ORIGINAL ARTICLE

Modeling human prostate cancer progression in vitro

Teresa T. Liu¹, Jonathan A. Ewald¹, Emily A. Ricke¹, Robert Bell², Colin Collins² and William A. Ricke^{1,3,4,*}

¹Department of Urology, University of Wisconsin–Madison, Madison, WI, USA, ²Vancouver Prostate Center, Vancouver Coastal Health Research Institute, Vancouver, British Columbia, Canada and ³Carbone Comprehensive Cancer Center and ⁴George M. O'Brien Center of Research Excellence, University of Wisconsin–Madison, Madison, WI, USA

*To whom correspondence should be addressed. Tel: +1 608 265 3202; Fax: +1 608 265 0614; Email: rickew@urology.wisc.edu

Abstract

Detailed mechanisms involved in prostate cancer (CaP) development and progression are not well understood. Current experimental models used to study CaP are not well suited to address this issue. Previously, we have described the hormonal progression of non-tumorigenic human prostate epithelial cells (BPH1) into malignant cells via tissue recombination. Here, we describe a method to derive human cell lines from distinct stages of CaP that parallel cellular, genetic and epigenetic changes found in patients with cancers. This BPH1-derived Cancer Progression (BCaP) model represents different stages of cancer. Using diverse analytical strategies, we show that the BCaP model reproduces molecular characteristics of CaP in human patients. Furthermore, we demonstrate that BCaP cells have altered gene expression of shared pathways with human and transgenic mouse CaP data, as well as, increasing genomic instability with TMPRSS2–ERG fusion in advanced tumor cells. Together, these cell lines represent a unique model of human CaP progression providing a novel tool that will allow the discovery and experimental validation of mechanisms regulating human CaP development and progression. This BPH1-derived Cancer Progression (BCaP) model represents different stages of cancer. The BCaP model reproduces molecular characteristics of prostate cancer. The cells have altered gene expression with TMPRSS2–ERG fusion representing a unique model for prostate cancer progression.

Introduction

Although the role of androgen signaling in prostate cancer (CaP) is well established, the specific molecular mechanisms involved in human CaP development and progression are not well understood (1,2). This is largely because of the lack of experimental models that reproduce important aspects of the biology of CaP as it occurs in human patients. These characteristics include the following: hormonally induced carcinogenesis of non-tumorigenic human prostate epithelial cells, intact stromal–epithelial interactions within a three dimensional *in vivo* context, tumor progression through distinct stages including non-malignant hyperplasia, carcinoma *in situ*, organ-confined localized tumors, indolent tumors with limited growth potential and aggressive metastatic tumors (2–4). Many established human CaP cell lines are derived from metastatic tumors that are too advanced to effectively address early stages of cancer progression experimentally. Although these advanced

CaP cell lines can be compared with immortalized ‘normal’ cell lines including RWPE1 and NHPRE1 (5,6), the patient-based variation between each of these genetically distinct independent cell lines confounds attempts to reliably compare normal versus tumor samples (3,7). Genetically engineered mouse models have been developed that produce prostate tumors, including the transgenic adenocarcinoma of mouse prostate (TRAMP) tumor model (4,8). Differences between the disease processes and characteristics of genetically engineered mouse tumors and human patient tumors make their relevance to human CaP development controversial (2,9). In the TRAMP model, for example, mice develop prostatic intraepithelial neoplasia lesions by 3 months of age, but the primary pathology is limited to neuroendocrine carcinoma with metastases (10). Analyses of patient prostate tumors and comparison to non-tumor controls frequently identify candidate genes and factors that may promote

Received: October 17, 2018; Revised: December 10, 2018; Accepted: December 12, 2018

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Abbreviations

BCaP	BPH1-derived Cancer Progression
CaP	prostate cancer
CNV	copy-number variation
E2	17 β -estradiol
miRNA	microRNA
mRNA	messenger RNA
qPCR	quantitative PCR
SNP	single nucleotide polymorphism
T	testosterone
TRAMP	transgenic adenocarcinoma of mouse prostate

or suppress prostate tumorigenesis or metastasis in humans (11). However, without a reliable model to compare with, or manipulate, their functional relevance in humans cannot be effectively investigated (3). As a result, the development of novel therapeutic targets and predictive biomarkers that may improve CaP patient treatment or prevention is limited. Currently, there are no models for human CaP that represent a singular genome involving key stages of CaP progression (e.g. non-tumorigenic, tumorigenic, metastatic), as well as, important genetic and epigenetic changes that are consistent with human disease. Moreover, there is a lack of models that have these features that are derived from the same genetic background and are available for manipulation *in vivo* and *in vitro*.

Previously, we described a tissue recombination model of hormonal carcinogenesis and progression from non-tumorigenic human prostate cells (12,13). In this model, tissue recombinants composed of a non-tumorigenic prostate epithelial cell line were combined with rodent urogenital mesenchyme stromal cells and renal capsule grafted into adult male athymic mice that received slow-releasing testosterone (T) and 17 β -estradiol (E2) implants. BPH1 cells are a non-tumorigenic human prostate epithelial cell line (14). After 3 months or more of growth *in vivo*, tissue recombinants developed into localized and invasive tumors and frank metastases could be observed. In this model, progression is dependent on the exposure of epithelium to stroma and hormones. When cells are derived from dissociation of these tumors and re-grafted into mice, the resulting tumors developed independent of stroma and hormones. Interestingly, these cells recapitulated the pro- or non-metastatic behavior of the tumors from which they were derived. These data suggest the hormonal carcinogenesis of BPH1 cells produce inherent changes that first promote tumorigenesis and later drive progression to metastasis. The objective of these experiments was to develop a model of CaP progression that represented key aspects of human disease that was easy to generate, manipulate and elucidate important cellular, genomic and epigenetic characteristics important in human disease.

Here we describe the development of cell lines from tumors of xenografted BPH1 cells. We refer to the model as the BPH1-derived Cancer Progression (BCaP) model with individual sublines representing separate stages of human CaP. The molecular characteristics of these cell lines and the sequential nature of the development allow for the interrogation of stage-specific medical interventions with a direct comparison to the normal control that has been unavailable to date.

Methods

Derivation and growth of BCaP cell lines

BPH1 and mouse UGM cells were combined and grafted under the renal capsule of intact adult male athymic mice receiving T+E2 slow-releasing

implants (12). Under differential conditions, the unique tumors from each mouse were allowed to form, thereby representing stage-specific characteristics similar to what is observed in human CaP patients (12). These tumors were dissociated and cultured *in vitro* under G418 selection medium to limit recovery to BPH1-derived cells (13,15). Bulk cell lines were cloned by serial dilution, isolation and propagation under G418 selective medium (16). Renal capsule grafting experiments assessing tumor behavior were performed using these cell lines as described previously (12).

BCaP cell lines were routinely maintained in RPMI supplemented with 5% fetal bovine serum, 2.5% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 0.2% normacin. To investigate whether the basal rate of *in vitro* proliferation varies in BCaP cell lines, equal numbers of cells were plated in triplicate 35 mm wells in growth medium and counted after 48 h, as described previously (17). To investigate whether cellular motility varies in the BCaP cell lines, cells were plated in triplicate wells of 24-well plates and grown to confluent monolayers. Wells were scratched, and the size of the scratch was measured immediately and after 6 h in growth medium, as described previously (18).

Global gene expression in BCaP cells

BCaP cell lines were cultured *in vitro* and messenger RNA (mRNA) was isolated from cells using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). Total mRNA was processed, labeled, hybridized to Affymetrix HuGene-1.0-st-v1 expression microarrays containing 22 211 individual gene probes, and data were collected and quantified by Affymetrix (Santa Clara, CA). Resulting data were analyzed to determine expression relative to parental BPH1 cells as described previously (19). Genes with expression levels up- or downregulated 2-fold were noted. Owing to limited sample size, statistics could not be incorporated into gene selection of this dataset.

Meta-analysis of publicly available datasets

To obtain global gene expression datasets to compare with BCaP, a search of the National Center for Biotechnology Information-Gene Expression Omnibus was performed to identify publicly available datasets of genome-wide mRNA expression data from human CaP tissues (20,21). This produced results that included the GSE6919 dataset generated by a previous study of normal prostate and CaP tumors of varying stages of progression collected from human patients (22,23). These data were segmented into groups of samples representing autopsy-normal, primary tumors with Gleason scores ≤ 6 , primary tumors with Gleason scores ≥ 7 , all metastases and lymph node metastases only. Data were analyzed using the GEO2R tool to identify differentially expressed genes relative to normal prostate obtained by autopsy. Only genes with greater than log2-fold change over control with an adjusted P-value < 0.05 were considered to be differentially expressed (19). Likewise, data generated from a study of TRAMP tumors (24) were identified (GSE10525), segmented into tumor and non-tumor samples and similarly analyzed.

To further interpret these data, bioinformatics pathway enrichment analysis was performed using WebGestalt to identify pathways and processes affected by differential gene expression relative to normal controls (25–27). Enriched pathways were identified using the hypergeometric test combined with the Benjamini-Hochberg procedure to adjust for the false discovery rate.

Analysis of select gene promoter methylation in BCaP cell lines

Genomic DNA was isolated from BCaP cell lines, subjected to bisulfate conversion of methylated cytosine bases (28) and quantified using real-time quantitative PCR (qPCR) with a Human Prostate Cancer EpiTect Methyl Signature qPCR Array (Qiagen, Valencia, CA). Data were analyzed using the raw C_T values and template provided by SABiosciences, Frederick, MD (version 1.7, 15 March 2011).

Detection of TMPRSS2-ERG fusion gene expression

To determine if TMPRSS2-ERG gene fusion occurs and is expressed in BCaP model cell lines, isolated mRNA was reverse-transcribed to complementary DNA using the OneStep RT-PCR Kit (Qiagen). Primers were designed to amplify a 150 bp region spanning a gene fusion site between the first exon of TMPRSS2 and the fourth exon of ERG, as described previously

(29,30). PCR was performed as described previously using the complementary DNA as template (31). The human CaP cell line, H660, was used as a positive control (29).

Single nucleotide polymorphism calling and copy-number variation in BCaP cell lines

Genomic DNA was isolated from BCaP cell lines and handled, processed, labeled and hybridized to Affymetrix SNP 5.0 tiled oligonucleotide microarrays according to manufacturer's instructions. For single nucleotide polymorphism (SNP) analysis, the Affymetrix CEL files were processed with the software GeneSpring version 12.0 (Agilent Technologies and Strand Life Sciences). Genotype analysis was performed using the Birdseed algorithm. The hg18 genome was used as a reference.

For copy-number variation (CNV) analysis, data were analyzed using Nexus Copy Number, discovery edition version 9.0 (BioDiscovery, El Segundo, CA). Affymetrix CEL files were processed using the SNP-FASST2 segmentation algorithm to make copy number calls (a circular binary segmentation/hidden Markov model approach). The significance threshold for segmentation was set at 5×10^{-9} , also requiring a minimum of three probes per segment and a maximum probe spacing of 1000 between adjacent probes before breaking a segment. The log ratio thresholds for single copy gain and single copy loss were set at +0.1 and -0.15, respectively. The log ratio thresholds for gain of two or more copies and for a homozygous loss were set at +0.7 and -1.1, respectively. Three percent of probes were removed as outliers. As the median ploidy of the samples was above diploid, normalizing probes to the median would have underestimated all copy number calls. Thus, the normalization was based on the median of the values of chromosome 4. This chromosome was judged diploid based on its allelic balance and copy number distributions.

Expression profiling of microRNA in BCaP model cells

The BCaP cell lines were cultured in growth media (RPMI, 5% fetal bovine serum, 2.5% *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid and 0.2% normacin) until near confluence. Trypsinized cells were washed and cell pellets were processed for RNA isolation according to miRNeasy kit (Qiagen). Total RNA (1 μ g) was reversed transcribed using RT² miRNA First Strand Kit (Qiagen). Real-time PCR was performed according to RT² miRNA PCR Array kit (Qiagen). Data were analyzed for expression profiling using the GeneGlobe Data Analysis Center by Qiagen (www.qiagen.com).

Expression of specific mRNA in BCaP model cells

To assess the expression of individual genes in BCaP cell lines, total RNA isolated from each cell line, was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad) and qPCR performed with specific primers designed and optimized as described previously (17). Normalization was performed using YWHAZ and TBP as housekeeping genes (Supplementary Table 9, available at *Carcinogenesis* Online). Relative mRNA expression was calculated using the previously described $\Delta\Delta C_T$ method and statistical significance was calculated using one-way analysis of variance with Dunnett's multiple comparisons test.

Authentication of BCaP cell lines

The BPH1 cell line was obtained from Dr. Simon Hayward and propagated under G418 antibiotic selection. SNP and CNV analyses were done for all cell lines and compared with publically available BPH1 SNP profile, and the BCaP cell lines were found to be derived from the same parental BPH1 cell line. In addition, all cell lines are routinely tested for mycoplasma as well as other mouse pathogen contamination using the IDEXX IMPACT III PCR.

Results

Development of BCaP cell lines

We established five human prostate cell lines representing different stages of CaP progression. A non-tumorigenic cell line (BCaP^{NT1}) was created by recovering cells from a grafted tumor consisting of UGM + BPH1 cells in an untreated adult male athymic mouse (Figure 1). Cells from UGM + BPH1 grafts in mice treated with T+E2 were collected at 2 months (BCaP^{T1}) and 4 months (BCaP^{T10}). Metastatic cell lines were created by recovering cells from renal lymph nodes of a mouse grafted with UGM + BPH1 and treated with T+E2 for 3 months (BCaP^{M1} and BCaP^{M2}). These sublines make up the BCaP cell line model.

Characteristics of BCaP cell lines

Previously, polyclonal populations of cells isolated from hormonally transformed BPH1 tumors maintained the metastatic potential of the tumors they were derived from (12,13,32). We

Figure 1. Derivation of BCaP cell lines. Diagram describing the derivation of BCaP cell lines from BPH1 cells that were renal capsule grafted with UGM. BCaP, BPH1-derived cancer progression.

Figure 2. Characterization of BCaP cell lines. (a) Renal capsule grafting of BCaP cell lines, independent of UGM or T+E2 implantation, produces growths similar to relevant associated CaP stage. (b) Cell proliferation was increased in BCaP^{T1} and BCaP^{T10} cell lines compared with BCaP^{NT1}. *n* = 3; ****P*-value < 0.001, *****P*-value < 0.0001 (c) There was a significant increase in cell motility in all BCaP cell lines compared with control BCaP^{NT1}. *n* = 3; ***P*-value < 0.01, ****P*-value < 0.001. BCaP, BPH1-derived cancer progression.

investigated whether the similarly derived BCaP cell lines also maintained these metastatic/non-metastatic behaviors when renal capsule grafted into mice. BCaP^{NT1} cells developed into small benign grafts. BCaP^{T1} cells produced small-localized non-metastatic tumors, whereas BCaP^{T10} and BCaP^{M1} cells produced a range of small to large primary tumors at the graft site and distant metastases (Figure 2a). Each carcinoma cell line maintains the growth and metastatic characteristics of the tumors they were originally derived from, reproducing the results originally observed with separate cellular populations (12).

Malignant transformation of cells frequently increases the rates of *in vitro* cellular proliferation and motility. In cellular proliferation assays, BCaP^{T1} and BCaP^{T10} cells proliferated at significantly higher rates than compared with BCaP^{NT1} (Figure 2b). Interestingly, BCaP^{M1} cells, though forming tumors and metastasizing *in vivo*, showed no increase in proliferation *in vitro*. In scratch assays, tumorigenic BCaP^{T1}, BCaP^{T10} and BCaP^{M1} cell lines were significantly motile whereas the non-tumorigenic BCaP^{NT1} cells were not (Figure 2c). These results suggest that rates of *in vitro* cellular proliferation are highest in localized and metastatic primary tumor cells, while cellular motility is consistently increased in tumorigenic cell lines independent of metastatic potential.

Comparative bioinformatics analysis of global gene expression

To evaluate the similarity of BCaP cell lines with human CaP development and progression, gene expression changes in

BCaP cell lines were compared with tumors from a highly published transgenic mouse model, TRAMP, and human patient samples. Gene expression in BCaP cells was measured and analyzed to identify genes differentially expressed in all BCaP cells compared with BCaP^{NT1} (Supplementary Table 1, available at Carcinogenesis Online). To compare the gene expression profile of the BCaP model to human CaP progression, a publicly available dataset (GSE6919) was analyzed (22). Human tumor samples were segregated as Gleason score ≤6, Gleason score ≥7 and metastases independent of secondary site; relative gene expression was calculated against autopsy-normal tissue (22) (Supplementary Table 2, available at Carcinogenesis Online). Likewise, dataset GSE10525 was analyzed to identify a list of differentially expressed genes in TRAMP tumors relative to normal mouse prostate (24) (Supplementary Table 3, available at Carcinogenesis Online).

To develop a broad, systems-level interpretation of differential gene expression data, pathway enrichment analysis was performed using WebGestalt (25) and the Kyoto Encyclopedia of Genes and Genomes pathways (33). Pathways represented in the differentially expressed gene data were identified for each BCaP cell line, each group of human CaP tumors and TRAMP tumors. We found that nine pathways are similarly affected in all three datasets including the general 'Pathways in cancer' as well as 'prostate cancer' (Supplementary Table 4, available at Carcinogenesis Online). Although these pathways are found within all three models of CaP, only genes up- or downregulated

within the human metastatic samples were represented. In addition, within the BCaP cell lines, many more differentially regulated genes were found in BCaP^{M1} and BCaP^{M2}, suggesting that the TRAMP tumors more closely models metastasis. To examine the capacity for BCaP cell lines to model early-stage prostate disease, we examined pathways that overlapped between human CaP tumors and BCaP cell lines. By excluding the TRAMP data, we identified 23 unique pathways including cancer-associated signaling pathways such as Wnt, MAPK and TGF β (Supplementary Table 5, available at *Carcinogenesis* Online). Moreover, although BCaP^{M1} and BCaP^{M2} differentially regulate genes within these pathways, we also have representation of genes in BCaP^{T1}, BCaP^{T10} and human tumor samples Gleason score ≤ 6 and ≥ 7 . This suggests that the BCaP cell lines can model changes seen within early CaP stages. In contrast, when human CaP tissues were excluded from analysis, TRAMP tumors and BCaP cell lines predominantly shared cellular metabolism pathways (Supplementary Table 6, available at *Carcinogenesis* Online). In addition, when TRAMP tumors were compared with human CaP tumors, there were only four pathways that were represented, and the genes were exclusively from human metastases with no representation in Gleason score ≤ 6 or Gleason score ≥ 7 (Supplementary Table 7, available at *Carcinogenesis* Online). Examination of the pathways significantly regulated within the BCaP cell lines alone revealed 95 unique pathways (Supplementary Table 8, available at *Carcinogenesis* Online). These data suggest that BCaP cells can model metastasis similar to that of the TRAMP model. However, BCaP^{T1} and BCaP^{T10} share a larger degree of similarity to non-metastatic human CaP.

In-depth gene expression analysis across all four BCaP cell lines compared with BCaP^{NT1} revealed 6990 genes differentially expressed in at least 1 cell line. Cluster analysis of these genes shows that BCaP^{T1} and BCaP^{T10} cluster together whereas BCaP^{M1} and BCaP^{M2} cluster together (Figure 3a). In the human CaP dataset obtained from GSE6919, 8212 genes were differentially expressed (adjusted *P*-value < 0.05) in at least one stage of progression. Cluster analysis of the patient-derived CaP samples shows that Gleason grade ≤ 6 tumors cluster with Gleason grade ≥ 7 , whereas samples derived from distant metastatic sites were different (Figure 3b). Further pathway analysis using gene ontology biological processes showed an overrepresentation of cell migration and cell motility genes throughout progression. However, cell cycle regulation and cell proliferation genes were only statistically overrepresented in BCaP^{T1} and BCaP^{T10} correlating with the *in vitro* proliferation data (Figure 2b). This clustering pattern in conjunction with the *in vitro* proliferation and migration data suggests that the BCaP cell lines can be classified as tumorigenic (BCaP^{T1}), metastatic (BCaP^{T10}) and metastasis derived (BCaP^{M1/M2}).

Given the potential of the BCaP cell lines to recapitulate CaP disease progression in patients, the overlap of gene expression between the patient and cell line model was examined. There is an overlap of 1408 genes between the two datasets, which represents 44% of the statistically significant genes found in the patient samples (Figure 3c). Further breakdown of each stage of progression showed an overlap of 23% of Gleason grade ≤ 6 , 54% of Gleason grade ≥ 7 and 54% of metastatic disease. In addition, the overlap of genes found in tumorigenic, metastatic and metastasis-derived stages shows distinct gene expression patterns (Figure 3d). This suggests that the BCaP model can be used to study not only the different stages of disease but may be useful in the study of transition from one stage to the next.

Methylation of CaP-related gene promoters regulating mRNA expression

Epigenetic regulation of gene expression by DNA methylation of the promoter is an important mechanism affecting CaP growth and progression, but its role in promoting tumorigenesis *in situ* is not well understood (34). To determine whether gene promoter methylation varies in the BCaP progression model, genomic DNA from BCaP cell lines was isolated and analyzed using a limited set of promoter-targeting PCR primers representing CaP-associated tumor suppressors whose expression is regulated by promoter methylation (Figure 4a). Promoter methylation of SLC5A8, a sodium transporter and tumor suppressor gene, has been previously shown to be important in the progression of CaP, and BCaP^{T10} was observed to be hypermethylated compared with BCaP^{NT1} (35). To correlate SLC5A8 gene expression with promoter methylation, reverse transcription (RT)-qPCR was performed; SLC5A8 was downregulated in BCaP^{T1} (*P*-value < 0.05) and BCaP^{T10} (*P*-value = 0.06) but not in BCaP^{M1} (*P*-value = 0.99) compared with BCaP^{NT1} (Figure 4b). ZNF185, a zinc finger protein with an LIM domain, may be a tumor suppressor in CaP that interacts with the actin cytoskeleton and is downregulated in CaP in part due to epigenetic regulation (36,37). In the BCaP cell lines, microarray analysis showed a downregulation of ZNF185 throughout progression compared with BCaP^{NT1}. The ZNF185 promoter is hypermethylated through progression with very low mRNA expression when assessed by RT-qPCR (Figure 4c). TIMP2, a metalloproteinase inhibitor, has been shown to be downregulated in CaP cell lines compared with normal, and plays a role in CaP invasion (38,39). This regulation in part is due to promoter methylation through CaP progression (40). TIMP2 mRNA expression is highest in BCaP^{T1} and lowest in BCaP^{T10} according to the microarray analysis. Methylation analysis of the BCaP cell lines shows little hypermethylation of the TIMP2 promoter and the expression of TIMP2 mRNA as measured by RT-qPCR correlates with the microarray data with BCaP^{T1} significantly upregulating TIMP2 expression (*P*-value < 0.05) when compared with BCaP^{NT1} (Figure 4d). These data provide evidence that promoter methylation and expression of genes are altered in CaP progression and metastasis of the BCaP model cell lines.

Copy-number alterations and TMPRSS2-ERG fusion with BCaP tumor progression

To interrogate the CaP-relevant genomic instability of the BCaP cell lines, SNP arrays were performed and compared with the non-tumorigenic BCaP^{NT1} cell line. The array showed 260 000 variances total among all five BCaP cell lines. The variances that were conserved across all the cell lines were filtered out leaving 59 608 variances. The number of conserved variances that occur across all the cell lines in addition to CNV analysis suggest, as expected, that the BCaP cell lines originate from one precursor cell line, the BPH1 cell (Figure 5a). In depth analysis of CNV alterations revealed changes on several chromosomes including a 50 Mb region loss on the p-arm of chromosome 1 in BCaP^{T10} and BCaP^{M1/M2} as compared with the BCaP^{NT1} and BCaP^{T1} cell lines (Figure 5b). Deletion of the distal short arm of chromosome 1 (1p) has been observed in a variety of human cancers with a loss at 1p36 strongly associated with familial CaP susceptibility (41,42). This region contains several putative tumor suppressor genes including CHD5 and mir34a.

In addition to CNV in chromosome 1, we also examined changes in chromosome 21, specifically in regards to the TMPRSS2 and ERG genes. In the BCaP^{NT1} and BCaP^{T1} cell lines,

Figure 3. Gene expression analysis through CaP progression. (a) Heat map of genes up- or downregulated in BCaP cell lines. Hierarchical clustering by row and column used one minus Pearson correlation and average linkage method. (b) Heat map of genes statistically significantly up- or downregulated in human CaP patient samples. Hierarchical clustering by row and column used one minus Pearson correlation and average linkage method. (c) Overlap (green) of genes differentially expressed in BCaP (orange) and human CaP (purple) datasets. The overall overlap was examined in addition to the overlap between the correlated stages of CaP. (d) Overlap of genes expressed at the different stages of CaP shows little overlap in gene expression overall. BCaP, BPH1-derived cancer progression.

there are heterozygous calls within the TMPRSS2/ERG locus, suggesting no loss of heterozygosity and no deletion. Within the more aggressive BCaP^{T10}, BCaP^{M1} and BCaP^{M2} cell lines, there are no heterozygous calls within the same locus, suggesting a high probability for deletion within this region. The gene fusion most frequently observed in aggressive CaP involves the ETS family of transcription factors, including ERG, ETV1, ETV4 and ETV5, and its fusion partner TMPRSS2 (43). Although TMPRSS2-ERG gene fusions have occurred in some CaP models *a priori*, or involve engineered expression of exogenous TMPRSS2/ERG, no model has yet been developed in which TMPRSS2-ERG gene fusion events occur spontaneously as a function of tumor progression. Owing

to the loss of heterozygosity identified in the SNP array along with an increased expression of ETV1, suggesting a possible duplication/rearrangement event in the metastatic cell lines in the BCaP model, we specifically interrogated each cell line for the TMPRSS2-ERG fusion. On PCR amplification, we identified the TMPRSS2-ERG fusion in BCaP^{T10} and BCaP^{M1} cells similar to human cell line H660 (CaP positive control) but not in non-tumorigenic BPH1 or lowly tumorigenic BCaP^{T1} cells (Figure 5c). These results suggest that the BCaP model spontaneously reproduces TMPRSS2-ERG gene fusion events, coincident with tumor progression. Taken together, the genomic alterations and gene fusions in the BCaP cell lines model the amplification/deletion/

Figure 4. Promoter methylation analysis of BCaP cell lines. (a) Heat map of promoter methylation in BCaP cell lines. (b) SLC5A8 promoter is hypermethylated in BCaP^{T10}, resulting in the lowest mRNA expression in that cell line. (c) ZNF185 promoter is hypermethylated, inversely correlating with the mRNA expression that is low throughout progression. (d) TIMP2 is unmethylated in BCaP^{T1} and BCaP^{M1}, resulting in high mRNA expression in those two cell lines. Blue is unmethylated and red is hypermethylated (right y-axis). BCaP, BPH1-derived cancer progression.

fusion events that occur in human disease processes and could provide novel insight into CaP disease and treatment.

Alterations in microRNA expression with BCaP progression

MicroRNAs (miRNAs) are short, non-coding RNAs that function as posttranscriptional regulators of protein expression, which is often dysregulated in CaP. miRNAs can function as repressors of gene expression by degrading mature target mRNA or as activators of gene expression through interactions with ribonucleoproteins. To examine changes in miRNA expression through progression, a CaP-associated miRNA qPCR array was used to interrogate changes in the BCaP cell lines (Figure 6a). miR-181a is upregulated in CaP tissues compared with that of normal adjacent tissue (44). In addition, miR-181a induction was shown to promote metastasis and is associated with poor clinical outcomes in patients with triple-negative breast cancer (45). Within the BCaP progression cell lines, miR-181a expression increases through progression and is significantly upregulated in

the metastatic cell line BCaP^{M1} (Figure 6b). miR-20a expression has been associated with an increase in invasion and migration and is upregulated in Gleason grade ≥ 7 CaP tissues (46,47). In the BCaP progression cell lines, miR-20a is significantly upregulated in the BCaP^{T1} cell line, but its expression decreases through progression (Figure 6c). miR-196a upregulation has been associated with various cancers including pancreatic cancer, breast cancer and osteosarcoma (48–50). Expression of miR-196a is highest in BCaP^{T1} with a slight decrease through progression (Figure 6d). The significant upregulation of miR-20a and miR-196a in BCaP^{T1} and the subsequent decrease of expression suggest that these two miRNAs may play a role in cell proliferation, invasion and migration of early-stage CaP.

Discussion

Understanding the initiation and progression of CaP in humans is a key field of interest to future advancements in the management of this disease. Currently, early detection of CaP

Figure 5. Genomic integrity in BCaP cell lines. (a) Acquired loss of heterozygosity occurs in the metastatic cell lines and not the non-tumorigenic and tumorigenic cell lines. (b) Expression of TMPRSS2-ERG fusion gene mRNA, detected by RT-PCR and complementary DNA copies of mRNA isolated from each cell line. BCaP, BPH1-derived cancer progression.

is a major factor in optimizing the effects of treatment strategies. At the same time, the treatment of advanced metastatic CaP is rarely curative and presents a major clinical challenge. Efforts to improve early detection and to prevent progression of CaP have been hindered by a limited understanding of early CaP tumorigenesis and progression to advanced disease in humans. Studies of normal prostate and CaP tumors from patients can provide a means to identify comparative changes that have occurred in cancer, but do not allow a means to observe or manipulate the processes that produce these changes (2,3,9). As established human CaP cell lines were derived from advanced metastatic tumors, they represent advanced CaP, with loss of androgen receptor expression and other changes, *a priori* of experimental conditions (3,7). These cell lines may be suitable to investigate whether a particular gene affects tumor growth and behavior but cannot be used to address whether the dysregulation of a particular gene is involved in early tumorigenesis, cancer progression or metastasis. Moreover, the establishment of xenograft tumors using these cell lines may be unrelated to hormonal carcinogenesis in human disease and may not be an appropriate human model for these studies (3,9).

Current animal and cell line models used to study CaP *in vivo* are limited by factors that differentiate them from CaP as it occurs in humans. The use of mouse models in the study of some human diseases is a controversial topic. The molecular physiology of humans and mice is widely divergent in some processes, including aging, pathobiology and tumorigenesis (51). Although human CaP is thought to be hormonally driven, mouse prostates do not typically develop spontaneous tumors (12). Instead, genetically engineered mouse prostate tumor models are induced by genetic manipulation to conditionally express oncogenes in androgen-responsive lobes of the prostate (4,8,52,53). Although this approach may generate *in vivo* mouse CaP, the effects of genetic engineering may demonstrate only

the relative tumorigenicity of a genetic event rather than a reproduction of human prostate pathobiology (53,54).

The BCaP cell lines were developed as a model to investigate changes that occur through human tumorigenesis and progression. As the parent cell line is the non-tumorigenic large T antigen-immortalized BPH1 human prostate epithelium cell line, the BCaP cell lines are a human-based model that may better reproduce the molecular biology of human prostate cells (14). As shown previously, when combined with mouse UGM stroma and renal capsule grafted into adult male athymic mice, with near-human serum levels of T+E2, BPH1 cells develop into tumors that progress from localized non-aggressive tumors to metastases, reproducing human hormonal carcinogenesis (12,13). Although the advantages of using immunocompromised mice in xenograft models are well documented, one potential drawback is an inability to examine the contribution of T cells to the tumor microenvironment or to examine how CaP progression evades immune surveillance (55). However, although T cells are deficient in these mice, the other immune components (e.g. B cells) are intact. Over time, T+E2 drives tumor progression from localized tumor to metastasis (12,56). Because the model was derived from these tumors, the differences between the BCaP lines are necessarily acquired *de novo* as a result of hormonal carcinogenesis involving stromal-epithelial interactions. These data suggest the BCaP cell lines are an appropriate model mirroring human CaP development and progression unlike any other model to date.

Initial renal capsule grafting experiments reproduced tumor behavior experiments previously performed with polyclonal tumor-derived populations. This shows that BCaP^{NT1}, BCaP^{T1}, BCaP^{T10} and BCaP^{M1} cell lines maintain tumor growth and metastatic behavior consistent with the tumors they were derived from (Figure 2a and b), suggesting these behaviors were acquired characteristics inherent to the cell line. The results of *in vitro* cellular proliferation and motility experiments showed

Figure 6. miRNA expression analysis of BCaP cell lines. (a) Heat map of miRNA expression array in BCaP cell lines. Blue is downregulated; red is upregulated. (b) RT-qPCR of miR-181a shows significant upregulation in BCaP^{M1}. $n = 3$; ** P -value < 0.01 (c) RT-qPCR of miR-20a shows significant upregulation in BCaP^{T1}. $n = 3$; ** P -value < 0.01 (d) RT-qPCR of miR-196a shows significant upregulation in BCaP^{T1}. $n = 3$; * P -value < 0.05 . BCaP, BPH1-derived cancer progression; miRNA, microRNA

that although both were increased in most BCaP cell lines compared with BPH1, these did not account for differences in tumorigenicity or metastatic activity (Figure 2c). Analysis of global gene expression shows that the BCaP model shares unique similarities in gene expression changes with stages of human CaP compared with normal prostates, which may be related to tumorigenesis and progression (Figure 3). Examination of promoter methylation and the epigenetic regulation of gene expression across BCaP progression shows consistency between the cell line model and CaP patient samples (Figure 4). Analysis of CNV in BCaP lines using SNP microarrays suggests the genomic integrity of BCaP cells deteriorates with progression, resulting in the development and expression of TMPRSS2-ERG gene fusions (30,57) (Figure 5). Finally, examination of miRNAs in the BCaP progression cell line shows similar changes in miRNA as it correlates to cell proliferation, tumor invasion and cancer metastasis (Figure 6). In each of these experiments, these data support the continued investigation of

BCaP cell lines as a model of human CaP development and progression. Moreover, these data suggest specific targets that will be the focus of future studies.

While providing an effective discovery tool for CaP research, the BCaP model also provides an experimental human model to allow investigation of candidate genes and proteins in the early stages of tumor development and later metastatic progression. In all, the BCaP model provides a relevant human-based model that uniquely reproduces notable aspects of CaP as it clinically manifests in humans. This model not only provides an observational tool with which to better understand the process of human CaP development and progression, but also allows manipulation of the model to experimentally investigate the relevance of candidate genes to this process. This model has the potential to promote innovative studies of CaP that will lead to improved management and treatment of patients with CaP.

Supplementary material

Supplementary material is available at *Carcinogenesis* online.

Funding

This work was supported by the NIH grants (U54DK 104310, K12DK 100022).

Conflict of Interest Statement: None declared.

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