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Three-Dimensional Cell Culture Models of Bone Metastatic Prostate Cancer

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by

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The Thesis of Theresa Jhoana Ramos Mendoza is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

I dedicate this thesis to my family especially, Tirso, Juanita, Tris, Augusto, and Liboria. Thank you for loving me unconditionally and supporting me throughout my academic endeavors. Without all of you, none of my success would be possible.

I would also like to thank the members of the Jamieson lab – Dr. Christina Jamieson for her mentorship and encouragement which inspired me to pursue what I love to do; Michelle Muldong for her irreplaceable assistance in my experiments (you are the best "lab sister" I could have ever asked for); Danielle Burner for her unwavering support and devotion to protecting our VWR lab markers; and to the rest of our lab members, I appreciate all the help and support that you have given me. Thank you is not even enough to express how grateful I am to have worked with all of you.

Epigraph

"The real voyage of discovery consists not in seeking new landscapes but in having new eyes."

- Marcel Proust

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List of Abbreviations

ADT	Androgen Deprivation Therapy
AR	Androgen Receptor
CRPC	Castration-Resistant Prostate Cancer
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FACS	Fluorescence Activated Cell Sorting
GLF	GFP-Luciferase Expressing Lentiviral Vector
GFP	Green Fluorescent Protein
IVIS	*In Vivo* Bioluminescence Imaging System
PDX	Patient-Derived Xenograft
PCa	Prostate Cancer
PCSD	Prostate Cancer San Diego
PSA	Prostate-Specific Antigen
RP	Radical Prostatectomy
RLU	Relative Luminescence Units

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Abstract of the Thesis

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Master of Science in Biology

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Professor Kimberly Cooper, Co-Chair

One in six men will be diagnosed with prostate cancer (PCa), making it one of the leading health problems affecting men in today's society. Patients diagnosed during the earlier stages are surviving longer due to improved therapies and the prevalence of prostate-specific antigen (PSA) testing. However, over 80% of advanced PCa patients develop bone metastatic prostate cancer for which there is no cure (Khan and Partin 2003). Cell lines,

widely used for the development of new drug treatments and therapies, fail to accurately represent the heterogeneity of prostate cancer. Thus, it is important to establish new patient-derived cell culture models for bone metastatic prostate cancer which could better recapitulate the physiological processes that occur *in vivo*. Through the incorporation of previously established methods into our own culturing methods, we optimized three-dimensional cell culture conditions which keep our patient-derived xenograft (PDX) and primary patient tumor cells viable *in vitro* for a month and a half without passaging. Furthermore, preliminary experiments showed that our three-dimensional cultures consist of heterogeneous cell populations that exhibit different responses to the androgen hormone, dihydrotestosterone (DHT), and the anti-androgen drug, Enzalutamide. In future experiments, we hope to further optimize our culturing conditions to improve the robustness and reproducibility of our three-dimensional cell cultures for patient-derived xenograft and primary prostate cancer tumor cells.

Introduction

Approximately 1 in 39 men will die of prostate cancer (PCa), making it the third leading cause of cancer death among men in the United States (American Cancer Society 2017). Recent therapeutic advancements and the prevalence of prostate-specific antigen (PSA) testing have increased the five-year survival rate of patients diagnosed with localized prostate cancer to nearly 100%. However, the five-year survival rate drastically decreases to 29% for patients that relapse and develop advanced metastatic cancer (American Cancer Society 2017; Siegel et al. 2017). The most common site of metastasis for prostate cancer is the bone, for which there is no cure. Therefore, bone metastatic prostate cancer is often associated with poor prognosis, malignant disease progression, and therapy resistance (Bubendorf et al. 2000; Lee et al. 2014).

Local and regional prostate cancer is frequently treated by radical prostatectomy (RP) or radiation therapy (Mayo Clinic 2017). Despite the removal of tumor cells in the prostate gland, approximately 20% of patients experience cancer progression (Roehl et al. 2004). Once relapse has occurred, surgical castration or androgen deprivation therapy (ADT) is used to decrease the level of androgen hormones in the body. Enzalutamide is an anti-androgen drug which inhibits androgen receptor (AR) signaling through competitive inhibition. The strong binding affinity of Enzalutamide to the AR prevents the binding of the AR to its ligands, testosterone and dihydrotestosterone (DHT). Because of the inhibited androgen receptor translocation to the nucleus, transcriptional processes associated with androgen regulated genes are disrupted (Tran et al. 2009; Scher et al. 2010). Enzalutamide is a potent androgen antagonist that has successfully reduced the

aggressive growth of prostate cancer cells while inducing fewer side effects compared to other available treatments (Boccardo et al. 1999; Iversen et al. 2000; Scher et al. 2012). However, Enzalutamide and other anti-androgen drugs are less effective for treatment of bone metastases in patients with castration-resistant prostate cancer (CRPC). Due to the ineffectiveness of current treatments, many patients with bone metastatic prostate cancer experience significant morbidity including debilitating fracture and severe bone pain. Thus, there is a clinical need to develop more effective therapies for bone metastatic prostate cancer. Yet, the paucity of *in vitro* prostate cancer models which accurately represent the complexities of the disease has hampered the development of therapies.

The intrinsic properties of cell lines used to model advanced prostate cancer *in vitro* largely affects the accuracy and reproducibility of results. Cell lines derived from human bone metastases, such as PC-3 and VCaP, are widely used for *in vitro* studies because of their cost-effectiveness and accessibility (Sobel and Sadar 2005; Goodspeed et al. 2015). While the capacity of cell lines to proliferate indefinitely has been beneficial, there are limitations. Cancer cells in our bodies develop genetic changes which disrupt normal cellular processes. Genetic alterations, such as somatic mutations and epigenetic silencing, enable tumor cells to proliferate aggressively and acquire drug resistance. Therefore, the ability of cell lines to effectively model prostate cancer is directly dependent on its capacity to accurately represent these genomic aberrations. Unfortunately, studies have shown that established cell lines fail to recapitulate the heterogeneous genotype of primary prostate cancer cells *in vivo* (Russel and Kingsley 2003; Peehl 2005).

Certain cell lines have been immortalized through the introduction of genes which induce telomerase activity and disrupt processes involved with normal cell growth and division (Rhim 2003; Russell and Kingsley 2003; Shay and Wright 2011). The introduction of these genetic modifications enables cancer cell lines to proliferate continuously *in vitro*, but they significantly alter the original genetic composition of the cells. Furthermore, cell lines established without immortalizing agents, often acquire new mutations after being continuously passaged (Peehl 2005; Masters and Stacey 2007; Marx 2014). Taken altogether, these findings illustrate some of the reasons why the use of established cell lines to discover genetic biomarkers and novel therapies often lead to inaccurate results. Yet, despite these limitations, many researchers continue to use cell lines for preclinical studies because propagating primary tumor cells has proven to be a challenging feat.

To address this problem, researchers have established patient-derived xenograft (PDX) models for prostate cancer through the transplantation of primary tumor samples into immunocompromised mice (Priolo et al. 2010; Raheem et al. 2011). Using this method, researchers successfully propagated primary patient tumor cells in mice while retaining their genetic integrity. Since PDX tumors closely resemble the original clinical cancer, patient-derived xenograft models for prostate cancer have been invaluable for evaluating the efficacy and toxicity of a drug (Siolas and Hannon 2013). However, extraneous variables inside an animal's complex internal environment pose several challenges for mechanistic studies. Therefore, in order to elucidate mechanisms that underlie cancer progression and resistance it is critical to develop *in vitro* models from primary and patient-derived xenograft tumor cells.

Despite the preconceived notions that primary and patient-derived tumor cells could easily be cultured due to their tumorigenic properties, the opposite proved to be true (Ellis et al. 1996; True et al. 2002; Corey et al. 2003). Some primary and patient-derived prostate cancer cells undergo senescence immediately after tumor harvesting, while other cells have approximately thirty divisions before becoming senescent (Iype et al. 1998; Schwarze et al. 2001; Peehl et al. 2005). The unsuccessful attempts at culturing primary and patient-derived tumor cells using traditional two-dimensional conditions resulted to the development of three-dimensional cell culture methods which better mimic the natural microenvironment of the tumor cells.

One of the three-dimensional culture methods used to propagate primary tumor cells is the multicellular spheroid culture. Spheroids are formed in culture through the aggregation of single cells (Weiswald et al. 2015). To promote the formation of spheroids, single cell suspensions are typically plated on ultra-low attachment plates that have a rounded bottom. Alternatively, inversion of small suspension aliquots placed on culture plates known as the "hanging drop method" could also be used to form spheroids (Kelm et al 2003). These methods led to the establishment of three-dimensional spheroid cultures derived from a set of metastatic prostate cancer xenografts known as the LuCaP series. In 2013, Young *et al.* demonstrated that long-term *in vitro* cultures of PDX tumor cells could be achieved through the maintenance of cell-to-cell contact (Young et al. 2013). By culturing PDX tumor cells on low-attachment plates and supplementing the cells with medium containing specific growth factors, tumor cells from various LuCaP xenograft models formed spheroids in culture (Young et al. 2013). Strikingly, these spheroids remained viable for months in culture and were successfully passaged multiple

times. The establishment of three-dimensional spheroid cultures from the LuCaP xenografts increased the accessibility and availability of patient-derived *in vitro* models for prostate cancer. However, the simplistic spheroid system lacks environmental cues from an extracellular matrix that is naturally present in the microenvironment of tumor cells.

In this regard, three-dimensional organoid cultures, initially developed for the growth and expansion of intestinal stem cells, better recapitulate the true microenvironment of cells. Through the incorporation of extracellular matrix analogues and specialized culture medium, colorectal cancer tumors were successfully established as organoids (Sato et al. 2011; van de Wetering et al. 2015). By embedding either primary or patient-derived tumor cells in a base membrane matrix and growing them with medium containing niche-specific growth factors, the tumor cells are cultured under conditions that closely resemble their physiological state i*n vivo* (Sato et al. 2011). Thus, tumor organoids established under these conditions retain genetic characteristics of the originating tumor.

The large success of colorectal cancer organoids, led to development of organoid culturing techniques for normal and malignant prostate cells (Drost et al. 2016). Metastatic prostate cancer tumor organoids cultured under these conditions maintained the histopathological traits of the originating tumor, exhibited expression of the androgen receptor, remained responsive to hormones, such as androgen, and demonstrated drug resistance (Chua et al. 2014; Gao et al 2016). However, unlike colorectal cancer, there has been limited success in the establishment of prostate cancer organoids from primary metastatic biopsies and patient-derived xenograft models. For this reason, the number and

availability of patient-derived *in vitro* models for prostate cancer remains low compared to other cancers. Since advanced prostate cancer, is a highly heterogeneous disease it is crucial to expand on the limited number of existing models to represent the full spectrum of the disease. Previously, our lab has developed patient-derived xenograft models for bone metastatic prostate cancer (Raheem et al. 2011, Godebu et al. 2014). In this study, we report establishment of three-dimensional cultures for our PDX and patient prostate cancer tumor samples through the adaptation and modification of previously established methods. So far, we have established *in vitro* cultures for both our primary and patient-derived xenograft tumor cells which remain viable for a month and a half without passaging. Furthermore, preliminary experiments showed that our three-dimensional cultures consist of heterogeneous cell populations that exhibit different responses to the androgen hormone, DHT, and the anti-androgen drug, Enzalutamide. In the future, we hope to further optimize our three-dimensional *in vitro* conditions for our PDX and primary tumor cells to improve the robustness of our three-dimensional cell culture model and ensure the reproducibility our results.

Methods

Patient-Derived Xenograft Model of Bone Metastatic Prostate Cancer

PCSD (Prostate Cancer San Diego) 1, 13, 16, 17, and 18 are surgical prostate cancer bone metastasis specimen donated by men who progressed to castrate resistant bone metastatic prostate cancer. Approval was received from the UCSD institutional review board (IRB) to collect surgical bone metastatic prostate cancer specimens for research purposes. PCSD1 and PCSD13 were previously established as patient-derived xenograft models for bone metastatic prostate cancer (Raheem et al. 2011). Briefly, the prostate cancer bone metastases obtained during surgery were resuspended in high concentration Matrigel (BD Biosciences, Inc.) at a 1:1 ratio and injected intra-femorally (IF) into the right femur of 6 to 8 week old male Rag2 $^{-/-}$;γc $^{-/-}$ mice. All animal protocols were performed under a UCSD animal welfare IACUC approved protocol. Prior to this experiment, the PCSD1 and PCSD13 tumor cells were propagated as high passage tumors in male Rag2 $^{-/-}$;γc $^{-/-}$ mice which remain localized in the femur. However, in one instance the PCSD1 cells that were injected into the right femur of a male Rag2 $^{-/-}$;γc $^{-/-}$ mouse spontaneously metastasized and developed as tumors in the abdomen and neck region. The cells from both the abdomen (PCSD1 SAM^{A}) and neck (PCSD1 SAM^{N}) metastases were isolated as previously described in Raheem *et al.* (2011) prior to culturing under three-dimensional conditions. The PCSD16, PCSD17, and PCSD18 samples used in this experiment were primary patient prostate cancer bone metastasis cells from orthopedic repair surgery of pathologic fractures that were directly cultured *in vitro* after surgery.

PCSD1 and PCSD13 GLF-lentiviral transduction and *in vivo* Bioluminescent Imaging (IVIS)

PCSD1 and PCSD13 cells were freshly isolated from intra-femoral xenograft tumors and transduced with a GFP-luciferase expressing lentiviral vector (GLF), a kind gift from Dr. Catriona Jamieson, UCSD, as previously described (Godebu et al. 2014). Cells were then sorted by flow cytometry for green fluorescent protein (GFP) positive cells on FACSAria prior to being intra-femorally injected into male Rag2 $^{-/-}$;γc $^{-/-}$ mice. Mice tumor volume was assessed using an *in vivo* bioluminescence imaging system (IVIS 200; Perkin Elmer Inc.) and using caliper measurements. When the tumors reached the maximal allowable size of 1.5-2.0 cm, the mice were sacrificed according to UCSD ACP standards.

PDX Tumor Cell Preparation

PCSD1 and PCSD13 cells were maintained as intra-femoral tumors in male Rag2 $^{-/-}$;γc $^{-/-}$ mice for this experiment. All experiments involving xenograft models were conducted under an IACUC approved protocol at the University of California, San Diego. On the day of harvest, tumors were removed immediately after sacrificing and submersed in a cold solution of DMEM/F-12 complete (DMEM/F-12 supplemented with 10% (v/v) FBS and 1% (v/v) penicillin streptomycin). The tumors were processed using a previously established by Raheem *et al.* (2011). Briefly, the tumor samples were minced to 1-3 mm^3 sized pieces and digested with Accumax Cell Dissociation solution (Stem Cell Technologies) for 45 minutes at room temperature. The enzyme solution was inactivated through the addition of DMEM/F-12 complete and filtered through a 70 μm

cell strainer (BD Biosciences). The filtered solution was centrifuged at 1200 RPM for 5 minutes at 4°C and the remaining cell pellet was washed three times. A magnetic mouse cell depletion kit catalog no. 130-104-694 (Miltenyi Biotec) was used to enrich for human cells in our PDX tumor samples. Final cell counts were determined using trypan blue dye and a hemocytometer.

Three-Dimensional (3D) Cell Culture of PDX Tumor Cells

The method to establish three-dimensional cultures was adapted from Drost *et al.* (2016). Primary and patient-derived xenograft tumor cells were resuspended in either growth factor reduced or high concentration Matrigel (BD Biosciences). Small aliquots of the cell suspension were domed on cell culture plates according to the seeding concentration listed in Table 1. To prevent cell adhesion to the bottom of the plate, the culture plates were inverted prior to being placed in the CO_2 incubator (5% CO_2, 37°C) for 15 minutes. Once the Matrigel domes solidified, the appropriate volume of medium, indicated in Table 2, was added to each of the wells. The exact formulation of medium used in these experiments is shown in Table 3. After 7 days, cultures were grown in medium without the Rho-kinase inhibitor, Y-27632. Medium was changed every 3 to 4 days and fresh medium was made every week.

To passage the three-dimensional cultures, the media was aspirated from the wells containing the Matrigel domes. The domes were then rinsed with cold passaging media (advanced DMEM/F12 containing 5% FBS, 1X penicillin/streptomycin, 10 mM HEPES, and 2 mM GlutaMAX) and collected into conical tubes. The three-dimensional cultures were mechanically dissociated with a syringe attached to a 25G needle. Once dissociated,

10 ml of cold passaging media was added to the disrupted cultures and the solution was centrifuged at 1200 RPM for 5 minutes at 4°C. The cultures were then incubated in a 37°C water bath with 1X TrypLE Express (ThermoFisher) to further dissociate the cultures into single cell suspensions. The TrypLE Express enzyme was inactivated through the addition of passaging media and the solution was centrifuged at 1200 RPM for 5 minutes at 4°C. The resulting cell pellet was resuspended in either growth factor reduced or high concentration Matrigel (BD Biosciences) and domed on cell culture plates as previously described.

Hormone and Drug Treatments

For the DHT and Enzalutamide experiments, PCSD1 tumor cells were embedded in growth factor reduced Matrigel (BD Biosciences) at a seeding density of 25,000 cells per 20 µl of Matrigel. Cultures were established from two independent mouse tumors (n=2) and given treatment during the initial day of plating. DHT was added to the medium at a final concentration of 1 nM. Three-dimensional cultures grown in medium without additional DHT served as a negative control. In the Enzalutamide drug experiment, cultures in both conditions were grown in medium that contained DHT at a final concentration of 1 nM. Enzalutamide was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium at a final concentration of 10 µM. The vehicle control conditions were treated with only 0.1% (vol/vol) DMSO in culture medium. Each of the independent experiments were performed in triplicates.

Cell Proliferation and Viability Assays

The morphological attributes, lumen size, and epithelial cyst counts, of the Matrigel embedded PDX tumor cells were observed using a microscope (Keyence Corporation). The scale bar on the Keyence microscope was placed in the center of the epithelial cyst and adjusted to measure the lumen diameter (Figure 5D). To compare the changes in proliferation over time, the fluorescence of GFP from the PDX tumor cells were imaged using a microscope (Keyence Corporation) on a weekly basis. Image J software was used to quantify the differences in fluorescence intensity between the treatment conditions. On day 28, the luciferase assay was performed according to a modified version of the manufacturer's instructions and the luminescence was quantified using the Tecan Infinite M200 plate reader (Männedorf, Switzerland).

Statistical Analysis

Statistical significance was determined using either unpaired or Welch's Student's t test on GraphPad Prism software version 7. The data shown in the graphs represent the mean ± standard error of the mean (SEM) unless otherwise stated.

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Results

Preliminary Optimization Experiments of Three-Dimensional Culture

Through the incorporation of previously established methods into our own culturing methods, we were able to optimize three-dimensional cell culture conditions which keep our PDX tumor cells viable in culture for a month and a half without passaging (Figure 1). Three-dimensional cultures consist of many interdependent procedures and components. Due to the long duration of each experimental trial, we only focused on two components during our preliminary optimization studies: (1) the tumor isolation process for our patient-derived xenograft tumor samples and (2) the cell culture medium. Most studies use cells that are solely of human or mouse origin for their organoid cultures. However, our patient-derived xenograft tumors consisted of both mouse and human cell populations because they were serially passaged in immunocompromised mice (Figure 1A-B). Therefore, we had to develop a method to enrich for the human cell population in our PDX tumors after dissociation. Some groups stain their cells using a human-specific antibody and perform fluorescence activated cell sorting (FACS) to enrich for human cell populations. While this method is highly selective, the low cell throughput rate of flow cytometry decreases the viability of cells. To maintain the viability of our cells during the enrichment process, we instead used a magnetic column kit (Miltenyi Biotec) to deplete the mouse cells in our isolated tumors (Figure 1C). The enriched populations obtained afterwards only contained a small number of mouse cells and the cell viability was not significantly reduced during this process. Therefore, depleting mouse cells using a magnetic column kit proved to be a

good alternative to enrich for human cell populations prior to culturing under three-dimensional conditions (Figure 1D).

For the medium and culture conditions, we adapted a method developed by Drost *et al*. (2016). In a preliminary experiment, we cultured our PCSD1 SAM^N and PCSD1 SAM^A tumor cells using previously established conditions to determine whether organoids would form from our patient-derived xenograft tumor cells. PCSD1 SAM^N and PCSD1 SAM^A are tumor cells that originated from intra-femorally injected PCSD1 cells which spontaneously metastasized to the neck and abdomen, respectively. Although these cells originated from the same PCSD1 tumor cells, they surprisingly formed two different phenotypes when cultured under three-dimensional conditions. The tumor cells that metastasized to the abdomen (PCSD1 SAM^A) remained as single cell suspensions (Figure 2A-D). These PCSD1 SAM^A cells did not proliferate *in vitro* and after four weeks most of the cells have undergone senescence. On the other hand, the tumor cells that metastasized to the neck (PCSD1 SAM^N) formed a large organoid structure that remained viable for months under the same three-dimensional culture conditions (Figure 2E-H). These cultures proliferated *in vitro* and formed a single structure that was approximately 400 µm in size. After four weeks, the organoids were successfully passaged through mechanical and enzymatic dissociation. However, prior to the third passage, the PCSD1 SAM^N organoid cultures started to exhibit slowed growth.

The decreased proliferation of the PCSD1 SAM^N organoid cultures along with the short lifespan of PCSD1 SAM^A demonstrated that the previously established medium conditions for prostate epithelial and cancer tissues may not be optimal for our patient-derived xenograft tumor samples. Therefore, to improve the viability of the PCSD1

SAM^N cultures, fetal bovine serum (FBS) was added to the culture medium. With this modified medium, the PCSD1 SAM^N organoid cultures remained viable *in vitro* and passaging resulted to the formation of multiple spherical organoids around 100 to 200 µm in size, contrast to a single, multilayered organoid structure (Figure 2I-L). Altogether, the results indicated that the addition of FBS in the culture medium could improve the survival rate of our PDX tumor cells in long-term cultures.

Using these optimized conditions, our localized PDX tumor cells (PCSD1 and PCSD13) were successfully cultured *in vitro* for the first time (Figure 3-4). The PCSD1 tumors consisted of heterogeneous cell populations, therefore various phenotypes were observed in the three-dimensional cultures. Unlike the PCSD1 SAM^N cultures which predominantly contained organoids, most of the cultures from the localized PCSD1 tumors comprised of single cells (Figure 3A), epithelial cysts (Figure 3B), and spheroids (Figure 3C). Furthermore, the viability and morphology of cellular aggregates formed by the PCSD1 cells were observed on a weekly basis using microscopy. During the first week in culture, the PCSD1 cells formed small cellular clusters which ranged from 50 µm to 80 µm in size (Figure 3D-G). After four weeks, some of these small clusters formed larger aggregates (spheroids) which were approximately 150 µm to 250 µm in size (Figure 3H-K). The PCSD1 cells remained viable in three-dimensional cultures for six weeks without passaging.

The PCSD13 tumor cells used in the preliminary experiments were frozen prior to culturing. Thus, high concentration Matrigel (BD Biosciences) was used to provide additional growth factors to the cells. After four weeks in culture, the PCSD13 tumor cells formed organoid structures that were phenotypically distinct from the PCSD1

SAM^N organoids structures. Some of the PCSD13 cell formed branched organoid cultures surrounded by hollow cysts (Figure 4A-D), while other cells formed spherical organoids that were approximately 100 µm – 150 µm in size (Figure 4E-H). Currently, tumor cells from our PDX models (PCSD1 and PCSD13) and primary patient metastases (PCSD18) have been successfully cultured using three-dimensional conditions (Figure 4I-J, Table 1).

Androgen Responsiveness of PCSD1 Tumor Cultures

One main advantage of maintaining cells in three-dimensional cultures is that they can better recapitulate the physiological processes that occur in the human body by retaining their ability to respond to signaling factors (Drost et al. 2016). To determine whether our cell cultures were responsive to the androgen hormone, Dihydrotestosterone (DHT), we cultured some of our PCSD1 cells with or without the addition of 1 nM DHT. Our PCSD1 tumor cultures consisted of heterogeneous cell populations, therefore we decided to observe the effect of DHT on the individual subpopulations of epithelial cysts and spheroids in addition to the tumor cell population.

Epithelial cysts are formed by normal and less malignant prostate epithelial cells (Debnath and Brugge 2005). Since prostate epithelial cells typically require DHT to function properly, it was hypothesized that the epithelial cysts formed in our tumor cultures would require DHT for normal development. The epithelial cysts grown with 1 nM DHT had a spherical shape, whereas the epithelial cysts grown without DHT had a distorted shape (Figure 5A). In addition, the average lumen diameter was significantly increased ($P < 0.05$) in the cultures that were grown with additional DHT (Figure 5A-B).

To determine whether additional DHT helped epithelial cysts form in culture, the number of epithelial cysts were counted in both treatment conditions. The addition of DHT significantly increased ($P < 0.05$) the number of epithelial cysts formed in culture (Figure 5C). These results demonstrated that the epithelial cysts were dependent on androgen for proper development.

The spheroids and tumor cells observed in culture showed a different response to DHT. Prior to injections in the femur of immunocompromised mice, our cells were transduced with a lentiviral vector which expressed green fluorescent protein (GFP) and luciferase under a constitutively active promoter (Figure 1B). Using this vector, the growth and viability of our PCSD1 cells could be analyzed through the measurement of GFP fluorescence intensity and luciferase activity. In preliminary experiments, we noticed that our PCSD1 tumor cells took approximately three to six weeks to grow in culture, unlike established cell lines which proliferate and divide within one week. Therefore, we maintained our cells in three-dimensional cultures for four weeks to ensure that our cells had enough time to grow during our *in vitro* experiments.

To determine whether DHT had a significant effect on the overall growth of our PCSD1 cells, the fluorescence intensity of the cultures in both conditions were compared at weeks 1 and 4. At week 1, the cultures from both conditions had low fluorescence intensity, but by week 4 the fluorescence intensity had greatly increased (Figure 6A). Small dots in the cross-section image represent cellular aggregates between 50 to 80 µm in size, whereas medium-sized circles represent spheroids greater than 100 µm (Figure 6A). The average intensity fold change of all the cultures were calculated using Image J analysis and the results show that there was no difference between the intensity fold

change of the cultures grown with additional DHT and the cultures grown without additional DHT (Figure 6B). These results indicated that the growth of our PCSD1 cells in three-dimensional cultures were not affected by DHT. To compare the viability of the PCSD1 tumor cells in the different conditions, a luciferase assay was performed during week 4. The average relative luminescence units (RLU) of the cells grown without additional DHT was not different than the RLU of the cells grown with additional DHT (Figure 6C). The comparable luciferase activity of cells grown with or without DHT implied that DHT did not affect the viability of the PCSD1 tumor cells in three-dimensional culture. Due to the small sample size in both analyses, it is possible that there was not enough power to detect the treatment effect. Therefore, this experiment should be repeated with a higher sample size to determine statistical significance. Furthermore, treatment with 1 nM DHT did not affect the spheroid formation of the PCSD1 tumor cells (Figure 6D).

Effect of 10 µM Enzalutamide on PCSD1 Tumor Cultures

To test our cultures for drug screening, we treated our PCSD1 cells with either vehicle (0.1% (v/v) DMSO) or 10 µM Enzalutamide. In preliminary experiments, we treated our three-dimensional cultures with increasing doses of Enzalutamide drug and found that the number of epithelial cysts was significantly reduced at a concentration of 10 µM. Therefore, we decided to only use 10 µM when we repeated the experiment. After four weeks in culture, the average lumen diameter of the epithelial cysts was significantly decreased ($P < 0.05$) in the cultures that were treated with 10 µM of Enzalutamide (Figures 7A-B). In addition, treatment with 10 µM Enzalutamide also

significantly decreased ($P < 0.05$) the number of epithelial cysts that formed in culture (Figure 7C). These results show that the development of epithelial cysts was negatively affected by the Enzalutamide drug.

However, similar to the findings from the cultures grown under different DHT conditions, the overall PCSD1 tumor subpopulations seemed to be unaffected by the Enzalutamide drug. At week 1, the fluorescence intensity of cultures that were treated with Vehicle or 10 µM Enzalutamide were weak, but by week 4 the fluorescence intensity increased (Figure 8A). The average intensity fold change of all the cultures were calculated using Image J analysis and the results show that there was no difference between the intensity fold change of the cultures treated with Vehicle and the cultures treated with 10 µM Enzalutamide (Figure 8B). These results indicated that the growth of our PCSD1 tumor cells in three-dimensional cultures was not affected by Enzalutamide. To compare the viability of the PCSD1 tumor cells in the different conditions, a luciferase assay was performed during week 4. The average relative luminescence units (RLU) of the cells treated with vehicle was not different than the RLU of the cells treated with 10 µM Enzalutamide (Figure 8C). The insignificant difference in RLU indicated that Enzalutamide did not affect the viability of the PCSD1 tumors cells which remained as single cell suspensions or formed multicellular aggregates (spheroids). Due to the small sample size in both analyses, it is possible that there was not enough power to detect the treatment effect. Therefore, this experiment should be repeated with a higher sample size to determine statistical significance. Furthermore, treatment with 10 µM Enzalutamide did not affect the spheroid formation of the PCSD1 tumor cells (Figure 8D).

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Discussion

In the last few years, significant efforts to develop more effective treatments for castrate-resistant prostate cancer has led to the discovery of potent therapeutic drugs, such as Enzalutamide. While Enzalutamide and other drugs have been successful in clinical trials, the response of advanced prostate cancer patients to these treatments widely varies and tumors often acquire resistance to established therapies. Thus, there is a clinical need to understand the molecular mechanisms the underlie therapy resistance. However, progress has been hindered by the limited availability of *in vitro* models which can fully recapitulate the heterogeneous disease. In this study, we report the establishment of new three-dimensional prostate cancer *in vitro* models using tumor cells from both patient-derived xenograft models and primary patient bone metastases.

Currently, there are several methods to establish three-dimensional *in vitro* models for cancer. In our initial attempts to culture our patient-derived xenograft and primary patient tumor cells, we adapted an organoid culture system established by Drost *et al.* (2016). Since the culture conditions in this system were primarily developed using epithelial progenitor cells from either the human or mouse prostate gland, we had limited success in propagating our PDX tumor cells under these conditions. In our preliminary experiments, the majority of our localized PDX and primary patient tumor cells did not grow and after four weeks in culture, most of the cells had undergone senescence. These results indicated that the cell culture conditions described by Drost et al. (2016) were not optimal for our tumor cells and other growth factors were needed to support the long-term culture of our tumors cells. Therefore, in contrast to the conditions used in other

organoid and 3D culture studies for prostate cancer, our culture medium contained serum to improve the viability of tumor cells in culture (Gao et al. 2014; Drost et al. 2016). With the addition of fetal bovine serum to our medium, our PDX and primary prostate cancer tumor cells remained viable in culture for a month.

Furthermore, we observed a wide range of phenotypes in our three-dimensional cultures. Since we adapted previously established organoid conditions, we expected to see the formation of tumor organoids from our PDX and primary patient cells. Instead, we observed mixed results. Some of our metastasized PDX tumor cells formed organoid structures, while our other cells formed a combination of epithelial cysts and spheroids in culture. Aside from mouse cell depletion, no additional enrichment or sorting procedure was performed to select for a specific cell population. Therefore, we speculated that different phenotypes were observed in our three-dimensional cultures because our isolated PDX tumors were highly heterogeneous.

An advantage of having heterogeneous three-dimensional cultures was that the effect of a treatment could be individually observed among subpopulations. Debnath *et al*. demonstrated that the morphology of epithelial cells grown in three-dimensional cultures is indicative of its cancerous properties (Debnath and Brugge 2005). Because prostate cancer cells are of epithelial origin, they typically exhibit polarization that contributes to lumen formation. Cancer cells which have normal or less malignant properties tend to form epithelial cysts or acini with a hollow lumen (Lang et al. 2001; Debnath and Brugge 2005). On the other hand, tumor cells with malignant properties tend to form cellular aggregates or filled acini (Debnath and Brugge 2005). Leveraging

this knowledge, we divided the observed phenotypes in our cultures into two categories (1) epithelial cysts and (2) multicellular aggregates.

The epithelial cysts represent the population of our tumor cells that are less malignant. Therefore, we hypothesized that these cell populations would be responsive to treatment with both DHT and Enzalutamide. Our results show that treatment with DHT increases the size and number of epithelial cysts, whereas treatment with 10 μM Enzalutamide decreases the size and number of epithelial cysts. Previous studies have shown that the presence of stroma and dihydrotestosterone increases the number of acinus-like spheroids formed by nonmalignant prostatic epithelial cells (Lang et al. 2001). Thus, consistent with previous findings, our results indicate that the formation of epithelial cysts (acini with a hollow lumen) by a subset of our PDX cells was dependent on androgen. Yet, to our knowledge, the sole effect of dihydrotestosterone and enzalutamide on the lumen size of epithelial cysts formed by patient-derived xenograft tumor cells has not been reported. To confirm whether these subpopulations are truly androgen dependent, further experimentation utilizing immunohistochemistry to analyze the localization of the androgen receptor is needed. Furthermore, the growth medium of our three-dimensional cultures contains fetal bovine serum which has a low concentration of the DHT hormone. Although the concentration of DHT in FBS is below the physiological concentration observed in male patients, charcoal stripped FBS could be used in future experiments to avoid potential confounding variables.

The multicellular aggregates represent the population of our tumor cells that are more malignant. Therefore, we hypothesized that these cell populations would be less responsive to treatment with DHT and Enzalutamide. Our results show that treatment

with DHT and 10 μm Enzalutamide had no significant effect on the overall viability and activity of our PDX tumor cells in culture. Fong *et al.* established three-dimensional models for patient-derived xenograft cells using a hydrogel construct and found that tumor cells cultured under these conditions are less sensitive to the chemotherapy, Docetaxel (Fong et al. 2014). Furthermore, organoid lines developed by Gao *et al.* also demonstrated a decreased sensitivity to drug treatments (Gao et al. 2014). Thus, similar to other findings, our results suggest that the presence of an extracellular matrix is important in understanding the mechanisms that underlie drug resistance in tumor cells. However, the results of our luciferase assay had high variability due to the inherent differences between the different tumor samples. In future experiments, luminescence measurements would be taken at weeks 1 and 4 to lessen variability between groups. In addition, a power analysis would be used to calculate the appropriate sample size needed to detect the treatment effect.

A limitation in utilizing heterogeneous *in vitro* models is the challenge of reproducibility. Treatment responses could greatly vary in a three-dimensional culture because of inter- and intra-tumor heterogeneity. Furthermore, there are limited number of commercial kits which accurately measure the growth and viability of the tumor cells in culture. Thus, in future experiments, we would optimize our method of performing *in vitro* experiments to make our system more robust. As of right now, we perform phenotype-specific analysis and an endpoint assay to measure the viability via luciferase activity of our cells. Although the phenotype-specific analysis of the cysts versus multi-cellular aggregates revealed the effect of treatments on the different tumor subpopulations, it would be difficult to perform high-throughput drug screens.

The results of our study demonstrated our initial attempts to establish three-dimensional culture models from our patient-derived xenograft and primary tumor cells. In this study, we have demonstrated that patient-derived xenograft and primary tumor cells could be maintained *in vitro* through modified organoid culture conditions, however further work is needed to improve the reproducibility and applicability of our *in vitro* models for high-throughput drug and molecular dissection experiments.

Acknowledgements

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Figures

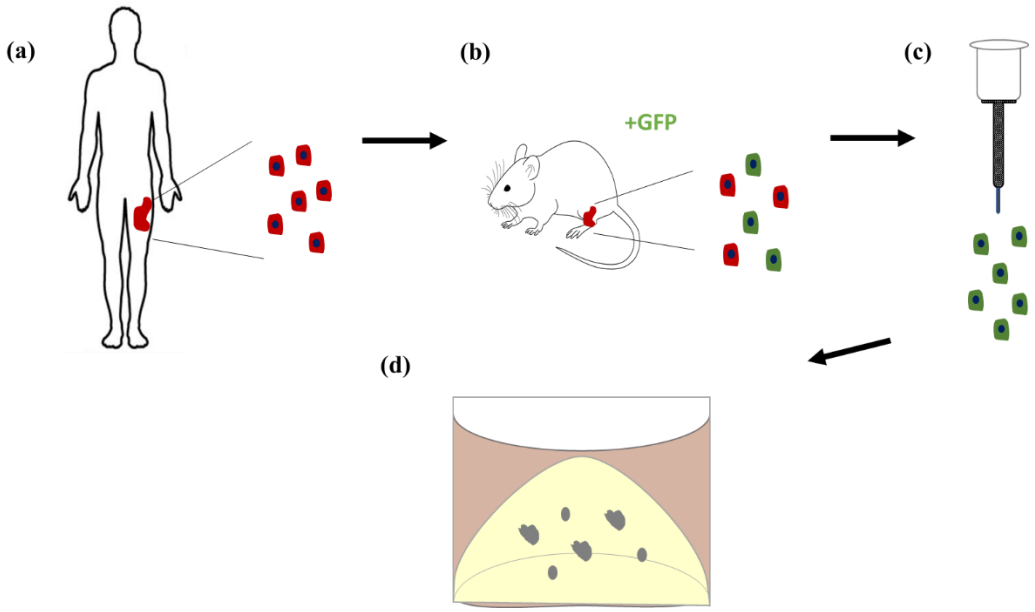

Figure 1. Workflow of three-dimensional (3D) cell culture from patient-derived xenograft tumors (PCSD).

To establish a xenograft (Xg) model for prostate cancer, (a) a tumor sample from an advanced prostate cancer patient was obtained, isolated, and then (b) injected intra-femorally into immunodeficient mice. The tumors harvested from the xenograft models were enzymatically isolated, transduced with a lentivirus containing GFP, and (c) enriched for human cells using a magnetic column which depletes mouse cells. (d) The enriched human cell populations were cultured using 3D cell culture conditions.

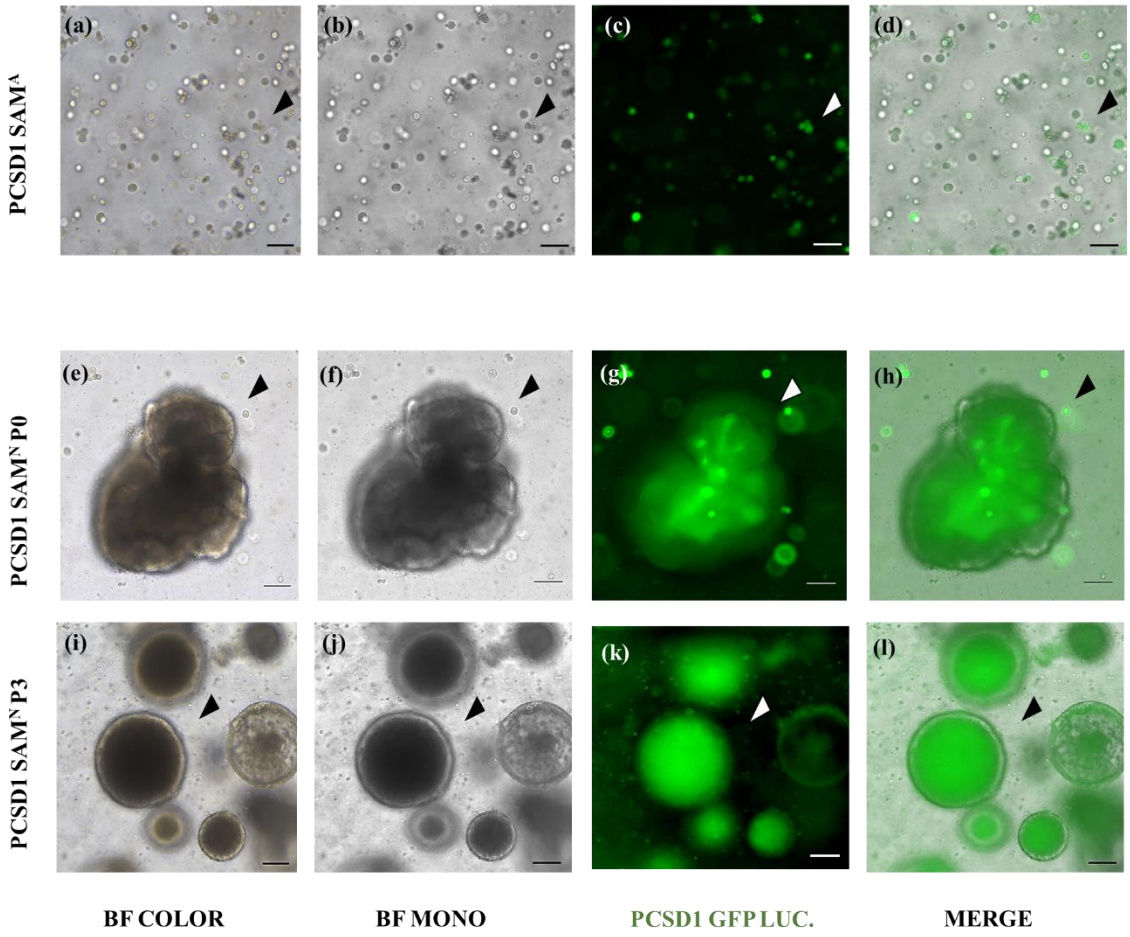

Figure 2. PCSD1 cells from both the abdomen (PCSD1 SAM^{A}) and neck (PCSD1 SAM^{N}) metastases of a mouse formed different morphologies under three-dimensional conditions.

(a-d) PCSD1 SAM^{A} tumor cells remained as single cell suspensions *in vitro* and became senescent after four weeks in culture. (e-h) PCSD1 SAM^{N} tumor cells formed a large single organoid structure that was approximately 400 µm in size. (i-l) The PCSD1 SAM^{N} cultures were successfully passaged and formed multiple spheroids around 100 µm to 200 µm after the third passage. The microscopy images are representative of the observed phenotypes and were taken at an objective of 10X. (a-l) Scale bars represent 100 µm. Black and white arrows indicate the described structures.

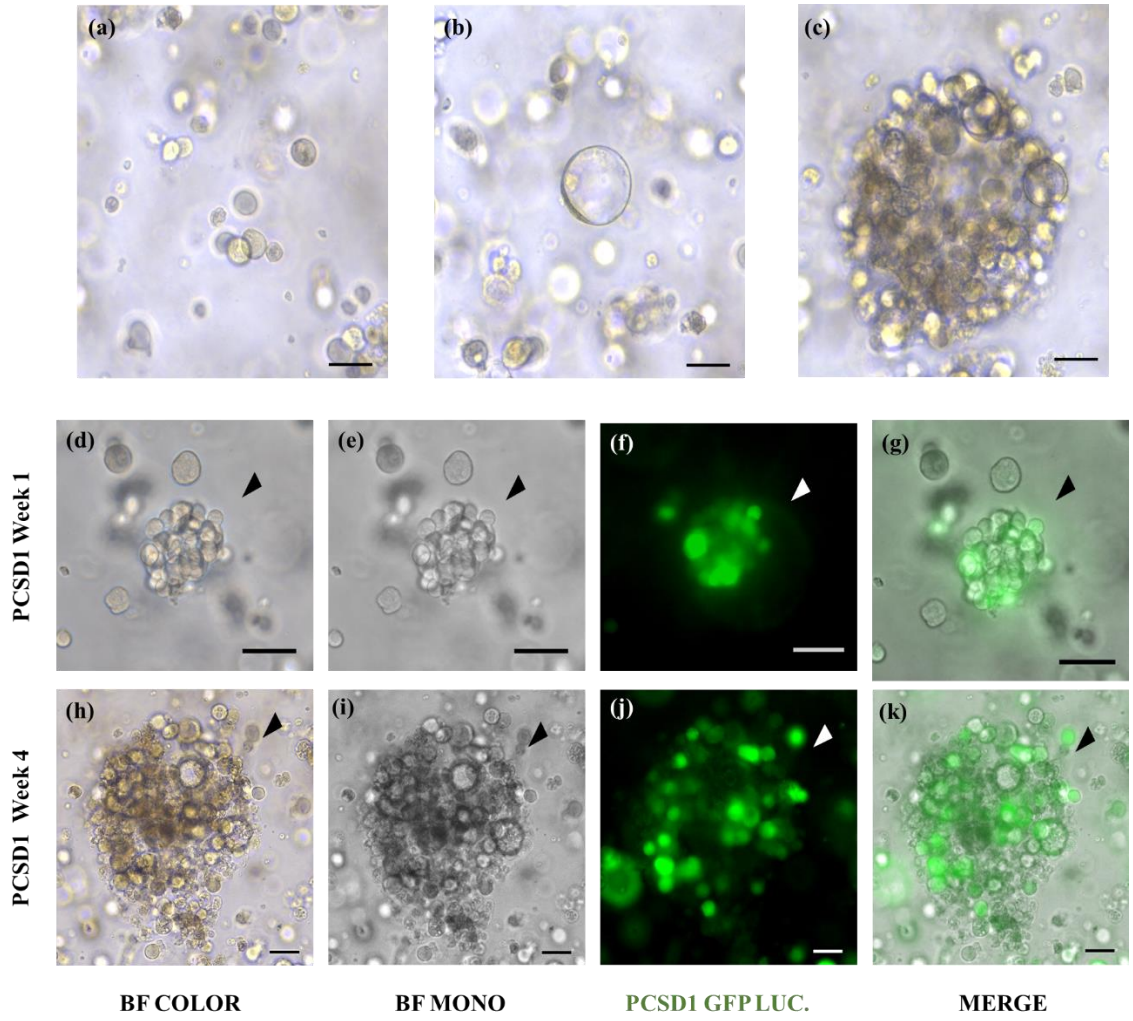

Figure 3. Morphology of PCSD1 Intra-femoral (IF) Tumor Cultures.

PCSD1 tumor cells form heterogeneous three-dimensional cultures consisting of (a) single cells suspensions, (b) epithelial cysts, and (c) spheroids. (d-g) After one week in culture, some PCSD1 cells formed small cell clusters approximately 50 µm – 80 µm in size indicated by black and white arrows. (h-k) After four weeks in culture, some of the cell clusters aggregated together to form larger spheroids that are approximately 150 µm to 250 µm in size indicated by black and white arrows. The microscopy images are representative of the observed phenotypes and were taken at an objective of (a-c, h-k) 10X and (d-g) 20X. (a-k) Scale bars represent 50 µm.

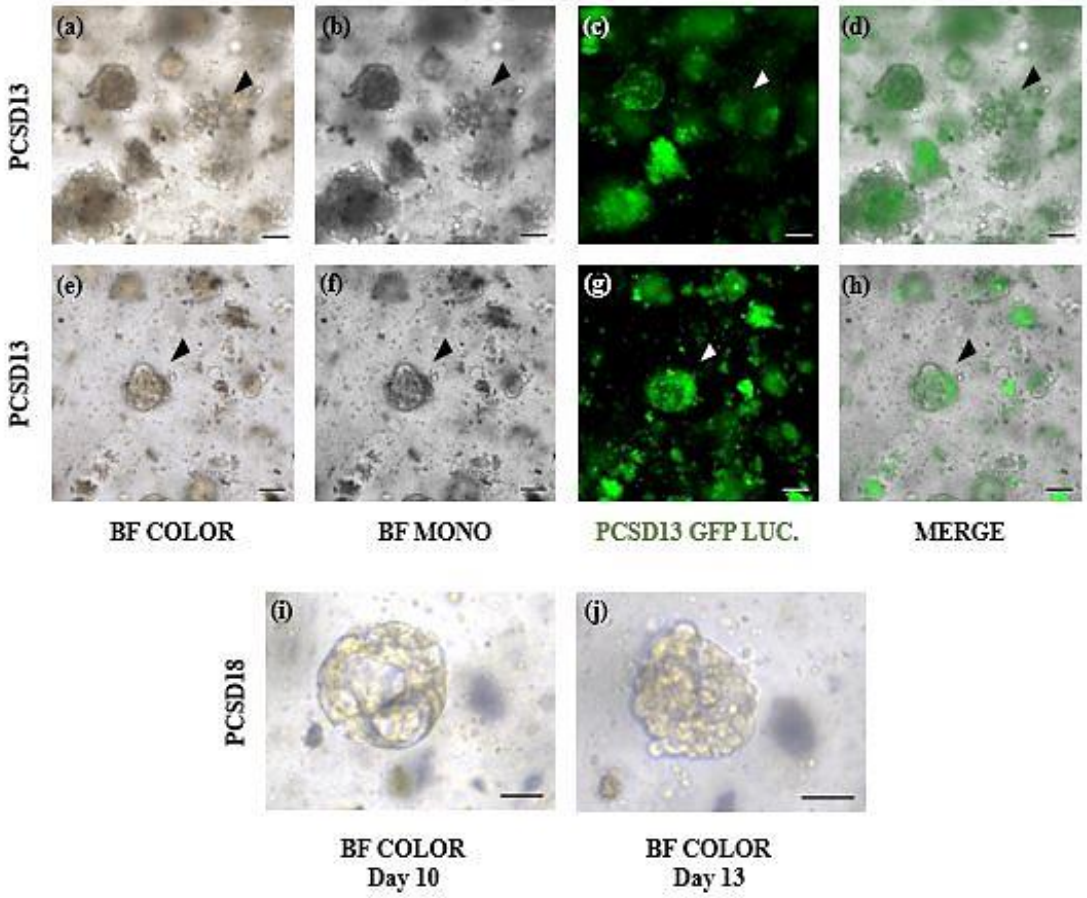

Figure 4. Morphology of PCSD13 Intra-femoral (IF) and PCSD18 Primary Patient Tumor Cultures.

PCSD13 xenograft tumor cells were previously frozen and PCSD18 tumor cells were freshly isolated from a patient bone metastases specimen prior to culturing under three-dimensional conditions. The PCSD13 organoid cultures formed both a (a-d) branched morphology surrounded by cysts and (e-h) a spherical morphology approximately 100 µm to 150 µm in size as indicated by black and white arrows. The PCSD18 tumor cells formed both (i) hollow and (j) filled organoid cultures greater than 100 µm. The microscopy images are representative of the observed phenotypes and were taken at an objective of 10X. Scale bars represent (a-h) 100 µm and (i-j) 50 µm.

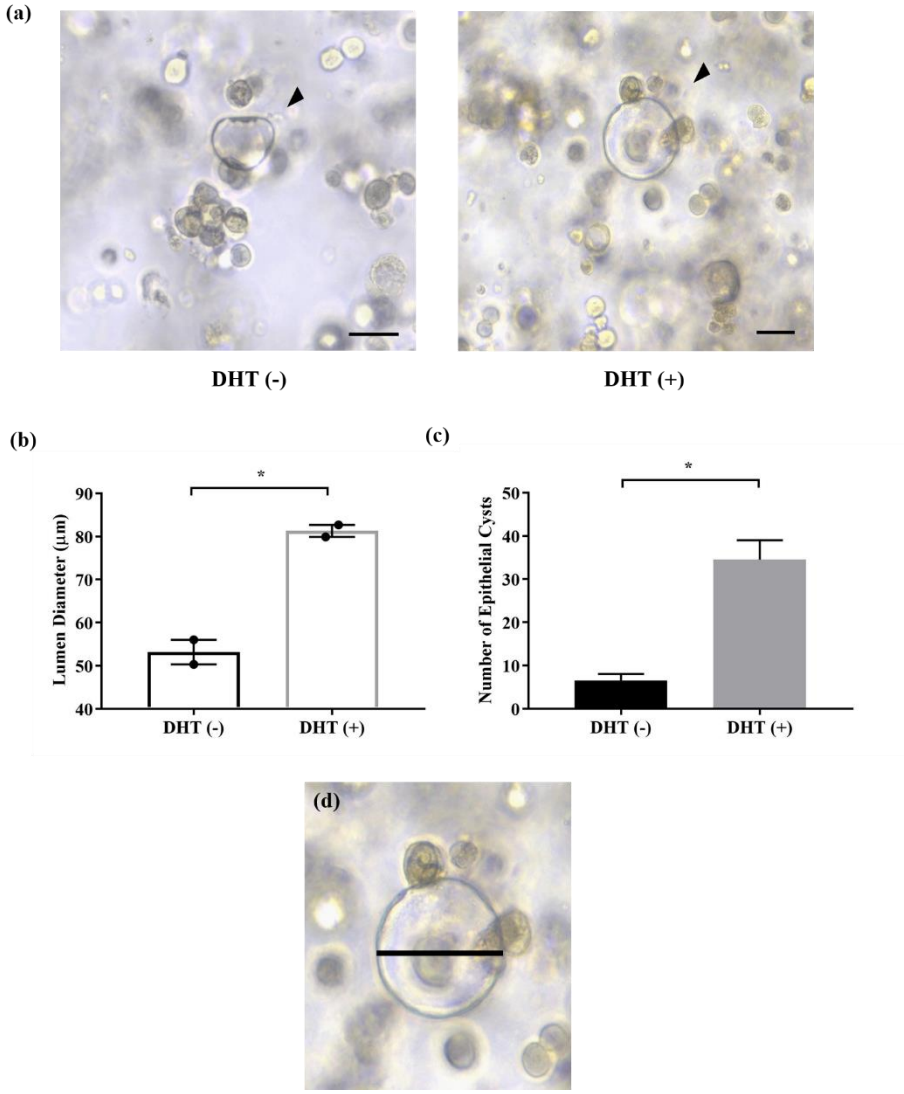

Figure 5. Treatment with DHT increases the lumen size and number of epithelial cysts formed in culture.

PCSD1 tumor cells were cultured with or without the addition of 1 nM DHT. After four weeks in culture, (a-b) the lumen of epithelial cysts grown with DHT was larger compared to the epithelial cysts grown without DHT. (c) The number of epithelial cysts greater than 50 µm in size were counted in both treatment conditions. Addition of DHT increased the number of epithelial cysts formed in culture. (d) Image of lumen diameter measurement. (a) The microscopy images are representative and were taken at an objective of 10X. Black arrows show epithelial cysts. Scale bars represent 50 µm. (b-c) Data represents the mean from two (n=2) independent experiments performed in triplicates ± SEM. Student's t-test was used to determine statistical significance (* indicates $P < 0.05$).

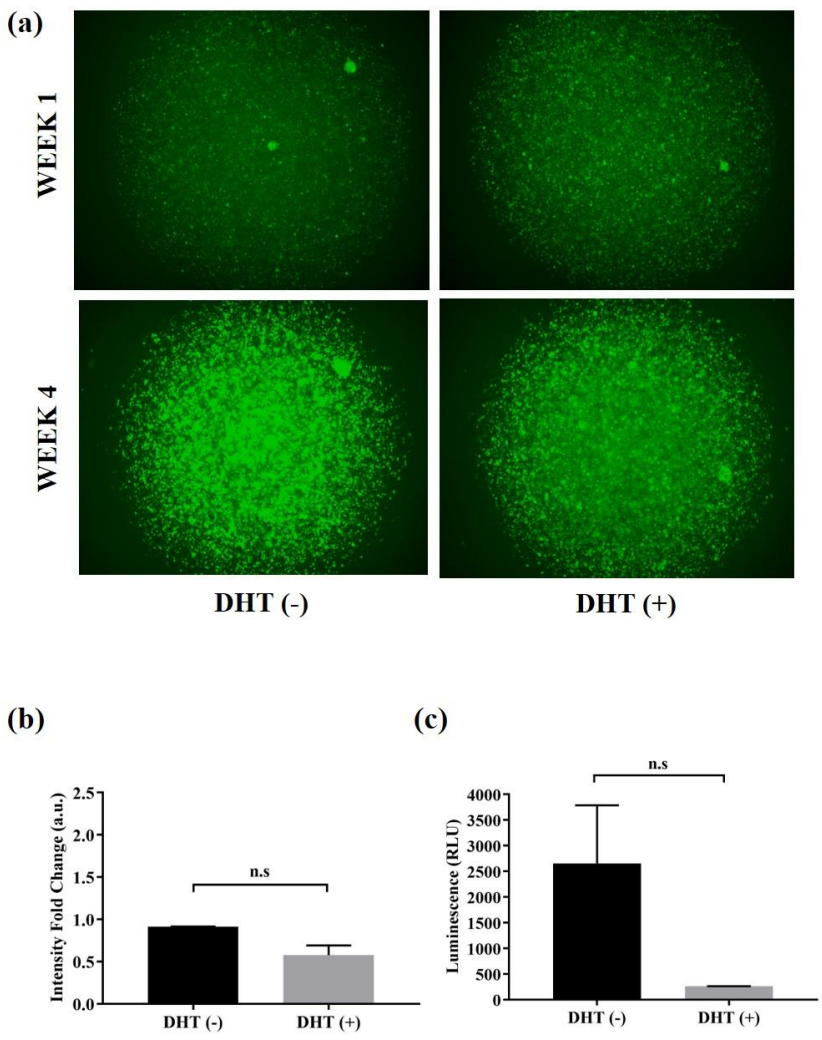

Figure 6. Treatment with DHT has no effect on overall tumor cell growth and viability.

PCSD1 tumor cells were cultured with or without the addition of 1 nM DHT. (a-b) At weeks 1 and 4, the fluorescent intensity of the cultures from each treatment condition were compared. The fold increase of fluorescence in both conditions were not different from each other, indicating that they had similar growth rates (c) The luciferase assay was performed on the cultures during week 4. The luminescence from the cultures treated with DHT was not different than the luminescence from the cultures that were not treated with DHT. (d) Image of spheroid formation in both treatment conditions. Scale bars represent 100 µm. (a) The fluorescent microscopy images are representative and were taken at an objective of (a) 2X and (d) 10X. (b-c) Data represents the mean from two (n=2) independent experiments performed in triplicates ± SEM. Student's t-test was used to determine significance (n.s indicates not significant).

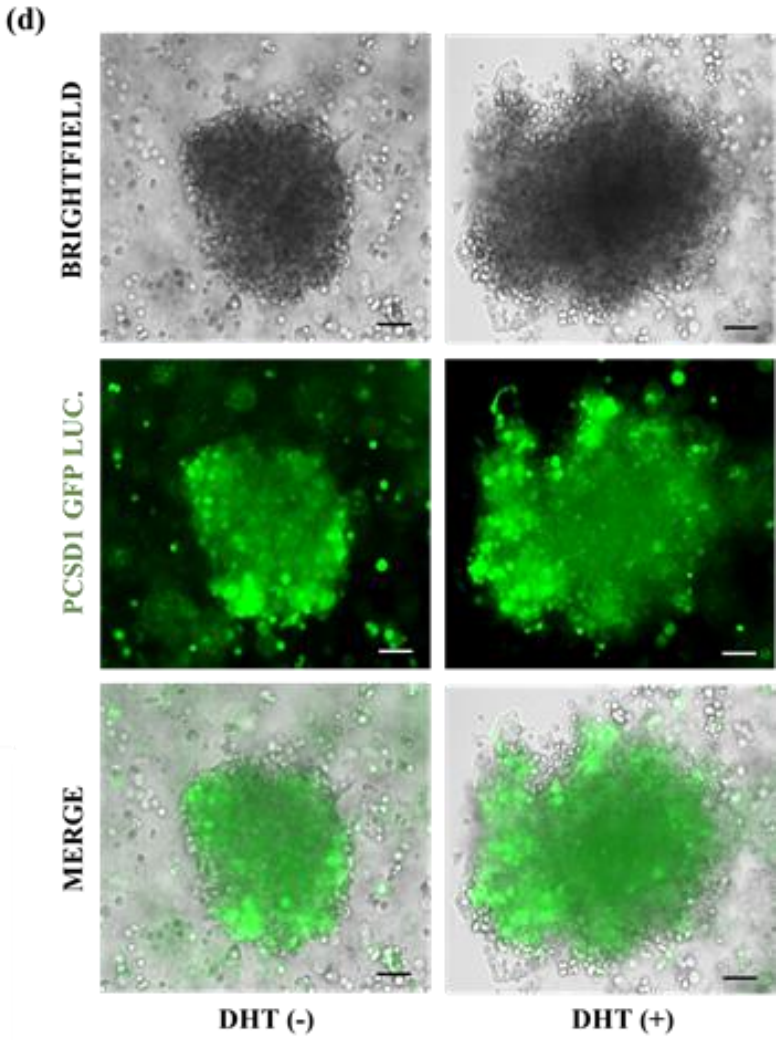

Figure 6. Treatment with DHT has no effect on tumor cell growth and viability. Continued.

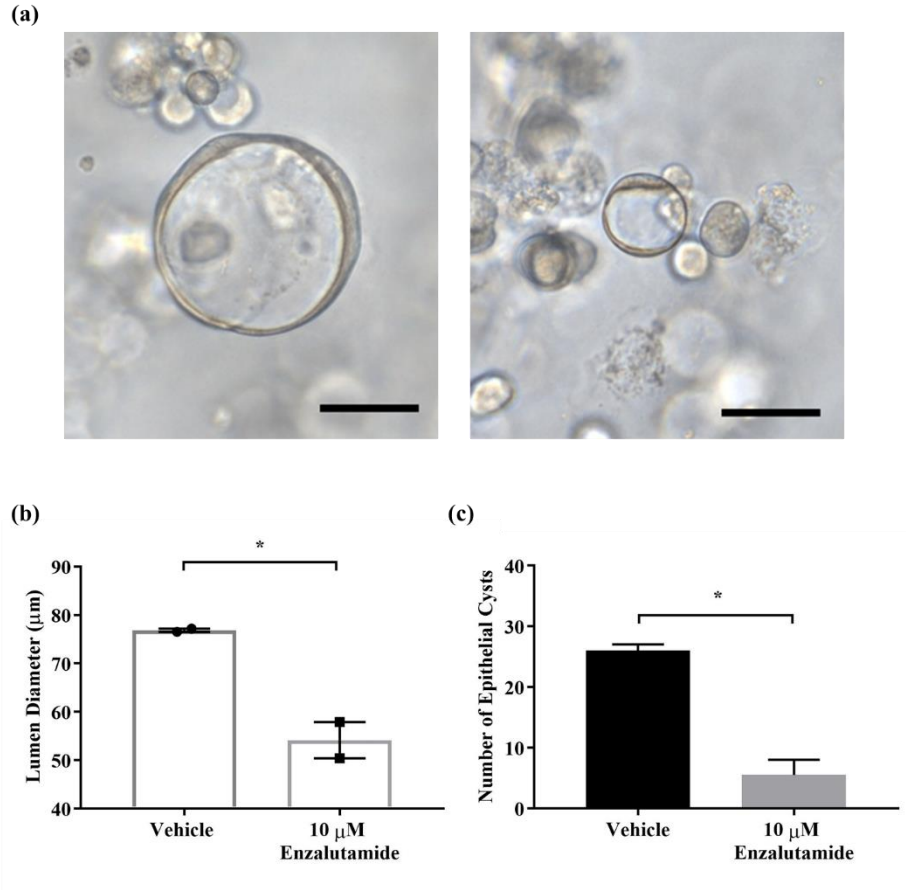

Figure 7. Treatment with 10 µM Enzalutamide decreases the lumen size and number of epithelial cysts formed in culture.

PCSD1 tumor cells were cultured with either Vehicle (0.1% DMSO) or 10 µM Enzalutamide. After four weeks in culture, (a-b) the lumen of epithelial cysts treated with 10 µM Enzalutamide was smaller compared to the epithelial cysts treated with Vehicle. (c) The number of epithelial cysts greater than 50 µm in size were counted in both treatment conditions. Treatment with 10 µM Enzalutamide decreased the number of epithelial cysts formed in culture. (a) The microscopy images are representative and were taken at an objective of 20X. Scale bars represent 50 µm. (b-c) Data represents the mean from two (n=2) independent experiments performed in triplicates ± SEM. Student's t-test was used to determine statistical significance (* indicates $P < 0.05$).

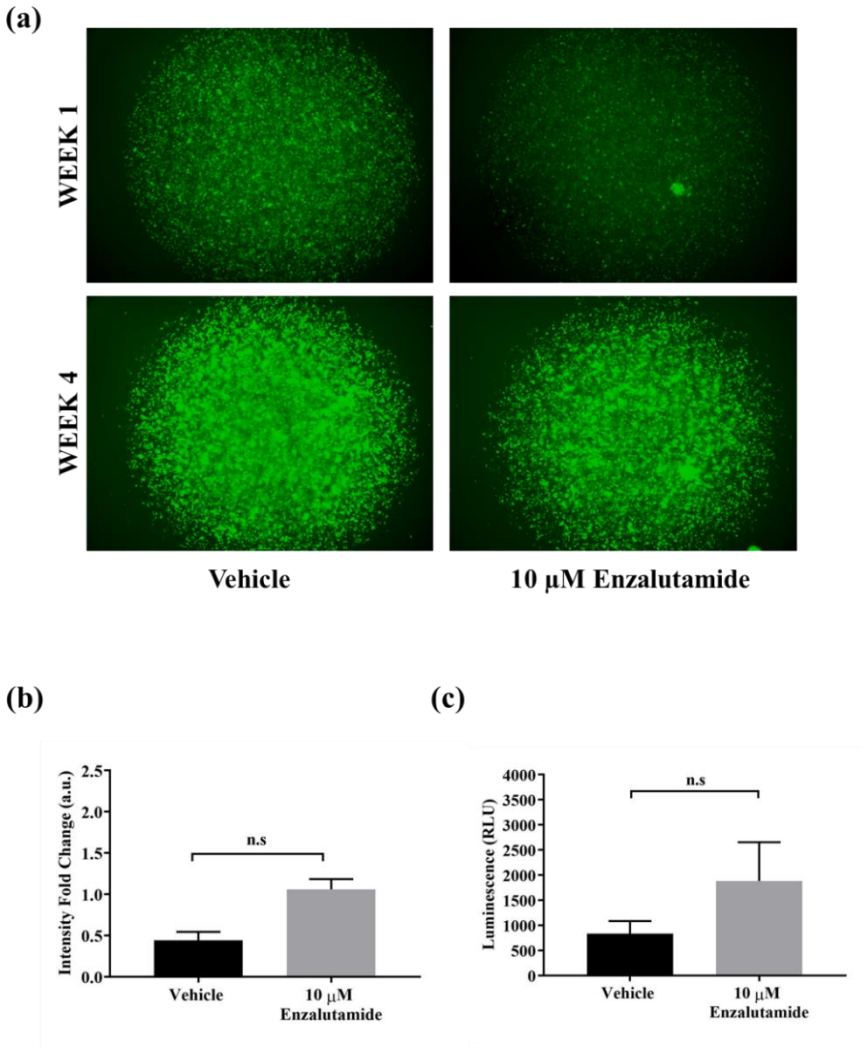

Figure 8. Treatment with 10 µM Enzalutamide has no effect on overall tumor cell growth and viability.

PCSD1 tumor cells were cultured with either Vehicle (0.1% DMSO) or 10 µM Enzalutamide. (a-b) At weeks 1 and 4, the fluorescent intensity of the cultures from each treatment condition were compared. The fold increase of fluorescence in both conditions were not different from each other, indicating that they had similar growth rates. (c) The luciferase assay was performed on the cultures during week 4. The luminescence from the cultures treated with vehicle was not different than the luminescence from the cultures that were treated with 10 µM Enzalutamide. (d) Image of spheroid formation in both treatment conditions. Scale bars represent 100 µm. The fluorescent microscopy images are representative and were taken at an objective of (a) 2X and (d) 10X. (b-c) Data represents the mean from two (n=2) independent experiments performed in triplicates ± SEM. Student's t-test was used to determine significance (n.s indicates not significant).

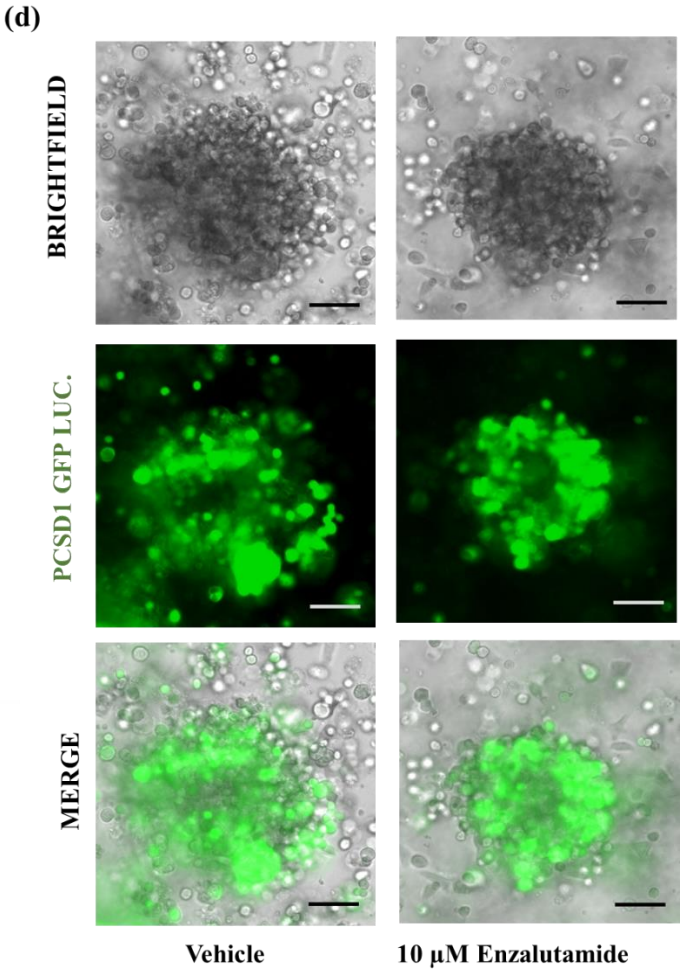

Figure 8. Treatment with 10 µM Enzalutamide has no effect on tumor cell growth and viability. Continued.

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Tables

Table 1. Characteristics of PDX and primary tumor cells used in the establishment of three-dimensional cell cultures.

Name	Tumor Cells Description	Samples Cultured	Tumor Growth Site	Morphology	Could be passaged?	Seeding Concentration (cells/µl Matrigel)	Matrigel Type
PCSD1	Localized PDX	4/4	Bone (Femur)	Single cells, cell clusters, epithelial cysts, spheroids	N	1,250	Growth Factor Reduced
PCSD13	Frozen Localized PDX	1/1	Bone (Femur)	Single cells, organoids	TBD	6,250	High Concentration
PCSD1 SAM	Metastasized PDX	1/1	Neck (N)	Single cells, epithelial cysts, organoids	Y	1,250	Growth Factor Reduced
			Abdomen (A)	Single cells, cell clusters	N	1,250	Growth Factor Reduced
PCSD (16-18)	Metastasized primary patient	1/3	Bone	Single cells, organoids	TBD	1,250	Growth Factor Reduced

Table 2. Plating conditions used for three-dimensional cell cultures.

Culture Plate	Seeding Density (cells)	Matrigel Volume (µl)	Medium (µl)
48 well	25,000 – 50,000	20	250
24 well	50,000 – 250,000	40	500
6 well	50,000 – 250,000	40	2,000

Table 3. Formulation of growth medium used for three-dimensional cultures.

Stock Component	Final Concentration	Volume (µl) for 50 ml solution	Volume (µl) for 25 ml solution
B27 (50X)	1X	1000	500
N-acetyl-L-cysteine (500 mM in H_2O)	1.25 mM	125	62.5
EGF (0.5 mg/ml in PBS + 0.1% BSA)	5 ng/ml	0.5	0.3
Noggin (100 µg/ml in PBS + 0.1% BSA)	100 ng/ml	50	25
R-Spondin 1	10% (v/v)	5000	2500
A83-01 (25 mM in DMSO)	500 nM	5	2.5
FGF10 (0.1 mg/ml in PBS + 0.1% BSA)	10 ng/ml	5	2.5
FGF2 (50 µg/ml in PBS + 0.1% BSA)	5 ng/ml	5	2.5
Prostaglandin E2 (10 mM in DMSO)	1 µM	5	2.5
Nicotinamide (1M in PBS)	10 mM	500	250
SB202190 (30 mM in DMSO)	10 µM	16.7	8.4
FBS	10% (v/v)	5000	2500
DHT (1 µM in ethanol)	1 nM	50	25
adDMEM/F12 +/+/+		Raise to 50 ml	Raise to 25 ml
Y-27632 Dihydrochloride (100 mM in H_2O)	10 µM	5	2.5

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