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Serum-free complete medium, an alternative medium to mimic androgen deprivation in human prostate cancer cell line models

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Background: Almost all men who present with advanced prostate cancer (CaP) and some men who fail therapy for clinically localized CaP are treated with androgen deprivation therapy (ADT). CaP cell lines are used to identify and characterize new agents for ADT or investigate mechanisms of ADT resistance. CaP cell lines are maintained in culture medium that contains fetal bovine serum, which contains testosterone (T). Androgen deprivation experiments are performed using media supplemented with androgen-free serum, such as charcoal stripped fetal bovine serum (CS-FBS). However, CS-FBS composition varies from batch-to-batch and variations may impact experimental reproducibility. Serum free media (SFM) may provide a better defined alternative to media supplemented with CS-FBS (CSM).

Methods: Cell growth of six human CaP cell lines was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Androgen levels were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: MTT assays showed 5 of 6 CaP cell lines grew after 6 days of culture in androgen- deprived SFM or CSM. LNCaP and VCaP growth was stimulated when cells were cultured in SFM or CSM supplemented with T. LNCaP, C4-2, LAPC-4, and VCaP cell growth was inhibited when cultured in SFM or CSM with T and bicalutamide. LC-MS/MS data showed LAPC-4 cells produced similar DHT levels when cultured in T-supplemented SFM or CSM. Dutasteride impaired T to DHT metabolism in LAPC-4.

Conclusions: Media composition contributed to growth differences observed between CaP cells cultured in SFM or CSM. However, the differences in media composition did not impair CaP cell response to T-stimulated growth, bicalutamide growth inhibition, metabolism of T, or dutasteride efficiency. SFM can be used as a better defined alternative to CSM for androgen deprivation experiments.

KEYWORDS
androgen deprivation, charcoal-stripped media, DHT, mass spectrometry, prostate cancer

1 | INTRODUCTION

Prostate cancer (CaP) growth and progression rely on transactivation of the androgen receptor (AR) by the testicular androgens, testoster-

one (T), and dihydrotestosterone (DHT). Almost all men who present with advanced CaP and some men who fail therapy for clinically localized CaP are treated with androgen deprivation therapy (ADT). The goal of ADT is to suppress circulating androgen levels and deprive

AR of T or DHT. However, ADT is not curative and CaP recurs as lethal castration-recurrent/resistant CaP (CRPC). One mechanism that contributes to resistance of CaP to ADT is intracrine metabolism, the conversion of weak adrenal androgens, dehydroepiandrosterone (DHEA) or androstenedione (ASD) to T or DHT.[1,2] Anti-androgens, such as enzalutamide, or anti-androgen metabolism drugs, such as the CYP17 inhibitor, abiraterone acetate extend patient survival by only several months.[3,4] Identification, development, and characterization of new therapeutic agents to inhibit CaP growth or recurrence using various forms of ADT rely on using combinations of in vitro and in vivo models.

In vitro models include human CaP cell lines, such as the androgen-sensitive LAPC-4[5] and LNCaP, and the androgen-independent LNCaP derivative, LNCaP-C4-2 (C4-2).[6] Different cell lines require different culture conditions. However, one common component used for cell culture maintenance is fetal bovine serum (FBS). Culture media typically contain 10% FBS, which yields T concentrations 0.03-0.097 nM T,[7] but serum composition varies from batch-to-batch.[8] One factor that contributes to FBS variation is that commercially available FBS compositions vary due to differences in genetic background and husbandry of cattle. Charcoal or charcoal and dextran are used to remove androgens and estrogens from FBS to produce charcoal-stripped FBS (CS-FBS), which is added commonly to growth media to produce androgen-free media. However, the stripping process removes molecules necessary for cell growth and metabolism. The stripping process is inconsistent and CS-FBS composition varies from batch-to-batch.[9] One alternative to using CS-FBS containing media for androgen deprivation is androgen-free defined media, such as serum-free media (SFM). The goal of these in vitro studies was to compare CaP cell growth and androgen metabolism in control media versus media altered to achieve androgen deprivation.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Human CaP lines, LAPC-4,[10] LNCaP-RPCI (LNCaP),[5,11] PC-3 (acquired from ATCC), and C4-2[6,12] were cultured in RPMI 1640 (Corning, Corning, NY). VCaP (acquired from ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning). RPMI and DMEM were supplemented with 10% FBS (Corning) and 2 mM glutamine (Corning). CWR-R1[13] was cultured in Richter's Improved media (Corning) supplemented with 2% FBS, 10 mM nicotinamide, 20 ng/mL epidermal growth factor, 5 µg/mL Insulin/transferrin/selenium (ITS) and 5 µg/mL linoleic acid. Media that contained FBS were considered control media (CM). SFM (Serum Free Complete Media, Corning) contains bovine serum albumin (1 g/L) and L-glutamine and other proprietary additives. Charcoal-stripped media (CSM) were identical to CM except that FBS was substituted with CS-FBS. CS-FBS was not purchased due to concerns for batch-to-batch variability. Instead, CS-FBS was generated in our laboratory from FBS that was incubated twice for 1 h with activated charcoal (Avantor, Center Valley, PA) and dextran (Amresco, Solon, OH) and sterilized using 0.2 µm filter (ThermoFisher Scientific,

Waltham, MA). Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) analysis confirmed CS-FBS androgen levels were below the limit of quantitation (BLQ). Cells were harvested using trypsin 0.05% (Corning). Dulbecco's phosphate buffered saline (PBS; Corning) was used for all cell washes.

All cell lines were authenticated using genomic profiling in the Genomics Shared Resource. DNA profiles were acquired using 15 short tandem repeat (STR) loci and an amelogenin gender-specific marker. Test and control samples were amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA) using the Verti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA) in 9600 Emulation Mode (initial denature: 95°C 11 min, 28 cycles of denature: 94°C 20 s and anneal/extend: 59°C 3 min, final extension: 60°C 10 min and hold: 12°C). PCR products were evaluated using the 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper v4.0 (Applied Biosystems). Eight of the 15 STRs and amelogenin from the DNA profile for the cell lines were compared to the ATCC STR database (https://www.atcc.org/STR%20Database.aspx?slp = 1) and the DSMZ combined Online STR Matching Analysis (http://www.dsmz.de/fp/cgi-bin/str.html). All matches >80% were considered the same lineage.

2.2 | Cell viability

A total of 1.0×10^4 LNCaP or LAPC-4 cells or 2.5×10^4 VCaP or 1.0×10^4 C4-2, CWR-R1 or PC-3 cells were plated per well in 24-well plates (Corning) and cultured in cell line-specific growth media for 24 h. All cell lines were cultured with CM, SFM or CSM alone or CM, SFM or CSM supplemented with 1 nM T (Steraloids, Newport, RI). Media were removed on day 0 (control) or 6 and 1 mg/mL 3-(4,5-dimethylthiazol-2-yl) methylthiazol-2-yl)-2,5-diphyltetrazolim bromide (MTT, Amresco) in fresh, respective media was added using 200 µL per well and incubated at 37°C for 4 h. After 4 h, equal volumes of 20% (w/v) SDS in double distilled H_2O (Quality Biological, Gathersburg, MD) were added to wells and plates were incubated at 37°C overnight. Plates were read at 590 nm using a Tecan F200 Series (Tecan Group Ltd., Mannedorf, Switzerland) and Magellian 7.2 software (Tecan Group Ltd.). Data were expressed as percent growth. Percent was calculated by subtracting the mean Day 6 OD590 from the mean Day 0 OD590 and dividing by Day 0 D590 and multipling by 100. OD590 data were reported in Supplemental Figures S1-5. Experiments were performed in triplicate.

2.3 | LC-MS/MS

LAPC-4 cells were plated, 1×10^5 cells per well into six-well plates for 6 days and 24 h experiments (two six-well plates were combined to generate one sample). LAPC-4 cells used for 24 h studies were cultured in CM, SFM or CSM alone, or CM, SFM or CSM supplemented with 1 nM T. LAPC-4 cells used for 24 h experiments were cultured in CM, SFM or CSM alone or CM, SFM or CSM supplemented with 1 nM T or 1 µM dutasteride (SelleckChem, Houston, TX) separately or in combination. Media (total 24 mL from 2 6-well plates) were collected

in 50 mL conical tubes (Corning) and cells were trypsinized and combined in 15 mL conical tubes (Corning). Cells were washed three times using PBS and centrifuged at 1200 RPM for 5 min. Cells were suspended after the last wash in 1 mL PBS and 50 μL of cell suspension were reserved to determine protein concentration using a BioRad Protein Determination Kit (BioRad Hercules, CA) and for Western blot analysis. The remaining 950 μL of cell suspension were centrifuged at 1200 RPM for 5 min, PBS was removed, and cell pellets were stored at −80°C until analyzed for androgens.

Cell pellets and media were analyzed over five runs using a modification of a validated LC-MS/MS assay[14] for five androgens, which included T, DHT, DHEA, ASD, and androsterone (AND). All androgens including the two internal standards, d_3-T and d_3-DHT, were obtained from Steraloids. Dutasteride and dutasteride-$^{13}C_6$ were obtained from Toronto Research Chemicals (Toronto, ON, Canada). The modified assay used aqueous-based spiked calibration standards to document calibration ranges and confirm quality control (Supplemental Table S1). Androgen or dutasteride quality control samples were prepared in charcoal-stripped human postmenopausal female plasma (Bioreclamation, LLC, Westbury, NY). Calibration ranges, QC concentrations and overall performance of the assay were listed in Supplemental Table S1. Cell pellet samples were resuspended to total volume 1 mL in water and vortexed or sonicated to obtain a homogeneous suspension.

Sample extraction involved mixing 250 μL calibrator, quality control, plasma blank, or study sample (cell pellet or media) with 750 μL HPLC-grade water, 100 μL internal standard (IS) solution (75.0/ 225 pg/mL d_3-T/d_3-DHT and 3.0 ng/mL $^{13}C_6$-dutasteride in 75% methanol) and 4.0 mL methyl-tert-butyl ether (MTBE, Omnisolve®, EMD Milipore, Billerica, MA) in 13 × 100 mm glass screw-top tubes. Androgens were analyzed in undiluted samples and dutasteride was analyzed twice, undiluted and diluted, to ensure concentrations were within range of each standard curve. Tubes were capped with teflon-lined caps, vortexed, rotated 15 min and centrifuged (Sorvall, model RT6000B, Thermo Fisher Scientific, Grand Island, NY) at 2800 rpm and 4°C for 15-30 min to separate liquid phases. The aqueous phase was frozen in dry ice/acetone bath and the MTBE layer was poured into a clean glass 13 × 100 mm conical tube and evaporated at 37°C under nitrogen. The residue was reconstituted with 100 μL 60% methanol. Suspensions were centrifuged (Heraeus Multifuge X3R, Thermo Fisher Scientific) at 2800 rpm at 4°C for 5 min to separate insoluble materials and approximately 13 μL of supernatant were injected for analysis. LC-MS/MS analyses of the extracted samples were performed using a Prominence UFLC System (Shimadzu Scientific Instruments, Kyoto, Japan), a QTRAP® 5500 mass spectrometer (AB Sciex, Framingham, MA), with an electrospray ionization source, and 2 10-port switching valves (Valco instruments Co. Inc., Houston, TX, model EPC10W).

Analytes were detected using multiple reaction monitoring (MRM) in positive ion mode controlled by AB SCIEX Analyst® software, version 1.6.2. Mass spectrometer conditions were ion spray voltage 5250 volts, turbo gas temperature 700°C, gas 1 = 65, gas 2 = 60, curtain gas = 20, collision-associated dissociation (CAD) gas medium and unit mass resolution for Q1 and Q3. Nitrogen was used for all gases

and voltages for maximum parent/fragment ion pair intensities were optimized using direct infusion and flow injection analysis. Calibration curves were generated using analyte/IS area response ratios versus nominal concentrations (ng/mL) and weighted linear regressions with weighting factor 1/concentration^2. The IS used for T, ASD and DHEA was d_3-T and d_3-DHT was used for DHT and AND. The IS used for dutasteride was $^{13}C_6$-dutasteride. Back-calculated concentrations were generated using the formula $x = (y−b)/m$, where x is the back-calculated concentration, y is the analyte/IS peak area ratio, b is the y-intercept, and m is the slope. Calibrator and quality control acceptance criteria required all acceptable concentrations to have accuracy deviations ≤15% from the nominal concentration, with relative standard deviations (% RSD) ≤15%, except at the lower limit of quantitation (LLOQ), which was allowed ≤20% deviation for both parameters. Values that were BLQ were treated as 0.0 ng/mL.

Analyte concentrations were represented as pmoles/mg protein. Cell lysates measured using LC-MS/MS (ng/mL) were multiplied by the total volume of lysate (1 mL), divided by 0.95 (to account for 50 μL removed for protein determination and Western blot), normalized by the total protein of the cell pellet (mg), divided by the molecular weight of androgen (ng/nmole) and converted to pmoles. Media androgen concentrations (ng/mL) were multiplied by the total volume of media (24 mL), normalized against total protein of the cell culture (mg), divided by the molecular weight of the androgen (ng/nmole) and converted to pmoles. Cell pellet and media androgen concentrations were reported as combined concentrations in the Results section and separately in Supplemental Figures S4 and S5. Experiments were performed in triplicate.

2.4 | Western blotting

Western blots analyses were performed on all three biological replicates generated for LC-MS/MS analyses. Cells removed from −80°C storage were re-suspended in ubiquitin extraction lysis buffer (150 mM NaCl, 50 mM Tris-HCL, pH7.4, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate [all reagents were purchased from Fisher, Pittsburgh, PA]) and 0.1% SDS (Quality Biological). Halt Protease Inhibitor Cocktail (Sigma, St. Louis, MO) was added to ubiquitin lysis buffer just before cells were lysed. Cells were freeze-thawed three times and centrifuged at 14 000g for 15 min. Supernatants were transferred to clean microfuge tubes. Protein was quantified using the Protein Determination Kit (BioRad) and analyzed in flat bottom 96-well plates using an EL800 University Microplate Reader (BioTek Instruments) and KC Junior software (Bio-Tek Instruments). SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 4-15% Mini Trans-Blot cell (BioRad) and protein was transferred to Immuno-Blot PVDF Membrane for Protein Blot (BioRad) and blocked in 5% milk in Tris-buffered saline with Tween 20 (TBST, Amersham Bioscience, GE Healthcare Bio-Sciences, Pittsburgh, PA) for 30 min.

Membranes were incubated with AR targeted antibody (BD Pharmagen, N-terminal targeted AR antibody [BD Biosciences, San Jose, CA]) at 1:1000 overnight at room temperature. After incubation, blots were washed three times with TBST (Amersham Bioscience) for

10 min each. Washed blots were incubated with goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:1000 for 1 h at room temperature. Blots were washed with TBST three times for 10 min each and protein expression was visualized using Pierce ECL Western Blotting Substrate (Life Technologies, Carlsbad, CA). Immunoblots were washed with TBST, blocked in 5% milk in TBST, re-probed for tubulin (1:1000 for 1 h at room temperature [Abcam, Cambridge, MA]) and incubated with goat anti-rabbit secondary antibody (1:1000 for 1 h at room temperature [Jackson ImmunoResearch Laboratories]). The western image was quantified using ImageStudio Lite (Li-Cor, Lincoln, NE) and data were expressed as relative AR/Tubulin expression.

2.5 | Statistical analysis

ASD, T, DHT, and dutasteride levels in LAPC-4 cell pellets and media were modeled as a function of treatment (CM, SFM, CSM +/− 1 nM T, +/− 1 μM dutasteride, or +/− 1 nM T and 1 μM dutasteride) using a general linear model (GLM). Cell growth (MTT data) was modeled for each cell line as a function of treatment (CM, SFM, CSM +/− 1 nM T or +/− 10 μM bicalutamide (B), treatment duration (day 0 or day 6) and their interaction using a GLM. Mean differences of interest were evaluated using Holm-Bonferroni adjusted F-tests about the appropriate linear contrasts of model estimates. All model assumptions were verified graphically using quantile-quantile and residual plots, and transformations (ie, Box-Cox) were applied as appropriate. All analyses were conducted using SAS v9.4 (Cary, NC) and nominal significance level 0.05. Statistical results were listed in Supplemental Tables 2–6.

3 | RESULTS

3.1 | CaP cell growth persisted when cultured in SFM or CSM media for 6 days

Six commonly used CaP cell lines were cultured in CM or were androgen deprived using SFM or CSM for 6 days and cell growth was measured. The 6 day time point was chosen because previous reports showed CaP cell growth slows or CaP cells undergo apoptosis 6 days after androgen deprivation.[15,16] All CaP cell lines grew (all P-values <0.05 that compared Day 6 to Day 0) after 6 days of culture in CM, SFM, or CSM (Figure 1C; Supplemental Figures S1A and S1B and Supplemental Table S2). All cell lines, except LAPC-4 cells cultured in CSM or PC-3 cells, grew slower in SFM or CSM compared to CM (all P-values <0.05). Only LNCaP and VCaP cell growth was slowed during androgen deprivation using either SFM or CSM (all P-values <0.05). However, LNCaP, VCaP, and PC-3 cells grew slower when cultured for 6 days in SFM compared to CSM (all P-values <0.05; Supplemental Table S2).

3.2 | T stimulated LNCaP and VCaP growth in SFM or CSM

Since LNCaP and VCaP cell growth differed in CM, SFM, or CSM, media were supplemented with T to determine if the differences were

abrogated. PC-3 cells were not analyzed because PC-3 cells lack AR and do not respond to T. LNCaP growth was stimulated after treatment with SFM with T (all P-values <0.05), but not CSM with T (Figures 2A and 2B; Supplemental Figure S2 A and B and Supplemental Table S3). LNCaP cells grew slower when cultured in SFM alone or SFM with T (all P-values <0.05) compared to LNCaP in CSM or CSM with T. VCaP cell growth was stimulated when cells were cultured in CM or SFM with T, but not in CSM with T, compared to VCaP cultured in CM or SFM without T (Figures 2E and 2F; Supplemental Figure S2 E, F and Supplemental Table S3). T-stimulated growth differed among VCaP cells cultured in CM, SFM or CSM with T, which suggests media composition impacted VCaP growth. Growth differences were not observed among LAPC-4 or C4-2 cells cultured in CM, SFM, or CSM alone or with T (Figures 2C, 2D, 2G, and 2H; Supplemental Figures 2C, 2D, 2G, and 2H and Supplemental Table S3).

3.3 | Bicalutamide treatment impaired T-stimulated CaP cell growth in SFM or CSM

MTT experiments were performed using LNCaP, LAPC-4, VCaP, and C4-2 cultured in SFM or CSM with T alone, bicalutamide alone or T and bicalutamide to confirm that cell growth observed after T treatment (Figure 2) was due to T-stimulated AR activation. All CaP cells grew after 6 days (all P-values <0.05; Supplemental Table S4), except for LNCaP cells cultured in SFM with bicalutimide, or CSM with bicalutamide (Figure 3A-H; Supplemental Figure 3A-H and Supplemental Table S4). T treatment stimulated cell growth in LNCaP and VCaP in both SFM and CSM, LAPC-4 cell growth in CSM, and C4-2 cell growth in SFM. Bicalutamide treatment stunted LNCaP (Figures 3A and 3B), LAPC-4 (Figure 3C and 3D; CSM with bicalutamide only), VCaP (Figures 3E and 3F) and C4-2 (Figures 3G and 3H). T-stimulated growth (all P-values <0.05; Supplemental Table S4). T-stimulated LNCaP or LAPC-4 growth responses were different when LNCaP or LAPC-4 cells were cultured in SFM with T or CSM with T (P-values <0.05). Bicalutamide treatment produced similar responses among LNCaP, LAPC-4, or VCaP treated with SFM with bicalutamide or CSM with bicalutamide, but not C4-2 cell cells treated with SFM with bicalutamide or CSM with bicalutamide (Figure 3A-H; Supplemental Figure 3A-H and Supplemental Table S4).

3.4 | Androgen levels were similar in LAPC-4 cells cultured for 6 days in SFM or CSM

MTT assays revealed that CaP cells produced different growth responses when cultured in SFM or CSM. Therefore, LC-MS/MS was performed to determine if media composition impacted T metabolism. LAPC-4 cells were used because LAPC-4 cells express SRD5A enzymes that metabolize T to DHT (Figure 4A, modified from [17]), unlike LNCaP or C4-2 cells, which cannot metabolize T to DHT.[5] LC-MS/MS revealed that LAPC-4 cells cultured for 6 days in CM alone produced higher levels of DHT (all P-values <0.05; Supplemental Table S5) than LAPC-4 cells cultured for 6 days in SFM or CSM alone (Figures 4B and 4C, and Supplemental Table S5). DHT levels were
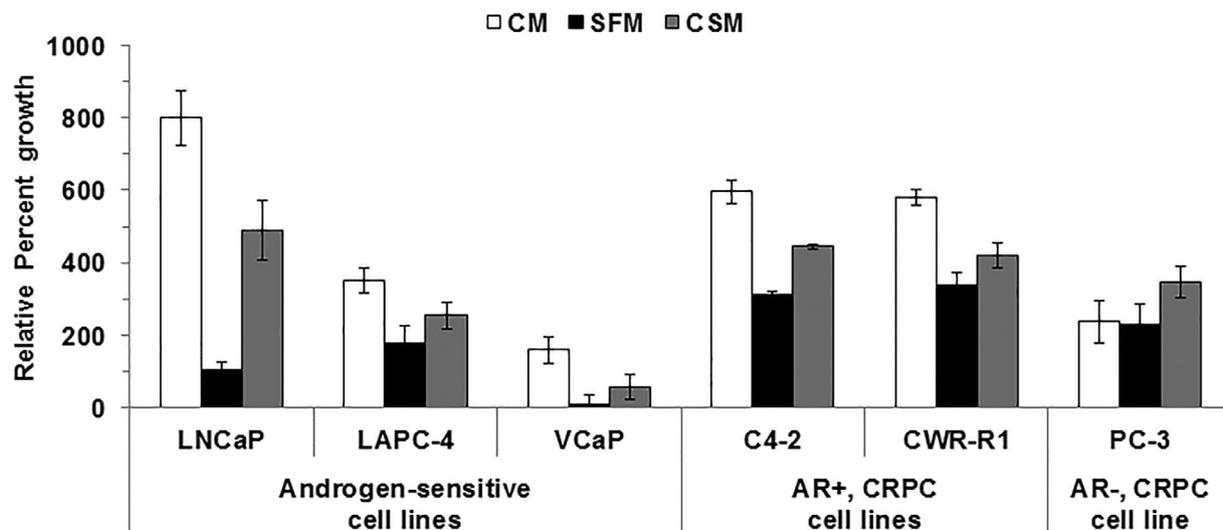
FIGURE 1 Androgen deprivation using SFM or CSM slowed LNCaP and VCaP cell growth. CaP cells were cultured in CM, SFM, or CSM. Cell growth was measured on days 0 and 6 using MTT assay (OD590). Cell growth was expressed as percent growth. Data were presented as mean +/− SEM. P-values for statistical comparisons among CaP cells cultured in CM, SFM, or CSM were listed in Supplemental Table S2. P-values for statistical comparisons between Day 0 OD590 and Day 6 OD590 were listed in Supplemental Table S2

higher (all P-values <0.05) in LAPC-4 cells cultured with CM with T compared to LAPC-4 cells cultured with SFM with T or CSM with T (Figure 4C and Supplemental Table S5). LAPC-4 cells cultured in SFM alone or CSM alone produced similar T or DHT levels (all P-values >0.05). LAPC-4 cells cultured for 6 days in SFM alone or CSM alone or SFM with T or CSM with T produced similar T or DHT levels (Figures 4B and 4C and Supplemental Table S5), which suggested media composition did not affect LAPC-4 cell metabolism of T to DHT. The data suggested various nutrients or molecules present in CM and not in SFM or CSM facilitated metabolism of T to DHT.

ASD levels were measurable when LAPC-4 cells were cultured for 6 days with CM with T, SFM with T, or CSM with T, but not when cells

were cultured with CM, SFM, or CSM alone (Figure 4D and Supplemental Table S5). The data suggested LAPC-4 cells metabolized T to ASD when T or DHT levels were sufficient to activate AR.

3.5 | LAPC-4 cell T metabolism to DHT or dutasteride activity did not differ between LAPC-4 cells cultured with SFM or CSM media for 24 h

LC-MS/MS was used to assess acute changes in LAPC-4 cell androgen levels after LAPC-4 cells were cultured for 24 h in CM, SFM or CSM with T, dutasteride or T and dutasteride. Dutasteride, a dual 5α-reductase inhibitor, was used to determine if SRD5A enzymes contributed to
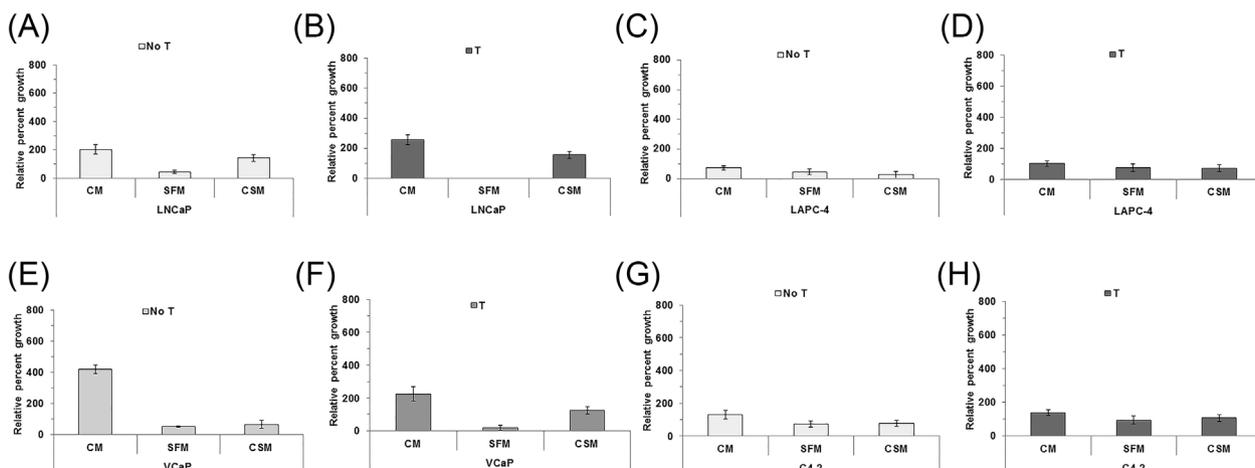

FIGURE 2 SFM or CSM supplemented with T stimulated LNCaP and VCaP cell growth. LNCaP (A and B), LAPC-4 (C and D), VCaP (E and F) or C4-2 (H and I) cells were cultured in CM, SFM, or CSM alone or CM, SFM, or CSM with 1 nM T. Cell growth was measured on days 0 and 6 using MTT assay (OD590). Data were presented as mean +/− SEM. P-values for statistical comparisons of cell growth between Day 0 and Day 6 were listed in Supplemental Table S3. Comparisons among CaP cells cultured in CM, SFM, or CSM alone or CM with T, SFM with T, or CSM with T were listed in Supplemental Table S3
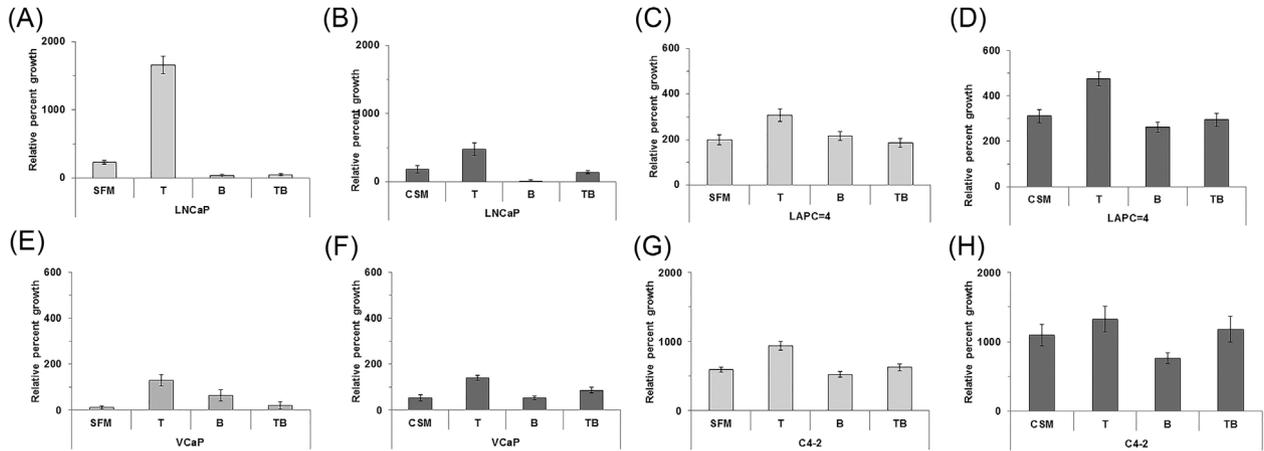
FIGURE 3 Bicalutamide treatment using SFM or CSM blunted T-stimulated growth in LNCaP, LAPC-4, VCaP, or C4-2 cells. LNCaP (A and B), LAPC-4 (C and D), VCaP (E and F), or C4-2 (H and I) cells were cultured in SFM or CSM alone, or SFM or CSM with 1 nM T, SFM, or CSM with 10 μM bicalutamide (B) or SFM or CSM with 1 nM T and 10 μM B. Cell growth was measured on days 0 and 6 using MTT assay read at OD590. Data were presented as mean +/− SEM. P-values for statistical comparisons of cell growth between Day 0 and Day 6 were listed in Supplemental Table S4. Cell growth comparisons among T and B and SFM versus CSM treatments were listed in Supplemental Table S4

intracellular DHT production. LAPC-4 cell T or DHT levels increased when CM, SFM, or CSM were supplemented with T (Figures 5A and 5B; note different Y-axis and Supplemental Table S6). These data were consistent with reports that show LAPC-4 cells 5α-reduce T to DHT.[5] DHT levels were lower (all P-values <0.05) when LAPC-4 cells were treated with CM,

SFM, or CSM supplemented with T and dutasteride (Figure 5B and Supplemental Table S6) compared to LAPC-4 cells cultured with CM, SFM, or CSM with T alone. These data suggested dutasteride inhibited SRD5A conversion of T to DHT. The data provide further evidence to show SRD5A isozymes were involved in T to DHT conversion. ASD levels
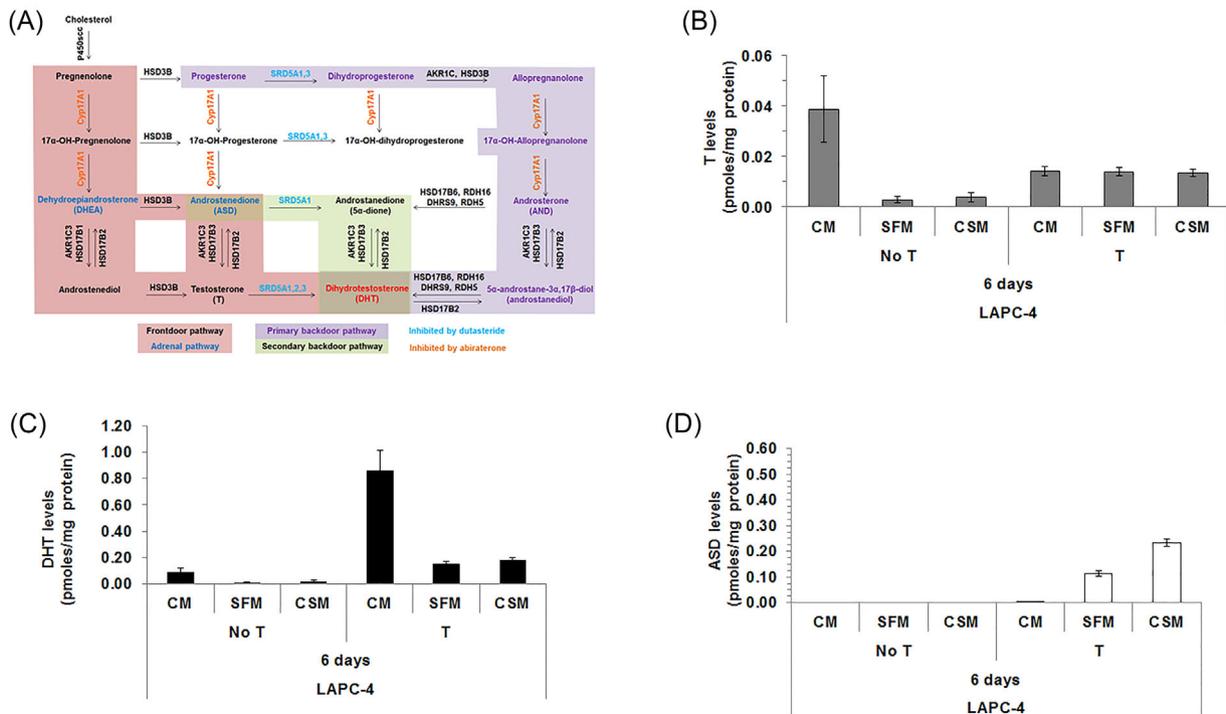

FIGURE 4 Androgen levels were similar among LAPC-4 cells cultured 6 days in SFM or CSM. Androgen metabolism pathways for DHT synthesis (A). LAPC-4 cells were analyzed using LC-MS/MS to measure T (B), DHT (C), and ASD (D) levels after LAPC-4 cells were cultured for 6 days in CM, SFM, or CSM alone or cultured in CM, SFM, or CSM with 1 nM T. Cell pellet and media androgen levels were combined. Cell pellet and media androgen levels were shown in Supplemental Figure S1. Data were presented as mean pmoles/mg protein +/− SEM. P-values for statistical comparisons among LAPC-4 cells treated with or without T were listed in Supplemental Table S5
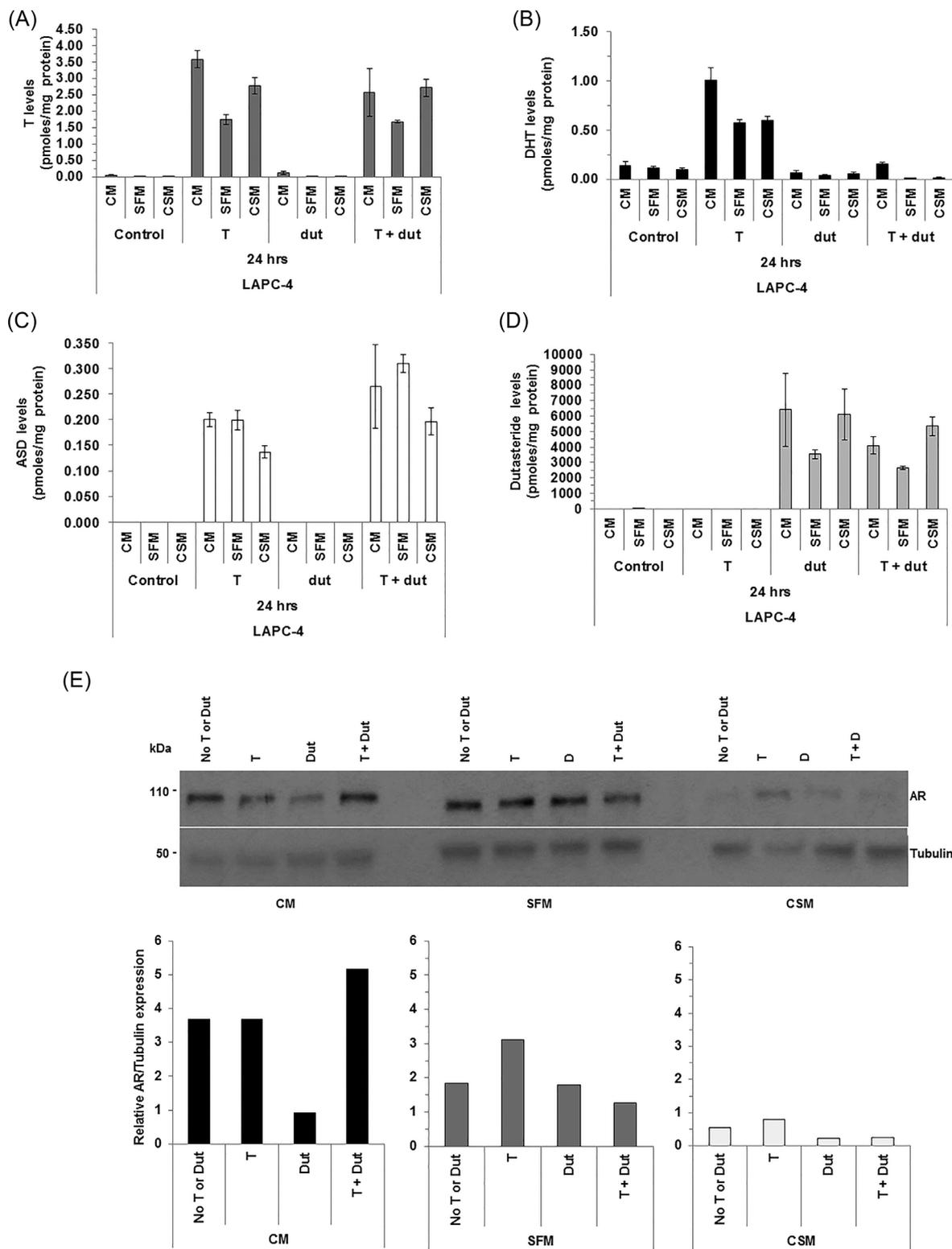
FIGURE 5 Metabolism of T to DHT was impaired in LAPC-4 cultured with dutasteride. LAPC-4 cells were analyzed using LC-MS/MS to measure T (A), DHT (B). ASD (C), and dutasteride (dut; panel D) levels after LAPC-4 cells were cultured for 24 h in CM, SFM, or CSM alone or cultured in CM, SFM, or CSM with 1 nM T or CM, SFM, or CSM with 1 µM Dut or CM, SFM, or CSM with 1 nM T and 1 µM Dut. Cell pellet and media androgen levels were combined. AR protein expression was measured using western blot and quantitated using densitometry (E). Cell pellet and media androgen levels were shown in Supplemental Figure S2. Data were presented as mean pmoles/mg protein +/− SEM. *P*-values for statistical comparisons among LAPC-4 cells treated with T, dut, or T and dut combined were listed in Supplemental Table S6

were measurable when LAPC-4 cells were cultured with CM, SFM, or CSM supplemented with T. LAPC-4 ASD levels were higher (all P-values <0.05; Supplemental Table S6) after treatment with CM, SFM, or CSM with T and dutasteride treatment compared to LAPC-4 cells cultured with CM, SFM, or CSM supplemented with T (Figure 5C and Supplemental Table S6). Dutasteride levels were similar among all LAPC-4 cells treated with dutasteride alone or T and dutasteride (Figure 5D and Supplemental Table S6).

Quantitative Western blot analysis revealed AR expression levels were lower in LAPC-4 cells treated with CM with dutasteride compared to CM alone, or CM with T (Figure 5E). AR expression levels appeared to recover when LAPC-4 cells were treated with T and dutasteride. AR expression levels were lower in LAPC-4 cells treated with CSM alone, or CSM with T, dutasteride, or T and dutasteride compared to SFM alone, or SFM with T, dutasteride, or T and dutasteride. However, AR expression levels appeared to increase when LAPC-4 cells were treated with SFM with T or CSM with T.

4 | DISCUSSION

The goal of the study was to determine if SFM could be used as an alternative to CSM to androgen deprive CaP cells. MTT assays revealed that media composition affected CaP cell growth, but did not impair CaP cell response to androgen deprivation, T treatment or bicatalumide activity. T metabolism to DHT was similar between LAPC-4 cells cultured in SFM or CSM and dutasteride activity was not affected by differences in SFM or CSM media composition.

CaP cell lines are used to study ADT resistance and androgen metabolism, and to characterize enzymatic pathways or properties because clinical specimens are difficult to acquire. Cell lines are readily available, easy to manipulate, and easy to used for molecular or biochemical approaches to address various hypotheses. However, characterization and identification of the most suitable androgen-free tissue culture media is important for proper use of various CaP cell line models and interpretation of results. These studies showed that SFM, a defined androgen-free media with a consistent formula, can be used as an alternative to CSM to perform androgen deprivation experiments. SFM use avoids numerous potential confounders associated with CSM that are difficult to identify and control.

The LC-MS/MS method used to analyze LAPC-4 cell pellets and media measured five adrenal and testicular androgens, but did not include all potential metabolites and androgen-conjugates (glucuronides or sulfated metabolites). Therefore, DHT, T, or ASD may have been metabolized further, which may account for some of the differences among treatment groups and 6 day or 24 h experiments. Dutasteride metabolism was not measured using this LC-MS/MS method; the metabolites generated from dutasteride metabolism may account for some of the observed changes in dutasteride levels.

The data showed LAPC-4 cells produced similar DHT levels after 6 days or 24 h of culture in SFM or CSM supplemented with T. However, overall androgen levels differed between short and long-term experiments, which suggested treatment duration may impact androgen metabolism. Androgen metabolism may reach steady state as CaP cells adjust to changes in media androgen levels, which suggests treatment time should be considered carefully for androgen deprivation or androgen metabolism experiments. The studies suggest CaP cells should be pre-cultured in media used for androgen deprivation experiments that contain T or DHT. CaP cell cultures propagated in SFM that contains androgen may provide a more reliable model because data generated using an adapted CaP cell line will not be influenced by acute changes that occur as CaP cells adapt to media composition changes.

5 | CONCLUSIONS

SFM may be used as an alternative media to CSM for androgen deprivation or androgen metabolism studies. Variability in media composition could impact baseline CaP cell line growth and androgen metabolism, and response to androgen deprivation. Time points for androgen deprivation or androgen metabolism studies should be considered carefully because CaP cell lines adjust to androgen deprivation by 6 days in culture in androgen-deprived media.

CONFLICTS OF INTEREST

This manuscript is an original unpublished work that was completed by all listed authors and authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.