Published in final edited form as:

Cancer Lett. 2014 September 1; 351(2): 272–280. doi:10.1016/j.canlet.2014.06.014.

Spheroid culture of LuCaP 147 as an authentic preclinical model of prostate cancer subtype with SPOP mutation and hypermutator phenotype

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Abstract

LuCaP serially transplantable xenografts are valuable preclinical models of locally advanced or metastatic prostate cancer. For the first time, we recently succeeded in establishing and serially passaging spheroid cultures of several LuCaP xenografts. Here, we characterized in depth the molecular and cellular phenotype of LuCaP 147 cultures and found faithful retention of the characteristics of the original xenograft, including immunophenotype, genetic fidelity, gene expression profile and responsiveness to androgen. Furthermore, we demonstrated capabilities for high-throughput drug screening and that anti-cancer agents induced cell cycle arrest and apoptosis in spheroid cultures. Finally, we showed that cells formed tumors when re-introduced into mice, providing an authentic *in vitro* - *in vivo* preclinical model of a subtype of prostate cancer with a hypermutator phenotype and an SPOP mutation.

Keywords

Prostate cancer; Preclinical model; Spheroids; Drug testing; SPOP

1. Introduction

One of the main roadblocks to developing effective treatments for advanced prostate cancer (PCa) is the lack of preclinical models that comprehensively and authentically depict the

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phenotypic diversity of this malignancy [1]. Of available models, the LuCaP serially transplantable xenografts are among the most multifarious. Derived at the University of Washington from metastatic tissues obtained from a “rapid autopsy” program or from radical surgical specimens, the ~30 xenograft lines encompass the molecular and cellular heterogeneity of advanced PCa [2]. The LuCaP xenografts portray the different stages of malignancy, ranging from androgen-dependent to castration-resistant PCa (CRPC) and including lines that model progression to CRPC.

While invaluable for *in vivo* studies, the LuCaP models have not reached their full potential due to historic inability to establish *in vitro* cell lines from the xenografts. Recently, we adapted methodology originally described for the culture of primary colon cancer cells to grow LuCaP cells *in vitro*. By retaining cell-cell contact at all points of the spheroid culture process, as described by Kondo et al. for colon cells [3], we successfully established cell cultures from several LuCaP xenografts and serially passaged these cultures multiple times [4]. A key component of the culture system that we devised for LuCaP cells was the addition of a ROCK (Rho-associated protein kinase) inhibitor, Y-27632, during digestion and for several days after passage. ROCK inhibition appears to enhance cell survival through increased E-cadherin-mediated cell adhesion [5] and is essential for maintaining stem cells [5-7] as well as for “conditional immortalization” of cells [8]. LuCaP cells from 6 different xenografts, including LuCaP 147, were grown as spheroids in suspension culture using a serum-free medium (StemPro® hESC SFM) supplemented with a synthetic androgen (R1881) and Y-27632.

Here, we characterized in detail the cell line derived from the LuCaP 147 xenograft. Initially derived from a liver metastasis, this xenograft is castration-sensitive and resistant to low-dose docetaxel. It has a mutated androgen receptor (AR) as well as an E3 ubiquitin ligase adaptor speckle-type poxvirus and zinc finger domain protein (SPOP) mutation [9]. The latter mutation is of particular interest because SPOP mutations were recently recognized as defining characteristics of a new molecular subtype of PCa [10], and no other currently available PCa cell lines endogenously possess this mutation. LuCaP 147 also possesses a strikingly high number of novel nonsynonymous single-nucleotide variants, nearly 10-fold more than other LuCaP tumors [9]. In unpublished studies, tumors from the original patient from whom LuCaP 147 was derived have now been sequenced and found to be hypermutated, suggesting that a “mutator phenotype” developed during the initial stages of tumorigenesis and was propagated by clonal selection. We confirmed the presence of the AR and SPOP gene mutations in the cultured LuCaP 147 cells and demonstrated that the spheroid cultures retained the immunophenotype of the xenograft of origin. Similar to the parental xenograft, the LuCaP 147 cell cultures were androgen-sensitive and not responsive to low-dose docetaxel. The potential for high-throughput drug screening with spheroid cultures was tested in both standard 96-well format as well as in NanoCulture® plates, specifically designed for assays of spheroid cultures. Responses to drugs under development for PCa, including a tyrosine kinase inhibitor and a dual mTOR inhibitor, were observed in both assay formats. Flow cytometry analysis revealed both cell cycle arrest and induction of apoptosis by these anti-cancer agents in LuCaP 147 cells. Finally, we showed that LuCaP 147 spheroids formed tumors when implanted subcutaneously into SCID mice, and that cell

cultures could be re-established *in vitro* from these tumors. Gene expression profiles and copy number variations (CNVs) of the original LuCaP 147 xenograft, its derived cell culture, and the tumors formed by implanting the cultured cells back into mice were remarkably similar. Our studies authenticate LuCaP 147 spheroid cell cultures as a faithful *in vitro* counterpart to the original xenograft, with the potential to broaden the experimental capabilities of this important preclinical model of PCa.

2. Materials and methods

2.1. Establishment, serial passage and cryopreservation of LuCaP 147 cell cultures

The establishment, propagation, freezing and thawing of LuCaP 147 spheroid cell cultures were previously described [4]. Cells were cultured in StemPro® hESC SFM (Invitrogen) supplemented with 10 nM of R1881 and 2 µM of Y-27632. The LuCaP 147 cell line has been passaged 18 times and proved to be unique and of human male origin by short tandem repeat analysis [4]. Tests for mycoplasma and murine viruses were negative.

2.2. MTS assay

The MTS viability assay was performed as previously described [4].

2.3. Immunohistochemistry and immunofluorescence staining

LuCaP 147 spheroids were digested to single cells, deposited on glass slides by cytocentrifugation, and fixed with paraformaldehyde. Xenografts were fixed in 10% buffered formalin, paraffin-embedded and sectioned at 5-µm. Immunohistochemistry and immunofluorescence staining with antibodies listed in Table 1 were performed as previously described [4].

2.4. Tumor-forming capability of cultured LuCaP cells

All animal studies were done in compliance with the regulations at Stanford University. Intact LuCaP 147 spheroids were collected, suspended in cold Matrigel diluted 1:3 in HEPES-buffered saline, and injected subcutaneously (100 µl per site) into each of three 6- to 8-week-old male CB17 SCID mice (Charles River Laboratories International, Inc.). A 25-mg testosterone pellet with a release rate of 0.2 mg/day was inserted into a small incision made under the skin between the shoulder blades to bring androgen levels to those typical of human males. Tumors were measured twice a week with a caliper and the volume was calculated using the standard ellipsoid formula ($L \times W \times H \times / 6$) [11]. Tumor doubling time was calculated using GraphPad prism 5 software. After sacrifice, tumors were resected, fixed in 10% buffered formalin and embedded in paraffin for immunohistological analysis or preserved in Allprotect tissue reagent (Qiagen) for genetic analyses.

2.5. DNA copy number variation (CNV) and gene expression profiling

Genomic DNA and total RNA were extracted from cells at passage 8 and xenografts preserved in Allprotect tissue reagent using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen) according to the manufacturer's directions. For CNV analysis, 1 µg of genomic DNA from each sample was labeled by random priming using the Agilent Genomic DNA

Enzymatic Labeling Kit (Cy3-dUTP) (Agilent). A pool of reference normal DNA (Promega) was labeled with Cy5-dUTP. Cy3 and Cy5 probes were combined and hybridized to Agilent 2×400K SurePrint G3 CGH Microarrays (Agilent) and washed following the manufacturer's specifications. Fluorescent array images were collected using the Agilent DNA microarray scanner G2505C and Agilent Feature Extraction software. Data analysis was performed with Nexus Copy Number 6.0 software (BioDiscovery). The FASST2 segmentation algorithm and default Agilent settings for significance, gain and loss thresholds, with at least six probes per segment, were used to identify regions of CNV for each sample.

For gene expression analysis, probe labeling and hybridization were performed following the protocols suggested by Agilent and fluorescent array images were collected using the Agilent DNA microarray scanner G2505C. Data were loess-normalized within arrays and quantile-normalized between arrays in R using the Limma Bioconductor package [12]. Unsupervised, average-linkage hierarchical clustering was performed on the 5000 most variable genes, computed as those with the highest interquartile range of the mean-centered log₂ ratios across samples.

2.6. Gene sequencing

cDNA was generated from total RNA as previously described [13]. Exon 8 and the 3' UTR of AR and exon 3 of SPOP were selected for polymerase chain reaction (PCR) amplification and direct sequencing. PCR primers for AR and SPOP are listed in Table 2.

2.7. Evaluation of response to drugs

Docetaxel was purchased from Sigma-Aldrich. Sunitinib was purchased from LC Laboratories and MLN0128 was purchased from ChemieTek. Spheroids were digested into small clusters of cells and resuspended in medium so that 60 µl aliquots of 2×10⁴ cells could be added to each well of 96-well ultralow attachment plates (Corning). Care was taken to agitate the cell suspension during aliquotting to ensure equal cell distribution. After overnight incubation, drugs in a total volume of 40 µl of medium were added to each well to give final concentrations of 0.03 – 10 µM for sunitinib, 0.1 – 100 nM for MLN0128 and 1 – 20 nM for docetaxel. The MTS assay was used to measure cell viability after 48 hours. For flow cytometry analysis, spheroids were digested into single cells, fixed in 95% ethanol at 4°C for at least 1 hour and stained with 25 µg/ml of 7-aminoactinomycin D. Fluorescent signal was collected using a LSR II Flow Cytometer (BD Bioscience) and analyzed using FlowJo (Tree Star Inc.).

2.8. Formation of adherent spheroids and drug response

Fifty µl of StemPro medium were placed into each well of a 96-well NanoCulture® plate (SCIVAX USA, Inc.). Plates were spun at 300g and cells were then seeded at a final density of 2×10⁴ cells in 60 µl. The next day, cells were incubated with various concentrations of MLN0128 and cell viability was evaluated after 48 hours as described above.

3. Results

3.1. Spheroid cultures of LuCaP 147 retain the immunophenotype of the xenograft of origin

To characterize LuCaP 147 spheroid cultures, we evaluated the expression of a panel of markers as shown in Fig. 1. The human origin of the original xenograft and spheroid culture was verified using human-specific antibodies against Ku70 and HuNu, respectively, due to different working conditions of these antibodies (Fig. 1A-B). PCNA staining demonstrated that the cells were highly proliferative (Fig. 1D-E). The majority of cells in spheroid cultures expressed androgen receptor (AR) (Fig. 1G-H) and keratin 18 (K18) (Fig. 1J-K), exhibiting the predominantly luminal-like phenotype of the original xenograft. Prostate-specific antigen (PSA) was expressed weakly by the cultured cells (Fig. 1N), as is characteristic of the xenograft of origin (Fig. 1M).

The normal prostatic basal epithelial cell marker p63 was absent in both the original xenograft and in the cultured cells, as is typical of PCa (Fig. 1P-Q). However, rare cells in both the culture and original xenograft expressed the basal cell marker keratin 5 (K5) (Fig. 1S-T). CD44 (a basal cell/stem cell marker) was heterogeneously expressed in cultured cells (Fig. 1W), but positive in the original xenograft (Fig. 1V). Gene expression profiling (described in detail in sections 2.5 and 3.4) showed that CD151 and ABCG2, two putative prostate stem cell markers, were expressed at higher levels in LuCaP 147 spheroids compared to normal prostate tissue, while expression of other stem cell markers (Oct4, Sox2, Nanog, Trop-2, ALDH1A1, ALDH1A3, CD166, and CD49f) were lower in LuCaP 147 spheroids compared to normal prostate tissue (supplemental Fig. 1). Moreover, expression of CD133, a well-known prostate stem cell marker, was undetectable in LuCaP 147 spheroids, suggesting lack of stem cell properties. Of interest, EpCAM expression was lower in the spheroid culture than the original xenograft (Fig. 1Y-Z). Finally, intermediate staining for PTEN protein was observed in the original xenograft, which is known to retain both alleles of the PTEN gene (Fig. 1AB), and was weakly positive in the spheroid culture (Fig. 1AC). These results demonstrated that spheroid cultures of LuCaP 147 largely retained the immunophenotype of the xenograft of origin.

3.2. LuCaP spheroids formed tumors similar to the xenograft of origin

Intact LuCaP 147 spheroids at passage 9 were suspended in Matrigel and injected subcutaneously into male SCID mice. Three mice were each implanted with an estimated 2, 4 or 6 million cells. Tumor doubling times were calculated to be 15-22 days, which is slightly faster than the original xenograft (23 days). The resultant tumors (culture-derived xenograft) displayed a similar expression pattern of Ku70 (Fig. 1C), PCNA (Fig. 1F), AR (Fig. 1I), and K18 (Fig. 1L) to the xenograft of origin. PSA expression was higher in the culture-derived xenograft (Fig. 1O) compared to the original xenograft, possibly due to androgen supplementation in mice carrying the culture-derived xenograft. Similar to the original xenograft and the spheroid culture, the culture-derived xenograft was negative for p63 (Fig. 1R) and positive for PTEN (Fig. 1AD) with rare K5-expressing cells (Fig. 1U). Interestingly, the culture-derived xenograft showed more homogenous expression of CD44 (Fig. 1X) and higher expression of EpCAM (Fig. 1AA) than the spheroid culture, similar to the original xenograft. Overall, the culture-derived xenograft exhibited a similar

immunophenotype to the original xenograft as well as to the spheroid culture. Importantly, applying the same methods of digestion and culture as used for the initial xenograft, we successfully isolated cells from the spheroid culture-derived xenograft and re-established them in culture. The cells retained the same immunophenotype as exhibited prior to formation of tumors in mice (not shown).

3.3. Mutations in AR and SPOP genes are retained in LuCaP 147 spheroid cultures

DNA sequencing revealed a missense mutation (Y83C, A>G) in exon 3 of SPOP carried by the original xenograft in both the spheroid culture and culture-derived xenograft (Fig. 2A). In addition, a missense mutation (H874Y, C>T) in exon 8 of AR and a single base pair deletion at T4037 in the 3'UTR of AR, both carried by the original xenograft, were also found in the spheroid culture and culture-derived xenograft (Fig. 2A). These results demonstrated that spheroid cultures of LuCaP 147 cells maintained genetic fidelity with respect to the xenograft of origin.

3.4. Microarray analysis showed fidelity of CNV and gene expression profile among the original xenograft, spheroid cultures, and culture-derived xenograft

We compared regions of CNV in LuCaP 147 spheroid cultures to those in the original and culture-derived xenografts at a genome-wide scale. Overall, most CNVs were conserved (Fig. 2B). One exception was large gains in chromosome 7p in the culture-derived xenografts and one original xenograft but not in the spheroid cultures (Fig. 2B). After removing variations that overlapped with normal tissue from the same patient from whom LuCaP 147 was derived, 119 and 129 CNVs were identified in the culture-derived xenografts and 51 and 58 in the spheroid cultures (Fig. 2C). The two original xenografts exhibited the most variability with 192 and 73 CNVs identified. In addition, one original xenograft had large gains in chromosome 5q while the other had gains on chromosomes 9q and 18. This may be due to the fact that these two tumors were from an earlier and later passage.

Gene expression profiles of three LuCaP 147 spheroid cultures, three culture-derived xenografts, two original xenografts and three samples of laser-capture microdissected benign epithelia from radical prostatectomy tissues were compared. Unsupervised clustering analysis using 5000 genes that showed most variable expression among the samples distinguished LuCaP 147 samples from normal prostate samples (Fig. 2D). The majority of the variability was contained in shared gene expression profiles between LuCaP 147 spheroid cultures, culture-derived xenografts and the original xenografts in contrast to normal prostate tissue. We identified Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were enriched in the list of genes that showed similar expression in LuCaP 147 cells in contrast to normal prostate tissue (original xenograft vs. normal prostate, spheroid culture vs. normal prostate, or xenograft derived from spheroid culture vs. normal prostate) using Gene Set Enrichment Analysis (GSEA). Pathways with GSEA q-value <0.05 in at least one comparison are shown in Fig. 2E. In addition, the spheroid cultures clustered together with culture-derived xenografts and diverged slightly from the original xenografts due to a small portion of varied gene expression. Together, these results demonstrated that

LuCaP 147 spheroid cultures maintained highly similar CNVs and global gene expression profiles compared to the culture-derived xenograft tumors and the original xenografts.

3.5. Growth rate and response to androgen

We previously determined that the MTS assay was suitable for quantifying growth of LuCaP spheroid cultures [4]. Here, we determined the growth rate of LuCaP 147 cells in spheroid culture over a period of 7 days. As shown in Fig. 3A, LuCaP 147 cells showed an increase in number over time. Since the growth rate was relatively slow with a doubling time of 5 days, we tested other basal media supplemented with the same factors as in the StemPro® hESC SFM-based formulation, but none resulted in a significantly improved growth rate (not shown).

We evaluated the responses of LuCaP 147 cells to androgen (R1881) and bicalutamide, an AR inhibitor. Cells were first cultured in StemPro® hESC SFM medium without R1881 for 5 days and then treated with 1-100 nM R1881 or 1-100 μ M bicalutamide. LuCaP 147 spheroids did not show any significant response to low-dose R1881 or bicalutamide (Fig. 3B). However, cell proliferation was significantly higher in the presence of 10 and 100 nM R1881. Moreover, 10 μ M bicalutamide significantly increased cell proliferation while 100 μ M bicalutamide dramatically inhibited cell growth. These results suggested that high concentrations of androgen promoted LuCaP 147 growth while bicalutamide had a bi-phasic effect on LuCaP 147 proliferation. Representative untreated spheroids in ultra low-attachment dishes at day 0 and spheroids in the presence of R1881 and bicalutamide at day 7 are shown in Fig. 3C. Untreated spheroids were smaller in size, fewer in number and more transparent at day 0 compared to day 7. In the presence of 10 nM R1881 or 10 μ M bicalutamide, spheroids were significantly larger, denser, and more numerous than in the absence of either compound. Moreover, spheroids were smaller and disorganized in the presence of 100 μ M bicalutamide compared to control, consistent with its bi-phasic effects on cell proliferation observed by MTS assay.

3.6. Response of LuCaP 147 spheroids to therapeutic drugs

The MTS assay was used to quantitate responses of LuCaP 147 cells to several drugs of interest. The growth of LuCaP 147 cells was not affected by 1 nM docetaxel, a standard-of-care drug for CRPC. Although the growth rate was significantly decreased by higher-dose docetaxel (3 - 20 nM), IC₅₀ values were not reached after 48 hours of exposure to the taxane (Fig. 4A). Next, we tested sunitinib, a tyrosine kinase inhibitor that recently has been shown to have direct apoptotic effects on PCa cells [14]. Sunitinib at 7 μ M and higher significantly reduced growth of LuCaP 147 spheroids (Fig. 4B). Finally, a dual mTOR inhibitor, MLN0128, also of interest for treatment of advanced PCa [15], showed significant inhibition of LuCaP 147 cell growth at concentrations of 30 nM and higher (Fig. 4C).

To determine whether the observed drug responses were due to induction of apoptosis or inhibition of cell cycle or both, we examined the proportion of cells at apoptosis, G0/G1, S, and G2/M phase of the cell cycle after 48 hours exposure to different concentrations of sunitinib, MLN0128, and docetaxel. Cells treated with these drugs all showed an increase in the percentage of apoptotic cells (Fig. 5). In addition, sunitinib and docetaxel treatment led

to an increase in cells at G2/M phase with a corresponding depletion of cells in G0/G1 and S phase, while MLN0128 induced an accumulation of cells at G0/G1 phase (Fig. 5). These results demonstrated that sunitinib, MLN0128, and docetaxel induced both apoptosis and cell cycle arrest in LuCaP 147 cells.

Nonadherent spheroid cell cultures pose certain challenges for high-throughput assays. Based on a recent publication using colon cancer cells [16], NanoCulture® plates were tested to assess their suitability for high-throughput drug screening of LuCaP cells. Using the MTS assay to evaluate cell viability, we found that survival and growth of LuCaP 147 spheroids were maintained over a period of 9 days on the 96-well NanoCulture® plates. Next, we used this platform to evaluate the response of LuCaP 147 spheroids to MLN0128 for a 48-hour period. Response was comparable to that measured in the suspension cultures in standard ultralow attachment 96-well plates, with a significant decrease in cell viability at concentrations of 30 nM MLN0128 in the microhoneycomb NanoCulture® plates (Fig. 4D). These results demonstrated capabilities for high-throughput drug screening with LuCaP 147 spheroid cultures.

DISCUSSION

There are several advantages to LuCaP 147 spheroid cultures as a new model of advanced PCa. First, the model is authentic since the cells largely retained the immunophenotype and genetic fidelity of the original xenograft. Second, the spheroids responded to drug treatments as expected, demonstrating the potential to be used in high-throughput drug screening. Third, three-dimensional spheroid cultures have been shown to better model *in vivo* behavior than monolayer cell cultures [17]. For example, another PCa cell line, LNCaP, showed similar gene expression profiles between the subcutaneously grafted tumors and multicellular spheroids, which differed from gene expression in the monolayer analog [18]. Finally, subcutaneous injection of LuCaP 147 spheroids into mice resulted in the generation of tumors very similar in nature to the original xenograft from which the cell culture was derived, and cells could be recovered from these xenografts and established in spheroid culture once again. These capabilities will expand the utility of the model. Cells grown *in vitro* are amenable to many experimental manipulations compared to cells in xenografts. The ability to introduce or silence genes will enhance mechanistic and other studies with LuCaPs; creation of luciferase-expressing cells, for instance, will greatly facilitate *in vivo* imaging capabilities.

Performing quantitative assays with spheroid compared to monolayer cultures can be challenging. We previously determined that the MTS assay proved suitable for measurement of viable LuCaP cells in spheroids [4]. Using this assay, we measured the growth rate of LuCaP 147 cells over a period of one week. The growth rate was relatively slow, and may be improved by more intensive optimization efforts in the future. The growth rate was not altered by low-dose R1881, however, proliferation of LuCaP 147 is responsive to high-dose androgen, in accordance with the known sensitivity of the original xenograft to androgen manipulation (unpublished data). The missense mutation (H874Y, C>T) in exon 8 of AR has been shown to alter its ligand-binding specificity [19], which may explain the agonistic rather than antagonistic effect of bicalutamide at low concentration. In addition, LuCaP 147

cells express low levels of PSA, a known downstream target of AR [20], and showed a significant increase in PSA mRNA expression in response to R1881 [4], consistent with the higher expression of PSA protein in the culture-derived xenograft when the murine host was supplemented with testosterone.

The LuCaP 147 xenograft also has a mutation in SPOP, one of the most frequently mutated genes in PCa with mutations found in 6-15% of cancers [10]. The mutation at Y83C (A>G) was confirmed to be present in LuCaP 147 cultures, providing a valuable and unique model for further investigation of the biological and therapeutic significance of this genetic aberration. Recent studies showed that PCa-associated SPOP mutants lose the ability of the wild-type protein to interact with the p160 steroid receptor coactivator SRC-3 and promote ubiquitination and degradation [21]. Consequently, the tumor suppressor effect of promoting turnover of SRC-3 and suppressing AR transcriptional activity is attenuated by mutation of SPOP. LuCaP 147 cells have been described as having a hypermutated phenotype [9]. Despite this, CNV and gene expression profiles of the spheroid cultures remained remarkably similar to the xenograft of origin, providing a unique preclinical model of a new subtype of PCa with an SPOP mutation and a hypermutated phenotype.

One of the major advantages to increasing the number of PCa cell lines is to provide a larger repertoire with which to screen experimental drugs. We found that LuCaP 147 cells are only moderately responsive to docetaxel. While statistically significant growth inhibition was observed after 48 hours of exposure to 3 nM of docetaxel, IC₅₀ was not reached even with 20 nM of docetaxel. In contrast, many monolayer cultures of PCa cells typically show 50% apoptosis at ~5 nM of docetaxel [22]. One explanation is that three-dimensional cultures more accurately mimic the *in vivo* situation of solid tumors than two-dimensional cultures [23, 24], and therefore spheroid cultures of LuCaP cells may better predict response to therapeutics when translated to clinical application. Indeed, the original LuCaP 147 xenograft is not responsive to low-dose docetaxel (10 mg/kg, unpublished data), similar to the spheroid culture. Alternatively, spheroid cultures are generally less responsive to drugs compared to monolayers [18, 25] and antimitotic drugs might be less effective in such a culture [26]. For example, low cytotoxicity of tubulin inhibitors in PCa spheroids has been reported [18, 27]. Whether this low sensitivity of LuCaP 147 spheroids to docetaxel is due to inherent resistance to docetaxel, as suggested by the lack of response of the original xenograft, or to decreased sensitivity of spheroids compared to monolayer cultures to drugs is unknown, since LuCaP 147 cells cannot be tested in monolayer culture. In any case, the similar response of LuCaP 147 cultures compared to the original xenograft suggests that cell culture has not modified the phenotype.

We are confident that LuCaP 147 spheroid cultures provide a valuable platform for drug testing. First, the cells responded to sunitinib as expected. A recent publication showed that PTEN expression is inversely associated with sunitinib resistance in PCa cells [28]. Since LuCaP 147 cells stained PTEN-positive, we predicted that LuCaP 147 would be sensitive to sunitinib treatment. Indeed, sunitinib inhibited the proliferation of LuCaP 147 spheroids in a concentration-dependent manner, reaching IC₅₀ values at 7 μ M. This is consistent with the observation that the PTEN-positive PCa cell line DU 145 showed IC₅₀ of 2.5 μ M for sunitinib in monolayer culture [29], while PTEN-negative PCa cell lines LNCaP and PC-3

are resistant to 25 μ M sunitinib [28]. We also predicted that MLN0128, a second generation, dual mTOR inhibitor would be effective in LuCaP 147 cells. Rapalogs preferentially inhibit only mTORC1 [30] and have been shown to be ineffective on human PCa cell lines [29]. In contrast, MLN0128 inhibits both mTORC1 and mTORC2 and induced apoptosis in LNCaP cell monolayers [15]. In our study, MLN0128 inhibited growth of LuCaP 147 spheroids with an IC₅₀ of 30 nM, providing supportive data suggesting that second-generation mTOR inhibitors might be more effective in PCa treatment.

Advanced PCa has proven extremely difficult to treat, and the heterogeneity of metastases is part of the challenge. The availability of cell lines that span the molecular and cellular heterogeneity of the disease could have an enormous impact on developing better treatment strategies. Perhaps the development of a high-throughput assay is the most significant advantage of the availability of cell lines. It is encouraging that our model can be used with the NanoCulture® platform, a multi-well plate for three-dimensional (3D) spheroid cell culture, with similar results to the suspension cultures in the ultralow-attachment 96-well plates. This new technology will provide sufficient throughput for high content applications using our model [25]. Tight spheroid attachment was observed because of the nano-scale pattern imprinted on the well-bottom film using a novel nanoimprint technology and cells could be fed by simple medium changes every three days. Literally thousands of novel and promising therapeutics could be tested in LuCaP cell lines, overcoming the long-standing problem that while LuCaP xenografts themselves provide the diversity of PCa, they are not amenable to high-throughput assays as are cell lines. For example, in the future, LuCaP cell lines could be screened with libraries of small molecules and responses correlated with the genetic features of the cell lines, as done with cells in the Cancer Cell Line Encyclopedia [31]. Investigators in Finland have assembled a panel of 29 prostate cell lines grown in spheroid culture in Matrigel, with use for cancer drug discovery and target identification [26], but the NanoCulture® platform is perhaps more facile than a Matrigel-based drug screen.

To discover biomarkers of sensitivity and resistance to cancer therapeutics, it will likely be necessary to screen hundreds of cell lines that in total represent the genetic diversity of cancer, as done initially with the NCI60 cell line panel and more recently with larger panels [31, 32]. It is noteworthy that PCa is conspicuously under-represented in these cell line panels, despite the fact that PCa is the second leading cause of death from cancer in men. Currently, three cell lines are typically used for the vast majority of PCa research [33]. These lines are PC-3, LNCaP and DU 145, each established more than 30 years ago [33, 34]. Regrettably, these lines are not typical of the majority of prostate cancers. For instance, PC-3 and DU 145 are used as models of “androgen-independent PCa” because they do not express AR, respond to androgen or require androgen for growth. However, it is now recognized that most “androgen-independent PCa” is in fact “castration-resistant PCa” (CRPC), retaining expression of AR, with growth and survival still maintained through androgen-mediated signaling [35]. Thus, PC-3 and DU 145 do not have characteristics typical of CRPC and are not the best models for studying this stage of disease. The availability of new, well-characterized LuCaP cell lines, with phenotypes reflecting

advanced PCa, will facilitate the ability of researchers to carry out experiments with informative and representative *in vitro* cell culture models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jennifer Santos for assistance with this study. The work was supported by a Prostate Cancer Foundation Challenge Award, P50CA097186, P01CA085859, and the Ferdinand Eisenberger Grant of the German Society of Urology ID SaM1/FE-11. We thank the Richard M Lucas Foundation and the Prostate Cancer Foundation for their long term support in the generation, maintenance and characterization of the LuCaP series of PCa xenografts in the Department of Urology at the University of Washington. We also thank the many patients and their families who donated tissues for this endeavor and to Eva Corey, Ph.D., and Holly Nguyen who have been intimately involved in their propagation, characterization and distribution.

Abbreviations

AR	androgen receptor
CNVs	copy number variations
CRPC	castration-resistant PCa
K5	Keratin 5
K18	Keratin 18
PCa	prostate cancer
PCR	polymerase chain reaction
PSA	Prostate-specific antigen
ROCK	Rho-associated protein kinase
SPOP	speckle-type poxvirus and zinc finger domain protein

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Highlights

- We established and characterized spheroid cultures of LuCaP 147 xenografts, models of advanced prostate cancer.
- LuCaP 147 cultures faithfully retained the characteristics of the original xenograft.
- We demonstrated capabilities for high-throughput drug screening using LuCaP 147 cultures.
- LuCaP 147 cultures formed tumors when re-introduced into mice.
- LuCaP 147 cultures provided an authentic *in vitro* preclinical model with a hypermutator phenotype and an SPOP mutation.

Fig. 1.

Immunohistochemistry of the original LuCaP 147 xenograft, cultured LuCaP 147 cells at passage 6 and xenograft derived from implanting cultured LuCaP 147 cells into mice. The original xenograft, the cultured cells and the culture-derived xenograft showed strong expression of human-specific Ku70 (A and C) and HuNu (B), PCNA (D-F), AR (G-I) and K18 (J-L). PSA expression was weak in the original xenograft and spheroid culture (M and N), but stronger in the culture-derived xenograft (O). There was no expression of basal cell marker p63 (P-R) and rare expression of keratin 5 (K5) (S-U). CD44 expression was positive in the original xenograft (V), heterogeneous in spheroids (W) and more homogenous in the culture-derived xenograft (X). EpCAM expression was positive in the original and the culture-derived xenografts (Y and AA) but low in spheroids (Z). Positive-PTEN staining could be confirmed as well (AB-AD).

Fig. 2. LuCaP 147 spheroid cultures maintained genetic fidelity and the global gene expression pattern of the original xenograft. (A) The same mutations in AR and SPOP reported in the original xenograft were detected in both the spheroid culture and the culture-derived xenograft. (B) Regions of CNV in LuCaP 147 spheroid cultures, xenografts derived from the cultured cells and the original xenograft overlapped at a genome-wide scale. The frequency of CNV for each region was plotted on the top. (C) The number of copy number gain and loss in LuCaP 147 spheroid cultures, xenografts derived from the cultured cells and the original xenograft. (D) Unsupervised clustering of gene expression profiles of LuCaP 147 spheroid cultures, xenografts derived from the cultured cells, the original xenograft and normal prostate tissues. (E) KEGG pathways that were enriched in the list of genes that showed similar expression in LuCaP 147 cells in contrast to normal prostate tissue using GSEA. Pathways with GSEA q-value <0.05 in at least one comparison were shown and ordered by the mean q-value across the three analyses (lowest to highest).

Fig. 3.

In vitro growth of LuCaP 147 cells at passage 12 and response to androgen and anti-androgen at passage 18. (A) LuCaP cells proliferated *in vitro* as shown by MTS assay every two days over seven days (* $p < 0.05$ compared to day 1). (B) Cell viability was significantly increased by exposure to 10 and 100 nM R1881 as well as 10 μM bicalutamide, and decreased by exposure to 100 μM bicalutamide for 7 days, as assayed by MTS. (C) Representative pictures of untreated spheroids in ultra low-attachment dishes at day 0 and spheroids in the presence of R1881 and bicalutamide at day 7. For (A) and (B), each bar represents results from one single experiment with triplicate samples.

Fig. 4.

Response of LuCaP 147 cells at passage 13 to drugs. (A) Docetaxel significantly decreased growth of LuCaP 147 cells at 3 nM and higher, but IC_{50} was not reached even at 20 nM. (B) Significant growth inhibition of LuCaP 147 cells was seen for 7 and 10 μ M sunitinib (* p <0.005 compared to control). (C) Significant growth inhibition of LuCaP 147 cells occurred with 30 and 100 nM of MLN0128 (* p <0.005 compared to control). (D) Response of LuCaP 147 cells to MLN0128 when spheroids were adhered to Nanoculture® plates was similar to that of spheroids in suspension, with significant growth inhibition at 30 and 100 nM (* p <0.005 compared to control). All experiments were done in triplicates and repeated at least twice.

Fig. 5.

Cell cycle distribution of asynchronous LuCaP 147 cells 48 hours after drug treatment as determined by flow cytometry. Treatment condition and percentage of cells at apoptosis (Ap), G0/G1, S and G2/M phase of the cell cycle are given for each graph.

Table 1

Primary antibodies used for immunochemistry

Antibody	Dilution	Source
anti-keratin 5	1:50	Imgenex (San Diego, CA)
anti-p63	1:50	Abcam (Cambridge, MA)
anti-CD44	1:100	Abcam
anti-keratin 18	1:100	Santa Cruz Biotechnology (Santa Cruz, CA)
anti-AR	1:100	BD Biosciences (Bedford, MA)
anti-EpCAM	1:100	Millipore (Billerica, MA)
anti-PCNA	1:100	Santa Cruz Biotechnology
anti-HuNu	1:100	Millipore
anti-PSA	1:100	Santa Cruz Biotechnology
anti-PTEN	1:100	Santa Cruz Biotechnology
anti-Ku70	1:200	Abcam

Table 2

Primer sequences used in this study

Name	Source
SPOP-Exon3-Forward	TTTGCGAGTAAACCCCAAAG
SPOP-Exon 3-Reverse	TGGCCAGAAATGTTGACAGA
AR-Exon 8-Forward	ATCTCTTGGGAGCCCTCAGT
AR-Exon 8-Reverse	GCTTCACTGGGTGTGGAAAT
AR-3'UTR-Forward	ATTTCACACCCAGTGAAGC
AR-3'UTR-Reverse	ACTGGGCCATATGAGGATCA