism version 8.4.1 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses. The Welch *t*-test was used for direct comparisons, and one-way analysis of variance with the Dunnett test was used for multicomparison statistics. For survival analysis from meta-data (accession number GSE16560), a Kaplan-Meier curve was used to represent survival in days with the robust multi-array average—normalized expression divided at the median into high ($n = 140$) and low ($n = 140$) expression. The hazard ratio was calculated using a Mantel-Haenszel test, and the *P* value was calculated using a log-rank Mantel-Cox test.

Results

BOP1 Expression Is Increased in Advanced CaP

A meta-analysis of publicly available data sets shows significant *BOP1* gene expression changes in CaP progression and recurrence. In data acquired from Oncomine, *BOP1* expression was increased significantly in multiple data sets when comparing prostate cancer with normal prostatic tissue (Table 1). Data mined from a GEO study assessing expression through CaP progression showed that *BOP1* expression was significantly higher in CaP tumors with higher Gleason scores (Gleason score, >6 ; $P < 0.01$) and

Table 1 *BOP1* Expression in Prostate Cancer versus Normal

Data set	FC	N	P value	Significance
Taylor prostate 3	1.125	185	1.97E-6	****
Grasso prostate	1.365	122	1.56E-5	****
TCGA prostate (acinar)	1.041	187	0.001	***
Welsh prostate	1.790	34	0.003	**
Luo prostate 2	1.601	30	0.004	**
Singh prostate	1.598	102	0.006	**
TCGA prostate (adeno)	1.051	106	0.008	**
Varambally prostate	1.415	19	0.033	*
Liu prostate	1.068	57	0.044	*

All data are publicly available through the Oncomine database (<https://www.oncomine.org>).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

adeno, adenocarcinoma; BOP1, block of proliferation 1; FC, fold change; TCGA, The Cancer Genome Atlas.

metastatic samples (mets; $P < 0.0001$), compared with that in the benign tissue (Figure 1A). Furthermore, data from cBioPortal shows *BOP1* copy number amplification in 8 independent data sets, with amplification ranging from approximately 1% of samples to more than 28% of samples within each study (Figure 1C). Three of the top four most amplified data sets for *BOP1* represent metastatic CaP (Figure 1C), with a significant increase in copy number amplification in metastases versus primary CaP ($P < 0.0001$) (Figure 1D). In another GEO study assessing expression in CaP recurrence, *BOP1* expression was increased significantly in recurrent samples compared with nonrecurrent samples ($P < 0.05$) (Figure 1B). Finally, *BOP1* expression was assessed in survival using a third GEO data set (Figure 1E). Here, overall survival was decreased significantly in samples with high *BOP1* expression compared with samples with low *BOP1* expression. Taken together, these data show increased *BOP1* expression through CaP progression and higher expression in recurrence and samples with decreased survival, suggesting that *BOP1* may be a biomarker for advanced CaP.

BOP1 Expression and Localization in CaP Patient Samples

To assess the expression and localization of BOP1 protein in CaP progression, tissue samples from several stages of CaP progression (benign, high-grade prostatic intraepithelial neoplasia, CaP, and mets) were co-stained for BOP1, nucleolar marker NOP56, E-cadherin, and hematoxylin. Multiplexing these factors allows for the following: i) comparison between expression and localization of two nucleolar proteins (BOP1 and NOP56), ii) segmentation of tissue types using E-cadherin as a marker for epithelial cells, and iii) subcellular compartment segmentation with hematoxylin as a nuclear stain (Figure 2). Representative images of BOP1 expression in CaP progression show increased expression in later stages of progression, consistent with metadata (Figure 3A). When quantified, total epithelial BOP1 expression was increased significantly in CaP

compared with that in benign tissue ($P < 0.05$) (Figure 3B). Interestingly, this increase in expression through progression appeared to coincide with a localization change from nuclear to cytoplasmic (Figure 3A). Quantification within subcellular compartments of epithelial cells showed no significant change in nuclear BOP1 through progression whereas cytoplasmic BOP1 increased significantly in CaP ($P < 0.001$) and mets ($P < 0.05$) compared with benign tissue (Figure 3B).

Because BOP1 is a nucleolar protein, it is possible that its expression and localization through CaP progression is indicative of overall nucleolar changes in CaP. To investigate the possibility that BOP1 is representative of overall nucleolar changes, tissue samples were co-stained with another nucleolar protein, NOP56. This protein has been established as part of the nucleolar fibrillar center and has been further validated as a marker for the nucleolus by the Human Protein Atlas initiative (www.proteinatlas.org, last accessed August 17, 2020). Consistent with prominent nucleoli in CaP progression and BOP1 expression, NOP56 expression increased in later stages of progression (Figure 3C). When quantified, total epithelial NOP56 expression was increased significantly in CaP ($P < 0.05$) and mets ($P < 0.05$) compared with that in the benign tissue (Figure 3D). However, unlike BOP1, this increased expression through progression appeared to occur in the nucleus with a significant increase of nuclear NOP56 in CaP ($P < 0.05$) and mets ($P < 0.05$) compared with that in the benign tissue, whereas cytoplasmic NOP56 did not change (Figure 3D). Taken together, these data indicate a cellular localization change of BOP1 from nuclear to cytoplasmic in CaP progression. This localization change did not coincide with overall nucleolar changes because nucleolar marker NOP56 increased in the nucleus through CaP progression. Therefore, cytoplasmic BOP1, independent of nucleolar prominence, may serve as an independent biomarker for advanced stages of CaP.

Cytoplasmic BOP1 Expression and Survival

To assess BOP1 as a biomarker for aggressive CaP, an additional set of patient samples was stained and analyzed using clinical data for patient survival outcomes. These samples contain CaP cores with associated clinical data, including years of survival. In these CaP samples, BOP1 expression was primarily cytoplasmic, supporting data from the progression TMA (Figure 4A). In addition, BOP1 expression appeared to increase in samples with shorter survival, consistent with meta-analysis (Figure 4A). Quantification indicated that total BOP1 expression was increased significantly in the group with the shortest survival (<6 years) compared with the group with the longest survival (8+ years) ($P < 0.0001$) (Figure 4B). Importantly, quantification of cytoplasmic BOP1 showed a significant increase in expression in the group with survival of fewer than 6 years compared with the group with survival of 8 or more years ($P < 0.0001$) (Figure 4C). A scatterplot showing BOP1 expression versus survival in non-binned samples is shown in Supplemental Figure S1C. In addition, cytoplasmic linear regression analysis of BOP1 versus

Figure 1 Block of proliferation 1 (*BOP1*) expression in meta-analysis of human prostate cancer (CaP). **A:** Gene expression analysis of publicly available Gene Expression Omnibus data showed a significant increase of *BOP1* expression in aggressive tumors (Gleason score, >6) and metastases compared with benign tissue. **B:** Gene expression analysis of publicly available GEO data showed a significant increase of *BOP1* expression in recurrent tumors versus nonrecurrent tumors. **C:** Analysis of *BOP1* copy number amplification accessed via cBioPortal showed copy number amplification of *BOP1* present in all data sets, with amplification frequency ranging from less than 5% to more than 25%. Prostate adenocarcinoma samples are shown with black bars, and metastatic samples are shown with red bars. **D:** Amplification of *BOP1* is increased significantly in metastatic samples compared with primary adenocarcinoma. **E:** Survival analysis of *BOP1* expression showed a significant decrease in survival in samples with high *BOP1* expression, compared with samples with low *BOP1* expression ($P = 0.0047$). ** $P < 0.01$, **** $P < 0.0001$ versus benign tissue; † $P < 0.05$ versus nonrecurrent; #### $P < 0.0001$ versus primary adenocarcinoma. AU, arbitrary units; DFCI, Dana-Farber Cancer Institute; FHCRC, Fred Hutchinson Cancer Research Center; HR, hazard ratio; MCP, metastatic prostate cancer project; MCTP, Michigan Center for Translational Pathology; Mets, metastatic tissue; MSKCC, Memorial Sloan Kettering Cancer Center; PCF, prostate cancer foundation; SMMU, Second Military Medical University; SU2C, stand up to cancer.

Gleason scores showed an increase of *BOP1* in samples with higher Gleason scores ($P = 0.0731$) (Supplemental Figure S1B). Taken together, these data suggest that *BOP1* expression, specifically in the cytoplasm, is associated with decreased overall survival in CaP. Moreover, these data provide a foundation for investigation of *BOP1* as a potential biomarker for aggressive CaP with poor overall survival.

BOP1 Expression, Localization, and Function in Models of CaP

Because cytoplasmic *BOP* expression is increased in CaP progression and associated with decreased survival, it may be informative to understand the role of *BOP1* in the cytoplasm. To address the functional role of cytoplasmic

Figure 2 Multiplexed immunohistochemistry (IHC) and segmentation. **A:** Representative images of optically isolated images from a multiplexed IHC on tissue microarray samples from various stages of prostate cancer (CaP) progression [benign, high-grade prostatic intraepithelial neoplasia (HGPIN), CaP, and metastases (mets)]. **B:** Representative images of cellular compartment segmentation: nucleus (green) versus cytoplasm (multicolored) using inForm software. Similarly, tissue compartments were segmented to identify epithelium (red) versus stroma (green). Original magnification, $\times 20$. BOP1, block of proliferation 1; NOP56, nucleolar protein 56.

Figure 3 Block of proliferation 1 (BOP1) and nucleolar protein 56 (NOP56) expression in prostate cancer (CaP) progression. **A:** Representative images of BOP1 expression in CaP progression. **Boxed areas** in **top row** are shown at higher magnification in **bottom row**. **B:** Total BOP1 expression increased in CaP compared with benign tissue ($P < 0.05$). There was no significant change in nuclear BOP1 expression, but there was a significant increase in cytoplasmic BOP1 expression in CaP and metastases (mets) compared with benign tissue. **C:** Representative images of NOP56 expression in CaP progression. **Boxed areas** in **top row** are shown at higher magnification in **bottom row**. **D:** Total NOP56 expression and NOP56 nuclear expression were increased significantly in CaP and mets compared with benign tissue. There was no significant change in cytoplasmic NOP56 expression. * $P < 0.05$, *** $P < 0.001$. Original magnification: $\times 20$ (**A** and **C**, **top row**); $\times 60$ (**A** and **C**, **bottom row**). HGPIN, high-grade prostatic intraepithelial neoplasia.

Figure 4 Block of proliferation 1 (BOP1) expression and survival. **A:** Representative images of BOP1 expression in human prostate cancer outcomes tissue microarray. Cores were divided into years of survival: 8+ years, 7 to 8 years, 6 to 7 years, and fewer than 6 years. **Boxed areas** in **top row** are shown at higher magnification in **bottom row**. **B:** Quantification of total BOP1 expression in samples stratified by years of survival showed a significant increase of BOP1 expression in samples with the shortest survival (<6 years) compared with the samples with the longest survival (8+ years). **C:** Quantification of cytoplasmic BOP1 expression showed a significant increase of cytoplasmic BOP1 in samples with the shortest survival (<6 years) compared with the samples with the longest survival (8+ years). **** $P < 0.0001$. Original magnification: $\times 20$ (**A**, **top row**); $\times 60$ (**A**, **bottom row**). OD, optical density.

BOP1, its expression and localization were assessed in several CaP cell line models. At the RNA level, BOP1 expression was increased significantly in the CaP cell lines metastatic/aggressive primary CaP (BCaP^{T10}; $P < 0.05$), BCaP^{M1} ($P < 0.05$), DU145 ($P < 0.01$), LNCaP ($P < 0.01$), and PC3 ($P < 0.01$) compared with the nontumorigenic cell line BPH1 (Figure 5A). To assess BOP1 protein expression changes through CaP progression, the BCaP model was used because of its derivation from a single patient, allowing maintenance of syngeneity for meaningful comparisons between disease stages.³¹ In this model, BOP1 protein expression and localization were assessed in nonmetastatic/indolent primary CaP (BCaP^{T1}), compared with BCaP^{T10} and BCaP^{M1}.³¹ Western blot analysis showed an increase in total BOP1 protein expression in BCaP^{T10} and BCaP^{M1} compared with BCaP^{T1} (Figure 5, B and C). Similar to data from patient samples, the increase in BOP1 expression

occurred in the cytoplasmic fraction, although nuclear BOP1 decreased (Figure 5, B and C). Using immunofluorescence, BOP1 localization was visualized in the BCaP model. Here, BOP1 co-localized with nucleolar marker NOP56 in BCaP^{T1} and BCaP^{T10} in puncta in the nucleus, suggesting localization to nucleoli (Figure 5D). However, in BCaP^{T10} and BCaP^{M1}, BOP1 was localized to the cytoplasm, consistent with patient data for metastatic CaP (Figure 5D). To assess the functional role of BOP1 in advanced CaP, siRNA was used to genetically knock down BOP1 in BCaP^{M1} cells. Western blot confirmed a robust knockdown of BOP1 with siRNA targeting BOP1 (*siBOP1*) compared with nontargeting scramble siRNA control (Figure 5E). Results from a proliferation assay showed that BOP1 knockdown decreased BOP1 proliferation significantly compared with controls (Figure 5F). Similarly, *siBOP1* decreased motility significantly compared with

control (Figure 5G and Supplemental Figure S1D), suggesting that *BOP1* also may play a role in mechanisms of cell motility. In addition, because the BOP1 expression in BCaP^{M1} is predominantly cytoplasmic, the decrease of proliferation and motility with BOP1 knockdown may be attributed to a loss of cytoplasmic *BOP1* function. Taken together, these data suggest that the BCaP model accurately represents human CaP progression for BOP1 expression/localization and provides a foundation for further assessment of the functional significance of cytoplasmic BOP1.

Discussion

Distinguishing between indolent and aggressive CaP has surged to the forefront of CaP research in recent years in an attempt to better inform treatment strategies and improve patient survival. Although some histologic and biochemical changes have been associated with advancement of CaP [ie, Gleason score/grade, clinical T stage, prostate-specific antigen (PSA), and prostate health index], biomarkers differentiating indolent versus aggressive CaP are scarce and lack ubiquitous use in the clinic.³⁵ Nucleolar prominence has been recognized as a histologic marker for CaP and CaP progression for decades^{9,10}; however, the functional implications of expression/localization changes for specific nucleolar proteins has not been studied in detail. Here, expression and localization of nucleolar protein BOP1 was explored both in CaP progression and in its association with clinical outcomes such as survival. To the best of our knowledge, this is the first publication to investigate the expression and localization of BOP1 expression in CaP, and its potential implications as a biomarker.

Historically, biomarkers for the prognosis of CaP have been difficult to identify and incorporate into clinical use. Although several CaP biomarkers have been characterized including PSA, phosphatase and tensin homolog (*PTEN*),³⁶ transmembrane protease serine 2:v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2:ERG*),³⁷ and androgen receptor variant 7 (ARv7), their prognostic values are limited, presenting a need for additional biomarkers. PSA is a serum biomarker that, when increased, indicates the presence of CaP; however, because PSA cannot stratify CaP prognosis, its widespread clinical use has contributed to overdiagnosis and overtreatment of CaP. Loss of *PTEN* has also been associated with CaP progression, with *PTEN*

mutation or deletion identified in 20% of primary CaP and increasing to up to 50% of aggressive/recurrent CaP with *PTEN* loss.³⁶ More recently, a gene fusion allowing *TMPRSS2*-driven *ERG* expression was identified in approximately 50% of CaP with some controversy around its prognostic significance.^{38,39} Emergence of *Arv7* expression was also identified in advanced CaP in approximately 18% to 30% of samples, which has been shown to provide a mechanism for therapy resistance.⁴⁰ Finally, several preliminary studies have identified potential biomarkers for advanced disease including protein tyrosine phosphatase 4A3 and prostate cancer–associated 3.^{41,42} Despite exhaustive efforts, the panel of biomarkers for aggressive disease is incomplete, and identification of additional biomarkers is necessary to improve treatment strategies.

In the present study, the expression, localization, and prognostic significance of BOP1 in CaP progression was investigated. Recently, nucleolar proteins were implicated in CaP with the investigation of small nucleolar RNA.¹⁵ Because BOP1 has been identified as part of a nucleolar complex, its expression and localization in CaP could provide insight into the histologic phenomenon of nucleolar prominence in CaP. However, these data indicate a localization change of BOP1 from nuclear to cytoplasmic that appears to be independent of nucleolar integrity. This localization change was associated with advanced disease and decreased survival. Although little is known about the significance of cytoplasmic BOP1, one study showed that BOP1 expression in the cytoplasm can occur owing to a molecular imbalance of BOP1 in relation to other components of the PeBoW complex. Nucleolar localization of BOP1 was dependent on PES1, and overexpression of BOP1 without PES1 resulted in accumulation of BOP1 in the cytoplasm.¹⁷ In other studies, BOP1 was implicated in increasing cell motility by activating RhoA²⁰ or decreasing apoptosis by inhibiting p53,⁴³ potentially supporting a role for BOP1 in advanced/metastatic disease.

This potential role for cytoplasmic BOP1 in metastasis can be addressed using the BCaP cell lines. This model is ideal for studying CaP progression for several reasons. First, the cell lines that represent each stage of CaP progression (nontumorigenic, indolent tumor, aggressive tumor, metastases) in this model are all derived from a single cell taken from a patient sample.³¹ Therefore, these cell lines are syngeneic, allowing meaningful gene expression

Figure 5 Block of proliferation 1 (*BOP1*) expression, localization, and function in prostate cancer (CaP) progression models. **A:** *BOP1* mRNA expression was assessed in a panel of prostate cell line models including benign [benign prostatic hyperplasia cell line 1 (BPH1), prostate epithelial transformed by HPV (RWPE-1)] and CaP [nonmetastatic/indolent primary CaP (BCaP^{T1}), metastatic/aggressive primary CaP (BCaP^{T10}), BCaP metastatic 1 (BCaP^{M1}), prostate cancer brain metastasis (DU145), lymph node carcinoma for the prostate (LNCaP), and prostate cancer bone metastasis (PC3)], where *BOP1* was increased significantly in BCaP^{T10}, BCaP^{M1}, DU145, LNCaP, and PC3 compared with BPH1. **B:** BOP1 protein expression was assessed in the BCaP progression cell lines, where total BOP1 and cytoplasmic BOP1 was increased in aggressive CaP (BCaP^{T10}) and metastatic CaP (BCaP^{M1}) compared with indolent CaP (BCaP^{T1}). **C:** Quantification of subcellular fractionation Western blot analysis from panel B. **D:** Immunofluorescent staining for BOP1 (green) and nucleolar marker nucleolar protein 56 (NOP56) (red) in the BCaP cell lines showed a BOP1 localization change from nucleoli in indolent CaP to the cytoplasm in metastatic CaP. Nuclei were stained with DAPI (blue). **E:** Western blot showed robust BOP1 knockdown in metastatic CaP cell line BCaP^{M1} with siRNA targeting *BOP1* (*siBOP1*) compared with nontargeting scramble siRNA control (*siSCBL*). α -tubulin (α -tub) was used as a loading control. **F:** *siBOP1* significantly decreased proliferation of BCaP^{M1} cells compared with control. **G:** *siBOP1* significantly decreased motility of BCaP^{M1} cells compared with control at 24 hours. **P* < 0.05, ***P* < 0.01 versus BPH1; †*P* < 0.05, †††*P* < 0.0001 versus *siSCBL*. Scale bar = 25 μ m (**D**). FC, fold change; H3, histone 3.

comparisons between stages.³¹ Second, important genetic and molecular changes are associated with disease progression, including the TMPRSS2:ERG gene fusion, were observed in this model, this being the only CaP model to conserve this fusion event.³¹ Finally, not only do the BCaP cell lines accurately model human BOP1 expression and localization change from the nucleolus to the cytoplasm, but the genetic inhibition of BOP1 also decreased proliferation and motility in this model. Taken together, these characteristics and expression profiles suggest the BCaP model would be ideal for further functional analysis of BOP1 in CaP progression.

This study characterized BOP1 expression and localization in CaP progression, and further suggested a prognostic value for BOP1 cytoplasmic expression in metastatic CaP in association with decreased survival. Based on a search of the literature, there may be a functional role of cytoplasmic BOP1 to induce cell motility via RhoA, which may lead to metastasis. Similarly, BOP1 in CaP may be functioning to inhibit apoptosis by associating with p53. The present results show that genetic knockdown of BOP1 expression decreased proliferation and motility in advanced CaP. There is a clinical need for additional prognostic biomarkers to differentiate indolent versus aggressive CaP, and observation from the current study of BOP1 localization to the cytoplasm in metastatic CaP provides a basis for further investigation of BOP1 as a biomarker in this context. Follow-up studies addressing the activity of BOP1 in the cytoplasm may provide additional insight into the molecular changes that occur as CaP progresses, and potentially serve as a foundation for targeting BOP1 in metastatic CaP.

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Supplemental Data

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