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14. ABSTRACT The purpose of this DOD NIA is to develop a novel method to detect circulating tumor cells (CTCs) from men with metastatic castration resistant prostate cancer (CRPC) based on a range of CTC phenotypes. The novel method employs a nanoparticle polymersome that contains near-infrared emissive porphyrins and permits antibody conjugation for target engagement and flow sorting in the infrared spectrum for specificity. We are developing near infrared emissive polymersomes (NIR-EPs) that contain antibodies to EpCAM, N-cadherin, OB-cadherin, and PSMA which permit the isolation and enumeration ev vivo of CTCs bearing these antigens in the circulation of men with CRPC. These specialized CTC detection nanoparticles permit the isolation of specific CTC phenotypes including epithelial, mesenchymal, and prostate cancer specific targets. In year 3 we have continued to optimize the methodology and chemistry for the creation of these NIR-EPs, including chemical synthesis, antibody conjugation, optimal reagents and processing, positive and negative control cell applications, and methods for red cell lysis, leukocyte depletion, and flow sorting of CTCs in the infrared spectrum. Challenges have included specificity due to antibody affinity during conjugation. This work in the Therien laboratory provides a potential clinical grade reagent for the ex vivo capture and identification of CTCs without binding to leukocytes nonspecifically.					
15. SUBJECT TERMS Circulating tumor cells, polymersomes, antibody conjugation, EpCAM, near-infrared, prostate cancer, metastasis, nanoparticle, epithelial plasticity, epithelial to mesenchymal transition, lethal phenotype					
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1. INTRODUCTION:

In 2013, over 80 U.S. men will die every day from metastatic prostate cancer (PC).¹ Many deaths could potentially be prevented or delayed through identification and treatment directed at high risk disease prior to the development of metastases. Currently, clinical/pathologic measures (i.e. PSA, stage, grade) provide little biologic insight into the process by which PC cells metastasize and become lethal. The measurement of circulating tumor cells (CTCs) in men with PC represents one biomarker with prognostic and predictive implications.² Many patients with metastatic PC, however, have undetectable CTCs, limiting clinical utility. We have identified epithelial-mesenchymal transitions (EMT) in experimental models of PC in which the cellular phenotype undergoes reversible (plastic) changes from an epithelial to a mesenchymal nature facilitating metastatic spread, followed by epithelial reversion in the target metastatic organ.³ While in the active process of metastasis, CTCs may possess a mesenchymal/plastic phenotype, and thus may not be captured by existing epithelial-based CTC technologies. We have also developed a novel CTC capture method, termed the near-infrared emissive polymersome (NIR-EP) which permits antibody conjugation to this light-emissive nanoparticle for tumor-specific binding and sorting from normal blood cells. In this DOD IDA/NIA 2014-2015 final report, we provide an update on our progress to develop NIR-EPs capable of binding prostate cancer cells with a range of phenotypes, to distinguish these cells from normal leukocytes, to isolate these cells using flow sorting based on near-infrared emission spectra, and to customize these nanoparticles based on the target cancer protein of interest. In Year 3 we have reevaluated our protocols for the fabrication of antibody-conjugated NIR-EPs, to optimize performance characteristics for NIR-EPs against N-cadherin, O-cadherin and PSMA for the isolation of cells that have lost EpCAM expression. These efforts have delayed testing of these NIR-EPs in healthy volunteers and men with metastatic castration resistant prostate cancer to provide proof of principle that these NIR-EPs provide similar or greater isolation of CTCs as compared with conventional ferrofluid-based assays such as the Veridex Cellsearch test. However, we are now confident that with our optimized protocols, we will be able to rapidly and reproducibly be able to generate these materials to provide insight into metastasis biology in PC and lead to the identification of relevant targets for therapies directed against this lethal metastatic process.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).* Circulating tumor cells, polymersomes, antibody conjugation, EpCAM, near-infrared, prostate cancer, metastasis, nanoparticle, epithelial plasticity, epithelial to mesenchymal transition, lethal phenotype

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

3.1 What were the major goals of the project?

Task 1: To develop and optimize a novel polymersome-based CTC capture method using NIR-EPs bearing conjugated antibodies to EpCAM, N- and O-cadherins, and PSMA.

Targeted NIR-EPs will be developed and tested in control cells, in experiments that exploit spiked tumor cells in whole blood, and in 22 healthy volunteers and patients with benign medical conditions. See below for progress in the development of these NIR-EPs.

Task 2: Assessment of circulating tumor cell capture using novel antibody-targeted NIR-EPs in men with mCRPC.

We will conduct a translational study of this novel antibody-targeted NIR-EP-based capture and imaging technology, using samples taken from 50 men with progressive mCRPC. This pilot study will compare in parallel the enumeration of CTCs derived from human blood samples using each capture method: Cellsearch® EpCAM-based standard FDA approved method, EpCAM-polymersome, N- and O-cadherin polymersome, and a PSMA-polymersome conjugate.

3.2 What was accomplished under these goals?

Task 1: To develop and optimize a novel polymersome-based CTC capture method using NIR-EPs bearing conjugated antibodies to EpCAM, N- and O-cadherins, and PSMA.

Therien Group: We initially developed the anti-EpCAM NIR-EP in Year 1 as EpCAM forms the basis for the Cellsearch CTC capture method, the only FDA cleared CTC isolation and enumeration method and thus has proven prognostic importance in men with CRPC.^{2,4} We were able to successfully construct an anti-EpCAM NIR-EP and tested this in cancer cells known to highly express EpCAM (T47D cells) and cells that lack EpCAM (PBMCs). As shown in **Figure 1**, these NIR-EPs exhibited excellent discriminatory abilities and sensitivity for EpCAM+ cells, with low non-specific binding to control cells. This discriminatory ability was noted with concentrations between 1.3–2.0 nM and different incubation periods for the cell lines (1 hour and overnight). We found that room temperature incubation provided the optimal temperature to maintain specific binding.

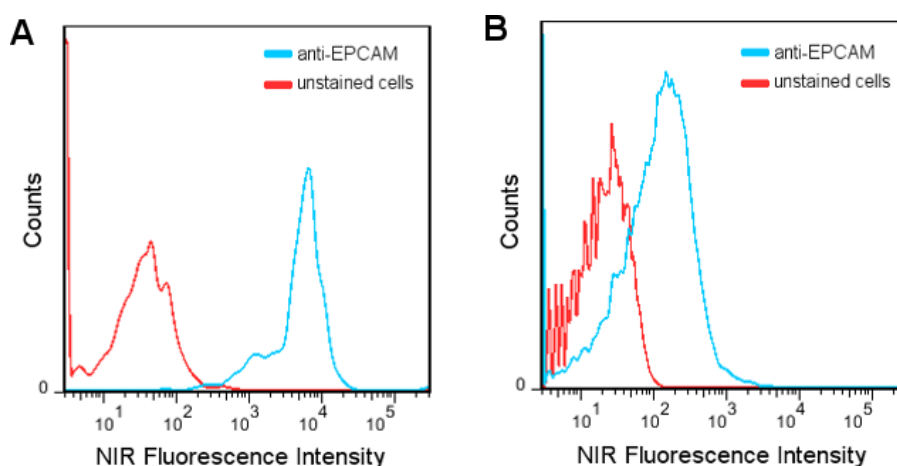


Figure 1. Flow sorting of EpCAM+ cancer cells (T47D cells, left) using the EpCAM-conjugated NIR-EP demonstrates clear signal separation vs. unstained cells using the Cy7 (>790 nm near infrared wavelength) channel. Peripheral blood mononuclear cells (PBMCs) that lack EpCAM expression demonstrate very little non-specific binding (right).

In later samples of EpCAM-targeted polymersomes however, batch-to-batch variability for binding to T47D cells was observed by flow cytometry. In some batches, high uptake of NIR-EPs per cell were observed; in others, uptake levels matched that of control NIR-EPs conjugated to

an isotype-matched IgG antibody (**Figure 2**). This prompted us to reinvestigate our protocols in detail. Our previously used bicinchoninic assay (BCA) used to determine the degree of antibody functionalization on the surface on the NIR-EP was unable to distinguish between covalently bound-antibody and surface associated antibody. Therefore, the use of this assay to determine the efficiency of different antibody-coupling chemistries in some cases may have yielded inaccurate data.

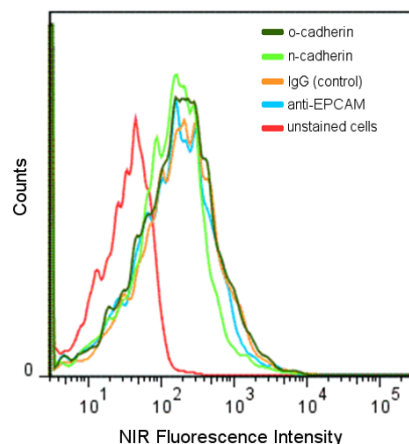
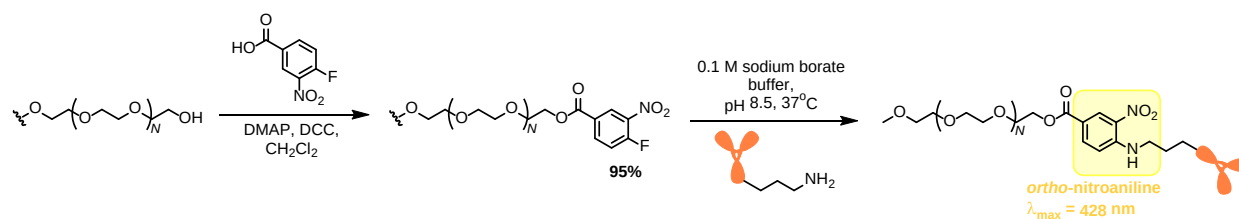


Figure 2. A later batch of anti-EpCAM NIR-EPs when incubated with EpCAM+ cells T47D showed no uptake relative to control NIR-EPs conjugated to an isotype-matched IgG antibody.

We have therefore invested time optimizing our coupling protocols and characterization methods as described below.

Antibody Conjugation Strategies

We have developed fluoronitrobenzoic acid (FNB)- based chemistry for the chemical conjugation of bioligands to the surface of NIR-EPs. The FNB group can be introduced onto the hydroxyl-terminus of a polymer in a single, high yielding step,⁵ and unlike maleimide chemistries, is stable to hydrolysis at mild pHs (**Scheme 1**).



Scheme 1. Functionalization of PBD-*b*-PEO diblock copolymer OB18 with FNB. Conjugation of a bioligand *via* a lysine residue yields a chromophoric *ortho*-nitroaniline linker highlighted in yellow.

FNB is reactive to primary amines, which in the case of protein-based ligands, is readily available *via* the N-terminus or surface lysine residues. The abundance of these residues in proteins such as antibodies (Abs) means prior chemical modification of Abs is unnecessary. The Therien group has previously used FNB-based chemistry to successfully functionalize NIR-EPs with the cell-penetrating Tat peptide for tracking dendritic cells (DCs) *in vivo*.⁶ In this work, conjugation of Tat to the FNB-functionalized polymer was carried out *prior* to polymersome formation, allowing the conjugation to be carried out in organic solvents; progress of the reaction could be monitored by

UV-vis absorption as the resulting *ortho*-nitroaniline chromophore absorbs at 428 nm (**Scheme 1**).⁵ Presence of the peptide did not adversely affect the resulting polymersome morphology.

We have adapted this FNB-based chemistry for the conjugation of whole antibodies to the surface of polymersomes with these considerations: Conjugation to FNB must 1) be carried out under mild, aqueous conditions in order to preserve antibody functionality; 2) occur post-polymersome formation, as the large, hydrophilic nature of antibodies would likely disrupt the polymer's ability to form stable vesicle structures due to a vastly altered hydrophobic fraction.⁷ It has already been demonstrated that polymersomes, once formed, are stable to surface decoration with Abs.^{8–10}

Covalent modification of one of the many surface lysine residues present in Abs has the potential to affect antigen-recognition properties, either directly (through modification of residues in the complementarity determining region (CDR)) or indirectly through allosteric effects. IgG antibodies contain between 80–95 lysine residues which can potentially act as a chemical handle for direct attachment to FNB-NIR-EPs. Thus, the conjugation of anti-EpCAM Ab CD326 with FNB-terminated tri(ethylene glycol) monomethyl ether (FNB-TEG) as a PEO- diblock copolymer model was explored. CD326 was incubated with FNB-TEG at various molar ratios (40:1, 20:1, and 10:1 FNB-TEG to antibody) in sodium borate buffer, and the resulting Ab-TEG conjugates were

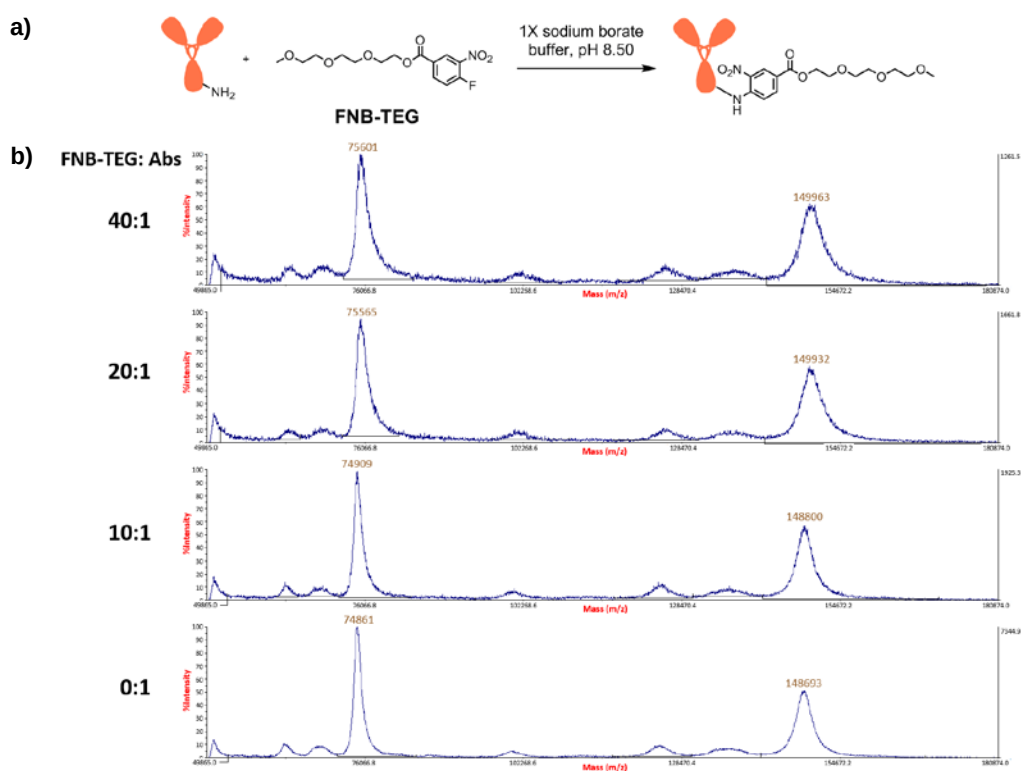


Figure 3. (a) Synthetic scheme for the modification of anti-EPCAM Ab CD326 with FNB-TEG. (b) MALDI-MS of the resulting Ab-TEG conjugates where CD326 was incubated with FNB-TEG in a (from top to bottom) 1:40, 1:20, 1:10 and 1:0 (blank) molar ratio. Spectra were acquired in linear detection mode with a sinapinic acid matrix; peaks were calibrated to BSA.

analyzed using a statistical MALDI-MS method as reported previously (**Figure 3**).¹¹ Briefly, by acquiring a mass spectrum of each Ab-TEG preparation in replicates greater than 3, it is possible to detect a minimal mass difference of 119 Da between IgG and its corresponding conjugates to

a 95% confidence level, even in linear detection mode. The average number of moles of the conjugate attached per mole of IgG can be subsequently calculated from the difference in the observed masses of Ab-TEG conjugates and the unconjugated trastuzumab blank (0:1 preparation), divided by the molecular weight of the FNB-TEG conjugate. The results, summarized in **Table 1**, showed that at the higher concentrations screened (40:1 and 20:1 preparations), an average of 3 surface lysine residues were accessible on the CD326 surface for FNB-TEG modification. This encouraging result demonstrates that despite the FNB-TEG group being present in vast stoichiometric excess, modification of the Ab is limited by steric access to reactive lysine side-chains, thus indiscriminate modification of CDR residues does not occur.

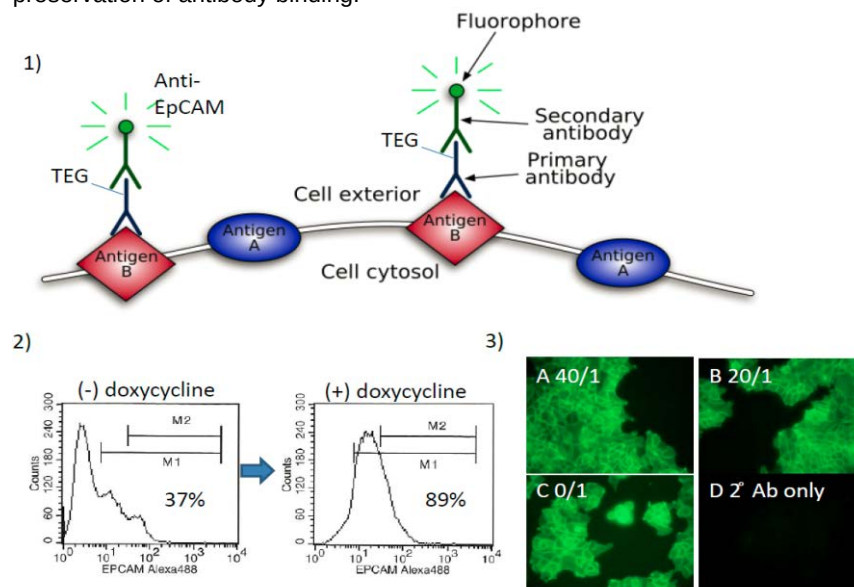
FNB-TEG/Ab ratio	Mean mass by MALDI /Da [†]	Standard deviation /Da	Mass difference to native Ab /Da	Number of FNB-TEG conjugates	<i>p</i> value
40:1	149286	586	1394	3.3	0.02
20:1	149238	602	719	3.1	0.02
10:1	148356	387	368	0.3	0.02
0:1[‡]	148273	365	0	0	-

Table 1. MALDI-MS results from the coupling of FNB-TEG model with CD326. The degree of covalent modification at the concentration ranges explored was limited to 3 TEG chains/Ab. [†] Data an average of three analyses. [‡] Blank sample. CD326 was treated with sodium borate buffer under identical reaction conditions.

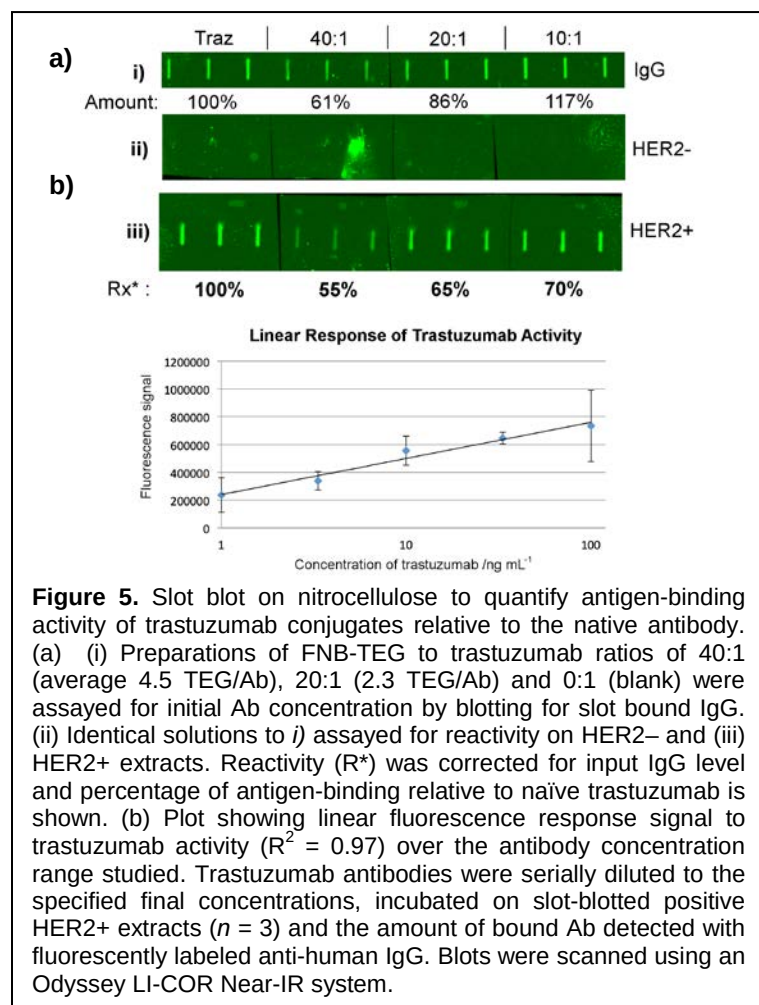
The antigen-recognition properties of the anti-CD326 conjugates were assessed in an immunofluorescent assay using a HeLa cell line (which has low EpCAM expression) following induction of EpCAM expression using a doxycycline regulated promoter. The lentiviral doxycycline-inducible system was originally used to express short-hairpin RNA for gene expression suppression¹² but has been modified by Neil Spector's group (Duke) for regulated expression of cloned cDNAs. The results showed that

covalently modified CD326 retained antigen recognition properties by FACS sorting. In addition, we found that the use of TEG-conjugated antibodies did not interfere with antigen recognition as measured by immunofluorescent (IF) staining (**Figure 4**).

Figure 4. Assessment of TEG-conjugated antibodies to EpCAM. 1) Experiment design using indirect immunofluorescence and FACS sorting of positive and negative controls. 2) FACS sorting of TEG-conjugated anti-EpCAM without doxycycline (EpCAM low) and with doxycycline induction (EpCAM high) demonstrated clear preservation of antibody affinity in HeLa cells. 3) IF staining of T47D cells (EpCAM high) under different TEG concentrations demonstrates preservation of antibody binding.



Similar experiments have been carried out with the humanized anti-HER2 monoclonal antibody trastuzumab, as a proof-of-concept experiment with research materials made available to us by Genentech. The degree of FNB-TEG modification on trastuzumab was similar to that found for CD326, and an immunofluorescent assay with doxycycline-regulated HER2+ expressing MCF10A cells again showed retention of antigen recognition properties. The antigen-binding activity of trastuzumab conjugates was further quantified by MCF10A HER2+ extract nitrocellulose slot blot using a fluorescently labeled secondary anti-human IgG (**Figure 5**); trastuzumab coupled to an average of 2 TEG groups retained ~65% of its antigen reactivity, which was comparable to the blank sample incubated with conjugation buffer under the reaction conditions (~70%). These results showed that the reduction in antigen binding affinity is associated with handling the antibody (e.g. temperature, buffer, purification steps) rather than through modification of the CDR region. The conclusions from these experiments show that we are able to control the number of modifications made on the surface of Abs, and covalent attachment of a TEG chain does not occur in the antigen binding region. It is our expectation that we will be able to couple many copies of CD326 onto the surface of polymersomes. This, coupled with the demonstrated ability of surface-conjugated bioligands such as antibodies to cluster on fluid membrane polymersomes to maximize antigen binding affinity means that the multi-ligand effect of Ab-conjugated polymersomes should more than compensate for a ~30% drop in antigen binding affinity.



NIR-EP construction

Using our newly validated functionalized OB18 polymer, we can construct NIR-EPs using our well-established methodology (**Figure 6**).

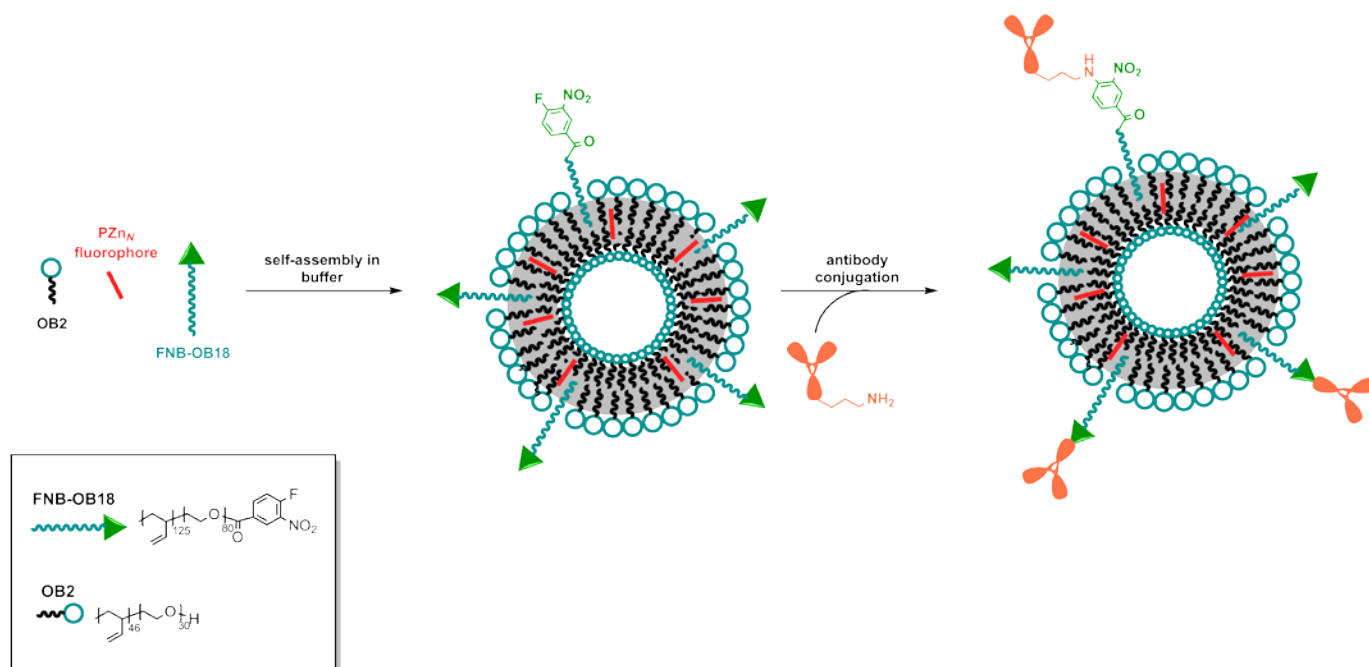


Figure 6. Construction of antibody-conjugated NIR-EPs.

NIR-EP synthesis was carried out using the thin-film hydration method as previously described.^{13–22} NIR-EP vesicle size and morphology was characterized by cryogenic transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS), and confirmed formation of vesicle morphologies of diameter 100–200 nm.

Reaction protocols for the formation and analysis of Ab-NIR-EPs

Having validated that the variation in batches of Ab-NIR-EPs is not due to inadvertent deactivation of CD326 through covalent modification or conjugation conditions, we began optimizing our reaction protocols for the coupling of CD326 to FNB-functionalized polymersomes, first using trastuzumab in proof-of-principle experiments.

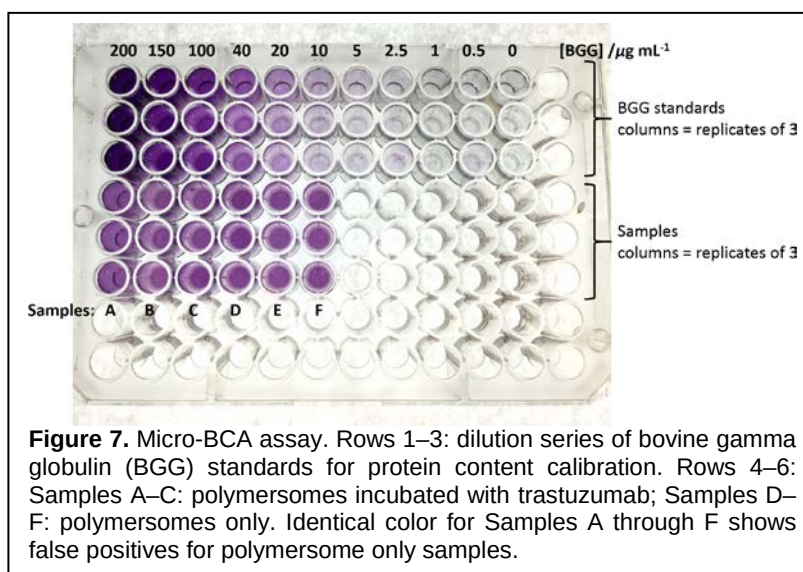
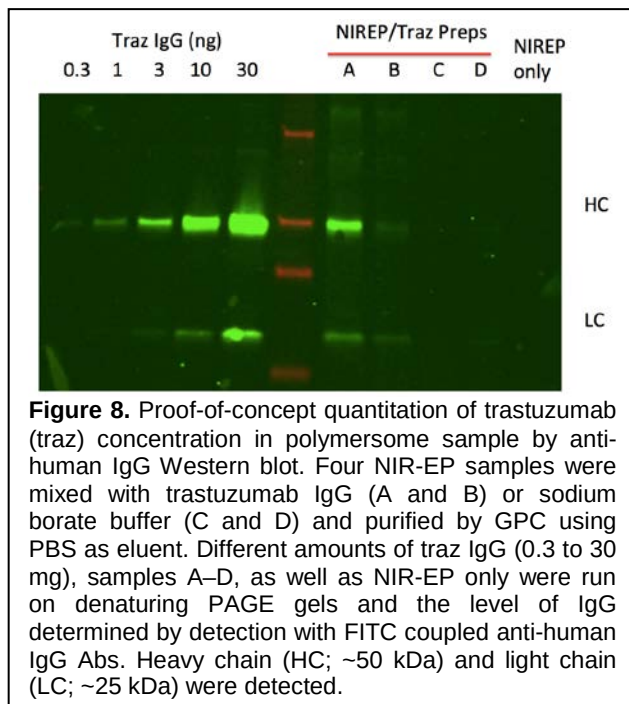


Figure 7. Micro-BCA assay. Rows 1–3: dilution series of bovine gamma globulin (BGG) standards for protein content calibration. Rows 4–6: Samples A–C: polymersomes incubated with trastuzumab; Samples D–F: polymersomes only. Identical color for Samples A through F shows false positives for polymersome only samples.

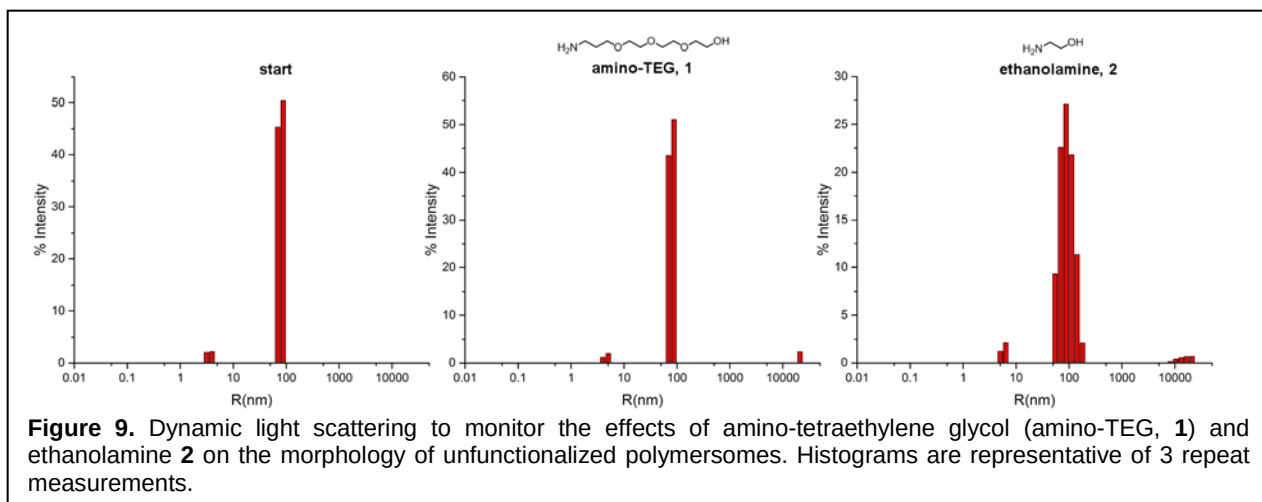
Surface antibody quantification

Previously, we used the enhanced BCA assay to determine the protein content of our polymersome samples, which has a sensitivity range of 5–250 $\mu\text{g/mL}$ protein. However, on re-exploring our protocols, we have determined that at a polymersome concentration of 5 mg/mL , the lower detection limit of 5 $\mu\text{g/mL}$ protein corresponds to approximately 19 antibodies/polymersome; we thus conclude that this assay is too insensitive for our needs. Unfortunately, we found that the micro-BCA assay, with an ideal detection range of 0.5–200 $\mu\text{g/mL}$ protein (corresponding to a lower detection range of 2 Abs/NIR-EP), is incompatible with polymersomes resulting in false positives (**Figure 7**). We developed a sensitive methodology for the detection of antibody concentration by Western blot analysis; **Figure 8** shows a proof-of-principle experiment analyzing unfunctionalized NIR-EPs incubated with (Samples A–B; 1:1 molar ratio trastuzumab/OB18) and without (Samples C–D) trastuzumab. The fluorescently labeled anti-human secondary IgG antibody shows linear sensitivity over a wide range of trastuzumab concentrations and is capable of detecting as little as 0.3 ng Ab, corresponding to <0.2 antibodies per NIR-EP.



Capping agents

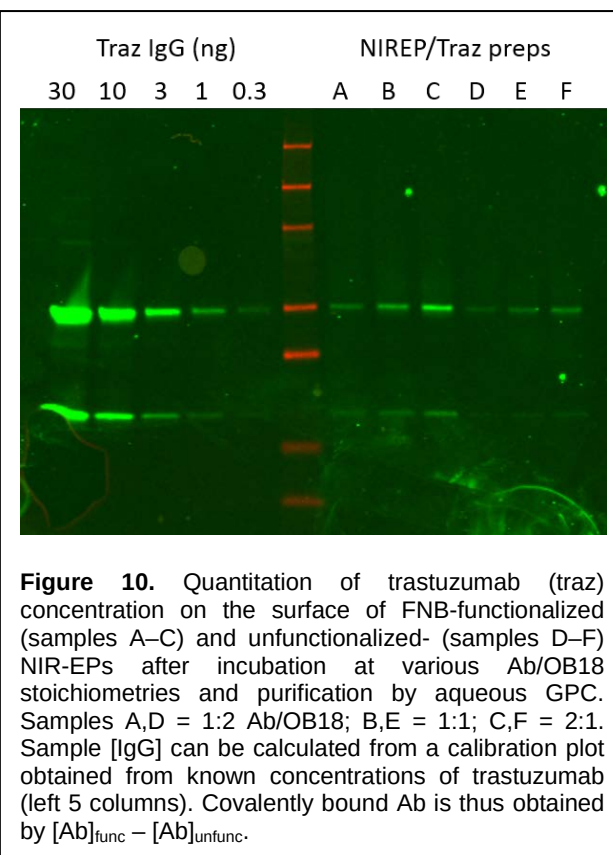
Even at high stoichiometric ratios of Ab to FNB-OB18, it is not expected that all of the available FNB surface groups will react due to steric crowding at the NIR-EP surface. For this reason, we sought a small molecule capping agent that would quench any remaining FNB groups after Ab-coupling, while preserving the stealth-like properties of the PEO surface. As molecules that fulfil these requirements will by nature be amphiphilic, it was necessary to ensure that the capping



reagent used would not act as a chaotropic reagent and disrupt the polymersome morphology. Commercially available amino-tetraethyleneglycol (amino-TEG; **1**) and ethanolamine **2** were each assessed by incubation with unfunctionalized polymersomes under the reaction conditions, and monitoring any changes to the polymersome structure by DLS. Polymersomes incubated with ethanolamine saw immediate disruption of the vesicle morphology, while no effect was seen for those treated with amino-TEG (**Figure 9**).

Antibody functionalization of NIR-EPs

With these protocols in hand, test couplings of trastuzumab to NIR-EPs were carried out at various ratios of Ab to FNB-OB18. To quantify the degree of non-specific absorption of Ab to the polymersome surface, unfunctionalized OB18/OB2 polymersomes were incubated with trastuzumab under identical conditions and purified in the same manner. Our best results to date, obtained at 10 mg/mL polymer concentration are shown in **Figure 10**. At the highest stoichiometry of Ab/OB18 screened (2:1), an average of 2.2 antibodies per polymersome were obtained, at a 5% FNB-OB18/OB2 loading. We are currently in the process of optimizing this reaction to increase the degree of NIR-EP surface functionalization, primarily by varying the percentage FNB-OB18 loading per polymersome, but also investigating other parameters such as pH or temperature. We are confident with our preliminary results showing successful functionalization of the polymersome surface, that we will be able to achieve this goal soon. Once in hand, we will be able to apply the results from trastuzumab-functionalized polymersomes to fabricate an array of Ab-polymersomes targeted to therapeutically relevant cell surface antigens.

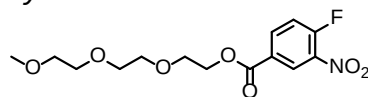


Task 2: Assessment of circulating tumor cell capture using novel antibody-targeted NIR-EPs in men with mCRPC.

Given that the duration of this 3 year proposal was spent in optimizing the chemistry of NIR-EP creation and conjugation, we were unable to move to human subjects testing of this NIR-EP for CTC isolation. Thus, this task will remain incomplete. However, we are encouraged by our progress to develop and synthesize NIR-EPs based on task 1 that should lend itself to testing in cell lines and spiked human blood samples and on CTCs *ex vivo* from patients in future proposals and work. Thus, no human subjects were enrolled in this study as of the time of this writing. We anticipate using the preliminary data generated from Task 1 of this NIA toward to development of new proposals and grants to fund the clinical studies of this approach for CTC detection and characterization.

Experimental Methods

Synthesis of FNB-TEG



Triethylene glycol monomethyl ether (292 μ L, 1.8 mmol), 4-fluoro-3-nitrobenzoic acid (405 mg, 2.2 mmol), *N,N'*-dicyclohexylcarbodiimide (490 mg, 2.4 mmol) and dimethylaminopyridine (27 mg, 0.21 mmol) were dissolved in dry dichloromethane (12.0 mL) and stirred at rt for 4 h until shown to be complete by TLC (5:100 MeOH/ CHCl_3 , Me-TEG R_f 0.35; product R_f 0.85; visualized by KMnO_4 stain). The reaction mixture was filtered, and the resulting filtrate was washed consecutively with DI water, 5% AcOH in water, and water. The organic layer was dried with MgSO_4 , concentrated then columned (silica, 9:1 $\text{CHCl}_3/\text{Et}_2\text{O}$, R_f 0.29) to obtain the product as a colorless solid (548 mg, 91%); δ_{H} (400 MHz, CDCl_3), 8.73 (dd, 1H, $J_1 = 7.2$, $J_2 = 2.2$ Hz, Ar-H), 8.33 (ddd, 1H, $J_1 = 8.7$, $J_2 = 4.2$, $J_3 = 2.2$, Ar-H), 7.37 (dd, 1H, $J_1 = 10.2$, $J_2 = 8.7$, Ar-H), 4.55–4.47 (m, 2H, CH_2), 3.87–3.80 (m, 2H, CH_2), 3.74–3.60 (m, 8H, CH_2), 3.56–3.49 (m, 2H, CH_2), 3.35 (s, 3H, OCH_3); δ_{F} (400 MHz, CDCl_3) –110.58 (ddd, 1F, $J_1 = 10.4$, $J_2 = 7.2$, $J_3 = 4.3$); m/z (ESI MS⁺) 332.1 ($[\text{M}+\text{H}]^+$, $\text{C}_{14}\text{H}_{19}\text{FNO}_7^+$, requires 331.1).

Modification of antibodies with FNB-TEG

Trastuzumab (Genentech) was desalted into 1X sodium borate buffer (285 mOsm, pH 8.50) using a Amicon Ultra 0.5 mL 100 kDa MWCO Centrifugal Filter using the manufacturer's instructions. The desalted antibody was diluted to a 4 mg/mL concentration with buffer, and the concentration checked by UV-vis absorption at 280 nm. FNB-TEG, dissolved in buffer, was added to the desalted antibody at molar ratios of 40:1, 20:1, or 10:1. The reactions were shaken at 750 rpm at 37 °C for 20 h, and then desalted into 1X PBS (285 mOsm, pH 7.40) for immunofluorescence studies, or MilliQ water for MALDI analysis.

MALDI-MS analysis of trastuzumab-TEG conjugates

MALDI-MS spectra were recorded on an AB Sciex 4800 Plus MALDI-ToF instrument in linear detection mode using a sinapinic acid matrix. For each sample, 3 separate mass spectra were obtained, and the peak maxima were averaged, and subtracted from that obtained from unmodified trastuzumab. The mass differences were divided by the molecular weight of the FNB-TEG conjugate to obtain the average degree of substitution per antibody.

Immunofluorescence

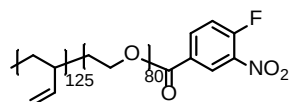
Cells were plated in complete media on 15 mm round uncoated glass coverslips. Cells were fixed in 4% formaldehyde/1X phosphate buffered saline (PBS), and permeabilized by incubation with a solution of 0.2% Triton/PBS. For staining of cellular expressed EpCAM, cells were incubated with 2.5 μ g/mL Mouse anti Human EpCAM antibody (clone VU-1D9, AbD Serotec MCA 1870G) 0.09% Sodium Azide/PBS. Protein was visualized with 2 μ g/mL AlexaFluor 488 goat anti-mouse secondary antibody, (ThermoFisher, cat#:A-11029) and imaged on Olympus Fluorescence Microscope.

Slot blot

A protein solution of either HER2+/- cellular extracts or trastuzumab antibody was diluted in PBS and 200-400 μ L was added to a slot blot manifold applied under vacuum onto pre-wetted Protran

nitrocellulose membranes. Membranes were dried and then incubated with a 5% non-fat dry milk (5% Blotto) in PBS for 1 h at room temperature to block unoccupied binding sites then incubated with either anti-human IgG-800 near-IR antibody (Molecular Probes/Live Technologies) or trastuzumab followed by anti-human IgG-800 in 1% Blotto. Following incubation with antibody solutions, non-specifically bound antibodies were removed by 3 × 15 minute washes with PBS containing 0.05% Tween 20. The level of secondary antibody was determined by scanning on a LI-COR Odyssey near-IR blot scanner.

Synthesis of FNB-functionalized OB18 (PEO(3.9k)-b-PBD(6.5k))



OB18 (100.0 mg, 9.6 μmol) was dissolved in toluene and dried by azeotropic distillation using a Dean-Stark trap to remove residual water. In a separate dried flask, 4-fluoro-3-nitrobenzoic acid (7.1 mg, 38.4 μmol), *N,N'*-dicyclohexylcarbodiimide (8.4 mg, 40.4 μmol) and dimethylaminopyridine (0.1 mg, 0.7 μmol) were dissolved in dry dichloromethane, stirred at rt for 30 min, before cannulation into the OB18 distillate. After stirring for 2 d at rt, the solvent was removed under reduced pressure, and the resulting residue dissolved in THF and purified by size exclusion column to obtain the title compound as an off-white waxy solid.

NMR:

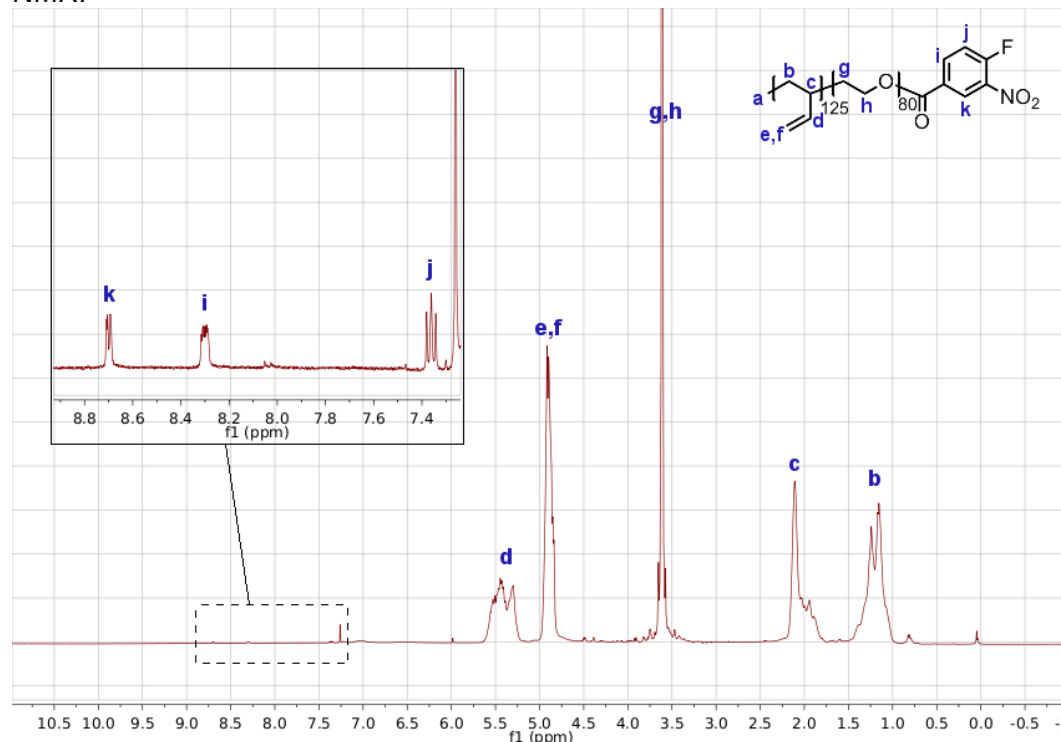


Figure 11. ^1H NMR (500 MHz, CDCl_3 , 298 K) of FNB-functionalized OB18.

NI-REP construction

An organic solution of FNB-functionalized PEO(3600)-b-PBD(6800) diblock copolymer (**FNB-OB18**), short-chain PEO(1300)-b-PBD(2500) (**OB2**) and PZn_N NIRF at a 5:95:5 molar ratio was spotted onto a roughened Teflon plate and allowed to evaporate to form a uniformly coated surface. After residual solvent removal under high vacuum for >24h, 1X PBS buffer (285 mOsm, pH 7.40) was added and the films incubated at 60 °C for 24 h, followed by 90 minutes of sonication in a bath sonicator. Nanoscale, unilamellar NIR-EPs were subsequently obtained using procedures analogous to those used to formulate liposomes (sonication and freeze-thaw). Ten freeze-thaw cycles are carried out by alternatively placing the samples in liquid N₂, followed by a 5 minute sonication in a water bath at 60 °C.

NIR-EP functionalization

5:95 FNB-OB18/OB2 NIR-EPs were desalted into 1X sodium borate buffer (pH 8.50, 285 mOsm), and concentrated to ~10 mg/mL concentration by centrifugal filtration (Amicon Ultra 4 mL 100 kDa MWCO centrifugal filter). The polymersome concentration was determined by UV-vis absorption of the sample corrected for scattering, using previously determined extinction coefficients.¹⁸ Similarly, trastuzumab (Genentech) was desalted into 1X sodium borate, and concentrated to a ~20 mg/mL concentration by centrifugal filtration; concentration was determined by absorption at 280 nm. Desalted trastuzumab and NIR-EP were combined at fixed molar ratios (e.g. 2:1 molar ratio of OB18 to trastuzumab) and agitated at 750 rpm at 37 °C. After 22 h, a 5 mM solution of amino-TEG in sodium borate buffer was added at a 5:1 amino-TEG/OB18 molar ratio, and the mixture agitated for a further 2 h. The reaction mixture was purified on a Sephacryl S-500 column with 1X PBS (1 mL/min, detection at 280, 426 and 700 nm). Purified NIR-EP fractions were combined and concentrated to ~5 mg/mL concentration by centrifugal filtration. To assess the degree of antibody non-specific absorption to the polymersome surface, the protocol was repeated with unfunctionalized 5:95 OB18/OB2 NIR-EPs.

Assessing protein content of NIR-EPs

The amount of trastuzumab bound to a NIR-EP was determined by Western blotting. Samples, and a dilution series of known quantities of pure trastuzumab antibody were denatured with Laemmli denaturing buffer, sized on denaturing PAGE gel, and transferred to nitrocellulose using an iBlott transfer apparatus (LifeTechnology). The membrane was blocked with 5% Blotto for 1 h at room temperature then incubated with anti-human IgG-800 antibody in 1% Blotto for minimum 2 h at room temperature. Non-specifically bound antibodies were removed by 3 × 15 minute washes with PBS containing 0.05% Tween 20 and the level of trastuzumab signal was determined by scanning on a LI-COR Odyssey near-IR blot scanner. A calibration plot of the diluted trastuzumab was determined and the amount of trastuzumab in each NIR-EP preparation was calculated.

Final NIR-EP concentration, determined by UV-vis as described above, was converted into average number of particles per unit volume, N , using the following equation.²³

$$N = \frac{6 \times W \times 10^{-3}}{\pi \times (D_o^3 - D_i^3) \times 10^{-21} \times \rho} \quad (1)$$

Where W is the polymer mass (in mg/mL), D_o is the average outer diameter of the polymersome (obtained by DLS), D_i is the diameter of the polymersome lumen (calculated $D_o - 2l$, where l is the polymersome membrane thickness, determined by cryo-TEM),²⁴ and ρ is the density of OB2

(previously determined as 1.08 g/cm³).²⁵ The concentration of trastuzumab in the sample, Y , determined by Western blot analysis, is converted to number of antibodies, M :

$$M = \frac{Y \times 10^{-6}}{mw} \times N_A \quad (2)$$

Where mw is the molecular weight of trastuzumab (145,531 g/mol) and N_A is Avogadro's constant. The number of antibodies per polymersome is thus calculated M/N . Samples were measured in triplicate.

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3.3 What opportunities for training and professional development has the project provided?

- **Training**

Melanie O'Sullivan (Postdoctoral Research Associate)

Training in colloidal synthesis and characterization including cryogenic transmission electron microscopy; biological techniques.

- **Professional development**

Melanie O'Sullivan (Postdoctoral Research Associate)

Attendance at conferences and seminars listed under major activities. Grant writing experience.

In addition, attended the following professional development workshops:

- "Writing a Convincing Research Plan" (Duke University, 04/2015)
- "The Academic Application Process" (Duke University, 09/2015)
- "The Academic Interview" (Duke University, 09/2015)
- "Negotiating the Academic Job Offer" (Duke University, 09/2015)
- "The Teaching Statement" (Duke University, 10/2015)

3.4 How were the results disseminated to communities of interest?

Nothing to report.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

N/A, this is the final report.

4. IMPACT:

4.1 What was the impact on the development of the principal discipline(s) of the project?

In Task 1, we have developed methods over the past 3 years for the creation of NIR-EPs using a novel methodology that permits for the sensitive detection of antibody conjugate on the surface of our polymersomes. Such a chemistry approach should allow for future testing of antibody-bound polymersomes as a diagnostic in prostate cancer or breast cancer ex vivo, given the specificity of these antibodies for EpCAM or HER2 targets, respectively. Such a diagnostic test, if sufficiently sensitive and specific, could be useful to help track both the number of circulating tumor cells in the circulation over time during anti-cancer therapy, but also be useful in isolating CTCs for downstream molecular characterization and functional studies. However, future grants will be needed to define the performance characteristics of these NIR-EPs.

4.2 What was the impact on other disciplines?

Nothing to report.

4.3 What was the impact on technology transfer?

Nothing to report.

4.4 What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

5.1 Changes in approach and reasons for change

Nothing to report beyond the summary in task 1.

5.2 Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report beyond the technical hurdles addressed in Tasks 1 and 2, which prevented enrollment of human subjects for testing of the performance characteristics of NIR-EPs as a CTC detection method.

5.3 Changes that had a significant impact on expenditures

Nothing to report.

5.4 Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report. No human subject or animal enrollment.

5.5 Significant changes in use or care of human subjects

Nothing to report.

5.6 Significant changes in use or care of vertebrate animals.

Nothing to report.

5.7 Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

6.1 Publications, conference papers, and presentations

1. Li J, Gregory SG, Garcia-Blanco MA, **Armstrong AJ***. Using circulating tumor cells to inform on prostate cancer biology and clinical utility. Critical Rev Clin Lab Sciences, Epub ahead of press Jul 25, 2015. *corresponding author

2. Bitting RL, Somarelli JA, Schaeffer D, Garcia-Blanco MA, and AJ Armstrong*. The Role of Epithelial Plasticity in Prostate Cancer Dissemination and Treatment Resistance. Cancer and Metastasis Rev Epub ahead of press Jan 11, 2014.

*corresponding author

6.2 Website(s) or other Internet site(s)

Nothing to report.

6.3 Technologies or techniques

Nothing to report.

6.4 Inventions, patent applications, and/or licenses

Nothing to report.

6.5 Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1 What individuals have worked on the project?

Name:	Andrew J. Armstrong
Project Role:	<i>PD/PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0001-7012-1754</i>
Nearest person month worked:	<i>1, annually</i>
Contribution to Project:	<i>PI, oversaw grant and collaborations with the Therien lab</i>
Funding Support:	<i>multiple</i>
Name:	Daniel J George
Project Role:	<i>Co-investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0002-0836-8542</i>
Nearest person month worked:	<i>0.1 annually</i>
Contribution to Project:	<i>PI, oversaw grant and collaborations with the Therien lab</i>
Funding Support:	<i>multiple</i>
Name:	Michael J. Therien
Project Role:	<i>Co-investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-4876-0036</i>
Nearest person month worked:	<i>0.1</i>
Name:	Melanie C O'Sullivan PhD
Project Role:	<i>Research Associate</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0001-8689-1059</i>
Contribution to Project:	<i>Conducted experiments in polymersome creation and chemistry, conjugation chemistry</i>
Name:	Gabor Kemeny MS

Project Role:	<i>Research Analyst</i>
Researcher Identifier (e.g. ORCID ID):	0000-0003-1567-3497
<i>Nearest person month worked:</i>	6
Contribution to Project:	<i>Tested reagents in cell lines by FACS, immunofluorescence, optimized reagents for testing in vitro and in vivo.</i>

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

See current other support document.

7.3 What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

- n/a

9. APPENDICES: Two references added (publications) and *attached CV*.

REVIEW ARTICLE

Using circulating tumor cells to inform on prostate cancer biology and clinical utility

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Abstract

Substantial advances in the molecular biology of prostate cancer have led to the approval of multiple new systemic agents to treat men with metastatic castration-resistant prostate cancer (mCRPC). These treatments encompass androgen receptor directed therapies, immunotherapies, bone targeting radiopharmaceuticals and cytotoxic chemotherapies. There is, however, great heterogeneity in the degree of patient benefit with these agents, thus fueling the need to develop predictive biomarkers that are able to rationally guide therapy. Circulating tumor cells (CTCs) have the potential to provide an assessment of tumor-specific biomarkers through a non-invasive, repeatable “liquid biopsy” of a patient’s cancer at a given point in time. CTCs have been extensively studied in men with mCRPC, where CTC enumeration using the Cellsearch® method has been validated and FDA approved to be used in conjunction with other clinical parameters as a prognostic biomarker in metastatic prostate cancer. In addition to enumeration, more sophisticated molecular profiling of CTCs is now feasible and may provide more clinical utility as it may reflect tumor evolution within an individual particularly under the pressure of systemic therapies. Here, we review technologies used to detect and characterize CTCs, and the potential biological and clinical utility of CTC molecular profiling in men with metastatic prostate cancer.

Keywords

Androgen receptor, biomarker, castration resistant prostate cancer, EpCAM, liquid biopsy, microfluidic, PSA

History

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Abbreviations: ADT: androgen deprivation therapy; AR: androgen receptor; CRPC: castration resistant prostate cancer; CTC: circulating tumor cell; ctDNA: cell free circulation DNA; DAPI: 4',6-diamidino-2-phenylindole; EMT: epithelial mesenchymal transition; EpCAM: epithelial cell adhesion molecule; EPISPOT: epithelial spot; FSMW: structured medical Seldinger guidewire; FACS: fluorescence-activated cell sorting; FISH: fluorescence assisted *in situ* hybridization; GEDI: geometrically enhanced differential immunocapture; GR: glucocorticoid receptor; ISET: isolation by size of epithelial tumor cells; MCC: microfluidic Cell Concentrator; mCRPC: metastatic castration resistant prostate cancer; OS: overall survival; PSA: prostate specific antigen; PSMA: prostate specific membrane antigen

Introduction

Prostate cancer is the most common non-cutaneous cancer in men and the second most common cause of cancer-related death in the United States, with 29480 deaths in 2014¹. Six therapies improve overall survival (OS) in men with

metastatic castration-resistant prostate cancer (mCRPC), including the taxane chemotherapeutics docetaxel and cabazitaxel, the hormonal agents abiraterone acetate and enzalutamide, the immunotherapeutic sipuleucel-T and the bone-targeting radiopharmaceutical radium-223^{2–7}. However, many men with mCRPC do not respond to these therapies. For example, prostate-specific antigen (PSA) response to enzalutamide or abiraterone in the pre-chemotherapy mCRPC setting is 60–70%. Approximately 30–40% of patients have no response to these agents with respect to PSA levels (primary resistance), and among patients who initially have a response to enzalutamide or abiraterone, virtually all eventually acquire secondary resistance^{3,6,8}. Furthermore, cross-resistance between enzalutamide and abiraterone is clinically evident: for instance, PSA response to treatment with abiraterone after enzalutamide, or vice versa, is reduced by nearly half, with

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most responses lasting only a few months^{9–11}. The primary cause of resistance is genetic alterations in the androgen receptor (AR) gene that re-activate the AR pathway. Additional genetic lesions in *PI3K*, *RAS*, *MYC*, *WNT* or genes in DNA repair pathways may also contribute to resistance¹². Recent data suggest that bypass from AR blockade can be mediated by activation of the glucocorticoid receptor (GR), which drives expression of AR target genes¹³. In addition, emerging data suggest that certain AR variants (i.e. AR-v7) that lack the ligand binding domain may not only convey resistance to abiraterone acetate and enzalutamide, but may also promote taxane resistance given that these variants do not require microtubule-dependent AR nuclear translocation¹⁴. Understanding the molecular mechanisms that underlie the development of resistance in men with mCRPC may permit the rational selection of therapies that are better able to address these resistance mechanisms. CTCs present an opportunity to carry out non-invasive real-time tumor sampling.

Hematogenous metastasis of solid tumors involves migration and invasion of carcinoma cells from the primary tumor into blood vessels, circulation in the bloodstream, dissemination to distant sites, extravasation and colony establishment in metastatic niches. CTCs are tumor cells released from the primary tumor or metastatic site into the periphery, and are believed by many researchers to be essential in the hematogenous spread of malignancy and establishing metastases^{15–17}. CTCs can be detected and captured via different technologies from peripheral blood, which is in contrast to metastatic biopsies which require an invasive procedure that may not be possible in certain locations or present too high a risk. Therefore, the ability to collect and analyze CTCs from peripheral blood for tumor-specific molecular aberrations is an attractive alternative to standard biopsies. In addition, with the continuous evolution of tumors, which involves genetic and epigenetic alteration of cancer cells and tumor heterogeneity, primary tumors and individual metastases likely provide a limited snapshot of the molecular status of a given cancer in a given patient at that time. CTCs could provide real-time and sequential “liquid biopsy” for patients with cancer, and CTC biomarker analyses from peripheral blood can be conducted repeatedly to allow real-time monitoring of cancer progression and response to therapies in patients who have sufficient CTCs.

Recent studies have demonstrated that CTC molecular analysis is feasible and may provide important information on therapeutic targets and drug resistance mechanisms in patients with carcinoma, including prostate cancer^{18–27}. The goal of CTC molecular profiling is to identify and select therapeutic targets, and to match individual patients with therapies designed to address the molecular lesions present (accurate medicine). In addition, longitudinal assessments of CTC biomarkers may permit the changing of therapy as cancer evolves or undergoes treatment selection. The application of novel next-generation sequencing technologies in the area of CTC molecular characterization, in combination with development in CTC detection technologies, should provide important areas of growth and clinical utility for the personalized treatment of men with prostate cancer and many other cancers.

Currently, the Cellsearch® platform is the only FDA-approved CTC detection method in patients with metastatic breast, prostate and colorectal cancer. The platform, which isolates CTCs from whole blood using an epithelial cell adhesion molecule (EpCAM)-based ferromagnetic antibody, defines a CTC to be a nucleated (determined by DAPI staining) cell larger than 4 µm in diameter that lacks the common leukocyte marker CD45, and expresses cytokeratins¹⁵. Using the EpCAM capture reagent coupled with three biomarkers, CTCs are reliably defined in patients with a range of solid tumors, but are absent in normal individuals^{15,28,29}. Enumeration of CTCs has been shown to be prognostic for overall survival in many tumors including breast, colorectal and metastatic prostate cancer³⁰. Unlike PSA changes, CTC flare (enumeration surge after starting chemotherapy) has not been observed to date, and CTC enumeration changes may occur earlier than PSA declines, with some studies suggesting improvements in survival association with early CTC changes as compared with PSA declines³⁰. The measurement and enumeration of CTCs in cancer also plays a critical role in the early diagnosis of metastatic disease, in prediction as it relates to systemic therapy selection, in risk-stratification for clinical trials or clinical practice or as a surrogate biomarker for decision-making in research studies or clinical practice. CTC characterization may also offer use as a pharmacodynamic biomarker in drug development for rapidly assessing drug activity.

Circulating tumor cells (CTCs) provide real-time and easy access to tumor cells; however, there are limitations with CTC studies. One major limitation is the lack of detection of CTCs in many men despite the presence of progressive, mCRPC (>50% non-detection rate)^{30,31}. Some CTCs may lack epithelial biomarkers entirely³². This CTC heterogeneity may partly explain the escape of detection of CTCs in CRPC and other solid tumors using the standard epithelial antigen based technology. Detecting CTCs with high sensitivity and specificity is an important goal of CTC studies in prostate cancer and other solid tumors. Improvements in CTC capture by novel capture antibodies (e.g. mesenchymal antigens), negative selection methods and novel CTC chip designs that improve CTC yield, and improved CTC molecular profiling technologies will help further exploration in CTCs and its implication in metastatic prostate cancer.

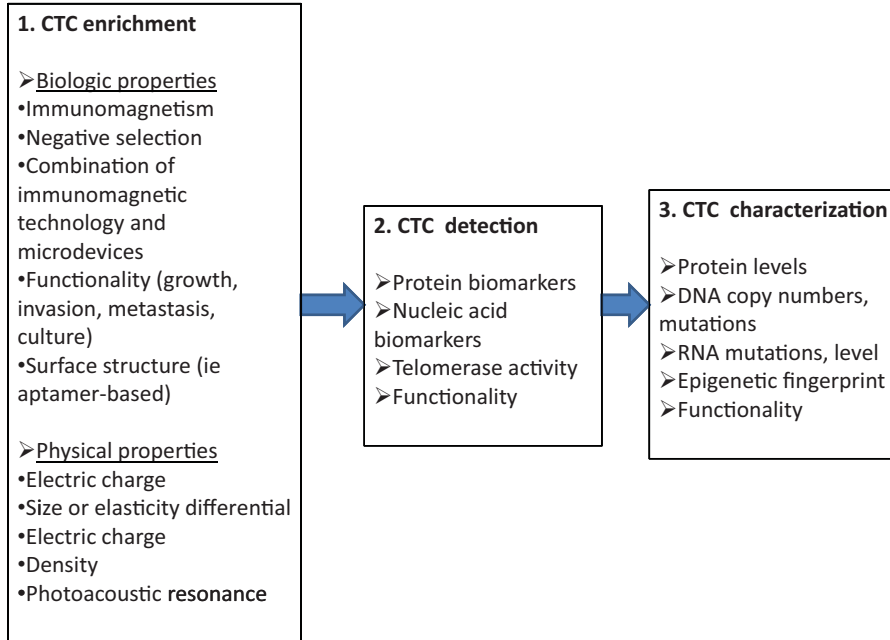
This review is mainly focused on existing CTC capture or isolation technologies, methods for the molecular characterization of these CTCs from men with metastatic prostate cancer and the biological and clinical utilities of these approaches (Figure 1).

Methods: CTC isolation approaches and molecular characterization

CTC isolation approaches

A variety of techniques for CTC isolation have been studied, each has specific advantages and limitations, and most methods have suffered from a lack of robust clinical data to inform on clinical utility. The rarity of CTCs is the key technical challenge for CTC capture, with some men having no CTCs evaluable for profiling despite metastatic disease. Methods that are more sensitive may additionally suffer from

Figure 1. Schematic illustration of the three major steps of a CTC assay. Due to CTC rarity in peripheral blood, most capture techniques require CTC enrichment, followed by detection. (1) There are multiple approaches to enrich CTCs based on physical or biological properties that distinguish CTCs from other circulating cells. (2) After enrichment, CTCs can be detected by different techniques, including protein-based detection, nucleic acid-based and telomerase activity-based detection. (3) Successful molecular characterization of CTCs could provide a real-time assessment of cancer metastasis biology, tumor biomarkers such as mutations or epigenetic signatures or gene expression levels and avoid the necessity of repeated invasive biopsies.



low specificity (false positives) due to the isolation of other cell types rarely found in the circulation, such as endothelial cells, CD45 negative leukocytes or circulating mesenchymal or bone marrow derived stem cells^{33,34}. In addition, the CTCs derived from different types of tumors have variations in size, shape and immunophenotyping (technique used to study the proteins expressed by cells). Even CTCs from the same origin have heterogeneity in morphology and immunophenotype³⁵. Therefore, the accurate detection of CTCs based on morphology and immunophenotype is challenging. Here, we summarize the current CTC capture and detection technologies, and their advantages and limitations.

CTC enrichment based on biological properties

Due to their rarity in peripheral blood, most capture techniques require CTC enrichment prior to detection. There are multiple approaches to enrich CTCs based on physical or biological properties that distinguish CTCs from other circulating cells (Table 1). After enrichment, the CTC fraction will likely contain a substantial amount of leukocytes and therefore CTC characterization is needed to distinguish CTCs from leukocytes and other circulating normal cells. The different approaches for CTC enrichment are based on the different properties of CTCs that distinguish them from the normal hematopoietic cells (and other rare circulating cells such as endothelial cells and mesenchymal stem cells), including different biological properties and physical properties.

Immunomagnetic-based CTC enrichment assays.

Immunomagnetic-based assays are based on the identification of cells with antibodies against specific antigens for positive selection, or against leukocyte antigens for negative selection. Positive selection with an antibody that recognizes EpCAM is most commonly used and the only currently FDA approved CTC assay. EpCAM is a transmembrane glycoprotein

which is consistently expressed by epithelial derived tumor cells, but not by leukocytes. Anti-EpCAM antibody is coupled to magnetic ferrous beads and the resulting CTC–antigen–antibody complex is isolated subsequently by being exposed to an external magnetic field. CTCs are then detected immunocytologically.

CellSearch® (Janssen Diagnostics, LLC) is the only FDA-cleared CTC isolation technology¹⁵. Detection by CellSearch® is dependent on EpCAM expression on CTCs, which are subsequently identified as nucleated cells positive for cytokeratin 8, 18 or 19 expression and negative for leukocyte antigen CD45 expression by immunofluorescence staining. This CTC detection technology has been widely used in prostate cancer research. CellSearch® is highly reproducible between laboratories and the results are stable for samples shipped for as long as 72 h¹⁵. In general, a threshold of 5 cells per 7.5 ml peripheral blood has been used to estimate prognosis. However, the relatively low yield of CTCs recovered with this method limits the ability to further refine prognosis among men with mCRPC and low CTC counts. There are several explanations for this lack of CTC detection, including loss of rare cells through multiple capture and purification steps, the strict characterization definitions and the inefficient magnetic separation of labeled cells throughout a large population of unlabeled cells. There are also other limitations with CellSearch®. For example, captured CTCs lose their viability after fixation, which is the step required for immunofluorescence-based detection, and thus it is not possible to culture collected cells or proceed to functional studies. In addition, review and interpretation of the CellSearch® data are somewhat subjective and CTCs may be called based on the operator's subjective decision and interpretation of cell morphology, size, fluorescent intensity and the presence of apoptosis; thus, a valid result requires a trained pathologist or technician. Attempts have been made to develop an automated Cellsearch® approach⁶⁶, which may reduce the variability and turnaround time for analysis

Table 1. CTC enrichment based on biological properties or physiological properties.

CTC enrichment	Mechanism	Examples	Selected references
Biological properties-based			
Immunomagnetic-based	Enrichment with magnetically labeled antibody	CellSearch AdnaTest MagSweeper VeriFAST GILUPI MACS [®] MicroBeads	36–40,70
Microdevices	Microfluidic cell sorting	^{HP} CTC-Chip ^{HB} CTC-Chip CTC-iChip NanoVelcro GEDI OncoCEETM Biofluidica LiquidBiopsy [®] Ephesia CTC-chip	26,41,42,43–46,47
Negative selection	Hematologic cells depletion	CTC-iChip Microfluidic Cell Concentrator (MCC) EPIC platform Multicellular rosettes	48,42,49,50
Combination of immunomagnetic technology and microdevices	Combined enrichment with magnetically labeled antibody and microfluidic cell sorting	Ephesia CTC-chip IsoFlux	47,51
Functional based	PSA secretion by CTC Invasion of CTCs into collagenous matrices	EPISPOT assay Cell-adhesion matrix (CAM)-based Vita-Assay TM	52,53,54
Aptamer based	Aptamer binding to cell surface specific protein	Aptamer modified microfluidic device	52,55,50
Physical properties based			
Density based cell separation	Differential migration according to difference in buoyant density	Oncoquick	56
Size or elasticity separation	Isolation based on size and/or deformability	ISSET Slanted spiral microdevices MetaCell [®] ScreenCell VyCAP Elasticity-based microfluidic device	57,58,59,60–62
Electric charges	Different polarizability and electrical properties	ApoStream TM DEPArray	63,64
Photoacoustic resonance	Photoacoustic resonance	Photoacoustic flow cytometry	65

while preserving prognostic significance. Further evaluation of this automated method in prospective studies is warranted.

In addition to these technical issues that may lead to the under detection of CTCs, some CTCs exhibit evidence of epithelial plasticity and have low EpCAM expression; instead, they possess a mesenchymal or stem-like phenotype^{35,67}. These cells would not be detected by CellSearch[®] if EpCAM was completely lost⁶⁷. To address this problem, our lab developed a cadherin-11 (OB-cadherin)-based ferrofluid capture method³³. In this platform, we enrich CTCs using an OB-cadherin antibody and captured nucleated cells identified by expression of beta-catenin, which is commonly expressed in both epithelial and mesenchymal cell types, and lack of CD45. Indeed, we have identified CTCs in some men with bone-metastatic CRPC, which lack cytokeratin and are able to be captured with an OB-cadherin ferrofluid. These cells have a different morphologic appearance when compared with EpCAM-captured CTCs, yet in some cases have been shown to be clonally derived from the epithelial population, sharing common genetic lesions such as PTEN

loss and the TMPRSS2-ERG fusion gene³³. We are also currently developing other modified CTC platforms using novel ferrofluids with CTCs enriched by expression of N-cadherin, O-cadherin or c-MET as a means of identifying potentially important subpopulations of disseminating tumor cells. Actin bundling protein, platin 3, a novel marker for CTCs undergoing the epithelial mesenchymal transition (EMT) that is not expressed in blood cells, could recognize both epithelial and mesenchymal CTCs and may overcome the limitations of CTC capture platforms that only detect either epithelial CTCs or mesenchymal CTCs^{68,69}. CTCs captured based on expression of platin 3 are associated with poorer prognosis of colorectal cancer⁶⁹, but this method has not yet been evaluated in prostate cancer. Finally, recent data suggest that cell surface vimentin may be commonly expressed in mesenchymal CTCs in multiple solid tumor types, and may be useful to examine changing CTC phenotypes in prostate cancer¹⁶².

Other immunomagnetic-based systems, such as the AdnaTest (AdnaGen, Langenhagen, Germany), MagSweeper

device (Stanford University, Stanford, CA), immiscible phase filtration platform VerIFAST (University of Wisconsin, Madison, WI) and the GILUPI cell *in vivo* collector, are all EpCAM expression dependent^{36–39}. The AdnaTest system combines immunomagnetic enrichment of epithelial cells using a cocktail of several antibodies against EpCAM, HER2, EGFR and other proteins with a polymerase chain reaction for cancer or prostate cancer specific transcripts to detect CTCs³⁹. In a recent predictive study by Antonarakis and colleagues, investigators used a modified Adnatest capable of detecting the AR variant v7 in CTC enriched samples from men with mCRPC, and demonstrated potential clinical utility without the need for enumeration⁸. In this study, AR-v7 expression correlated strongly with prior abiraterone or enzalutamide exposure, and the presence of AR-v7 was strongly associated with poor outcomes and low response rates to either agent. This study suggested that men with mCRPC who harbor an AR-v7 dominant CTC population should not be treated with these agents and should consider other systemic options⁸.

The MagSweeper device uses magnetic rods to collect CTCs that bind to anti-EpCAM-antibody coated magnetic beads, while non-specifically bound blood cells are released through a controlled shear force produced by movement of the magnetic rods in wash buffer³⁸. MagSweeper was reported to be able to gently extract live CTCs with high purity from unfixed, unfractionated blood^{38,71}. The VerIFAST technique uses magnets to selectively and rapidly move the EpCAM positive cells bound to paramagnetic particles between immiscible liquids, where only the cells bound to immunomagnetic beads can cross between phases to enable rapid isolation of CTCs⁴⁸. VerIFAST avoids the multiple transfer or wash steps required in many other CTC isolation methods, which can cause loss of rare cell populations. This approach is currently under evaluation prospectively in CRPC for the characterization and enumeration of CTCs. The GILUPI cell detector uses a functionalized structured medical Seldinger guidewire (FSMW) coated with a chimeric monoclonal antibody directed to EpCAM to collect CTCs *in vivo* during blood passage. The FSMW is inserted through a standard venous cannula for the duration of 30 min while the patient rests. After removal, CTCs are identified by immunocytochemical staining of EpCAM and/or cytokeratins and staining of their nuclei and counted. This approach is being tested in early stage high risk prostate cancer as a prognostic biomarker through the European TRANSCAN multicenter trial⁷². All above discussed systems are subject to the problem of a lack of capturing CTCs that have mesenchymal or stemness phenotypes and/or lack of EpCAM expression. Furthermore, these methods have not been prospectively studied in large cohorts of men with PC, and the clinical utility is lacking.

Microfluidic device based CTC enrichment assays. With the improvement in microfluidic engineering over the past years, innovative microfluidic devices have been rapidly developed for efficient CTC isolation. Microfluidic devices (chips) for CTC enrichment are also dependent on the CTC's biological characteristics with specific cell surface antigen expression. ¹¹⁹CTC-Chip consists of 78 000 microposts coated with

anti-EpCAM antibodies to capture EpCAM-expressing CTCs coming into contact with the microposts as blood flows through the microfluidic chip⁴¹. ^{HB}CTC-Chip is a second generation chip and consists of microfluidic channels etched in herringbone patterns, which induce formation of microvortices as blood flows through the chip and therefore increases the contact time between cells and the channel wall coated with anti-EpCAM antibodies. CTC Chips are not limited to EpCAM capture. Instead, the capture antibodies used to coat microfluidic channels could be tailored to target different CTC specific antigens, e.g. non-epithelial markers^{41,73}.

Different from the positive selection based microfluidic devices discussed above, the third generation CTC-Chip, CTC-iChip, is a device that uses both a positive selection and negative selection strategy to purify CTCs independent of antigens present on the tumor-cell surface⁴². The CTC-iChip enriches CTCs through three steps: first, a size-based hydrodynamic sorting removes red blood cells and platelets using a laminar flow microfluidic device; second, the chip aligns the remaining cells in a single file in the flow channel; last, magnetophoresis to remove antibody coated magnetic bead labeled cells, either CTCs (positive selection) or leukocytes (negative selection). The advantage of the negative selection mode of CTC-iChip is the ability to collect unlabeled CTCs, which are assumed to be mixed population of both epithelial and mesenchymal/stem-like or antigen negative CTCs⁴². The limitation of this device is that large clumps of tumor cells may be filtered out, and very small (<8 micron) stem-like CTCs may flow with the leukocytes. Prospective studies are required to determine the clinical utility of measuring CTCs by the iChip; however, initial publications suggest the ability to molecularly profile CTCs that are collected for mutational analysis, and thus this method may enable predictive medicine⁷⁴.

The NanoVelcro microfluidic device incorporates anti-EpCAM-antibody-coated silicon nanowires with an overlaid polydimethylsiloxane chaotic mixer to generate vertical flows, and enhances contacts between CTCs and the capture substrate⁴³. The NanoVelcro device has demonstrated its consistency for CTC enumeration in metastatic prostate cancer and established that continuous monitoring of CTC enumeration could be employed to examine disease progression and to follow prostate cancer patients' responses to different treatments⁴³.

Another microfluidic device, the geometrically enhanced differential immunocapture (GEDI), combines an anti-prostate specific membrane antigen (PSMA) antibody with a 3D geometry to capture CTCs while minimizing non-specific leukocyte adhesion⁴⁶. This GEDI microfluidic device was directly compared with CellSearch[®] and demonstrated a 2–400 fold higher sensitivity⁴⁶. Another advantage of this device compared with CellSearch[®] is its independence of EpCAM expression, and the potential ability to grow CTCs *ex vivo* on the chip and to molecularly profile CTCs on the chip for biomarker analysis, such as AR or microtubule biomarkers⁷⁵.

The microfluidic cell concentrator (MCC) is another microfluidic device to enrich CTCs by negative selection. In this device, CTCs are negatively selected after bulk

erythrocyte and hematopoietic cell removal with the OncoQuick buffycoat isolation method, followed by removal of peripheral hematopoietic blood cells, identified as CD45+ cells. MCC will then process the cell suspensions following pre-processing steps for enrichment and downstream processing³⁶. This method does not rely on positive selection based on surface markers; therefore, it enables the isolation of EpCAM negative cells. In addition, isolated cells through this device are free of antibodies or other tethering molecules.

Ephesia CTC-chip and IsoFlux are two CTC isolation platforms, which combine magnetic bead technology and microfluidic devices. Ephesia CTC-chip uses columns of biofunctionalized superparamagnetic beads self-assembled in a microfluidic channel onto an array of magnetic traps prepared by microcontact printing⁴⁷. IsoFlux uses immunomagnetic beads coated with EpCAM antibody to target CTCs, then, the sample passes through a microfluidic device that contains an isolation zone to capture CTCs on the upper surface of the cartridge in an externally applied magnetic field⁵¹. These two systems combined the advantages of microfluidic cell sorting, notably the application of a well-controlled, flow-activated interaction between cells and beads, and those of immunomagnetic sorting, notably, the use of well-characterized antibody-bearing beads. Other microfluidic platforms which have not been explored in prostate cancer are summarized in Table 1.

Functional-based CTC enrichment assay. CTCs could also be enriched by approaches dependent on the viable CTCs functions, e.g. invasiveness and secretion of specific proteins. A functional enzyme-linked immunosorbent epithelial spot (EPISPOT) assay was reported to be able to detect prostate-specific antigen (PSA) secreting CTCs from men with metastatic prostatic carcinoma⁷⁶. In another study, CTCs were detected in 59% of patients with metastatic breast cancer using the EPISPOT assay⁴⁹. CTC enumeration through the EPISPOT assay in this study was evaluated on a large cohort of metastatic breast cancer patients and demonstrated prognostic relevance of the presence of viable CTCs⁴⁹. The EPISPOT assay offers several advantages compared with other CTC isolation approaches: because the secreted specific proteins are immunocaptured by the membrane in the immediate vicinity of the cells before being diluted in the culture supernatants, EPISPOT has a greater resolution than that of flow cytometry and immunometric assays. The EPISPOT assay enumerates only viable functional CTCs targeted by the proteins they secrete. However, the limitations of this PSA EPISPOT assay are relatively low yield for CTCs, the dependence on certain secreted proteins and the requirement of 48 h cell culture.

The cell-adhesion matrix (CAM)-based Vita-Assay™ (Vitatex, Stony Brook, NY) is another functional-based CTC isolation assay, which isolates CTCs from metastatic prostate cancer patients using the propensity of tumor cells to be able to invade into collagenous matrices⁵². Friedlander et al. compared CTC recovery efficiency of Vitatex versus CellSearch® by isolating and enumerating CTCs simultaneously from 23 men with mCRPC using the Vitatex and CellSearch®. This study reported that more CTCs were recovered using the

CAM platform than the CellSearch® platform, and the CAM platform allowed for the detection of CTC clusters, CTCs expressing EMT and stem-cell markers⁷⁷. The advantages of Vitatex are its independence of the status of EpCAM expression on CTCs, and that it allows for the capture of epithelial-like CTCs and CTC clusters, as well as for those not expressing epithelial markers.

There are, however, limitations with all of these functional based approaches. These methods are dependent on CTC viability under the artificial *in vitro* cell-culture conditions, and are also dependent on the assumption that these specific culture conditions are sufficient to recapitulate the *in vivo* biological behavior of CTCs.

Aptamer-based CTC enrichment assays. Aptamers are single-stranded RNA or DNA molecules that bind to a specific ligand. Aptamers have been demonstrated in multiple studies to be able to target extracellular membrane proteins on cancer cells, e.g. prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 3 (HER-3), RET, tenascin-C and muc1⁷⁸. Therefore, incorporation of aptamer technology with microdevices has important potential implications for CTC isolation. Highly efficient capture and enumeration of low abundance prostate cancer CTCs using PSMA aptamers immobilized to a polymeric microfluidic device have been reported^{55,79}, however, there is little published clinical data to date on this approach.

Negative enrichment. The majority of CTC enrichment approaches are based on positive selection. However, positive selection is based on the expression of tumor associated cell surface antigens and this approach encounters the problem of tumor heterogeneity and may miss a subpopulation of CTCs. Negative selection of CTCs by depletion of leukocytes is an alternative approach for CTC enrichment to avoid this problem. Magnetic beads binding to CD45+ leukocytes could remove leukocytes and negatively select CTCs^{80,81}. Bi-specific antibodies against antigens on leukocytes and erythrocytes will induce the formation of large multicellular rosettes to help remove hematologic cells from the blood sample by Ficoll density centrifugation⁴⁹.

In the EPIC platform^{50,82}, nucleated cells are plated onto glass slides and subjected to immunofluorescence staining for specific markers, e.g. pan-cytokeratin (CK), CD45 and AR, and CTCs can then be identified by fluorescent automated scanners. In this manner, EpCAM and/or CK negative cells may be identifiable based on additional biomarkers. Prospective evaluation of the EPIC System's clinical utility using AR-specific and other molecular probes in the context of novel hormonal and other systemic therapies in CRPC is ongoing. The CTC-iChip and MCC discussed earlier are examples of microfluidic devices to enrich CTCs by negative selection^{36,42}.

Negative selection does not rely on surface markers, therefore, it is believed valuable to harvest all possible CTCs without biases relevant to the properties of surface antigens on the CTCs. However, under such a negative cell isolation strategy, the CTC purity may be compromised. Not all CD45[−] cells in the blood are tumor cells, e.g. circulating endothelial cells are CD45[−].

CTC Enrichment based on physical properties

CTCs have physical properties that can help distinguish them from normal peripheral blood cells, e.g. electric charge, size, deformability, elasticity and density (Table 1). The advantage of physical property-based CTC enrichment is to permit CTC separation without labeling. A microfiltration platform, e.g. ISET® (Isolation by Size of Epithelial Tumor cells) system (RARECELLS, France), is an approach to CTC isolation by size, based on the assumption that CTCs are larger than leukocytes⁵⁷. CTCs are enriched by filtering blood through membranes with pores 8 µm in diameter, followed by staining of cells retained on the filter for cytomorphological examination or immunocytochemistry. This size-based platform has the advantage of ease to use and independence of specific cell surface protein expression. The disadvantage of the ISET system is that the size of CTCs varies and a subset of CTCs may be smaller than leukocytes⁸³. A prospective trial of 60 patients with metastatic carcinomas of breast, prostate and lung, compared CellSearch® and ISET directly, and demonstrated concordant results between two assays⁸⁴. Slanted spiral microdevices are novel microfluidic devices with a trapezoidal cross-section for ultra-fast, label-free enrichment of CTCs based on the larger CTC size compared with hematologic cells. In this device, the smaller hematologic cells migrate along the Dean vortices (pairs of counter-rotating vortices) towards the inner wall, and then back to the outer wall again; in contrast, the larger CTCs stay along the microchannel inner wall due to additional strong inertial lift forces⁵⁸. There are other size-based CTC enrichment platforms, e.g. MetaCell®, ScreenCell and VyCAP^{60–62}. All these size-based platforms are subjected to the limitation of variable CTC sizes with some smaller than leukocytes. The Metacell approach has been studied in 55 men with localized prostate cancer and demonstrated that a subset (~30%) of patients were able to generate stable CTC cultures that exhibited proliferative potential, which did not correlate with Gleason score or T stage in the patients. The long-term clinical significance of these findings, however, is unknown and CTC culturing has not yet been associated with clinical outcomes⁸⁵.

Dielectrophoresis separates CTCs from peripheral blood cells based on intrinsic differences in the polarizability and electrical properties between CTCs and normal peripheral blood cells. ApoStream™, an example of a dielectrophoretic device, was reported to have linearity of recovery of viable

cancer cells independent of their EpCAM expression level, and avoided the step of antibody labeling and enables the isolation of minimally modified CTCs for future analysis⁶³. In one study of patients with metastatic prostate cancer the ApoStream CTC enrichment platform isolated a greater number of CTCs from eight patients compared to CellSearch®, and all cell counts obtained by the ApoStream technique were higher than CellSearch®. These results indicated that ApoStream platform is well suited for detection and recovery of CTCs, including the tumor cells missed by CellSearch®⁸⁶.

Multiple other physical properties based on CTC enrichment technologies have been explored. The DEPArray™ technology is a CTC isolation assay, which combines microfluidic technology and a dielectrophoretic approach^{64,87}. Density-based cell separation, for example differential migration according to difference in buoyant density, is utilized by the Oncoquick CTC enrichment system (Greiner Bio One, Frickenhausen, German)⁵⁶. A photoacoustic flow cytometry system was reported to be able to detect melanoma CTCs by exploiting the broadband absorption spectrum of melanin within CTCs⁶⁵. Recent advancements in the development of elastomer (polymer having both viscosity and elasticity) microparticles bound to target tumor-specific antigens permit the acoustic separations of rare cells based on these properties⁸⁸. Cancer cells are more deformable compared with red blood cells (RBCs) and white blood cells (WBCs) and are able to squeeze through very small pores. Using a reverse-selectivity approach, an elasticity-based microfluidic device consisting of a large number of channels to reduce shear stress on each cell was reported to be able to detect CTCs from metastatic renal cell cancer patients with an efficiency of more than 78%⁵⁹. However, the importance of these devices in prostate cancer needs to be further validated.

CTC detection after enrichment

After enrichment, the CTC fraction will still contain a substantial amount of leukocytes, which are a major source of contamination for downstream high-throughput molecular studies. CTC detection is needed in many cases to distinguish CTCs from leukocytes to prevent false-positive signals following the initial enrichment step. After enrichment, CTCs can be detected by different techniques, including protein-based detection, nucleic acid-based and telomerase activity based-detection (Table 2).

Table 2. CTC detection by protein based, nucleic acid based or telomerase activity based technologies.

CTC detection	Detection principle	Advantages	Limitation	Selected references
Protein-based CTC detection				
Immunofluorescence staining	Antigen expression	Many parameters can be simultaneously measured	Low throughput and loss of CTC viability	30
Flow cytometry	Antigen expression	Fast	Dependent on antigen expression; and decreased CTC viability	89
Nucleic acid-based CTC detection				
RT-PCR	Expressed cell specific makers	High sensitivity	High frequency of false positive	90
FISH	DNA sequence detection	Highly accurate	Labor intensive	91
Telomerase activity based CTC detection				
Telomerase–PCR–enzyme-linked immunosorbent assay	Telomerase activity	Highly accurate	All CTCs are destroyed during whole blood lysis	92

Protein-based CTC detection. Cytometric approaches permit protein-based detection of CTCs, do not require cell lysis, and allow subsequent morphological identification of CTCs and molecular characterization. Cytometric approaches involve two major technologies: digital microscopy and fluorescence-activated cell sorting (FACS). Digital microscopy uses a computerized microscope with an image processing system that allows for the automatic screening of samples on the basis of nuclear and cell surface characteristics. The Cellsearch[®] method utilizes this step for manual or automated screening of cellular events. Flow cytometry identifies cells labeled with fluorochrome tagged antibodies after activation by the corresponding wavelength, which enables the analysis of thousands of cells quickly⁸⁹. FACS has been used by many investigators, including our group, for the enrichment and detection of CTCs for downstream molecular characterization⁹³. FACS is antigen-based, and multiple channels and biomarkers can be used for either positive or negative selection of cellular events. The limitations of FACS include the potential for loss of cells during processing, the impact on gene expression and RNA quality and cell viability and the marker-based requirements for CTC identification.

CellSearch[®] and many others CTC detection assays use immunofluorescence staining of CK, nuclear dye with DAPI and the common leukocyte antigen CD45 to detect CTCs by digital microscopy and differentiate CTCs from leukocytes. CTCs are defined in the Cellsearch[®] method to be CK⁺/CD45⁻/DAPI⁺ and meeting additional size and quality controls. These assays can be associated with false positive and false negative results. False-positive results are generated either by non-specific binding of antibodies to non-cancer cells in the blood, or to circulating non-tumor epithelial cells found in blood due to inflammation, tissue trauma, surgical interventions or benign epithelial proliferative diseases. Autofluorescence may contribute to false positive staining of cells for a range of biomarkers as well. In addition, some CTCs may not express epithelial antigens. Loss of expression of CK8, 18 and 19 was reported in micro metastatic cancer cell lines and in cells undergoing an EMT^{35,94}. In men with advanced prostate cancer, loss of CK was highly associated with relapse after surgery and resistance to chemotherapy, and the prevalence of CK loss increased during bone metastatic progression⁹⁵, emphasizing that the property of stemness inherent in aggressive solid tumors may lead to the under detection of CTCs that rely on epithelial biomarkers. Therefore, some CTCs, and particularly clinically important subsets of CTCs, may be missed by these assays.

CytoTrack technology (Lyngby, Denmark) combines the virtues of flow cytometry (high capacity scanning) and scanning microscopy (detailed image analysis). It is an alternative type of flow cytometry where cells are attached to the surface of the CytoTrack disc, instead of being carried by a fluent buffer string. Fluorescently labeled cells are captured by antibodies on glass discs and imaged for further analysis. This system eliminates the need for EpCAM pre-enrichment because it is based on a fluorescent scanning principle that has an extreme high capacity. The CytoTrack can scan 100 million blood cells within 1 min.

Nucleic-acid based CTC detection. Detection of specific mRNAs expressed by CTCs is an alternative to immunologic assays. Reverse transcription polymerase chain reaction (RT-PCR) is a frequently used nucleic acid-based method for CTC detection and characterization. Several studies reported that RT-PCR-based CTC detection is more sensitive than immunocytochemistry⁹⁰. As described earlier, the Adnatest CTC test, modified to detect the AR variant AR-v7 by RT-PCR, was shown to have potential predictive clinical utility in determining resistance to conventional AR-directed therapies in men with mCRPC⁸, suggesting that an RT-PCR based detection method may have clinical utility without the need for enumeration or protein characterization. RT-PCR offers high sensitivity and specificity and may be less limited by the above subjective and technical limitations inherent in protein-based assays. However, since there is no tissue specific marker in the great majority of solid tumors including prostate cancer, use of markers with poor specificity to individual tumors may result in false positives. For example, loss of PSA or PSMA is common in CTCs from men with mCRPC and RT-PCR probes against these differentiation antigens may miss important CTC events⁹⁶.

Fluorescence-assisted *in situ* hybridization (FISH) uses fluorescent nucleic acid probes to detect the presence and copy number of specific DNA sequences on chromosomes. FISH has been used to detect probes against AR, PTEN, TMPRSS2-ERG fusions and other key cancer-specific probes to permit the detection of CTCs and distinguish them from normal circulating cells. These studies have shown that CTCs may often have an underlying clonal element to them for certain probes (i.e. the TMPRSS2-ERG translocation), but heterogeneity for other events (i.e. PTEN loss)¹⁸. We have also shown that some CTCs that have lost their epithelial character may possess the same clonal FISH signature in the same individual patient, suggesting epithelial plasticity³³. A study reported high accurate detection of CTCs by FISH in prostate cancer, colorectal cancer and ovarian cancer⁸⁹. However, FISH is labor-intensive, interpretation of results can be subjective, and it requires ongoing validation work with suitable controls to account for the normal levels of FISH positivity for a given probe in normal white blood cells (such as PTEN loss in leukocytes).

Telomerase activity-based CTC detection. Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric repeats on to chromosomal ends using its own RNA component as a template, and has been found to be activated in many cancer types, e.g. prostate cancer, ovarian cancer, breast cancer and non-small-cell lung cancer (NSCLC)⁹⁷. Fizazi et al. developed a CTC detection method in prostate cancer patients based on telomerase-PCR-enzyme-linked immunosorbent assay⁹². Using telomerase-specific replication, selective adenovirus for CTC detection was reported in breast cancer and gastric cancer^{98,99}. This assay was reported to be highly accurate, and in a large prostate cancer Phase III trial, CTC derived telomerase activity was prognostic for OS in a significant subset of patients¹⁰⁰. However, telomerase activity-based assays require whole blood samples to be

lysed to measure the enzyme activity. Therefore all CTCs are destroyed during the processing. Future studies demonstrating clinical utility of this detection measure are needed.

Limitations for all CTC tests. Most CTC technologies remain dependent on EpCAM or some epithelial biomarker expression by CTCs. However, for CTCs that lose, down-regulate or lack EpCAM expression, EpCAM-based capture will fail to enrich an important subpopulation of CTCs. And, cells that lose CK expression or change CK patterns may escape detection. Therefore, CTC detection by alternate tumor cell surface markers is needed, e.g. markers that are able to detect CTCs with mesenchymal or stemness phenotypes. Our lab has been working on novel CTC capture methods by using mesenchymal markers, e.g. N-cadherin, OB-cadherin and c-MET to identify potentially important subpopulations of CTCs. Meanwhile, there are also some other potential CTC surface markers to be used for capture, e.g. tumor specific CD44, protocadherin family members, cell surface vimentin and plastin 3 as potentially useful for some mesenchymal-like CTCs or neuroendocrine prostate cancer CTCs^{69,101–104}. In addition, the limited blood sample volumes available from patients limit the number of CTCs available for downstream analyses and the number of CTCs detectable in early stages of the disease. CTC capture approaches with potentially higher sensitivity and specificity are under development and may permit a greater ability for molecular and functional characterization of CTCs.

CTC molecular characterization

CTCs captured from peripheral blood provide the potential for a greater overall reflection of tumor biological heterogeneity at a given point in time than from site directed individual metastatic biopsies. CTCs may be analyzed as populations or as single cells depending on the context and scientific question. Pooled CTC analyses offer the potential for assessment of the dominant circulatory clone at a given time point in a specific patient and permit the tracking of clonal selection during systemic therapies. Individual CTC profiling offers the ability to reconstruct complex evolutionary trees of tumor molecular changes from the primary or

metastatic sites and within the circulation, and may offer the ability to detect rare resistant clones before they become dominant²⁴.

The importance of CTC studies is not limited to detection and enumeration. Successful molecular characterization of CTCs could provide a real-time assessment of cancer metastasis biology, tumor biomarkers and avoid the necessity of repeated invasive biopsies. CTC biomarkers may reflect the tumor resistance and viability to treatment, and interrogation of the molecular profile of CTCs for expression of protein biomarkers, genetic variants and gene expression provides opportunities to use this information to monitor therapy and detect emerging resistance¹⁰⁵. With the development of novel technologies, there has been a rapid development in CTC high-throughput molecular assessments at various levels (protein, DNA, RNA and epigenetic).

In prostate cancer, dissemination to bone, which is challenging to biopsy, is common, and tumor cell growth, biomarker expression, survival prognosis and response to treatment changes over time. While metastatic prostate cancer is thought to be a monoclonal process initially¹⁰⁶, subclonal evolution of a fraction of tumor cells into genetically distinct subpopulations is likely to occur, with loss of protein expression and differentiation (epithelial plasticity) layered on as an additional adaptation during therapy resistance and progression³⁵. Invasive repeat biopsies are not practical for the majority of men with bone-metastatic prostate cancer. In addition, molecular profiling at different metastatic sites of prostate cancer may vary significantly^{107,108}. Therefore, blood-based CTC molecular assays may provide essential information about the current tumor biology at a given point in time, which may be assessed longitudinally in men with prostate cancer. Many studies have demonstrated the feasibility of molecular characterization of CTCs from men with metastatic prostate cancer (Table 3).

Protein level-based molecular characterization of CTCs from men with metastatic prostate cancer

Immunophenotyping, used as a common basis for CTC detection, is also used for CTC molecular characterization at the protein level and may provide important insights

Table 3. CTC characterization through molecular profiling studies in prostate cancer.

Molecular profiling	Target	Molecular studies	Selected reference
Protein level	PSA and Ki-67	Immunofluorescence	73
	AR localization	Immunofluorescence	19
	Microtubule dynamics	Immunofluorescence	46
	Multi-target: AR, ERG, and PTEN	Immunofluorescence	109
DNA and RNA levels	AR copy number	FISH	23
	TMPRSS2-ERG gene fusion	FISH	73,93
	Multi-target: ERG break-apart, AR copy number, and regional deletion of PTEN	Multi-color FISH	18
	MYC amplification	FISH	110
	AR gene mutation	PCR and direct sequencing	23
	Whole genome	aCGH	25
	Whole exome	WES	24
	Target RNA (e.g.Wnt2)	single-molecule RNA sequencing	27
Epigenetic level	Telomerase activity	qPCR-based telomeric repeat amplification	100
	DNA methylation	Methylation array	77

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into the molecular biology of human metastatic prostate cancer. For example, in a pilot study, dual staining of captured CTCs from men with metastatic prostate cancer for PSA and the cell division marker Ki67, indicated a broad range for the proportion of proliferating cells among CTCs (1–81%), and an increased Ki67 proliferative index in CTCs was associated with resistance to castration therapy⁷³. PSA loss and gain of PSMA expression in CTCs also has been found to correlate with progression to castration resistance, although there is great heterogeneity between patients and loss of both proteins is possible⁹⁶. AR protein nuclear localization by immunofluorescence staining was investigated in CTCs derived from patients with CRPC and the result demonstrated a significant correlation between AR cytoplasmic sequestration and clinical response to taxane chemotherapy¹⁹. Visualization and measurement of microtubule bundling in CTCs were performed by immunofluorescence staining in CTCs captured by the geometrically enhanced differential immunocapture (GEDI) microfluidic device from men with CRPC. The results demonstrated that visualization and measurement of microtubule bundling in CTCs could be used to monitor the drug-target engagement of docetaxel chemotherapy; this suggested a novel-mechanism of action of taxanes in reducing AR transport, which could be useful in predicting the response of docetaxel in individual patients⁴⁶. Nagy et al. reported a platform of CTC molecular analysis using multiplex Quantum Dot immunofluorescence staining and FISH procedures with anti-AR, -ERG and -PTEN antibodies and 5'ERG, 3'ERG, PTEN and Cen10 probes, respectively, on an automated slide-staining platform (Ventana Medical Systems, Inc., Tucson, AZ)¹⁰⁹. As others have shown ERG status by FISH in CTCs to be associated with response to abiraterone acetate¹⁸, there may be some clinical utility in measuring ERG status at the protein level. This method offers a high-sensitivity, multiplex molecular characterization of critical CTC biomarkers in mCRPC patients and might assist oncologists to identify which patients with mCRPC are likely to respond to combination therapy with targeted PI3K/AKT inhibitors and anti-androgens/Cyp17 inhibitors.

We have used protein biomarkers in CTCs to characterize a panel of epithelial plasticity biomarkers in CTCs from men with mCRPC, and found the common expression of N-cadherin, vimentin, OB-cadherin and CD133 in CTCs from these men, as well as individual cells that have lost E-cadherin and gained N-cadherin expression or possessed dual expression, suggesting phenotypic plasticity³⁵. These findings provided evidence for plasticity during CRPC progression, and have been validated by others using other RNA-based methods¹¹¹.

In summary, protein expression by CTCs may provide a useful biomarker for metastatic prostate cancer biology and have clinical utility if linked to specific therapeutic decisions. For example, assessment of AR status (N- or C-terminal) may be useful in selecting patients for AR-directed therapy, and assessment of the glucocorticoid receptor (GR) may be useful in determining one potential mechanism of enzalutamide resistance¹³. Additional methods to improve on the number of protein biomarkers that can be reliably assessed in individual CTCs are needed.

DNA or RNA level-based molecular characterization of CTCs from men with metastatic prostate cancer

Screening tumors for genomic aberrations (mutations, translocations and copy number variation) is essential for understanding tumor progression and resistance development for guiding specific clinical therapies. Characterization of specific mutations, gain or loss of genes or genomic regions and changes in gene expression patterns in CTCs from men with metastatic prostate cancer has been reported to be feasible and useful by many studies. FISH, RT-PCR, aCGH (array comparative genomic hybridization) and high throughput genome or exome sequencing have been reported to reveal genomic aberrations in CTCs from men with prostate cancer^{22,24,112,113}.

Cytogenetic studies based on FISH have been widely utilized in CTC analysis in prostate cancer. AR copy number changes assessed by FISH were complemented in CTCs from men with CRPC²³. Detection of the TMPRSS2-ERG gene fusion in CTCs from men with prostate cancer was reported using FISH and RT-PCR⁷². Using multicolor-based FISH on CTCs from CRPC patients, one study demonstrated AR copy number gain, PTEN loss and rearrangement of ERG in CTCs¹⁸, and a potential clinical association between response to abiraterone and ERG amplification in CTCs. Amplification of MYC has also been reported from CTC studies in prostate cancer by FISH¹¹⁰.

RT-PCR is highly sensitive and specific, and can detect the expression of individual genes even at the single cell level. Therefore, RT-PCR is widely used in CTC-enriched blood for the study of cancer biomarkers. Using global gene expression profiling with microarray and quantitative RT-PCR of CTC specific expression of selected genes, Smirnov et al. demonstrated that gene expression profiles of CTCs may be used to distinguish normal donors from advanced cancer patients with metastasis¹¹⁴. Mutations in the AR gene were detected in CTCs from patients with metastatic prostate cancer using PCR amplification and direct sequencing²³. RT-PCR has been used for the study of EMT related genes in CTCs from prostate cancer patients and identified a heterogeneous pattern of expression in EMT-related genes¹¹¹. The TMPRSS2-ERG fusion transcript was detected by RT-PCR from CTCs captured by the microvortex-generating herringbone-chip from patients with metastatic prostate cancer²⁶. There are limitations to the RT-PCR analysis of CTCs due to the fact that CTC-enriched fractions still contain leukocytes, which interfere with CTC-specific gene expression profiling and create a lower signal to noise ratio and reduce the ability to observe less common RNA events. Efforts to improve upon the purity of CTCs through novel detection/capture approaches should facilitate improved downstream RNA studies.

Whole genome amplification (WGA) and gene copy number analysis via aCGH have been utilized in CTC genomic studies in a variety of cancers, including prostate cancer. High-level copy number gains in the AR locus were reported in CTCs from mCRPC patients^{25,27}. In our lab, we successfully analyzed DNA of CTCs from four men with mCRPC by aCGH and revealed loss of AR copy number gains, MYCN copy number gain and ABL1/2 copy number

gain in enzalutamide resistant mCRPC patients¹¹⁵. In this study, we found that loss of genomic AR copy gain and gain of the MYCN region developed during enzalutamide resistant visceral progression and was observed with longitudinal CTC profiling. Additional common gains and losses of known oncogenic pathways were also commonly observed in our study.

With the rapid development of next generation sequencing and the ability to perform single cell whole genome sequencing, CTCs could provide a non-invasive source for genomic DNA and RNA for whole exome or genome sequencing and analysis²². Lohr et al. developed a modular set of protocols for census-based whole-exome sequencing (WES) and confident calling of somatic single nucleotide variants (SSNVs) from prostate CTCs²⁴. Their results demonstrated that WES could provide a window into the genetic analysis of metastatic prostate cancer and the evolutionary progression of metastatic disease from a small locus in the primary cancer, and this may provide a potential use in the clinic²⁴. Our lab has also sequenced the whole exome of CTCs from a man with enzalutamide resistant CRPC and revealed many SSNV and insertion/deletions (INDELs), whose importance in enzalutamide resistance needs further study (manuscript in preparation). An essential point for all of these methods is the need for adequate internal controls including matched leukocytes to determine the somatic nature of the genomic changes versus germline changes, validation of WES variants by Sanger sequencing and mechanistic studies to determine the validity and relevance of genomic findings to the clinical care of patients.

In addition to DNA sequencing, RNA sequencing of CTCs has also been reported in breast cancer¹¹⁶. The authors identified changes in epithelial and mesenchymal target genes during response and progression in women with metastatic breast cancer, suggesting the importance of this plasticity to therapeutic response. Yu et al. reported single-molecule RNA sequencing of CTCs from an endogenous mouse pancreatic cancer model and identified Wnt2 as a candidate gene enriched in CTCs²⁷. RNA sequencing on single CTCs isolated from patients with metastatic prostate cancer and on single prostate cancer cell line LNCaP cells spiked into the blood of healthy donors were reported¹¹⁷. These results demonstrated that RNA-sequencing is feasible to be carried out on small numbers of CTCs isolated from men with mCRPC.

Epigenetic level based molecular characterization of CTCs from men with metastatic prostate cancer. CTCs have also been used in cancer epigenetic studies. Comprehensive profiling of whole genome DNA methylation status at CpG sites were performed on CTCs from CRPC and the result demonstrated that CTCs epigenetically resemble CRPC tissue taken at autopsy²¹. Larger studies of the CTC epigenome and how it changes over time during systemic therapy and metastatic dissemination in prostate cancer are needed.

With the sparse number of CTCs, robust and accurate genetic profiling of CTCs is challenging. Most genetic studies of CTCs are done on DNA or RNA extracted from enriched CTCs, which is contaminated by WBC wild-type DNA or RNA, and which may lead to misclassification of epigenetic

signatures. Single CTC genomic analysis overcomes this limitation but this is technically and financially daunting in high numbers from patients. Despite the challenges of pure CTC capture, limited CTC enumeration and complex downstream processing appears feasible by select laboratories. The development of a platform that allows isolation of highly pure individual CTCs will offer opportunities to advance understanding of gene expression in individual CTCs to be used in clinical setting.

Biologic utilities of CTCs

Molecular profiling of CTCs can help elucidate the mechanisms involving invasiveness, aggressiveness, plasticity, tumor dissemination and metastasis in prostate cancer. CTCs may also provide a source of phenotype and tumor functionality, and may provide a source of renewable tumor tissue itself that may have clinical utility.

Metastasis in prostate cancer

Tumor initiating cells are cancer cells that are thought to have stem cell-like properties and are capable of initiating tumor growth^{118–120}. To develop metastasis, the tumor initiating cells have to survive passage through the circulation and then be able to exit the circulation and invade into the micro-environment of metastatic sites¹²¹. The tumors cells travel through the circulation as CTCs, which likely are continually repopulated by the metastases themselves. Therefore, CTC research could facilitate the identification of dominant tumor metastasis initiating cells, and offer the prospect of understanding these initiating cells' functions. Only three groups to date have reported success in the ability to culture CTCs from patients with metastatic cancer^{101,122,123}. These cultures permitted drug sensitivity analyses of cultured CTCs that could be used to predict clinical response and benefit for a wide range of agents. Further studies are needed to help optimize CTC cultures and profiling for drug sensitivity testing.

An additional method to culture CTCs from men with mCRPC is the organoid culture method, based on isolation of single cells and then using a growth-factor-based method established for colorectal cancer to isolate and propagate stem-like cells. In one study, a single CTC culture was developed using organoid methods in a patient with mCRPC who had >100 cells in 8 mL of blood, and this organoid culture recapitulated the histology and molecular genetics of the patient's primary tumor¹²⁴. These data suggest that organoid culturing methods may provide a useful framework for the functional and genomic characterization of CTCs from men with mCRPC, but larger studies are needed to test this suggestion.

EMT in metastatic prostate cancer

Epithelial mesenchymal transition (EMT) plays essential roles in mesoderm development, and in wound healing and fibrosis^{125,126}. EMT has been hypothesized to play a critical role in the cancer metastasis process¹²⁷. The hypothesis is that tumor cells transition from epithelial to mesenchymal at migration, then revert back to epithelial at site of distant

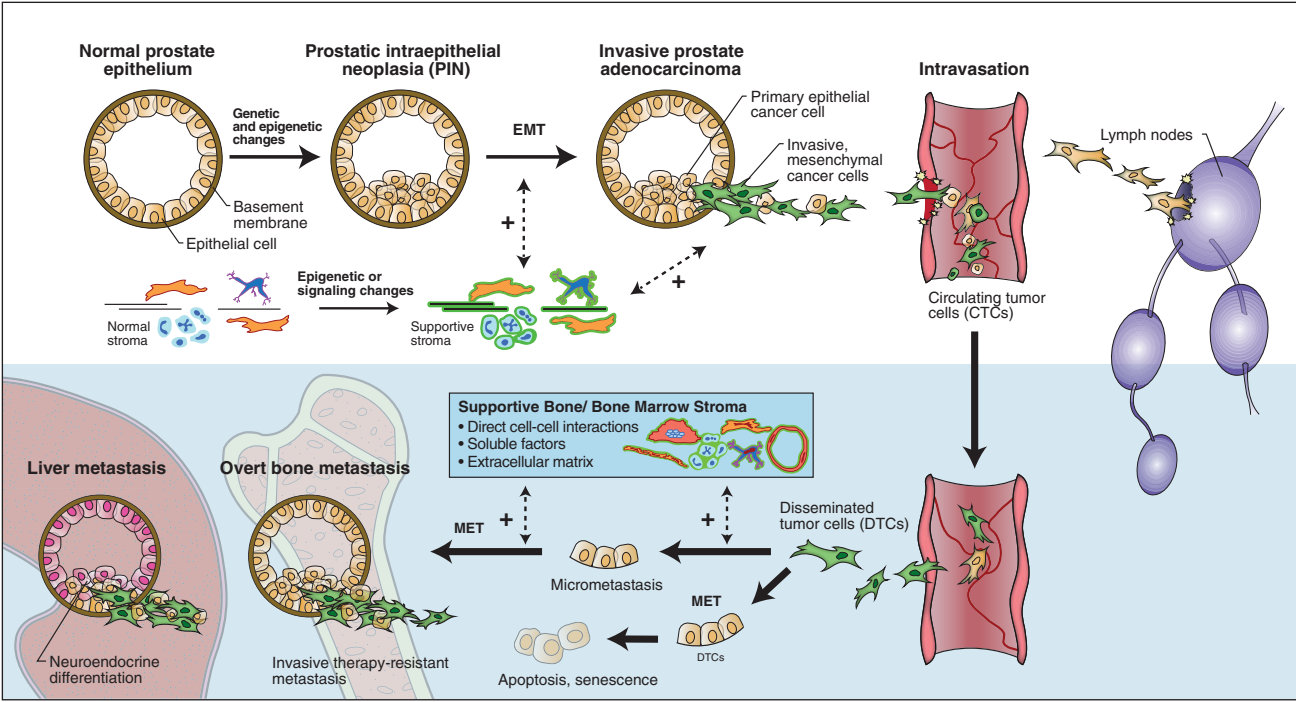


Figure 2. Epithelial plasticity during prostate cancer dissemination. Reproduced from Bitting et al.¹²⁸. Due to genetic or epigenetic changes, normal prostate cells begin to grow un-controllably, a premalignant process known as prostate intraepithelial neoplasia (PIN). In response to signaling from the surrounding stroma, some of these cells undergo an epithelial–mesenchymal transition (EMT) and invade through the basement membrane. These invasive cells enter the bloodstream and may exist as epithelial circulating tumor cells (CTCs), mesenchymal CTCs, or CTCs with a dual phenotype. Upon exiting the vasculature, disseminated tumor cells (DTCs) may sit dormant or undergo apoptosis. Other DTCs undergo a mesenchymal–epithelial transition (MET) and grow as detectable macrometastases. In prostate cancer (PC), bone metastases are typical and are initially AR dependent, progressing through a range of AR mutations or splice variants and other oncogenic and tumor suppressor mutations. Visceral metastases are atypical, are variably AR dependent, and generally involve loss of an epithelial phenotype (EP) and are enriched for a neuroendocrine or anaplastic phenotype. EP is not clearly linked to the process of lymph node metastasis; instead, nodal metastases likely involve other forms of invasion or migration.

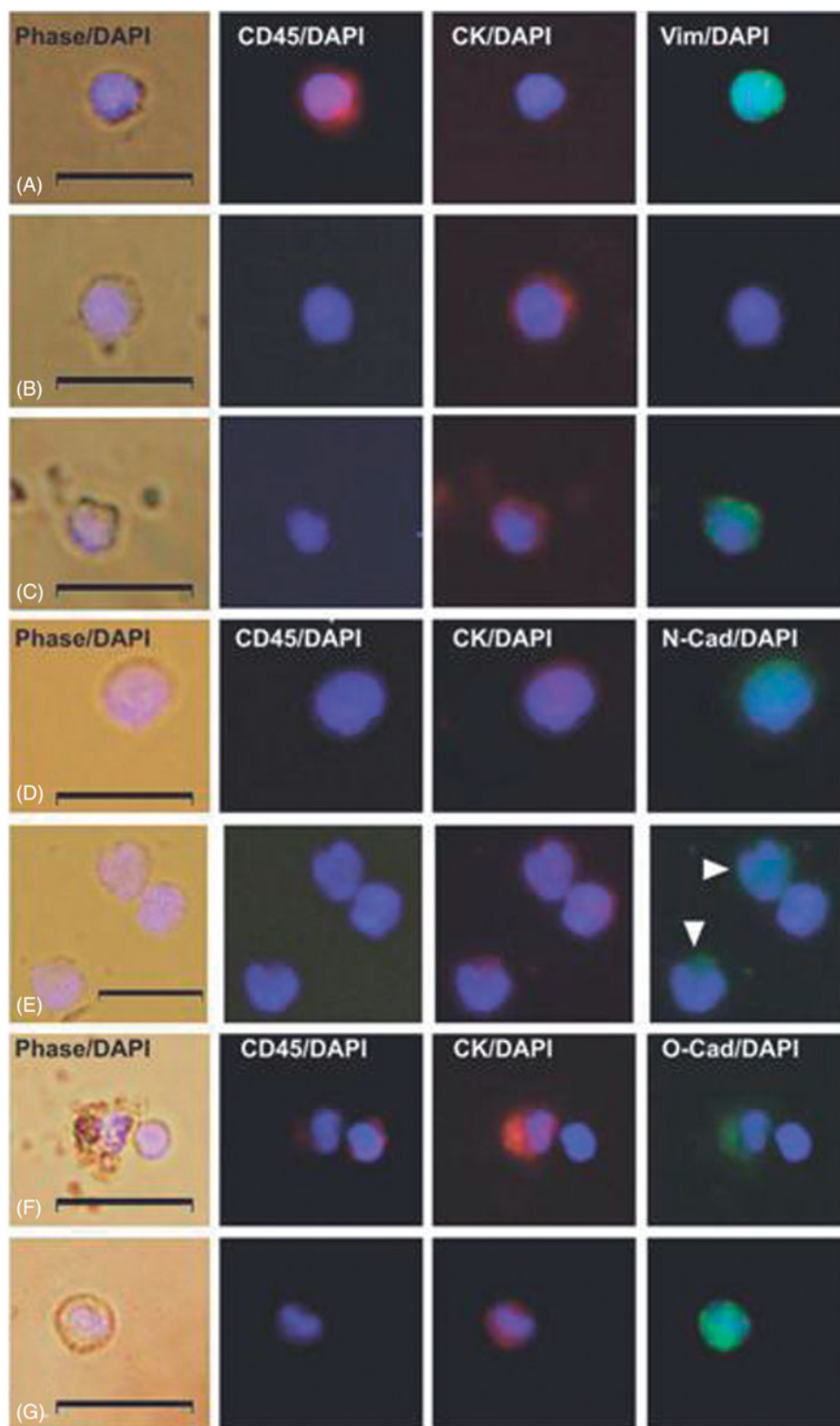
Table 4. Evidence of EMT by CTC studies in solid tumors including prostate cancer.

Cancers	EMT markers	Selected references
Prostate	Cytokeratins, vimentin, N-cadherin, O-cadherin, CD133	35
Prostate	Insulin like growth factor, epidermal growth factor receptor, forkhead box P3, transforming growth factor beta 3	111
Breast	Cytokeratins, vimentin, twist	135
Breast	Cytokeratins, vimentin, N-cadherin, O-cadherin	35
Breast	Panel of 7 epithelial and 3 mesenchymal genes by RNAish	116
NSCLC	E-cadherin, vimentin, N-cadherin	136
NSCLC	Cytokeratins, vimentin	137
SCLC	E-cadherin, vimentin, N-cadherin	136
SCCHN	Cytokeratins, vimentin, N-cadherin, CD44	138
Multiple solid tumors	Cell surface vimentin	103

metastasis (Figure 2). Though the concept of EMT is still a topic of debate in cancer, expression of mesenchymal markers in tumor tissue has been reported to be a poor prognostic factor in multiple cancers, including prostate cancer^{129–134}. Real-time analysis from CTCs should provide evidence for this process. The heterogeneous expression of both epithelial and mesenchymal markers on CTCs support previously reported partial EMT rather than “none or all”^{35,131}. Mesenchymal markers, including N-cadherin, O-cadherin, vimentin, twist, fibronectin, serpin peptidase inhibitor, have been explored in CTC EMT studies. Table 4 summarizes the reported evidence of EMT in different tumors including prostate cancer through CTCs studies.

Several groups reported the up-regulation of EMT markers in CTCs. In our study, N-cadherin and O-cadherin were shown to be commonly expressed in EpCAM-captured CTCs from mCRPC patients (Figure 3), which indicate the existence of transition of epithelial to mesenchymal phenotype³⁵. Expression of mesenchymal and stem cell markers in CTCs from a metastatic breast cancer patient was reported to be related to therapy resistance and metastasis development^{139,135}. Chen et al. demonstrated that a subset of EMT-related genes (e.g. *PTPRN2*, *ALDH1*, *ESR2* and *WNT5A*) were expressed in CTCs of CRPC, but less frequently in a small cohort of castration sensitive prostate cancer¹¹¹. This finding suggested that increased expression of EMT related

Figure 3. Co-expression of epithelial and mesenchymal proteins in CTCs from men with metastatic castration resistant prostate cancer (mCRPC). Reproduced from Armstrong et al.³⁵. All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI and either vimentin (Vim)/DAPI, N-cadherin (N-cad)/DAPI expression or O-cadherin (O-cad)/DAPI as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CTC with no vimentin expression, (C) a CTC with vimentin expression, (D) a CTC with N-cadherin expression, (E) 3 CTCs, 2 with N-cadherin expression (arrowheads), (F) a CTC with O-cadherin expression and a nearby leukocyte and (G) an additional CTC with O-cadherin expression. Scale bars represent 20 nm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in the Supplementary material.



genes in CTCs is associated with mCRPC, and these unique EMT related gene signatures may provide a new opportunity for patient stratification and personalized treatments. Given the association of EMT with taxane resistance and chemoresistance in general, the implications of these findings for improved outcomes with docetaxel in the metastatic castration-sensitive setting are intriguing¹⁴⁰.

The CTC studies in EMT have been limited by the fact that most CTC capture technologies are dependent on epithelial marker expression (e.g. EpCAM). With the development of

new CTC isolation technologies and improved CTC molecular profiling technologies, functional characterization of CTCs will help to elucidate the EMT process *in vivo* and clarify the cancer metastasis mechanisms.

Clinical utility of CTCs

In addition to biological utility, CTC analysis has the potential to be useful as a platform for clinical biomarkers. In the clinical setting, CTCs may provide prognostic, predictive,

Table 5. Potential CTC predictive biomarkers in prostate cancer.

Molecular profiling	Potential predictive value	Selected references
AR cytoplasmic sequestration	Response to taxanes	19
AR gene copy number changes	Sensitivity to second-generation AR antagonist	93
AR-V7 or loss of AR	Resistance to abiraterone and enzalutamide, possibly taxanes	8,14
AR mutation	Resistance to ADT	23
Ki-67	Resistance to castration therapy	73
TMPRSS/ERG fusion status	Sensitivity to abiraterone	18,93
PTEN loss	Response to small molecule inhibitors of the phosphatidylinositol-3-kinase (PI3K)/PTEN	18
Microtubule bundling	Response to docetaxel	46
MYCN amplification	Response to enzalutamide or sensitivity to Aurora Kinase Inhibition	115

pharmacodynamic, or surrogate uses in specific therapeutic disease states in prostate cancer, from localized disease and metastasis prevention to mCRPC and novel therapy development.

Prognostic biomarkers

A prognostic biomarker reflects disease outcome independent of therapy. CTC enumeration has been proven to be prognostic in several types of metastatic solid tumors, e.g. breast cancer, colorectal cancer, bladder cancer, NSCLC, SCLC and prostate cancer^{30,136,141–146}. CTC enumeration was reported to be an accurate and independent predictor of overall survival in mCRPC and it led to FDA clearance of CellSearch[®] for the evaluation of CRPC³⁰. Multiple studies demonstrated the independent prognostic role of CTC enumeration in CRPC: before treatment, four or fewer cells per 7.5 ml of blood were related to favorable prognosis, whereas five or more cells per 7.5 ml of blood were associated with an unfavorable prognosis. In addition, a decrease in the CTC counts to less than five after treatment was associated with improvement in OS^{30,145,146}. Favorable and unfavorable CRPC groups, stratified by CTC number, had more than 10 months difference in overall survival³⁰. Median overall survival in patients with unfavorable CTC counts at 2–5 weeks after initiation of the new chemotherapy regimen was >50% shorter than in the individuals with favorable CTC counts at this time point³⁰. The independent prognostic relevance has been confirmed in a randomized phase 3 trial of men with mCRPC treated with docetaxel ± atrasentan, in which CTCs were found to provide additional discriminatory value over PSA and other prognostic factors¹⁴⁷.

CTCs may also be useful as part of a biomarker panel in determining prognosis, and may complement other prognostic factors, such as visceral disease, PSA and pain³³.

A CTC and (lactate dehydrogenase) LDH biomarker panel was able to clearly separate survival outcomes in men with mCRPC treated with abiraterone acetate in the phase 3 post-docetaxel mCRPC trial¹⁴⁸, suggesting a role for post-treatment prognostic monitoring.

Potential predictive biomarkers

Predictive biomarkers are biological/molecular determinants or clinical parameters, which are associated with sensitivity (positive prediction) or resistance (negative prediction) to specific therapies. In CRPC, a number a prognostic biomarkers are available to guide risk stratification, however,

there are no confirmed or validated predictive biomarkers. Aside from their use as prognostic biomarkers in CRPC, the potential of CTCs to predict the response to treatment is especially attractive. The feasibility of detecting predictive molecular changes in CTCs could be applied as a non-invasive method to select patients who should receive or avoid a specific therapy. For example, detection of mutations in epidermal growth factor receptors (EGFR) in CTCs from NSCLC provides predictive value for EGFR-directed therapy in NSCLC¹¹². Demonstration of EML4-ALK fusion by FISH testing in CTCs from NSCLC patients can guide the treatment of NSCLC with ALK inhibitors^{149,150}. The presence of AR-v7 in CTCs may indicate the lack of benefit from novel hormonal therapies. Antonarakis et al. reported that none of the mCRPC patients with detectable AR-V7 in CTCs responded to enzalutamide or abiraterone, which was defined as a reduction in serum PSA levels of 50% or more⁸. These findings suggest a potential predictive value of AR-V7 in determining resistance to enzalutamide and abiraterone in CRPC patients, but this needs to be validated by large-scale prospective studies.

Molecular profiling of CTCs could help to discover predictive biomarkers in prostate cancer and to facilitate therapeutic decision making and individualization in treatment. Robust prospective randomized studies of CTCs to stratify CRPC patients are needed, and the relevance of CTC heterogeneity with treatment response and resistance development in prostate cancer needs to be unveiled. Table 5 summarizes selected potential predictive biomarkers that may be assessable in CTC studies in prostate cancer and are worthy of prospective validation.

CTCs from men with CRPC receiving taxane chemotherapy demonstrated a significant correlation between AR cytoplasmic sequestration and clinical response to taxane¹⁹. These results indicated that monitoring AR subcellular localization in the CTCs might predict clinical responses to taxane chemotherapy, and efforts are underway to test this hypothesis in the TAXYNERGY trial (NCT01718353). Resistance to androgen deprivation therapy (ADT) in general has been linked to the presence of AR variants that have truncated or spliced C-terminal regions, in which the ligand-binding domain for androgens has been disrupted. Evaluation of AR splicing variants in CTCs could help guide hormonal therapy selection and predict response or resistance to therapy¹⁵¹. Antonarakis ES et al. reported that AR-V7 was reliably detected in CTCs from men with mCRPC and detection of AR-V7 in CTC was strongly associated with enzalutamide and abiraterone resistance including lack of

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PSA declines and short progression free survival in approximately 60 patients⁸. This result indicated that AR-V7 could be used as a biomarker to predict de novo or acquired resistance to androgen pathway targeted therapies. Clinical validation of these predictive biomarkers is ongoing through a PCF-Movember global treatment sciences challenge award entitled “Development of Circulating Molecular Predictors of Chemotherapy and Novel Hormonal Therapy Benefit in Men with Metastatic Castration Resistant Prostate Cancer (mCRPC)” (Clinicaltrial.gov NCT02269982). This study will help to further validate the potential predictive role of AR-V7 across a range of platforms and in the context of whole genomic CTC analysis. With the development of next generation genome sequencing, AR mutations have been identified in CTCs from CRPC patients²³. The F876L agonist-switch mutation in AR was reported to confer genetic and phenotypic resistance to enzalutamide¹⁵².

CTCs isolated from men with CRPC exhibited wide variability in Ki67 positivity, and increased Ki67 proliferative index in CTCs was associated with resistance to castration therapy⁷². Danila et al. reported that the frequency of detection of the TMPRSS2-ERG fusion in CTCs by RT-PCR from patients with metastatic CRPC was 37%, and that androgen-driven TMPRSS2-ERG fusion in CTCs is a potential predictive biomarker of sensitivity to abiraterone⁹³. AR genomic amplification and copy number gain occurring under the selective pressure of androgen deprivation therapy have been documented in CTCs from men with CRPC and have potential predictive value for sensitivity to second-generation AR antagonists^{18,93}. PTEN loss in CTCs from men with CRPC was reported and the status of PTEN loss in CTCs may be predictive for patient response to small molecule inhibitors of phosphatidylinositol-3-kinase (PI3K)/PTEN¹⁸. Visualization and measurement of microtubule bundling in CTCs from CRPC patients can be used to monitor the drug-target engagement of docetaxel and might be useful in predicting the effectiveness of docetaxel in individual CRPC patients⁴⁶. The recently successful evaluation of docetaxel in the metastatic castration sensitive population (ECOG CHAARTED trial) suggests that CTC-based biomarkers of taxane sensitivity (such as EMT biomarkers, which can promote chemoresistance or AR-v biomarkers) may be able to identify the reason for improved outcomes in these men prior to the onset of castration resistance¹⁵³.

The amount of neuroendocrine differentiation in prostate adenocarcinoma increases with disease progression and predicts resistance to androgen deprivation therapy¹⁵⁴. MYCN amplification is seen in 40% of neuroendocrine prostate cancer (NEPC) and 5% of prostate adenocarcinoma, respectively, and has been found to induce a neuroendocrine phenotype in prostate cells^{154,155}. By aCGH analysis, MYCN gene copy number gain in CTCs from an enzalutamide resistant CRPC patient was demonstrated by our lab¹¹⁵. The result indicated that CTC assessments of MYCN expression might play a predictive role in CRPC response to enzalutamide.

With the development of novel CTC capture technologies and next generation sequencing, we anticipate a time when oncologists will detect the disseminating tumor burden as well as rapidly select targets and effective therapies

through blood-based CTC analysis. Rapid assessments of risk/benefit can be performed after brief therapeutic trials without the need to wait for radiographic evidence. However, this clinical application requires that all of the detection and characterization tools described above in this review be matched with prospective clinical trials testing this clinical utility.

CTCs as a potential surrogate biomarker in CRPC

A surrogate biomarker is a laboratory measurement or physical sign that is used in therapeutic trials as a substitute or intermediate for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions or survives and is expected to predict the full effect of the therapy on the gold standard endpoint¹⁵⁶. The gold standard for most phase 3 trials in CRPC remains overall survival; however, this endpoint is increasingly challenging to obtain given the number of newly approved agents and increasing survival times and cross-over effects. A surrogate marker is intended to substitute for overall survival and help to provide early decision-making at a trial level for the discovery of potentially active agents, and at the individual patient level for the detection of patients with a poor prognosis who need intensified or alternative treatments, or for responding patients who should remain on therapy.

Circulating tumor cell (CTC) enumeration and kinetics appear to be good candidates as OS surrogate biomarkers and are under intense investigation across multiple phase 3 trials in CRPC of abiraterone, enzalutamide, ipilimumab and other agent classes. The surrogate role of CTC enumeration in metastatic prostate cancer was evaluated in the phase III COU-AA-301 trial, which was the first phase III study to prospectively assess CTCs as a surrogate biomarker as part of a regulatory qualification process¹⁴⁵. CTC conversion, defined as converting from unfavorable (CTC ≥ 5) to favorable (CTC < 5) counts was predictive of OS as early as 4 weeks after treatment¹⁵⁷. In this study, the incorporation of CTC count changes with serum LDH demonstrated a level of individual level surrogacy for OS by correlating well with survival. Proof of CTC enumeration surrogate role requires reproduction in large clinical trials and future trials are needed to further evaluate the CTC based surrogate developed from COU-AA-301. Confirmation of the surrogate role of CTCs would help to speed up approval of novel therapies using CTC number as surrogate for OS, by increasing the efficiency and reducing the cost of novel therapeutic drug development and eliminating the OS induced bias introduced by treatment in a post-trial setting.

CTC molecular analysis for new therapeutic development

The molecular characterization of CTCs can be useful as a pharmacodynamics measure of drug effect or in selection of patients for various clinical trial designs. CTC analysis could permit the assessment of early efficacy or failure in clinical trials, as well as target engagement, which could lead to significant savings in drug development. For example, in phase I trials, novel markers in mCRPC could speed up the trial by facilitating predictive biomarker-driven patient

selection and surrogate biomarker-driven early read outs of novel drug effects. Meanwhile, access to CTC molecular profiling may offer a real-time sampling platform for pharmacodynamic studies, which allows for real-time monitoring of the drug effect on CTCs at different dose levels to determine pharmacokinetic/pharmacodynamics correlations and avoid repeated primary tumor or metastasis biopsies.

There are several challenges in the use of CTCs in new therapeutic development in CRPC. First, many men with metastatic prostate cancer lack CTCs. With the development of novel CTC technologies, more sensitive CTC capture devices may address this limitation. Second, only a limited number of CTCs are able to be detected and captured from peripheral blood while different CTC phenotypes likely exist. The ability to culture CTCs *ex vivo* for drug sensitivity testing may overcome this limitation but has not yet been demonstrated in prostate cancer^{101,122,123}.

Beyond CTCs

Cell free circulating tumor DNA (ctDNA) has been reported in various tumors including prostate cancer and is reported to be associated with unfavorable outcomes¹⁵⁸. ctDNA is believed to originate from apoptotic or necrotic tumor cells from primary tumors, metastatic lesions or CTCs^{158,159}. ctDNA has been detected in plasma and serum from prostate cancer patients, and the detection of increased DNA levels and tumor-specific DNA sequences may provide diagnostic and prognostic information^{160–162}. A higher level of ctDNA was reported to be associated with metastatic versus localized prostate cancer¹⁵⁸. CTC characterization and ctDNA analysis are complementary to each other. ctDNA analysis has the great advantage of easy collection and high throughput batched or real-time analysis, and provides the advantages of simplicity and sensitivity. However, its limitation is the restriction to measurable DNA aberrations and the uncertain source of ctDNA from viable vs. dying cells. CTC analysis can provide additional information, e.g. cell morphology, immunocytochemical phenotype, viability and the ability to reveal multiple molecular aberrations within the same cell. Further improvements in DNA sequencing technologies would allow improved genome analysis and associated gene discoveries by using both ctDNA and CTCs.

Conclusions

There is great promise in using CTCs as a platform for personalized medicine in advanced prostate cancer. The rate-limiting step for widespread use of CTCs is the lack of robust and high throughput CTC capture technologies and the lack of prospective studies on clinical utility. CTC molecular analysis is an exciting research area in CRPC and holds great promise for novel biomarker development and novel therapies development. CTC studies provide extraordinary depth in analysis of whole cell, DNA, RNA or protein based tests, and allow for cancer heterogeneity analysis for development of individualized therapies. Currently, many promising technologies for CTC isolation and analysis are ongoing, which will allow widespread use of CTCs in prostate cancer research and patient treatment. Finally, cell free methods to isolate genomic DNA and RNA are under evaluation and may

provide additional information beyond that available in CTCs¹⁶³.

Glossary

EMT: a process by which epithelial cells lose their cell polarity, and epithelial specific cell–cell adhesion and cell–matrix adhesions, and gain properties commonly found in mesenchymal cells, such as invasiveness.

Overall survival: the length of time from either date of diagnosis or start of treatment for a disease until death.

Predictive biomarker: a biomarker that identifies the likelihood of benefit from a specific therapy.

Prognostic biomarker: a biomarker that reflects disease outcome independent of therapy.

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Declaration of interest

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The role of epithelial plasticity in prostate cancer dissemination and treatment resistance

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Abstract Nearly 30,000 men die annually in the USA of prostate cancer, nearly uniformly from metastatic dissemination. Despite recent advances in hormonal, immunologic, bone-targeted, and cytotoxic chemotherapies, treatment resistance and further dissemination are inevitable in men with metastatic disease. Emerging data suggests that the phenomenon of epithelial plasticity, encompassing both reversible mesenchymal transitions and acquisition of stemness traits, may underlie this lethal biology of dissemination and treatment resistance. Understanding the molecular underpinnings of this cellular plasticity from preclinical models of prostate cancer and from biomarker studies of human metastatic prostate cancer has provided clues to novel therapeutic approaches that may delay or prevent metastatic disease and lethality over time. This review will discuss the preclinical and clinical evidence for epithelial plasticity in this rapidly changing field and relate this to clinical phenotype and resistance in prostate cancer while suggesting novel therapeutic approaches.

Keywords Epithelial plasticity · Prostate cancer · Metastasis · Epithelial–mesenchymal transition · Dissemination · Stem cell

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1 Introduction

In the USA, nearly 30,000 men die from prostate cancer (PC) each year, largely due to metastatic disease. Although the prognosis for patients with localized disease is good, for patients who develop metastatic disease, the 5-year survival rate is only approximately 30 % [1]. Androgen deprivation therapy (ADT) through either chemical or surgical castration is the first-line therapy for metastatic disease; however, response is temporary, and patients consistently progress to castration-resistant prostate cancer (CRPC), although at variable rates [2, 3]. The mechanisms underlying castration-resistant progression are likely diverse, but several key pathophysiological themes are emerging, including androgen receptor (AR) amplification, AR splice variants, and mutations in the ligand binding domain that render the AR constitutively active, as well as the induction of autocrine synthesis of androgen precursors within the PC itself [3–5]. In addition, key oncogenic drivers such as activation of the PI3K and Ras signaling pathways, loss of Rb and p53 function, and the emergence of epigenetic dysregulation and DNA repair defects underscore the complexity of advanced PC and the multifaceted genomic aberrations that promote treatment resistance.

Emerging from this genetic and epigenetic dysregulation is metastatic and hematogenous dissemination, frequently to bone, but also to other distant sites such as lung or liver. The clinical and pathological phenotype of lethal PC is quite heterogeneous, with autopsy studies demonstrating a high prevalence (>90 %) of bone metastases, and relatively high rates of visceral (liver, lung) metastases (>50 %)[6]. Histologically, metastatic PC is diverse, with some metastases exhibiting a neuroendocrine phenotype, others with poorly differentiated sheets of cells with or without spindle-like cells (sarcomatoid differentiation), and still others with a glandular well-differentiated epithelial appearance. Even

within patients, phenotypic heterogeneity is commonly observed in histological appearance and protein and RNA biomarker expression, despite an underlying monoclonal metastatic genotype and epigenome [6–9]. These findings suggest substantial cellular plasticity at the level of RNA and protein expression within a given patient that is uncoupled from mutations and chromosomal anomalies. This metastatic dissemination leads to pathological fractures, anemia, bone marrow failure, fatigue, cachexia, progressive pain, and failure to thrive, hallmarks of the lethal clinical phenotype in advanced PC. While available hormonal, immunologic, and chemotherapeutic agents provide palliation and incremental improvements in survival, treatment resistance inevitably emerges over time, and thus, novel approaches are needed in this disease.

One potential approach to understanding metastatic PC and novel therapeutic strategies is through the study of epithelial plasticity (EP). EP describes the ability of a cell to undergo reversible phenotypic changes during invasion and dissemination. EP encompasses not only the epithelial to mesenchymal transition (EMT) during initial invasion and hematogenous dissemination and its converse of mesenchymal to epithelial transition (MET) during metastatic growth and colonization but also the more general concept of loss of the epithelial phenotype and replacement with a novel phenotype. While EMT is thought to confer upon the carcinoma cell the ability to invade and seed metastatic sites, MET is proposed to enable the disseminated cells to establish macrometastatic colonies. EP is emerging as a common theme in solid tumor pathobiology that encompasses both metastatic dissemination and treatment resistance, with links to underlying embryonal stemness and invasion programs [10]. EMT pathways are causally associated with the acquisition of stem-like properties (the ability to de-differentiate and self-renew) and may link tumor dissemination with phenotypic heterogeneity. Evidence to support EP in cancer biology is robust and has been established in both preclinical models of carcinoma and in patients with carcinomas [11–15]. Furthermore, EP biology has been linked to the risk of metastasis [10, 16]. In breast cancer models, for example, the induction of an EMT results in the expression of stem cell markers, increased metastatic potential, and resistance to conventional chemotherapy [10, 17–19]. Figure 1 depicts the general concept of EP during PC cellular dissemination. This review describing the role of EP in PC progression will start with a case discussion of secondary neuroendocrine differentiation of prostate cancer.

The concept of EP is illustrated in the following clinical vignette. Patient X is a 75-year-old African American man, with prostate-specific antigen (PSA) levels that were rising for many years, who presented in March of 2009 with an extremely elevated PSA of 50. He previously

had two prostate biopsies that were negative for malignancy. His third prostate biopsy revealed Gleason 5+5=10 (high grade) adenocarcinoma with perineural invasion. Imaging revealed enlarged retroperitoneal lymph nodes up to 2 cm but no visceral or bony metastases. He was treated with combined androgen blockade, and PSA was undetectable within 9 months. Subsequent PSA and imaging progression was treated with sipuleucel-T immunotherapy followed by the novel androgen synthesis inhibitor abiraterone acetate, again with a good PSA response. However, after several months, rapidly enlarging lymph nodes in the setting of a stable PSA prompted a lymph node biopsy. The immunohistochemistry revealed strong staining for CD56 and synaptophysin with minimal PSA, prostatic acid phosphatase (PAP), or cytokeratin staining; together, these findings are suggestive of neuroendocrine differentiation. This neuroendocrine phenotypic transformation was not evident in his original prostate biopsy (Fig. 2). Evolving or secondary neuroendocrine transformation is increasingly recognized in advanced PC [20, 21] and may represent one form of EP similar to what has recently been described in lung cancer [22]. It is well documented from autopsy and pathology studies of human PC that many histological phenotypes emerge during hormonal therapy for PC, including squamous differentiation, neuroendocrine differentiation, and a general loss of markers of prostate differentiation [6, 23], as shown in Fig. 2.

Neuroendocrine differentiation (NED) occurs as one path to CRPC [24]. Although NED can arise *de novo*, it more commonly develops during hormonal therapy for PC [21]. NED does not have a strict clinical or pathological definition, but it is frequently defined histologically as the presence of neuroendocrine cells with chromogranin A or synaptophysin immunoreactivity. Chromogranin A also may be detectable in the plasma, where it correlates with the NED disease burden and is prognostic [20, 25]. The cells may also stain for synaptophysin or neuron-specific enolase, typically lack AR, and do not secrete PSA [26]. Clinically, NED is suspected when a patient has rapid disease progression, especially with visceral metastases, in the setting of a stable PSA. The presence of NED portends a poor prognosis, with frequent metastasis to the liver, transient response to chemotherapy, and survival often <1 year. While NED accounts for a large minority (perhaps 25 %) of aggressive CRPC [21], other mechanisms of EP leading to phenotypic changes are also likely to be important in human PC dissemination and treatment resistance.

This review focuses on the role of epithelial plasticity in the progression of prostate cancer, from both preclinical and clinical perspectives, and describes how EP may be associated with metastatic dissemination and treatment resistance. Additionally, we provide hypotheses and suggestions for therapeutic interventions to address EP in PC.

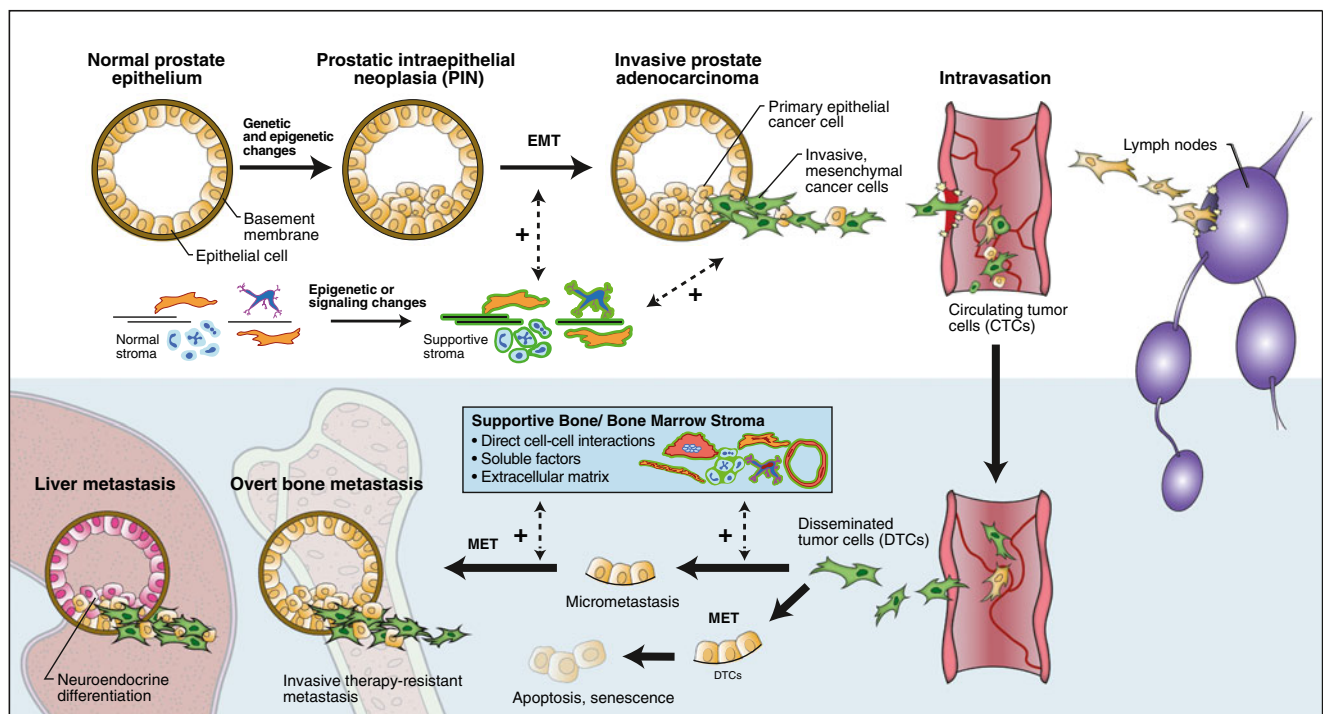


Fig. 1 Epithelial plasticity during prostate cancer dissemination. Due to genetic or epigenetic changes, normal prostate cells begin to grow uncontrollably, a premalignant process known as prostate intraepithelial neoplasia (PIN). In response to signaling from the surrounding stroma, some of these cells undergo an epithelial–mesenchymal transition (EMT) and invade through the basement membrane. These invasive cells enter the bloodstream and may exist as epithelial circulating tumor cells (CTCs), mesenchymal CTCs, or CTCs with a dual phenotype. Upon exiting the vasculature, disseminated tumor cells (DTCs) may sit dormant

or undergo apoptosis. Other DTCs undergo a mesenchymal–epithelial transition (MET) and grow as detectable macrometastases. In PC, bone metastases are typical and are initially AR dependent, progressing through a range of AR mutations or splice variants, and other oncogenic and tumor suppressor mutations. Visceral metastases are atypical, are variably AR dependent, and generally involve loss of an epithelial phenotype and are enriched for a neuroendocrine or anaplastic phenotype. EP is not clearly linked to the process of lymph node metastasis; instead, nodal metastases likely involve other forms of invasion or migration

2 Preclinical evidence of EP in PC

EP in epithelial-origin tumors (carcinomas) involves the reversible loss or reduction of epithelial biomarkers [e.g., E-cadherin, zona-occludens (ZO)-1, cytokeratin isoforms, fibroblast growth factor receptor-2 (FGFR2) isoforms, and miR-200 family] and the loss of differentiation antigens [27]. In PC, these differentiation antigens include PSA, PAP, and prostate specific membrane antigen, among others. Epithelial markers may be replaced by mesenchymal markers and transcription factors such as SNAIL, Slug, TWIST1, ZEB1/2, and others, and/or increased expression of stemness pathways, such as Hedgehog or NOTCH signaling. While NED is relatively common in PC progression, it may occur as a result of EP, a fixed evolution through novel mutations, or perhaps both [21, 28]. Suggesting the importance of plasticity, however, in lung cancer a change to a neuroendocrine-like phenotype can occur in response to treatment and is reversible when treatment is stopped [22]. Also implying the relevance of EP in dissemination and disease progression, at autopsy, many PC patients demonstrate histologic heterogeneity, in which

multiple phenotypes are evident despite an underlying clonally derived tumor, as shown in Fig. 2.

EMT and MET are highly dynamic and mediated by multiple proteins, microRNAs, and second messengers, including but not limited to those involved in transcription, posttranscriptional gene regulation, signal transduction, cytoskeletal remodeling, migration, invasion, and proliferation. Given the inherent complexity in such a system, it is likely that many incomplete or partial EP-like events take place in different contexts. One such example of an EP-like event is the mesenchymal to amoeboid transition, in which mesenchymal cells are able to alter their cellular shapes to pass through the basement membrane without degrading it [29].

Another type of EP is osteomimicry, in which PC cells can acquire bone-like properties [30]. PC most commonly metastasizes to bones, and the ability of PC cells to mimic the bone environment may enable them to survive and colonize in this new environment. The upregulation of β 2-microglobulin, an immune regulator protein, can induce EMT, promote osteomimicry, and lead to bone metastasis in mouse models of prostate and other cancers [31]. Furthermore, PC cell lines

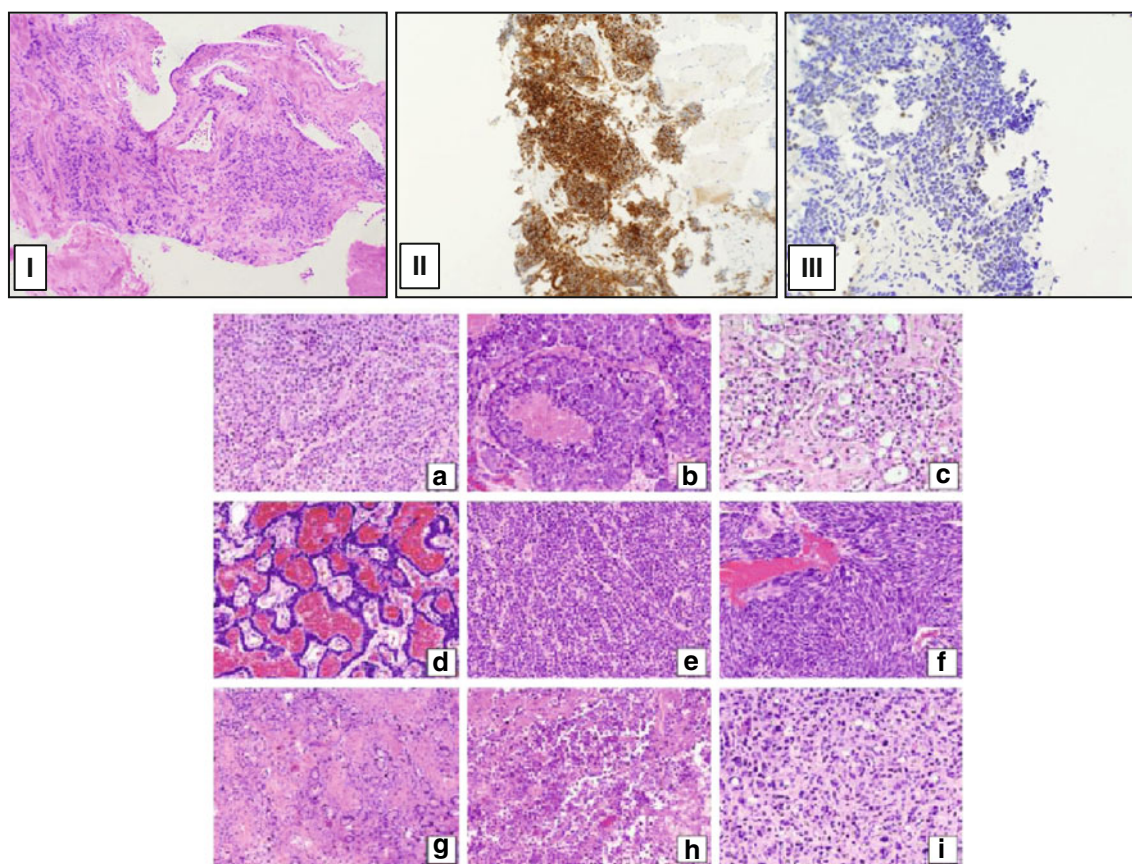


Fig. 2 Examples of prostate cancer phenotypic transformations that emerge with treatment. The *top panel* illustrates the phenotypic changes that arise during treatment of patient X, as described in the clinical vignette. His initial prostate biopsy showed high-grade prostate adenocarcinoma (*I*), but neuroendocrine differentiation emerged as his disease progressed, illustrated by strong synaptophysin (*II*) with weak PSA staining (*III*). All images are at $\times 100$ magnification. The *bottom panel*

shows the histological spectrum noted at autopsy of treated prostate cancer. **a–c** Variations of Gleason grade 4 and 5 adenocarcinoma. **d, e** Neuroendocrine differentiation. **f** Small cell carcinoma. **g** Well-differentiated Gleason grade 3 disease. **h** Undifferentiated growth pattern. **i** Signet ring differentiation. (Figure reprinted with permission from the American Association for Cancer Research: Rajal Shah et al. [6], p. 9211.)

can be forced to differentiate into osteoblast-like cells or adipose cells [32], suggesting that PC cells have the inherent capability to change phenotypes. Additional studies have established that PC cells produce soluble factors that lead to the expression of osteoblast-specific genes [33]. We have identified osteoblast (OB)-cadherin frequently in the circulating tumor cells (CTCs) of men with CRPC, illustrating the clinical relevance of this form of phenotypic change [15]. If the process of osteomimicry could be effectively targeted therapeutically, metastasis of PC to bone could potentially be prevented.

A variety of pathways and biomarkers have been confirmed to be associated with EP in cell lines and preclinical xenograft or genetically engineered models of PC; a smaller subset has been validated in human PC progression. Table 1 provides an overview of those pathways and biomarkers linked, preclinically and clinically, to EP in PC. In PC cell lines, EMT can be induced or may occur spontaneously. ARCaP cells, for example, were derived from a patient with metastatic CRPC and gave rise to stable epithelial, ARCaP_E, and mesenchymal,

ARCaP_M, sublines [34]. Other mesenchymal sublines have been generated from a parental epithelial PC line, including derived EPT1 lines, generated by *in vitro* passaging of the EP156T cell line [35] and the PZ-HPV-7T subline, generated by subrenal capsule xenografting of the PZ-HPV-7 cells [36]. PC-3 and DU145 cells additionally commonly express a range of mesenchymal and epithelial phenotypes [37]. These cell lines are valuable tools for studying EP in PC in the laboratory setting and provide further evidence for EP in clinical settings. The following sections discuss transcriptional activators or repressors of EMT/MET, signaling pathways, microenvironmental cues, microRNA regulators, stemness pathways, and other regulators of phenotypic change and the role that each play in promoting EP and dissemination in PC.

2.1 Transcriptional activation of EP

Several transcription factors have been shown to be sufficient for inducing EMT in carcinoma cell lines by repressing the E-

Table 1 Selected biomarkers and pathways associated with EP in preclinical models and patients with PC

Pathway and biomarker associated with EP in PC	Link to stemness	Link to AR signaling in PC	Validation in human PC	References
EMT-related transcription factors				
SNAIL	N	N	N	[38, 39, 41, 42]
TWIST1	Y	N	Y	[45–51]
Id-1	N	N	N	[56–61]
Slug/Snai2	N	Y	Y	[42, 43]
ZEB1/2	N	Y	N	[44, 228]
ETS-family (ERG)	N	Y	Y	[225, 227–232]
HIF-1 α	N	N	Y	[125–127]
Cell surface protein expression				
Loss of E-cadherin	Y	N	Y	[27]
N-Cadherin	Y	N	Y	[235]
OB-Cadherin	N	N	Y	[15]
EGFR	N	N	N	[109]
FGFR1	N	N	Y	[116, 122]
FGFR2 isoforms	Y	N	N	[115–117]
Stemness pathways				
Hedgehog/NOTCH-1	Y	N	Y	[173, 222]
WNT/ β -catenin	Y	Y	Y	[73–78]
NANOG	Y	N	N	[123]
BMI	Y	N	N	[199]
TGF- β signaling				
SMAD4	N	N	Y	[97]
TGF- β RIII	N	N	Y	[94]
COUP-TFII	N	N	Y	[98]
BMPs	Y	N	N	[99]
Intracellular protein signaling				
AR	N	Y	Y	[16, 64–67]
PTEN/PI3K pathway	Y	Y	Y	[68, 69]
DAB2IP	Y	Y	Y	[79–81]
EZH2	Y	Y	Y	[80, 166]
Ras pathway	Y	Y	Y	[69, 71]
NF- κ B pathway (IL-6/8)	Y	Y	Y	[82–87]
Micro-RNA species				
miR-200 family	Y	N	N	[172, 174, 177]
Chaperone proteins				
HSP27	N	Y	Y	[14, 108]

cadherin promoter; however, only a few of these transcription factors, including SNAIL, Slug, ZEB1, TWIST1, and Id-1 have been identified as having a role in EMT during PC progression. SNAIL is a zinc finger transcription factor that has been shown to induce EMT in many types of human cancers, including breast [38] and colorectal [39]. Forced expression of SNAIL in epithelial PC lines ARCaP_E and LNCaP is sufficient to induce at least a partial EMT, as evidenced by altered biomarker expression and migration. In contrast, SNAIL inhibition in mesenchymal PC-3 cells induces epithelial biomarker expression [40]. Consequently,

expression of SNAIL is thought to be both necessary and sufficient to induce EMT, but the relationship of SNAIL to human PC remains to be established. Of note, SNAIL expression also induces a neuroendocrine phenotype in PC cells [41], suggesting that SNAIL expression may play promote differentiation into several cell states. Another zinc-finger transcription factor required for the initiation of EMT in PC cells is Snai2, commonly known as Slug. Knockdown of Slug in PC-3 cells results in increased expression of E-cadherin, suggesting that Slug is required for maintenance of the mesenchymal phenotype [42]. Importantly, Slug acts as a

coactivator of AR and, in androgen-deprived conditions, provides a growth advantage to PC cells [43]. ZEB1 is another zinc-finger transcription factor that is both necessary and sufficient to induce EMT in PC [44].

Twist1, a basic helix loop helix (bHLH) transcription factor, has been most widely studied in EMT in breast cancer [45] but has also been shown to induce EMT in gastric [46] and head and neck cancers [47], and is clinically associated with distant metastasis and poor prognosis in these tumor types [48–50]. In PC cell lines, knockdown of Twist1 has been shown to induce a partial MET with an increase in E-cadherin expression, highlighting the importance of Twist1 in maintaining a mesenchymal phenotype [51]. Further supporting the role of Twist1 in EMT is the observation that epigenetic regulation of the Twist1 promoter is needed for a common p53 mutant to induce EMT. Wild-type p53 is a transcription factor that, when activated by cellular stress, promotes cell cycle arrest and apoptosis [52, 53]. Mutations in p53 are common in cancer cells, are responsible for the functional loss of the tumor suppressor, and may result in downregulation of the epigenetic regulator BMI-1 and resultant upregulation of Twist1 expression [54]. Dysregulation of p53 is common in metastatic PC, and loss of p53 function may promote EMT through Twist1 deregulation, or through a separate pathway involving microRNA deregulation [55].

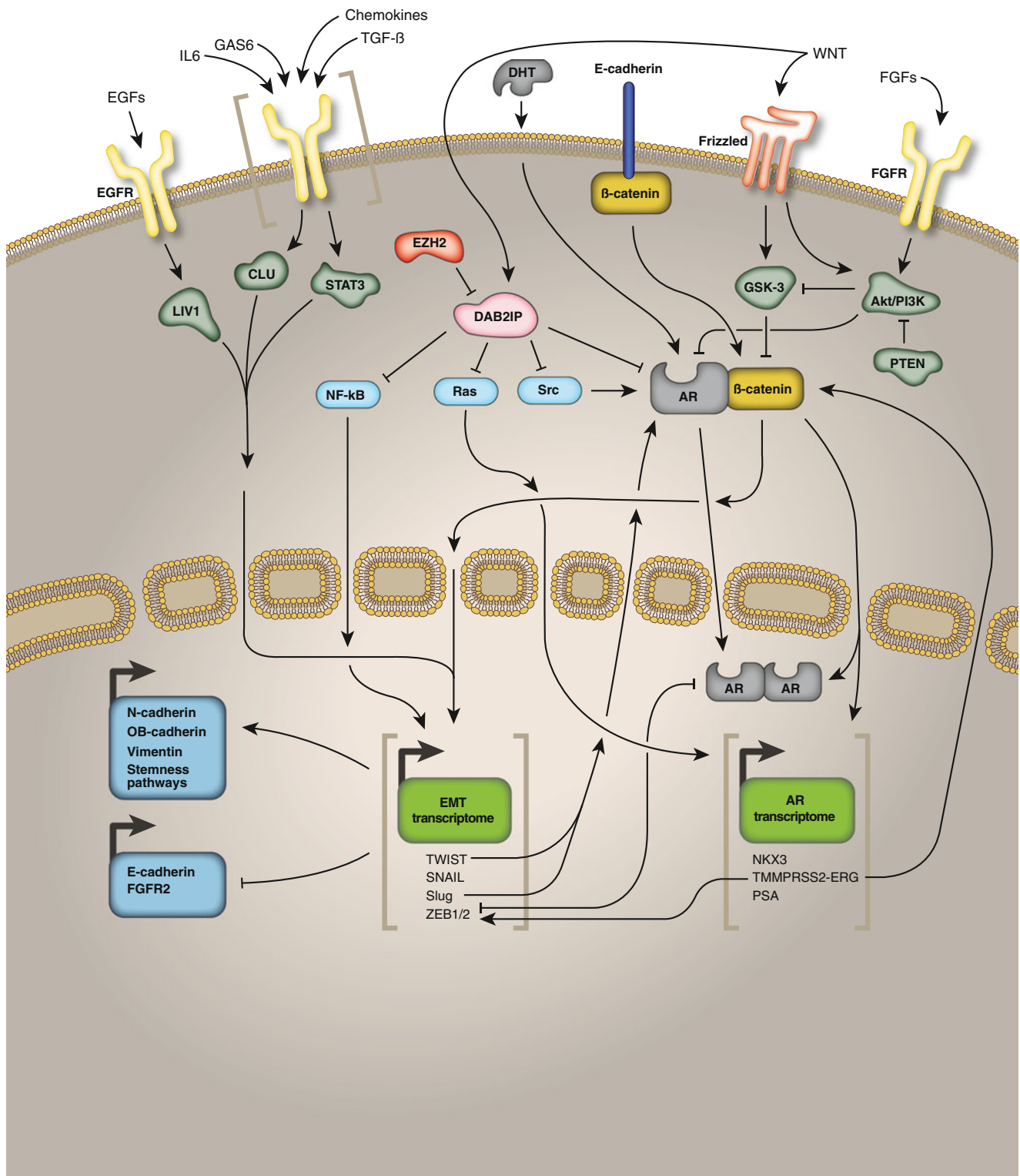
Inhibitor of differentiation/DNA binding (Id-1) is another bHLH transcription factor that has a dominant negative effect on other bHLH transcription factors because it lacks a DNA binding domain. Id-1 is involved in several physiological processes, including inhibition of differentiation and delayed senescence [56], and is upregulated in several carcinomas including prostate [57]. Id-1 interacts with caveolin-1 (Cav-1) [58], which is a membrane protein involved in signaling transduction and is upregulated in metastatic PC [59, 60]. Combined expression of Id-1 and Cav-1 induces cell migration and EMT in LNCaP and PC-3 cells. Specifically, the interaction of Id-1 and Cav-1 induces Akt activation, which is thought to be the mechanism of EMT induction [58]. Cav-1 promotes Akt activation by repressing the activity of a serine/threonine protein phosphatase, PP2A [61], and suppression of PP2A requires Cav-1 binding to PP2A [58]. Together, these results suggest that the interaction between Id-1 and Cav-1 activates Akt and subsequent EMT. Further work in human PC is needed to decipher the relationship between the Id-1 pathway and dissemination/differentiation. Interestingly, NED in human PC has been linked to deregulated PI3K/Akt/mTOR signaling, raising the possibility of a link between EP, the ID-1, and PI3K pathways, and phenotypic transformation [62, 63]. In summary, a range of transcription factors have been linked in PC cell lines and model systems to EMT and invasion and are typically accompanied by alterations in other cellular pathways important in cellular differentiation, survival, and DNA repair.

2.2 Signaling in EP

In preclinical models of PC, transcriptional activation of EP can be induced via a wide range of signaling pathways. Both intracellular activators and soluble growth factors can mediate phenotypic plasticity, and extensive crosstalk between multiple signaling pathways illustrates the importance of redundancy and feedback loops in regulating cellular survival, dissemination, and plasticity. See Table 1 for a select listing of the roles of these pathways in PC progression. In addition, Fig. 3 depicts key signaling nodes in PC that regulate EP.

AR signaling is required for normal development of the prostate [16] and is a common target for therapeutic intervention in PC. The AR pathway is activated by 5 α -dihydrotestosterone (DHT), a metabolite of testosterone, and binding of DHT to AR initiates translocation of the nucleus, where AR acts as a transcription factor to transcribe genes involved in cell cycle progression [64]. Importantly, androgens can also modulate EMT in some PC cell lines. For example, treatment of PC-3 and LNCaP cells with DHT leads to downregulation of E-cadherin and upregulation of N-cadherin and SNAIL [16]. Furthermore, knockdown of AR in LNCaP and CWR22 cells sensitizes cells to androgen-mediated EMT [16], suggesting that AR may protect PC cells from undergoing EMT in the presence of androgens, whereas AR inhibition may promote EMT. In normal mouse prostate tissue and LuCaP35 xenografts, ADT induces EMT and stemness features [65]. In LNCaP cells, AR represses ZEB1 expression and vice versa [65], indicating that a feedback loop

Fig. 3 Key signaling nodes in prostate cancer that regulate epithelial plasticity. This is a simplified and broad schematic describing the interplay of EP signaling and transcription with AR in aggressive PC. Signaling through multiple and interacting pathways leads to EMT through a variety of mechanisms. Signaling by EGFs, IL6, GAS6, chemokines, and TGF- β , through their respective receptors, can lead to increased expression of EMT transcription factors (TFs). EMT TFs, including but not limited to Twist, Snail, Slug, and ZEB1/2, can then upregulate mesenchymal biomarker expression (e.g., N-cadherin, vimentin, OB-cadherin) and downregulate E-cadherin expression. Twist also inhibits FGFR2 expression. These TFs can interact with AR in varying ways. For example, Twist and Slug can activate AR, while ZEB 1 and AR are reciprocal inhibitors of each other. AR also upregulates NKX3-1, which in turn represses Twist. When Wnt ligands are present, β -catenin moves to the nucleus and activates target genes linked to EMT and survival. β -Catenin can also act as a cofactor with AR. DAB2IP negatively regulates Ras and NF- κ B signaling and, when epigenetically silenced by EZH2, leads to EMT and PC metastasis through activation of the Ras and NF- κ B pathways. Loss of DAB2IP also activates AR through phosphorylation by Src kinase and β -catenin pathways. AR activation can lead to increased TMPRSS2-ERG fusion, which in turn can activate EMT through ZEB1/2 and increase β -catenin signaling. FGFs signal through the PI3K/Akt pathway to promote tumor proliferation, and the PI3K/Akt pathway also negatively regulates AR. DHT is the AR ligand, and when available to tumor cells, also promotes growth. Note that not all pathways discussed in the manuscript are shown in this figure



between these two proteins may exist. AR also upregulates NKX3-1, which represses TWIST1 via binding to the TWIST1 promoter [66]. Contrary to the above findings, which suggest that AR inhibits EMT, ectopic expression of AR in BPH-1 cells induces EMT, whereas knockdown of AR

downregulates EMT markers [67], suggesting that AR may play a different role in culture conditions than within the tumor microenvironment. The connections between AR signaling and EP are likely complex and context dependent, and many signaling pathways including β -catenin, Src kinase,

Akt/mTOR, and G-protein receptors can signal directly to AR independent of ligand, further adding to the complexity. Tables 1 and 2 provide an overview of these associations.

Loss of PTEN, a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and member of the Akt signaling pathway, is observed in approximately 40 % of human PC [68]. Genetic knockout of PTEN in mouse models mirrors the clinical disease course but without progression to metastatic disease [69]. To identify the additional components required for metastatic disease progression, an analysis of human PC microarrays revealed that the Ras pathway is significantly upregulated in both primary and metastatic PC tissue [69]. Interestingly, a prostate-specific Ras/PTEN-null mouse model results in PC, followed by EMT and metastasis in 100 % of mice. Models with PTEN null or Ras mutant tumors alone do not develop macrometastases, suggesting the importance of cooperative signaling in the promotion of dissemination [69]. PTEN loss is linked to the acquisition of stemness properties and loss of a differentiated phenotype in PC model systems [69, 70]. Given that aberrations in the PTEN/PI3K, AR, and Ras signaling pathways are present in nearly 100 % of metastatic PC [71], it is likely that drivers of EP are associated with these three key oncogenic pathways in CRPC.

The wingless/int1 (Wnt) gene was originally identified as a retroviral oncogene and a modulator of embryonic development in *Drosophila melanogaster* [72]. Decades later, it was shown that anomalous activation of the Wnt/ β -catenin pathway is a driver of multiple human cancers, including prostate [73]. The Wnt pathway is activated by the binding of Wnt ligands to their receptors. When Wnt ligands are present,

β -catenin moves to the nucleus and activates target genes linked to EMT, invasion, proliferation, and survival [74]. In PC, β -catenin may act as cofactor with AR [75], and increased β -catenin expression and change in localization have been observed in advanced disease [76, 77]. Another member of the Wnt family, Wnt5a, mediates EMT via activation of the membrane type I matrix metalloproteinase (MT1-MMP), which is a membrane-bound MMP involved in degrading the extracellular matrix, and is upregulated in breast and prostate cancers [78].

Also involved in the Wnt pathway, DAB2IP, a Ras GTPase-activating protein, has been shown to possess tumor suppressive properties via maintenance of an epithelial phenotype [79]. Knockdown of DAB2IP leads to EMT in PC-3 cells, while overexpression of DAB2IP decreases mesenchymal biomarker expression and migratory potential of PC cells via antagonism of the Wnt/ β -catenin pathway. Moreover, knockdown of DAB2IP in PC-3 cells leads to increased metastatic burden in a xenograft mouse model [79]. Importantly, DAB2IP levels positively correlate with E-cadherin and negatively correlate with vimentin in primary tumor tissue from PC patients [79], which supports the role of the Wnt pathway in mediating PC progression via regulation of EP. Epigenetic loss of DAB2IP has been linked to EMT and PC metastasis through overexpression of the epigenetic regulator EZH2 and subsequent downstream activation of nuclear factor κ B (NF- κ B) and Ras pathways [80]. Furthermore, the loss of DAB2IP is linked to enhanced AR activation and AR variant activity through phosphorylation by Src kinase and β -catenin pathways, providing a novel

Table 2 Selected clinical states of PC and evidence of associations with EP as a treatment resistance mechanism

Clinical Disease State of PC	Description of EP Association with Outcome	Direct evidence from men with PC	References
Localized disease			
Surgery (radical prostatectomy)	E/N cadherin switch associated with PSA recurrence, metastasis after surgery	Y	[223]
	Loss of CK or PSA expression, increased TWIST or vimentin in localized disease correlates with outcomes	Y	[221, 224]
Radiation therapy	Induction of WNT16B in stroma mediates radioresistance in PC	N	[150]
Active Surveillance	ERG overexpression in biopsy specimens associated with progression during surveillance	Y	[229]
PSA recurrent disease			
Androgen deprivation therapy	ADT induction of EMT transcription factors	Y	[65]
Metastatic PC			
Immunotherapy	Immunotherapy against epithelial targets leads to mesenchymal tumor escape	N	[240]
Docetaxel chemotherapy	Loss of CK, overexpression of stemness pathways (NOTCH/Hedgehog) in docetaxel-treated metastases, PC cell lines	Y	[200]
Cytotoxic DNA-damaging agents	Induction of DNA-stress response in stroma leads to WNT16b induction and EMT, treatment resistance to mitoxantrone	Y	[150]
Circulating tumor cell expression	Common expression of vimentin, N-cadherin, CD133, OB-cadherin in CTCs from men with metastatic CRPC	Y	[15]

link between EMT, dissemination, and AR signaling mediated through the epigenetic and thus reversible loss of DAB2IP [81].

NF- κ B transcription factors regulate a variety of immune and inflammatory responses and developmental processes (reviewed by [82]). Levels of NF- κ B correlate with prognosis in PC patients, and increased NF- κ B signaling correlates with disease progression in a subset of PC patients [83]. NF- κ B regulates EMT by directly or indirectly upregulating multiple EMT transcription factors, the mesenchymal intermediate filament protein vimentin, and matrix metalloproteases MMP2 and MMP9 [84]. In addition, IkappaB kinase alpha activation by receptor activator of NF- κ B ligand (RANKL) inhibits expression of the Maspin protein and metastatic dissemination. Maspin is a serpin family member, expression levels are inversely correlated with metastatic potential in human PC, and its signaling or epigenetic regulation may be causally related to dissemination [85]. In PC cell lines, induction of EMT leads to upregulation of RANKL [86]. Interestingly, RANKL activation results in osteoclastogenesis *in vitro* [86], suggesting that upregulation of RANKL via EMT induction may promote skeletal metastasis. NF- κ B also mediates EMT via downregulation of fibulin and activation of CXCL1/GRO α [87], a chemokine that promotes angiogenesis and enhances cancer cell proliferation [88]. These examples highlight the complexity of signaling networks that may cooperate to drive EMT and the metastatic cascade in advanced disease.

One of the best-studied initiators of EMT is the transforming growth factor beta (TGF- β) family of cytokines and their receptors, TGF- β RI, II, and III. TGF- β can induce EMT, as evidenced by increased expression of mesenchymal biomarkers in multiple PC cell lines [89]. Importantly, TGF- β can induce EMT in an androgen-independent cell line, PC-3, and in an androgen-dependent line, LNCaP, suggesting that the ability of TGF- β to induce EMT is independent of AR expression [90]. TGF- β treatment also induces clusterin (Clu) expression during EMT, with Clu functioning as a molecular chaperone to protect against cellular stresses [91]. Clu is transcriptionally activated by TWIST1, and this activation is required for TGF- β -induced EMT [89]. Clu has emerged as an important therapeutic target in men with CRPC, and given its role in mediating chemotherapy resistance, its link to EP may be equally important [92, 93]. In addition, loss of TGF- β RIII is common in human PC, through deletions or epigenetic dysregulation, and this is accompanied by enhanced invasion and relapse after surgery [94]. The paradox of TGF- β signaling in human PC, in which there is increased TGF- β expression and tumor suppression early in the disease, followed by tumor promotion during disease progression, may be explained through altered intracellular signaling. Specifically, TGF- β signaling may initially promote invasiveness and escape from the primary tumor microenvironment; however,

loss of TGF- β in distant metastasis may promote an epithelial phenotype and ultimately colonization [95, 96]. For example, loss of SMAD4 is consistently identified in metastatic as compared to localized PC, indicating that loss of this tumor suppressor may facilitate dissemination [97]. Importantly, SMAD4 was identified as a component of a four-gene signature, along with PTEN, cyclin D1, and SPP1, that is prognostic of biochemical recurrence and metastatic disease in human PC [97]. It has recently been shown that COUP transcription factor II (COUP-TFII) regulates SMAD4-dependent transcription in PTEN-null tumors, making a TGF- β dependent checkpoint ineffective and leading to EMT and metastasis [98]. Taken together, loss of SMAD4 signaling and altered TGF- β signaling is associated with the acquisition of an invasive phenotype and metastatic dissemination in PC. Finally, a TGF- β superfamily member, bone morphogenetic protein-7, protects against bone metastases in PC through the induction of epithelial differentiation [99], possibly by counteracting SMAD family members. However, the role of BMPs and TGF- β signaling in general in mediating EP and PC dissemination remains an area of active investigation.

The role of the interleukin-6 (IL-6)/STAT3 pathway, which activates inflammatory responses during infection and oncogenesis [100, 101], in EMT has been demonstrated in head and neck [102], nonsmall cell lung [103], and breast cancers [104]. This pathway may also be important in PC, as IL-6 can induce EMT in some PC cell lines. Importantly, induction of EMT by IL-6 requires Hsp27 expression. Specifically, knock-down of Hsp27, an ATP-independent molecular chaperone that is induced in response to stress [105–107], reverses the pro-EMT effect of IL-6. The role of Hsp27 in IL-6-induced EMT is likely through the transcriptional activation of TWIST1. Hsp27 expression is required for TWIST1 expression upon treatment with IL-6, and transcriptional activation is mediated by direct binding of STAT3 to the TWIST1 promoter [108]. Taken together, this suggests that Hsp27 is needed for IL-6-induced EMT but also can act independently to induce EMT. IL-6 has also been linked to activated stromal and immune cell cross-talk and induction of EP/stemness in PC [14], suggesting a complex relationship between the tumor microenvironment and EP.

Other pathways implicated in PC progression preclinically through an EP mechanism include the following: (1) the epidermal growth factor receptor (EGFR) pathway via expression of LIV-1, a zinc transporter [109]; (2) macrophage inhibitory cytokine-1, a member of the TGF- β superfamily that plays a key role in regulating growth and differentiation in response to stress [110–112]; (3) β 2-microglobulin mediation of the hemochromatosis protein, a member of the nonclassical major histocompatibility complex signaling pathway [31]; (4) the kallikrein family of serine proteases, which induce EMT and invasiveness [113]; and (5) ubiquitin C-terminal hydrolase-L1,

UCH-L1, a deubiquitinating enzyme, the expression of which is both necessary and sufficient to induce EMT [114].

2.3 Alternative splicing in EP

There is evidence that fibroblast growth factor receptor (FGFR) signaling plays a role in PC onset and progression. The FGFRs are a family of four receptor tyrosine kinases (FGFR1-4) that bind to a family of fibroblast growth factors (FGFs) (reviewed in [115]). Binding of FGFs induces dimerization of the receptors and signaling via MAPK and PI3K/Akt pathways. FGFR1-3 transcripts are alternatively spliced within their ligand binding domains to give IIIb and IIIc isoforms. The IIIb and IIIc isoforms are typically expressed exclusively in epithelial and mesenchymal cells, respectively [115]. A switch from FGFR2-IIIb to FGFR2-IIIc in nontumorigenic rat prostate epithelial cells leads to malignancy [116]. A subset of human PC specimens expresses the IIIc isoform, although metastatic samples predominantly express the epithelial IIIb isoform [117]. Work from our group and others has identified several splicing factors that regulate FGFR2 isoform switching, including PTBP1 [118], RBFOX2 [119], and ESRP1 [120]. Interestingly, RBFOX2 and ESRP1 have also been implicated in mediating numerous splicing events that help to maintain mesenchymal or epithelial phenotypes, respectively, in breast cancer cell lines [121]. It is possible that these splicing factors may play a role in EP during PC by inducing FGFR2 isoform switching and by regulating the splicing of a number of other EP-related transcripts.

Both FGFs and FGFRs are known to be upregulated in PC, including FGFs 1, 2, 6, 7, 8, and 9 and FGFR1 [115], and inducible expression of FGFR1 leads to adenocarcinoma and EMT in a mouse model of PC [122]. FGFR1-induced adenocarcinomas show loss of the epithelial-specific IIIb isoform, increases in Sox9, MMP15, and genes related to TGF- β signaling, and metastases to the liver and lymph [122]. The lack of validated FGFR isoform-specific antibodies has impaired the translation of these findings in human PC, and this work is ongoing.

2.4 Microenvironmental cues as mediators of EP

The effect of hypoxia within the tumor microenvironment on EP has been widely studied in human cancer. Hypoxia is capable of inducing EMT in PC-3 and LNCaP cells, as evidenced by a switch to a more mesenchymal morphology and increase in mesenchymal biomarker expression [90]. Additionally, PC-3 cells grown under hypoxic conditions have an increased migratory and invasive phenotype. Hypoxia also induces transcripts associated with stemness, including Nanog and EZH2 in PC-3 and LNCaP cells [123].

The molecular mechanism of hypoxia-induced EMT is through the stabilization of HIF-1 α , a transcription factor expressed at low oxygen concentrations [124]. Importantly, PC-3 cells grown under hypoxic conditions have increased HIF-1 α protein expression [123] and forced expression of HIF-1 α in LNCaP cells results in a partial EMT, as evidenced by a decrease in E-cadherin expression and an increase in vimentin [125, 126]. In addition, in other model systems, HIF-1 α expression increases TWIST1 transcription to promote EMT and metastasis [127, 128]. To date, however, regulation of TWIST1 by HIF-1 α in PC has not yet been studied.

Hypoxia also plays an indirect role in the initiation of the EMT cascade by stabilizing the Axl/GAS6 axis. Axl is a receptor tyrosine kinase that induces cell survival/proliferation upon binding its ligand, GAS6. The Axl/GAS6 pathway is important for metastasis of several carcinomas [129–133], and is adversely prognostic [134–138]. Axl is necessary for EMT, as evidenced by reduction in mesenchymal biomarkers and increased migration and invasion upon knockdown of Axl in PC cells [139]. GAS6 downregulates expression of its receptor, Axl, and hypoxia is sufficient to prevent GAS6-mediated downregulation of Axl. Therefore, hypoxia acts to stabilize Axl/GAS6 signaling, which ultimately results in induction of EMT [139].

Another mechanism by which the tumor microenvironment can contribute to EP is by fibroblasts in the host stroma, which secrete soluble factors, such as growth factors and extracellular matrix [140, 141]. Activated fibroblasts (AFs) are necessary for the growth and differentiation of PC cells [142, 143], and AF can induce EMT. Specifically, prostate-specific fibroblasts isolated from men with benign hyperplasia and can be activated by either TGF- β treatment or by exposure to conditioned media from PC-3 cells to induce EMT. EMT induction in PC-3 cells also promotes stemness, as evidenced by an increase in prostasphere formation, an increase in CD133 positive cells, and an increase in the percentage of CD44^{high}/CD24^{low} cells [14]. Furthermore, induction of EMT in PC-3 cells activates the COX-2 pathway and HIF1A, both of which are involved in the inflammatory response. Upon knockdown of COX-2 and HIF1A in PC-3 cells, EMT cannot be induced, suggesting that the proinflammatory axis is required for initiation of EMT. In addition to initiating an inflammatory response, induction of EMT in PC-3 cells also results in reactive oxygen species (ROS) production. With inhibition of ROS production by treatment with antioxidants, prostate AF can no longer induce EMT, stemness, or the inflammatory response pathway [144]. Together, these data suggest that prostate AF produce ROS and activate the proinflammatory response to induce EMT and stemness [14, 144].

The generation of ROS has been associated with EMT in several model systems, including human ovarian carcinoma

cells [145], renal tubular epithelium [146], and mammary epithelial cells [147]. In the context of PC, there are conflicting reports about the role of ROS in mediating EP. For example, ROS increase during SNAIL-induced EMT, and a ROS scavenger, *N*-acetyl cysteine, causes a partial reversion of EMT [148]. On the contrary, psoralidin, a natural pro-oxidant chemical, induces ROS production, but leads to downregulation of β -catenin and Slug, upregulation of E-cadherin, and inhibition of migration and invasion in PC cell lines [149]. While it remains unclear whether ROS stimulates or prevents EMT, it is possible that different ROS levels can have variable effects on the phenotypic status of a cell. For example, moderate ROS can induce cell proliferation, but higher levels lead to apoptosis ([149] and references therein). In addition to hypoxia and ROS, stromal cells can induce EMT through a range of soluble mediators such as chemokines and the soluble protein WNT16B. DNA damage from radiation or chemotherapy can induce WNT16B and promote EMT in the neighboring prostate epithelial cells, leading to invasion and treatment resistance [150]. Furthermore, activated fibroblasts and other stromal cells such as fat cells or bone marrow derived cells may be recruited into the prostate from distant sites to promote EP [151]. Thus, a number of microenvironmental and host insults can promote EP, dissemination, and treatment resistance in PC. In addition to microenvironmental drivers of EMT, there is also evidence that MET in metastatic colonization may be mediated by the microenvironment. For example, DU-145 cells re-express E-cadherin upon coculture with human hepatocytes, and re-expression of E-cadherin also leads to chemoresistance, suggesting that MET may serve a protective role against chemotherapeutics at metastatic sites [152]. Similarly, coculture of DU-145 and PC-3 cells with primary rat hepatocytes leads to re-expression of E-cadherin and cytokeratin and reduced levels of vimentin [153], and coculture of ARCaP_M cells with bone marrow stromal cells results in re-expression of E-cadherin in the ARCaP_M cells [154], lending further support for the idea that microenvironmental cues at the sites of metastatic dissemination may lead to MET. Using a reporter of MET based on alternative splicing of a mesenchymal IIIc exon of FGFR2, clusters of MET can be identified within AT3 Dunning rat mesenchymal prostate tumors [155]. These regions of MET also express E-cadherin and ZO-1 and localize to areas rich in collagen, suggesting that the interaction of tumor cells with collagen or some other microenvironmental driver may contribute to MET.

2.5 Epigenetics in EP

Histone deacetylase inhibitors (HDACI) have been studied as potential cancer therapeutic agents based on the increased expression and activity of HDACs in carcinomas (as reviewed in [156]). When evaluating the efficacy of HDACI in PC cell

lines, the cells unexpectedly undergo EMT upon treatment with both suberoylanilide (SAHA) and trichostatin A (TSA), as evidenced by a more mesenchymal morphology, upregulation of ZEB1 and vimentin, and increased stemness and migration. The mechanism by which HDACI induce EMT is thought to be via hyperacetylation of EMT promoters, which create a more relaxed chromatin state to promote transcription. Specifically, PC-3 cells treated with TSA and SAHA have an increased amount of acetylated histone 3 associated with the vimentin, ZEB2, and slug promoters, which results in increased EMT signatures [157]. These findings may explain the limited single agent activity of HDACIs in the clinic as therapy for CRPC and suggests that combination approaches are needed [158].

Despite the limited utility of HDACI in clinical treatment of PC, there is evidence for the importance of epigenetic changes in PC. For example, multiple myeloma SET domain (MMSET), a histone methyltransferase that is associated with the dimethylation of histone H3 lysine 36, a mark of active transcription [159], can be upregulated in PC [160], with high expression associated with PC recurrence [161]. Overexpression of MMSET in PC cells leads to increased expression of mesenchymal biomarkers and a more migratory and invasive phenotype. Conversely, knockdown of MMSET in PC cells leads to decreased migration and invasion. MMSET promotes EMT by binding the TWIST1 promoter and increasing TWIST1 transcription, suggesting that MMSET epigenetically regulates TWIST1 to induce EMT [162].

SIRT1 is another histone deacetylase, which is implicated in the stress response [163] and apoptosis [164] and induces EMT in PC cells. Moreover, knockdown of SIRT1 in PC cells induces MET. ZEB1 is required for SIRT1 to induce EMT, as ZEB1 recruits SIRT1 to the E-cadherin promoter for deacetylation of histone H3, which suppresses E-cadherin transcription. This suggests that ZEB1 interacts with SIRT1 to downregulate the E-cadherin promoter to induce EMT [165]. Likewise, enhancer of zeste homolog 2 (EZH2), which is involved in gene silencing by histone methylation, is overexpressed in advanced PC and can mediate the silencing of E-cadherin [166]. Interestingly, a survey of primary PC samples and metastatic bone biopsies showed that 70 % of primary PC samples have a methylated E-cadherin promoter with heterogeneous E-cadherin expression, while 87 % of metastatic bone biopsies contain an unmethylated E-cadherin promoter with homogenous E-cadherin expression [167]. Together, these results demonstrate that EMT can be epigenetically regulated and provide a mechanism linking EMT with PC progression.

2.6 MicroRNAs in EP

MicroRNAs (miRs) are important regulators of gene expression that play diverse roles in development, metabolism, and

the pathogenesis of cancer (as reviewed in [168, 169]). Several miRs have been shown to regulate EP, including miR-21, miR-31, miR-29a, miR-135, and the miR-200 family (reviewed in [170]). In PC, ectopic expression of miR-1 or miR-200 precursors reduces Slug-dependent EMT, restores E-cadherin expression, and significantly reduces the invasive potential of PC-3 cells [171]. Similarly, PC-3 cells overexpressing platelet derived growth factor D undergo EMT, which leads to reduced levels of miR-200 family members [172]. Re-expression of miR-200b induces MET [172] and represses NOTCH1, a driver of stemness [173]. Taken together, these data suggest that miR-200b acts as a tumor suppressor at least partially through regulation of NOTCH1 expression.

Loss of the ZEB1 and ZEB2 repressors, miR-200c and miR-205, has been shown in docetaxel resistant PC-3 and DU145 lines and re-expression of either miR led to E-cadherin upregulation [174]. This suggests that loss of these miRs during docetaxel-mediated EMT may contribute to chemotherapeutic resistance. Additional studies have shown that expression of miR-182, miR-203, and miR-29b in mesenchymal prostate cells can induce MET [175]. While many miRNAs have been associated with MET, miR-21 has been shown to induce EMT in RWPE-1 cells [176] and is the only mesenchymal-specific miRNA currently identified in human PC.

Although there are a number of *in vitro* studies on miRs in PC, few studies have investigated levels of EP-related miRs in PC specimens. While both miR-200c and miR-29 contribute to an epithelial phenotype *in vitro*, the correlation between these miRNAs and clinical outcome is less clear. For example, the epithelial specific miRNAs, miR-200c and miR-29b, are upregulated in men with CRPC compared to those with localized disease [177] and in patient-matched normal tissue compared to PC [178]. This is inconsistent given that an epithelial-specific miRNA is associated with both metastasis and healthy prostate tissue. Similarly, the mesenchymal-specific miRNA miR-21 is higher in CRPC tissues compared to localized PC [177]. One possible explanation is that an epithelial phenotype can be simultaneously associated with normal prostate tissue and also be needed for metastatic colonization via MET. It is conceivable that the mesenchymal miR-21 is associated with an early metastatic event, while miR-200c is associated with a late metastatic event that requires MET for colonization. Further complicating the relationship between miRs, EP, and clinical outcome, the loss of epithelial-specific miR-205 reduces time to biochemical recurrence in human PC [179].

2.7 Dietary and small molecule control of EP in PC

A number of dietary substances and small molecules can induce epithelial differentiation (MET) and possibly invasion in PC cell lines. One of the most frequently cited supplements

is silibinin, the active compound in milk thistle extract, which has shown some promise as a regulator of EP. Silibinin treatment inhibits growth of PC cell lines [180], synergizes with various chemotherapeutic compounds to induce apoptosis [181–185], and attenuates AR signaling by inhibiting AR nuclear localization [186]. Furthermore, silibinin also mediates MET in PC cells, as evidenced by reduced proliferation, adhesion, and migratory potential of PC cell lines [187], decreased expression of mesenchymal biomarkers [188], and upregulation of the epithelial biomarkers [189]. HIF-1 α , which induces EMT in response to hypoxia via upregulation of TWIST1 [128], is also inhibited by silibinin [190].

Dietary consumption of another compound, Genistein, an isoflavone found in soy beans, is associated with a lower risk of PC and PC metastasis ([191] and references therein). Interestingly, Genistein treatment results in MET of PC cells, as evidenced by altered biomarker expression and decreased invasion [191]. Exposure of Genistein also reduces CD44+ cancer stem cells, inhibits the Hedgehog-Gli1 pathway [192], and upregulates miR-574-3p, which decreases proliferation, migration, and invasion of PC cells [193]. Pathway analysis indicates that miR-574-3p controls several genes involved in the Jak-STAT and Wnt signaling networks [193]. This suggests that a small molecule, Genistein, controls EP via miR-mediated regulation of the Wnt and other signaling pathways.

Treatment with the proteasome inhibitor salinosporamide A (NPI-0052) also causes an MET in the mesenchymal-like DU-145 cells, with reduced levels of SNAIL and upregulation of E-cadherin. SNAIL repression is driven by inhibition of NF- κ B and upregulation of Raf kinase inhibitory protein (RKIP), a known inhibitor of metastasis [194]. RKIP expression in DU145 cells leads to reduced levels of SNAIL expression, whereas SNAIL overexpression in LNCaP cells antagonizes RKIP levels, leading to increased metastatic capacity. Moreover, treatment with a specific NF- κ B inhibitor, dehydroxymethylepoxyquinomicin, mirrors the EMT repression that is observed upon treatment with salinosporamide A [194]. Together, these results implicate the proteasome as a potential modulator of EMT via a SNAIL/NF- κ B/RKIP pathway.

2.8 Stemness as a mediator of EP

Work by the Weinberg laboratory and other groups has shown that EMT results in enrichment of cell populations with stem cell properties of self-renewal, clonogenic growth, and ability for differentiation in several cancer models [10, 195, 196]. In PC, CD44+ LNCaP and DU145 cells lose E-cadherin and are more invasive than their CD44- counterparts [197]. EMT has also been associated with the acquisition of a stem-like phenotype in PC-3 and ARCaP_M cells [173]. Similarly, knock-down of the ETS transcription factor ESE/EHF in immortalized prostate epithelial cells leads to EMT, acquisition of stem-

like properties, tumorigenic capability, and metastatic dissemination [198]. Association of cancer-associated fibroblasts with PC-3 cells also leads to EMT, along with upregulation of CD133 and an increase in CD44^{high}/CD24^{low} cells, which display self-renewal capacity and tumorigenicity [14]. In PC model systems, overexpression of the polycomb repressor BMI-1 is required for de-differentiation, prostate stem cell renewal, and has been linked to malignant transformation [199]. In the clinical context, evidence for EP and stemness can be found in the examination of CTCs from men with CRPC. CTCs have been found to coexpress epithelial and mesenchymal markers, and >80 % of CTCs from CRPC patients also express the stemness marker CD133, suggesting that stemness may play a role in modulating EP during metastatic dissemination through the vasculature [15]. Finally, evidence is strong for the loss of epithelial biomarker expression during castration and chemotherapy-resistant progression in human PC, and this EP is linked to upregulation of stemness pathways including Hedgehog and NOTCH signaling, suggesting the importance of the dual regulation of EP by these embryonic programs [200].

There is, however, also evidence of PC cell lines in which cancer stem cells are enriched for an epithelial phenotype. E-Cadherin positive subpopulations of DU145 and PC-3 cells express embryonic stem cell markers SOX2, OCT3/4, Nanog, and c-Myc. Furthermore, the E-cadherin positive populations form tumors, while E-cadherin negative sublines do not [201]. Additionally, DU145 cells treated with chemotherapy generates drug-tolerant lines with low tumor initiating capacity, and addition of 5'-aza-deoxycytidine to drug-tolerant cells leads to re-expression of E-cadherin and CD44+, with increased tumorigenic potential [202]. Moreover, it has been shown that an epithelial-like subpopulation of PC-3 cells is enriched in tumor initiating cells (TICs) while the mesenchymal subpopulation are depleted in TICs [37]. Overexpression of SNAIL in the epithelial-like TICs reduces their self-renewal and metastatic capacity, concomitant with an EMT-like event [37]. Conversely, combined knockdown of SNAIL, ZEB1, and TWIST leads to an epithelial phenotype, enhanced spheroid formation, and self-renewal programs [37]. In a review of CSCs in PC, a distinction is made between TICs and CSCs, highlighting that the existence of TICs suggests the clonality of tumor cells rather than a hierarchical structure of the tumor [203]. Yet, despite this distinction, the data surrounding CSCs and EP highlight the dynamic and complex relationships between stem-like programs and EP pathways and suggest that EMT may not be the sole driver of PC cell tumorigenicity and invasive potential.

Based on these findings, we hypothesize that it is the ability to interconvert reversibly between epithelial and nonepithelial stem-like phenotypic states (plasticity) that drives metastatic spread and lethality in PC (and likely other solid tumors), rather than the epithelial or mesenchymal state in isolation.

3 Evidence of EP in treatment-resistant and disseminated PC

The above sections suggest a role for EP in the development of invasiveness, treatment resistance, and dissemination in PC model systems. Observing this plasticity in the clinic is a greater challenge given that EP is transient, may occur in rare cellular populations at the invasive edges of the tumor, and that the gold-standard biomarkers of EP in PC are still being defined. Furthermore, metastatic tissue in PC is not collected or analyzed routinely, metastatic tissue architecture and phenotype can be heterogeneous, and the ability to observe EP biomarkers in patients is likely context dependent. EP is linked to drug resistance [204], and there is emerging evidence that EP mediates resistance to local therapy (surgery or radiation), hormonal therapies, immunotherapies, and chemotherapeutics commonly used to treat PC. The following sections detail the clinical evidence to supporting a causal relationship between EP and treatment failure due to resistance in human PC. Selected clinical states of PC and their associations with EP are highlighted in Table 2.

3.1 Detecting EP in PC

One of the challenges in establishing the existence and relevance of EP in PC metastasis is the difficulty visualizing the process. To establish distant metastases, invasive cancer cells likely circulate in the bloodstream and settle in other organs, which in CRPC is often bone. Evidence supporting EP is found through an analysis of circulating tumor cells (CTCs). CELLSEARCH® (Janssen/Veridex) is the only FDA-cleared technology for the detection of CTCs, which are defined as nucleated, cytokeratin-positive, and CD45-negative cells immunomagnetically captured from the bloodstream using antibodies against epithelial cell adhesion molecule [205]. CTCs can be enumerated to provide prognostic information in multiple tumor types [206–209], but more importantly, CTCs carry genotypic and phenotypic information about an individual's tumor at a discrete point in time. A substantial number (30–40 %) of men with advanced metastatic CRPC do not have detectable CTCs using the CELLSEARCH® epithelial-based method [210], and recent evidence indicates that there is phenotypic heterogeneity among CTCs, with some CTCs expressing not only epithelial proteins but also mesenchymal and stemness proteins, indicators of EP [15]. We have found that a range of EP biomarkers are expressed in CRPC CTCs, including loss of E-cadherin and gain of N-cadherin, vimentin, CD133, β -catenin, and OB-cadherin. Importantly, many CTCs have a dual epithelial and mesenchymal/stemness phenotype, suggesting the importance of this duality in treatment resistance and dissemination [15]. This EP biology is not unique to PC, as variable phenotypes have been observed in CTCs from other malignancies, such as

lung [211, 212], colorectal [213], and breast cancer [214], suggesting a broad conceptual parallel. Therefore, EP may explain the underdetection of CTCs in patients with advanced malignancy using the standard epithelial antigen-based technology [15, 215, 216]. There are a number of technologies under development that employ nonepithelial targets for CTC capture and characterization and may provide a noninvasive window into the role of EP in cancer metastasis [217].

Given its dynamic and transient nature, visualizing EP is a major challenge radiographically. EP may be routinely seen but not clinically recognized through tumor imaging. In PC, there is well-documented discordance between PSA measurements and imaging responses. For example, technetium-99 bone scans indirectly assess osteoblastic activity induced by PC metastases to bone, and the interpretation is often complicated by the “flare phenomenon,” which is an osteoblastic reaction that may occur in response to treatment where new or increased intensity of existing lesions is noted. The flare gives the appearance of worsening of bony metastatic disease, but is not adversely prognostic. For instance, in a phase II study of abiraterone plus prednisone in patients with metastatic CRPC, over half of the patients responding to abiraterone by PSA criteria had initial worsening of the bone scan, but more than 80 % of those scans improved subsequently, consistent with the flare phenomena [218]. We hypothesize that this initial flare may represent an element of EP induced by treatment, in which PC osteomimicry linked to induction of EMT becomes evident during the initial phases of treatment. During treatment-induced EMT, the mesenchymal, stem-like cells mimic osteoblasts and take up more technetium-99, accounting for these early changes on bone scans. Although this imaging flare temporarily stabilizes and often improves, the bone lesions typically progress at a later time point, indicating persistent viable tumor in these regions of bone scan activity. Given that a number of agents used to treat men with PC, such as hormonal therapies, can induce this reaction, and that osteomimicry markers may likewise emerge during ADT [15, 65], the bone scan flare may be imaging evidence of a shift toward a bone-forming mesenchymal state and thus plasticity.

In contrast to the flare phenomenon described above, in a phase II study of the c-met/VEGFR2 inhibitor cabozantinib in metastatic CRPC, nearly 80 % of patients had complete or partial resolution of bone scan lesions after 12 weeks of therapy, but bone scan response did not correlate with PSA or CTC response [219]. The initial imaging improvement with cabozantinib is typically short-lived, with the re-emergence of active bone lesions over time in the same regions, indicating persistent viable tumor despite the disappearance on scans. We hypothesize that the changes visualized on bone scan during the course of treatment with cabozantinib may be the result of cellular plasticity and induction of MET. This induced MET would shift away from the osteoblastic

mesenchymal state in bone metastases and toward a more epithelial, nonbone-forming state, and lead to a transient reduction in technetium-99 uptake. This may be accompanied by a rise in PSA due to this epithelial differentiation driven by AR activity [220], which is often disconnected from the radiographic changes. Thus, PSA changes reflecting epithelial biology and bone scan changes representing mesenchymal tumor biology may be clinical biomarkers of EP. Further studies to quantify these changes in the context of tumor biopsies during a range of therapies are needed.

3.2 EP in localized PC

Although advanced metastatic PC is known to be a heterogeneous disease [6], it has been demonstrated that most metastases arise from a single precursor lesion in the primary tumor, suggesting that lethal PC has a monoclonal origin [8]. Therefore, differences in phenotype rather than genotype must account for the heterogeneity, and even in localized PC, there is evidence for EP. For example, TWIST1 is absent in benign prostatic tissue but expressed in prostate adenocarcinoma cells, and higher levels of TWIST1 expression are associated with higher Gleason scores in the primary tumor [221]. By immunohistochemistry, higher expression of EMT markers can be seen at the invasive front of the tumor versus the center of the tumor. For example, E-cadherin expression decreases at the invasive front while vimentin and ZEB-1 expression increase [222]. Similarly, in the primary prostate tumor, the combination of weak E-cadherin and strong N-cadherin expression, or high vimentin or TWIST1 expression, predict early dissemination and clinical recurrence [223, 224].

A frequently observed genetic lesion in human PC is the TMPRSS2-ERG fusion, in which exon 1 of TMPRSS2, an androgen-regulated serine protease, is joined to exons 4–9 of the ERG gene, an erythroblast transformation-specific (ETS) transcription factor [225]. The fusion protein TMPRSS2-ERG is present in more than half of all PC [226]. Interestingly, TMPRSS2-ERG fusion can induce EMT via activation of the Wnt/ β -catenin pathway [227]. In addition, EMT can be induced *in vitro* and *in vivo* by overexpression of the TMPRSS2-ERG fusion. Here, EMT is mediated by ZEB1 and ZEB2, and chromatin immunoprecipitation assays revealed that TMPRSS2-ERG directly binds the ZEB1 promoter [228]. This suggests that the TMPRSS2-ERG fusion may be associated with more aggressive disease by controlling ZEB1-induced EMT and offers a biological explanation for the prognostic significance attributed to detection of the TMPRSS2-ERG protein. In a cohort of men with localized PC undergoing active surveillance, those men with the TMPRSS2-ERG fusion had a higher likelihood of PC-specific mortality [229]. Additional studies show that the presence of the fusion protein predicts for recurrence after prostatectomy [230] and portends a worse survival [231].

This is controversial, however, as a recent metaanalysis found no association between ERG overexpression via TMPRSS2–ERG fusion and recurrence or mortality [232], and the relevance of the genomic rearrangement may be variant dependent. For example, one variant found in approximately 5 % of PC is the TMPRSS2-ERG fusion together with the deletion of sequences 5' to ERG, and the presence of this variant confers a poor prognosis [231].

Radiation therapy is commonly used to treat localized PC and, in many men, is curative; however, greater than one third of men with high-risk disease will relapse after local radiotherapy. There is concern, however, that the emerging tumor clones in men who fail radiotherapy may undergo EMT and develop an associated treatment resistance. For example, ionizing radiation induces DNA double-strand breaks, and the DNA damage response can induce stromal cells to secrete WNT16B, a soluble protein that may induce EMT mediated through the NF- κ B pathway in neighboring PC cells. WNT16B overexpression has been observed during cytotoxic chemotherapy and radiation in PC patients and model systems and has been recently linked to treatment failure and dissemination [150]. Thus, EP is emerging as an adaptive stress-activated mechanism of resistance to radiotherapy and cytotoxic therapy that is induced by stromal signaling.

3.3 EP in metastatic PC

Gene expression analysis of single CTCs revealed increased expression of EMT-related genes in CRPC patients compared to castrate-sensitive patients, suggesting that activation of EMT-related genes may be associated with disease progression [233]. For example, NOTCH-1, which has been associated with an EMT and stem cell phenotype [173], is significantly upregulated in bone metastasis compared with the primary prostate tumors, suggesting that NOTCH-1 may be important for PC progression [222].

As discussed above, EP is increasingly recognized as a mechanism underlying drug resistance, and in PC, evidence exists for the upregulation of mesenchymal biomarkers during androgen deprivation in cell lines, animal models, and in patient tumor specimens. For example, expression of the mesenchymal marker N-cadherin increases after androgen deprivation in men treated prior to surgery [234]. Furthermore, ADT has been shown to induce an EMT, possibly by removing the inhibitory effect that AR signaling has on the transcription factor, ZEB-1. However, these cells are able to revert to an epithelial phenotype upon replacement of testosterone, indicating EP [65]. N-Cadherin expression is rare in untreated PC, increases with androgen deprivation, and is highest in the castration-resistant setting [235]. In the primary prostate tumor, the combination of weak E-cadherin and strong N-cadherin expression predicts for early biochemical failure and clinical recurrence [223]. N-Cadherin expression has been associated

with a more rapid progression to castration resistance, which may be circumvented preclinically through direct targeting with monoclonal antibodies to N-cadherin [235]. With this rationale, one could hypothesize that high N-cadherin expression would predict for resistance to agents that block AR signaling; however, clinical studies are needed to confirm the role of mesenchymal biomarkers in predicting treatment resistance to pathways that target androgen synthesis or signaling.

Metastatic sites may variably express EP markers, and this variability may exist within and between patients. For example, in a metastatic survey study of human PC, lymph node metastases frequently had lower E-cadherin expression levels than bone metastases in the same patient [236]. This heterogeneity may reflect different modes of invasion or migration, such as collective sheet migration to lymph nodes, which may be independent of EP, as compared to a TGF- β -mediated hematogenous dissemination that has a greater requirement for EMT/MET [237]. In PC, metastatic site has prognostic importance, as lymph node metastatic CRPC has the most favorable prognosis, followed by bone-metastatic and visceral metastatic CRPC [238].

Docetaxel, an antimitotic microtubule-stabilizing agent, is the most commonly used chemotherapy for PC, and resistance to this agent often emerges within 6–12 months of treatment initiation. Recent evidence shows that PC cells lacking the epithelial marker cytokeratin (CK18 and CK19) are able to survive treatment with docetaxel. These docetaxel-resistant cells are more abundant in metastatic sites as compared to the primary tumor [200]. In cell line and xenograft models, docetaxel-resistant cells are induced by activation of stemness pathways important for EP and can be depleted by combining docetaxel with agents that target the NOTCH and Hedgehog signaling pathways [200]. Loss of CK or PSA in prostatectomy specimens is associated with recurrence and metastasis as well [239], suggesting that identification of cytokeratin- or PSA-negative PC cells may predict for resistance to local or systemic therapies, but additional validation is necessary. Given that taxanes have been shown to induce EP and stemness in several model systems, accompanied by treatment resistance and dissemination, therapies that reduce this resistance mechanism are needed [204].

EP may also lead to resistance to immunotherapy. Treatment with an epithelial-based complementary DNA (cDNA) vaccine results in regression of prostate tumors in mice, but when resistant tumors eventually emerge, these tumors lack PSA expression and gain mesenchymal markers. Revaccination with a cDNA library derived from the resistant tumors eradicates the tumors and cures the mice. Reversal of the vaccination strategy, giving the mesenchymal vaccine followed by the epithelial vaccine, is ineffective [240]. This is further evidence for the role of EP in treatment resistance and may provide clues as to how to tailor treatment to target these resistance pathways.

Given that the FDA-approved PC immunotherapy sipuleucel-T and the investigational vaccine ProstateVaccin (Prostvac) utilizes epithelial differentiation proteins (prostatic acid phosphatase and PSA, respectively) to prime dendritic and T cells, and results in only modest survival benefits [241, 242], tumor escape from this immunotherapy may involve EP and loss of epithelial targets or upregulation of mesenchymal or stemness targets.

As discussed in the clinical vignette above, one path to CRPC may be through neuroendocrine differentiation (NED), in which PC cells no longer secrete PSA or express AR. Instead, the cells often express and secrete chromogranin A [20], and this may be another example of EP. Clinically, NED most often occurs after ADT or AR signaling inhibition. Likewise, preclinically, depletion of androgen in cell culture promotes NED of PC cells [243], and NED in response to androgen deprivation in cell lines is dependent on Akt activity [62]. Given the known crosstalk between the AR and PI3K–Akt pathways [244], there is rationale for a combination approach clinically, and PI3K–Akt pathway inhibitors are currently under investigation both as single agents and in combination (reviewed in [245]). PC tumors with NED often have high levels of EZH2, which as discussed above, leads to suppression of DAP2IP and subsequent activation of important oncogenic pathways and EMT, further supporting the hypothesis that NED is a result of EP [28]. To further classify NED in PC, next-generation RNA sequencing was performed on primary tumors and metastatic biopsy samples from men with NED and compared with tumors from men with classic prostate adenocarcinoma. Aurora kinase A and N-myc are overexpressed and amplified in 40 % of NED versus 5 % of classic prostate adenocarcinoma and cooperate to induce NED in prostate cells [28]. This suggests that aurora kinase inhibitors may be used alone or in combination with cytotoxic chemotherapy to treat NED in PC, and trials targeting aurora kinase A are ongoing. Finally, whether NED is associated with EP or genetic evolution in PC is not clear. However, small cell differentiation of nonsmall cell lung carcinomas has been reported during EGFR inhibition, which is reversible phenotypically upon withdrawal of the epithelial targeting agent [22]. This suggests that a similar phenomenon may be occurring in PC during ADT or with potent AR inhibition.

4 Therapeutic strategies directed toward EP

As described above, there is substantial evidence that one mechanism of drug resistance is through phenotypic plasticity. In the era of personalized medicine, combination anticancer therapies have fallen somewhat out of favor; however, rational combination approaches may eradicate PC, similar to the way combination therapy revolutionized treatment for leukemia and infectious diseases, such as tuberculosis and HIV/AIDS.

Combination strategies in preclinical models of malignancy have turned cytostatic activity into cytotoxic activity and resulted in durable remissions. Therefore, combining therapies based on the knowledge of resistance pathways inherent to the cancer cell and the tumor microenvironment is an emerging and essential step in oncology [246]. We hypothesize that targeting the underlying regulators of EP, such as stemness pathways, epigenetic regulators, or oncogenic pathways, will be more effective than single agent therapies directed against more traditional epithelial differentiation pathways. These epithelial differentiation pathways, including AR and androgen synthesis, are not likely to be causally related to PC survival, given the inevitable resistant escape observed clinically after relatively short intervals of time. While a PC stem cell has not been clearly identified, it is possible that AR is not present in this stem-like cell [247], and thus, strategies to target key stemness, invasion, and dissemination pathways may be of greater benefit than AR targeting. However, given the central role of AR in PC and its potential role in promoting survival of PC cells, targeting AR in the context of additional therapies directed against EP regulators may remain critical. Indeed, multiple pathways may require targeting in order to address the bulk of the differentiated cancer and its stem-like progeny [200].

There are several available drugs and therapies in development that specifically target the epithelial or the mesenchymal phenotype or stemness pathways, and potential therapeutic approaches to addressing EP in the clinic are listed in Table 3. Agents directed toward epithelial targets, such as androgen synthesis and AR signaling inhibitors, may need to be partnered with therapy against mesenchymal targets for maximal benefit. For example, in cell lines with constitutively active AR variants, there is increased expression of mesenchymal markers including N-cadherin, again implicating EP as a mechanism of treatment resistance [248]. Furthermore, there is a monoclonal antibody against N-cadherin that, in mouse models, prevents invasion and metastasis and delays the time to castration-resistance [236]. Combining a pure mesenchymal target such as this with an epithelial target may be a rational approach, such as combinations with enzalutamide or abiraterone acetate. Epithelial-antigen immunotherapies such as Prostvac (against PSA) or sipuleucel-T (against PAP) may lead to mesenchymal or stemness-based immune escape, similar to what has been observed preclinically, and thus novel targeting of mesenchymal or stemness antigens may be more productive long term. In addition, targeting of stromal cells directly through prodrugs, monoclonal antibodies, or chemokine inhibitors may reduce EP and invasion indirectly [249].

Approaches that target embryologic pathways important in regulating EP may provide clinical benefits similar to those observed preclinically. For example, treatment with a cytotoxic agent such as docetaxel may reduce the bulk of disease, but disease relapse is inevitable. Activation of Hedgehog or

Table 3 Potential therapeutic strategies directed toward EP

Therapy	Mechanism of action	Efficacy in human PC	References
Epithelial phenotypic targets			
Androgen receptor antagonist			
Enzalutamide	Blocks AR, targets epithelial cells	Enzalutamide prolongs survival; Multiple agents in phase II-III trials	[256–258]
ARN-509			
TOK-001			
Androgen synthesis inhibitors			
Abiraterone	Inhibits the CYP17 enzymes needed for testosterone synthesis, targets epithelial cells	Abiraterone prolongs survival; orteronel in phase II-III trials	[258–261]
Orteronel			
TOK-001			
Mesenchymal phenotypic targets			
N-Cadherin			
Anti-N-cadherin antibody ADH-1 (Exherin)	Block N-cadherin to slow tumor growth and inhibit EMT	Unknown	[235, 262]
Clusterin inhibition			
OGX-011 (custersin)	Antisense oligonucleotide against secretory clusterin, may inhibit EMT	OGX-011 in combination with docetaxel improved survival in a phase II of men with CRPC	[92, 93]
C-met			
Cabozantinib	Tyrosine kinase inhibitor against MET and VEGFR2	Bone scan and progression-free survival improvement	[219]
Sarasinoside A1	Induces MET, even in the absence of E-cadherin	Unknown	[263]
Stromal targets			
fibroblast specific protein (FSP)	Prodrug targets stroma and may prevent EMT	Unknown	[249]
FGFR family (mesenchymal isoforms)	Inhibits invasion, survival	Unknown	[264]
Aurora kinase A inhibitor (MLN8237)	Blocks neuroendocrine differentiation	MLN8237 in phase II trials	[28]
Combination approaches			
Immunologic therapies in combination			
Checkpoint/vaccine strategies	Target multiple antigens during escape from initial immunotherapy	Unknown	[240]
Epigenetic therapies in combination			
HDAC inhibitors	Induce EMT or MET	Unknown	[156]
Stemness pathway targets			
TGF-β pathway inhibitors	Kinase inhibition, neutralizing antibodies, or antisense oligonucleotides	Unknown	[252]
Hedgehog/Gli signaling inhibitors	Small molecule inhibition of Gli	GDC-0449 in phase 1-2 trials	[248]
NOTCH inhibitors	Gamma secretase inhibition	Unknown, ongoing	[251]
PI3K/PTEN pathway inhibitors	Reduced stemness, survival	BKM120, others in phase 1-2 trials	[245]

PSA prostate specific antigen, ADT androgen deprivation therapy, CRPC castration-resistant prostate cancer

NOTCH signaling in CRPC patients suggests that biomarkers of stemness may predict for benefit of agents that block stemness pathways. Hedgehog and NOTCH signaling inhibition is an active area of investigation in prostate and other cancers, and clinical trials with these agents alone and in combination are ongoing (reviewed in [250, 251]). Combination therapy with Hedgehog or NOTCH inhibition to address the stem-like cells with loss of epithelial differentiation may be more effective than treatment with either agent alone [200]. However, investigation of the

selectivity of these agents against tumor cells rather than normal hematopoietic and organ-specific stem cell niches will be imperative given the potential for stem-cell toxicity. In a high-throughput drug screen to uncover agents specific to EMT-induced stemness properties, there were only a handful of agents, such as salinomycin, that were specifically toxic to cancer stem cells over normal cells, illustrating the formidable problem of selectivity. In this screen, paclitaxel actually induced a greater metastatic burden and promoted stemness properties [204]. These surprising findings require further

validation in PC model systems, where new classes of agents more specific to the underlying biology of EP rather than differentiated cells may bear greater fruit.

Another stemness target under investigation is TGF- β and the differing roles of TGF- β in early versus late stage cancer and in mediating hematogenous versus lymph node metastases, as described above, highlights the need for biomarkers to help guide patient selection for treatment with these agents. Clinical trials with anti-TGF- β therapies will likely show different results depending on the clinical context and again may be more effective when given in combination (reviewed in [252]). A clearly defined biomarker or set of biomarkers for EP in PC is needed to track these phenotypically diverse cells as they progress and contribute to treatment resistance. For example, detection of AR variants may be predictive of treatment response or resistance [253]. As reviewed elsewhere, predictive biomarkers in CRPC require extensive validation and prospective qualification both preclinically and in clinical trials, before they can be incorporated into clinical practice [254]. AR-independent PC may also be important in the development of EP, and identifying biomarkers of the different PC disease states and their relationship with EP is crucial.

Finally, because disease stability and differentiation rather than rapid cytorreduction and tumor shrinkage may occur with these therapies, especially when investigated as single agents, clinical trial endpoints that adequately test the activity of antiplasticity or stemness agents are necessary. In CRPC, these endpoints may include metastasis-free survival, overall survival, and radiographic or clinical progression-free survival. Combination approaches leading to novel cure model based clinical trial designs would also provide fair tests of substantial long term activity while limiting sample size [255], similar to what has been observed in the treatment of tuberculosis and HIV infections. Thus, combination approaches of EP targeted therapy with more traditional hormonal, immunomodulatory, or chemotherapies may extend survival, similar to what has been observed preclinically.

5 Conclusions

Substantial improvements in outcomes have been realized with novel hormonal therapies used for the treatment of metastatic CRPC, including abiraterone acetate and enzalutamide, and with immunotherapies and chemotherapies, such as sipuleucel-T, docetaxel, and cabazitaxel. Despite these incremental advances, treatment resistance emerges within 1–2 years in most cases, suggesting that novel approaches are needed. With the clinical use of more potent androgen pathway inhibitors, the emergence of neuroendocrine and other variant phenotypes is predicted to rise. EP is clearly associated with dissemination in multiple solid tumors, and emerging evidence supports EP as a mediator of both hematogenous

dissemination (bone, visceral metastases) and therapeutic failure. To address this biology, novel agents that target stemness and embryonic pathways that influence cellular differentiation and invasion will be needed, likely in combination with current therapies that target the more differentiated epithelial bulk of the metastatic lesions. Rational combination therapies, based on the knowledge of feedback resistance pathways inherent to the cancer cell and tumor microenvironment, as well as on knowledge of immunologic escape due to loss of epithelial antigens, will likely be the most effective way to target EP in PC.

6 Key unanswered questions

- 1 How is AR regulation related to EP in PC and are these two pathways linked?
- 2 Can metastasis occur in human PC without loss of an epithelial phenotype or gain of a mesenchymal phenotype? Can other forms of migration/invasion, such as amoeboid invasion or collective sheet migration also explain dissemination and treatment failure?
- 3 Does EP explain treatment resistance to enzalutamide and abiraterone acetate or immunotherapy with sipuleucel-T based on studies of CTCs and metastatic biopsies over time in patients?
- 4 Can combination approaches targeting both epithelial and stem-like/mesenchymal compartments lead to eradication of established metastases or are these approaches more effective at preventing metastatic disease?
- 5 Does secondary neuroendocrine PC emerge due to genetic evolution and clonal selection over time or can this phenotype be reversed through systemic therapies, implying cellular plasticity?

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- metastatic prostate cancer without previous chemotherapy. *The New England Journal of Medicine*, 368, 138–148.
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111/11/15



DUKE UNIVERSITY MEDICAL CENTER

CURRICULUM VITAE

Date Prepared: November 11, 2015

Name: **Andrew J. Armstrong, MD ScM**

Primary academic appointment: **Associate Professor, Medicine (Medical Oncology) and Surgery**

Primary academic department: **Medicine**

Secondary appointments: **Surgery, Pharmacology and Cancer Biology**

Present academic rank and title: **Associate Professor with tenure**

Date and rank of first Duke Faculty appointment: **September 1, 2006**

Medical Licensure: North Carolina License # 2006-01418

Date of License (Month/Day/Year) 08/16/2006

Specialty certification(s) and dates (Month/Day/Year):

Internal Medicine, 2003, expires 12/31/2013, recertified April 23, 2013

Medical Oncology, 2006, expires 12/31/2016

Hematology, 2006, expires 12/31/2016

Date of birth: 02/21/1974

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Education	<u>Institution</u>	<u>Date</u> (Year)	<u>Degree</u>
High School	John Handley High School Winchester, VA	1988-1992	n/a
College	Duke University School of Engineering	1992-1996	BSE (distinction)
Graduate or Professional School	University of Virginia School of Medicine	1996-2000	MD
	Johns Hopkins School of Public Health	2004-2008	ScM (Masters of Science)

Scholarly societies:

Phi Eta Sigma
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 Delta Omega National Public Health Honor Society (nominee)
 American College of Physicians (Fellow, FACP)

Professional training and academic career:

<u>Institution</u>	<u>Position/Title</u>	<u>Dates</u>
Hospital of the University of Pennsylvania	Intern and Resident	2000-2003
Johns Hopkins School of Medicine	Oncology and Hematology Fellow	2003-2006
Johns Hopkins School of Public Health Graduate Training Program in Clinical Investigation (GTPCI)	Research Fellow	2004-2008
Duke University Department of Medicine	Assistant Professor of Medicine and Surgery	2006-2011
Duke University Department of Medicine	Associate Professor of Medicine and Surgery	2012-present
Duke University Department of Medicine	Associate Professor with tenure of Medicine and Surgery	2014-present
Duke University Department of Pharmacology and Cancer Biology	Associate Professor with Tenure	2016-present

Mission Statement

As a clinical and translational investigator, I have spent the last 8 years investigating tumor-host interactions in the context of experimental therapeutics for patients with advanced genitourinary malignancies, particularly with a focus on prostate cancer and the investigation of biomarkers of response and benefit. It is in this context that my research focus has turned to the investigation of circulating tumor cell biology, with a focus on epithelial plasticity (EP) in these cells and how this may relate to the lethal phenotype. This work has led to a Department of Defense Physician Research Training Award and Prostate Cancer Foundation Young Investigator Award, and findings confirming EP biomarker (N-cadherin, vimentin) expression in CTCs from these patients with metastatic cancer, as well as evidence of stem cell biomarker expression on these cells. These findings implicate epithelial plasticity in treatment resistance and metastatic dissemination in prostate cancer and also imply that CTCs inhabit a transitional or intermediate state and may possess stem-like properties. I have developed a number of experimental agents in prostate and renal cell cancer, including completed or ongoing trials of mTOR inhibitors and PI3 kinase inhibitors, immunomodulatory agents, hormonal therapies, and anti-angiogenic agents, and am heavily involved in the leadership of several phase 3 studies in advanced prostate cancer (dasatinib, tasquinimod, enzalutamide) in CRPC. I co-direct the Duke clinical research program in genitourinary malignancies, overseeing 8 research nurses, 4 clinical trial assistants, 2 regulatory specialists, and 2 budget and finance personnel, and provide mentorship to medical oncology fellows and post-doctoral laboratory based fellows. I am the principal investigator on 8 investigator-initiated clinical trials and approximately 12 industry or cooperative group sponsored clinical trials, as well as several correlative science studies including a large PCF-Movember Global Treatment Sciences Challenge Award that is developing and validating a range of predictive biomarkers in men with mCRPC. As an internationally recognized expert in prognostic and predictive biomarkers and outcome studies in men with castration-

resistant metastatic disease, my goals are to further therapeutic advances for men with advanced prostate cancer through a predictive and personalized approach driven by the known pathophysiology and inherent heterogeneity of this disease.

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Publications:

*=notable peer reviewed first/senior publications selected

Peer Reviewed First/Senior Author Publications (in order of chronicity):

1. Armstrong AJ, Eck SJ. EpCAM: A New Therapeutic Target for an Old Cancer Antigen. *Cancer Biology and Therapy*. 2003; 2(4): 320-5.

2. Armstrong AJ, Garrett-Mayer ES, Yang YC, de Wit R, Tannock IF, Eisenberger M. A contemporary prognostic nomogram for men with hormone-refractory metastatic prostate cancer (HRPC). *Clin Cancer Research* 2007;13:6396-6403.

Description: Developed an internally validated prognostic nomogram for men undergoing chemotherapy for castration-resistant prostate cancer. This nomogram has the highest predictive accuracy as compared to other available prognostic models in this disease and is widely recognized and used internationally.

3. Armstrong AJ, Garrett-Mayer E, Ou Yang YC, Carducci MA, Tannock I, de Wit R, Eisenberger M. PSA and pain surrogacy analysis in men with metastatic hormone-refractory prostate cancer (HRPC). *J Clin Oncol* 2007;29:3965-70.

Description: This paper refuted the evidence that declines in PSA following docetaxel therapy were surrogate endpoints (ie potential approvable endpoints) in men with CRPC, and also evaluated changes in pain. These data helped provide evidence that informed on the Prostate Cancer Working Group's criteria for endpoints in clinical trials, and fortified evidence that overall survival should remain the primary phase 3 endpoint in this disease.

4. Armstrong AJ*, George DJ. Satraplatin in the Treatment of Hormone-Refractory Metastatic Prostate Cancer. *Ther Clin Risk Mgmt* 2007; 3(5)

5. Armstrong AJ*, Creel P, Turnbull J, Moore C, Jaffe TA, Haley S, Petros W, Yenser S, Gockerman JP, Sleep D, Hurwitz H, George DJ. A phase I-II study of docetaxel and atrasentan in men with castration resistant metastatic prostate cancer. *Clin Cancer Res* 2008;14: 6270-76.

Description: This was a phase 1-2 report of the novel endothelin-A receptor inhibitor with docetaxel in men with CRPC, and provided evidence for an independent bone turnover modifying property of this agent; these data also set the stage for the eventual and ongoing phase 3 trial being currently conducted by the NCI cooperative groups.

6. Armstrong AJ*, Halabi S, de Wit R, Tannock IF, Eisenberger M. The relationship of body mass index and serum testosterone levels with disease outcomes in castration-resistant metastatic prostate cancer. *Prostate Cancer and Prostatic Diseases* 2008; 36:1-6.

7. **Armstrong AJ***, Tannock IF, de Wit R, George DJ, Eisenberger M, Halabi S. The development of risk groups in men with metastatic castration-resistant prostate cancer based on predictive risk factors for PSA decline and survival. *Eur J Cancer* 46:517-525, 2010.
8. **Armstrong AJ*** and PG Febbo. Using Surrogate Biomarkers to Predict Clinical Benefit in Men with Castration-Resistant Prostate Cancer: an Update and Review of the Literature. *Oncologist* 2009, 14: 816-27.
- *9. **Armstrong AJ***, Garrett-Mayer E, Tannock IF, de Wit R, and M Eisenberger. Prediction of Survival Following First Line Chemotherapy in Men with Castration-Resistant Metastatic Prostate Cancer, *Clin Cancer Res*, 2010;1:203-211.

Description: This is the first post-docetaxel nomogram to be developed, and has reasonable prognostic accuracy, incorporating several novel factors such as type of progression and duration of first line chemotherapy. The clinical utility of this nomogram in the post-docetaxel space has increased following the approval of novel agents in this setting in 2010.
10. **Armstrong AJ***, George DJ. Optimizing Docetaxel Chemotherapy for Men with Metastatic Castration Resistant Prostate Cancer. *Prostate Cancer Prostatic Diseases* 2010 (published online Jan 12)
- *11. **Armstrong AJ***, Netto GJ, Rudek MA, Halabi S, Wood D, Creel P, Mundy K, Davis SL, Wang T, Albadine R, Schultz L, Partin A, Jimeno A, Fedor H, Febbo PG, George DJ, Gurganus R, DeMarzo AM, Carducci MA. Pharmacodynamic study of pre-prostatectomy rapamycin in men with advanced localized prostate cancer. *Clin Cancer Res* 2010;16: 3057-66.

Description: This was a rigorous mechanistic study of the mTOR inhibitor rapamycin in men with localized prostate cancer that utilized paired tumor biopsy specimens, PK and PBMC data, and found that while rapamycin inhibited its intended downstream target, it had no effect on proliferation or apoptosis, thus providing the only published evidence of clinical mechanism of this class of agents in prostate cancer and suggesting methods to improve upon targeted therapy against the PI3K pathway in prostate cancer.
12. Sonpavde G, Pond GR, Berry WR, de Wit R, Eisenberger MA, Tannock IF, and **AJ Armstrong***. The association between radiographic response and overall survival in men with metastatic castration-resistant prostate cancer receiving chemotherapy. *Cancer* 2011 (online ahead of press March 1, 2011)
13. Bitting R, Madden J, and **Armstrong AJ***. Therapy for non-clear cell histologies in renal cancer. *Curr Clin Pharmacol* 2011, Epub ahead of press Aug 9, 2011.
14. Pili R, Häggman M, Stadler WM, Gingrich J, Assikis V, Björk A, Nordle Ö, Forsberg G, Carducci MA, **Armstrong, A.J.** Phase II randomized double blind placebo-controlled study to determine the efficacy of tasquinimod in asymptomatic patients with metastatic castrate-resistant prostate cancer. *J Clin Oncol* 2011 Sept 19 (epub ahead of press)
This large international phase 2 trial of the novel agent tasquinimod demonstrated a more than doubling of the progression-free survival of men with metastatic CRPC over placebo and has led to the launch of a global phase 3 registrational study. This agent is an S100A9 inhibitor with effects on angiogenesis and myeloid-derived suppressor cell function, each of which apparently has anti-tumor and anti-metastatic functions.
15. Antonarakis A and **Armstrong AJ***. Evolving standards in the treatment of castration-resistant metastatic prostate cancer. *Prostate Cancer Prostatic Diseases*. 2011; 14(3):192-205
16. Antonarakis A and **Armstrong AJ***. Emerging therapeutic approaches in the management of men with metastatic castration-resistant prostate cancer. *Prostate Cancer Prostatic Diseases*. 2011;14(3):206-18.
- *17. **Armstrong AJ***, Kemeny G, Marengo M, Oltean S, Chen L, Herold C, Turnbull J, Marcom PK, George DJ, and Garcia-Blanco MA*. Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. *Mol Cancer Res* 2011 (epub ahead of print July 26, 2011)

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This correlative study of human circulating tumor cells establishes the presence of markers of epithelial mesenchymal transition in CTCs taken from patients with metastatic breast and prostate cancer, as well as provides evidence for the expression of stemness antigens and epithelial plasticity in metastatic sites.

18. Pond G*, **Armstrong AJ***, Wood BA, Brookes M, Leopold L, Berry WR, de Wit R, Tannock IF, Sonpavde G. Evaluating the Value of Number of Cycles of Docetaxel and Prednisone in Men with Metastatic Castration-Resistant Prostate Cancer. Eur Urol 2011 (Epub ahead of print 6/22/11).

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4. Moul JW, **Armstrong AJ**, Hollenbeck BK, Lattanzi J, Bradley D, and Hussain M: Prostate Cancer. In: R Pazdur, LD Wagman, KA Camphausen, and WJ Hoskins (Eds). 11th Edition Cancer Management: A Multidisciplinary Approach, CMP Healthcare Media, Manhasset, NY, Chapter 17, pp. 393-423, 2008.
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- 9-12. Moul JW, **Armstrong AJ**, Lattanzi J: Prostate Cancer. In: R Pazdur, LD Wagman, KA Camphausen, and WJ Hoskins (Eds). 13th Edition Cancer Management: A Multidisciplinary Approach, CMP Healthcare Media, Manhasset, NY, Chapter 14, 2010. **Editions in 2012, 2013, 2014 were also published as a co-author.**
13. Section editor for (section 6), *Textbook of Prostate Cancer* (Springer), editor: Ashutosh Tewari, in press 2013. Involves editing and oversight for 9 chapters.
14. Li J, **Armstrong AJ**. Prognostic and Predictive Biomarkers for Castration Resistant Prostate Cancer. In *Biomarkers in Cancer*. Ed VR Preedy and VB Patel, Chapter 21, Springer Reference, 2015.
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a. Published scientific reviews (for mass distribution)

1. **Armstrong AJ**, Carducci MA. Chemotherapy for advanced prostate cancer: results of new clinical trials and future studies. *Curr Oncol Reports* 2005; 7:110-7.
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8. Li J, **Armstrong AJ**. Counterpoints: Which should be used first in symptomatic metastatic castration-resistant prostate cancer, docetaxel or radium? Radium-223 is the preferred therapy in bone-predominant symptomatic metastatic castration-resistant prostate cancer. *Clin Adv Hematol Oncol* 2015; 13: 293-8.

b. Selected abstracts

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2. **Armstrong AJ**, Kulesza P, Netto GJ, Rudek MA, Halabi S, Wood D, Creel P, Mundy K, Davis SL, Wang T, Albadine R, Schultz L, Partin A, Jimeno A, Fedor H, Febbo PG, George DJ, Gurganus R, DeMarzo AM, Carducci MA. A pharmacodynamic study of pre-prostatectomy rapamycin in men with advanced localized prostate cancer. Abstract presented at Cancer Education Consortium, Amelia Island April 2006.
3. Stoker CE, Adams RB, Slack JB, **Armstrong AJ**, Parsons JT. Integrin Mediated Migration of Prostate Cancer Cell Lines. Abstract presented at *Keystone Symposium on Motility and Metastasis*, Copper Mountain, CO, Feb. 25, 1998.
4. Nightingale RW, Camacho DL, **Armstrong AJ**, Robinette JJ, Myers BS. Cervical Spine Buckling: the Effects of Vertebral Mass and Loading Rate. Presented at 1997 *Advances in Bioengineering Conference*

(ASME), vol BED-36, 231-2.

5. **Armstrong AJ**, de Marzo A, Partin AW, Rudek M, Gurganus R, Beekman K, Hidalgo M, Carducci MA. A pharmacodynamic study of pre-prostatectomy rapamycin in men with advanced localized prostate cancer. *Cancer Education Symposium 2006*, Amelia Island, Florida (abstract).
6. **Armstrong AJ**, E. S. Garrett-Mayer, Y. Ou Yang, R. de Wit, I. Tannock and Eisenberger ME. A baseline prognostic model and nomogram incorporating PSA kinetics in hormone-refractory metastatic prostate cancer (HRPC). *Proceedings of the American Society of Clinical Oncology Prostate Cancer Symposium 2007*, abstract 222.
7. **Armstrong AJ**, Garrett-Mayer E, Ou Yang Y, Tannock IF, de Wit R, and Eisenberger R. Limitations of the current progression-free survival (PFS) definition in hormone-refractory prostate cancer (HRPC): Benefit associated with continuation of docetaxel beyond TAX327 protocol-defined progression. *Proceedings of the American Society of Clinical Oncology Prostate Cancer Symposium 2007*, abstract 223.
8. **Armstrong AJ**, Garrett-Mayer E, Ou Yang YC, Carducci MA, Tannock I, de Wit R, Eisenberger M. Analysis of PSA decline as a surrogate for overall survival in metastatic hormone-refractory prostate cancer (HRPC). *Proceedings of the American Society of Clinical Oncology Prostate Cancer Symposium 2007*, abstract 148.
9. Speca J, Mears A, Creel T, **Armstrong AJ**, George DJ. Phase I study of PTK787/ZK222584 (PTK/ZK) and RAD001 for patients with advanced solid tumors and dose expansion in renal cell carcinoma patients. *Proc Am Soc Clin Oncol 2007*, abstract 5039.
10. **Armstrong AJ**, E. S. Garrett-Mayer, Y. Ou Yang, R. de Wit, I. Tannock and M. Eisenberger. Analysis of PSA decline as a surrogate for overall survival in metastatic hormone-refractory prostate cancer (HRPC). *Proceedings of the American Society of Clinical Oncology 2007*, abstract 5009.
11. **Armstrong AJ**, E. S. Garrett-Mayer, Y. Ou Yang, R. de Wit, I. Tannock and M. Eisenberger.. A multivariate prognostic nomogram incorporating PSA kinetics in hormone-refractory metastatic prostate cancer (HRPC). *Proceedings of the American Society of Clinical Oncology 2007*, abstract 5058.
12. George DJ, A. J. Armstrong, P. Creel, K. Morris, J. Madden, J. Turnbull, M. Dewhirst, N. Major, P. G. Febbo. A phase 2 study of RAD001 in men with hormone-refractory metastatic prostate cancer. *GU Symposium (ASCO) 2008*, abstract 181.
13. **Armstrong AJ**, S. Halabi, I. F. Tannock, de Wit R, and M. A. Eisenberger. The relationship of body mass index and serum testosterone levels with disease outcomes in castration-resistant metastatic prostate cancer. *GU Oncology Symposium 2008*, abstract 44.
14. Y. E. Whang, C. N. Moore, **A. J. Armstrong**, W. K. Rathmell, P. A. Godley, J. M. Crane, G. I. Grigson, K. Morris, C. P. Watkins, and D. J. George. Phase II trial of lapatinib in hormone refractory prostate cancer. *GU Symposium 2008*, abstract 156.
15. G. Netto, **A. Armstrong**, D. Wood, P. Creel, A. Partin, A. Jimeno, M. Rudek, D. George, R. Gurganus, and M. A. Carducci. Pharmacodynamic (PD) study of pre-prostatectomy rapamycin in men with advanced localized prostate cancer (PC): *preliminary results of a Prostate Cancer Clinical Trials Consortium Trial, ASCO 2009 abstract 5001*.
16. **Armstrong AJ**, S. Halabi, I. F. Tannock, D. J. George, R. DeWit, and M. Eisenberger. Development of risk groups in metastatic castration-resistant prostate cancer (mCRPC) to facilitate identification of active chemotherapy regimens, *ASCO 2009 abstract 5137*.

17. J. Araujo, A. J. Armstrong, E. L. Braud, E. Posadas, M. Lonberg, G. E. Gallick, G. C. Trudel, P. Paliwal, S. Agrawal, and C. J. Logothetis. Dasatinib and docetaxel combination treatment for patients with castration-resistant progressive prostate cancer: A phase I/II study (CA180086), ASCO 2009 abstract 5061. (also presented as update at ESMO 2009).
18. **AJ Armstrong**, George DJ, and Halabi S. Serum lactate dehydrogenase (LDH) is a predictive biomarker for mTOR inhibition in patients with metastatic renal cell carcinoma (RCC). ASCO GU Symposium, San Francisco, CA 2010, abstract. Also: ASCO 2010, abstract 4631.
19. C.P. Hart, **A.J. Armstrong**, E.G. Chiorean, M. Borad, A. Mita, J.D. Sun, V.K. Langmuir, F. Meng, C. Eng, S. Kroll, M.D. Matteucci J.G. Curd. Bench to Bedside Experience with TH-302: a Tumor-Selective Hypoxia-Activated Prodrug as a Promising Treatment for Prostate Cancer. AACR-NCI-EORTC meeting November 2009, abstract.
20. **Armstrong AJ**, J. D. Turnbull, K. Morris, S. E. Yenser Wood, S. Voyles, Y. A. Fesko, and D. J. George. Impact of temsirolimus and anti-androgen therapy on circulating tumor cell (CTC) biology in men with castration-resistant metastatic prostate cancer (CRPC): A phase II study. ASCO 2010, abstract 47821.
21. **Armstrong AJ**, Oltean S, Kemeny G, Turnbull J, Herold C, Marcom PK, George DJ, Garcia-Blanco M, Circulating Tumor Cells from Patients with Metastatic Breast and Prostate Cancer Express Vimentin and N-Cadherin. AACR Conference on EMT, Washington DC 2010, abstract and oral presentation.
22. **Armstrong AJ**, Oltean S, Kemeny G, Turnbull J, Herold C, Marcom PK, George DJ, Garcia-Blanco M, Plasticity, stemness, and aggressive behavior in preclinical models and circulating prostate cancer cells: importance of the transitional phenotypic state to lethal cancer biology. ASCO GU Symposium 2010, abstract 172.
23. Sonpavde G, Pond GR, Berry WR, de Wit R, **Armstrong AJ**, Eisenberger M, Tannock IF. Changes in serum alkaline phosphatase predict survival independent of PSA changes in men with castration-resistant prostate cancer and bone metastasis receiving chemotherapy: a retrospective analysis of the TAX327 trial. ASCO GU Symposium 2010, abstract.34.
24. Pili R, Häggman RM, Stadler WM, Gingrich JR, Assikis V, Björk A, Forsberg G, Carducci MA, **Armstrong AJ**. A randomized multicenter international phase II study of tasquinimod in chemotherapy naïve patients with metastatic castrate-resistant prostate cancer (CRPC). ASCO 2010, abstract 4510. (oral presentation)
25. G. C. Trudel, F. Saad, **A. J. Armstrong**, J. Bellmunt, G. Wilding, E. Y. Yu, J. C. Araujo, S. Durham, P. Paliwal, C. Logothetis. Dasatinib or placebo combined with docetaxel in castration-resistant prostate cancer (CRPC): Design of CA180227, a phase 3, randomized, double-blind trial. ASCO 2011 abstract 80104
26. G. R. Pond, **A. J. Armstrong**, B. A. Wood, M. Brookes, L. H. Leopold, W. R. Berry, R. De Wit, M. A. Eisenberger, I. Tannock, G. Sonpavde. Evaluating the value of continuing docetaxel and prednisone [DP] beyond 10 cycles in men with metastatic castration resistant prostate cancer [mCRPC]. ASCO 2011 abstract 76830
27. M. T. Fleming, G. R. Pond, **A. J. Armstrong**, B. A. Wood, M. Brookes, L. H. Leopold, V. B. Matveev, J. M. Burke, J. R. Caton, G. Sonpavde. Ability of serum alkaline phosphatase [ALP] changes to complement PSA changes and predict survival in men with metastatic castration resistant prostate cancer [mCRPC] receiving docetaxel and prednisone [DP]. ASCO 2011 abstract 77014.
28. J. S. De Bono, K. Fizazi, F. Saad, M. E. Taplin, C. N. Sternberg, K. Miller, P. Mulders, K. Chi, **A. Armstrong**, M. Hirmand, B. Selby, H. I. Scher. Primary, secondary and quality-of-life endpoint results from the Phase 3 AFFIRM study of MDV3100, an androgen receptor signaling inhibitor. Proc ASCO 2012

- 29. Armstrong AJ**, J.R. Gingrich, M. Häggman, W.M. Stadler, J.E. Damber, L. Belkoff, R. Clark, S. Brosman, O. Nordle, G. Forsberg, M.A. Carducci, R. Pili. Long term safety and efficacy in a randomized multicenter international phase II study of tasquinimod in chemotherapy naïve patients with metastatic castrate-resistant prostate cancer. European Association of Urology, February 2012, Paris, abstract.
- 30. Armstrong AJ**, R. Kaboteh, M.A. Carducci, J-E Damber, W.M. Stadler, M. Hansen, L. Edenbrandt, G. Forsberg, Ö. Nordle, R. Pili, M. Morris. Tasquinimod and effects on bone scan index in men with metastatic castration-resistant prostate cancer (mCRPC): results of retrospective follow up of a randomized phase 2 placebo-controlled trial. Proc Am Soc Clin Oncol 2013 abstract 5081.
- 31.** Rhonda L. Bitting, Rengasamy Boominathan, Chandra Rao, Elizabeth Embree, Daniel J. George, Mark Connelly, Gabor Kemeny, Mariano A. Garcia-Blanco, and **Andrew J. Armstrong**. Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method. Proc Am Soc Clin Oncol 2013, abstract 5031.
- 32. Armstrong AJ**, Halabi S, Eisen T, Stadler WM, Jones RR, Vaishampayan UN, Garcia JA, Hawkins RE, Kollmannsberger C, Lusk C, Broderick S, George DJ. ASPEN: A randomized phase II trial of everolimus versus sunitinib in patients with metastatic non-clear cell renal cell carcinoma. Proc Am Soc Clin Oncol 2013 abstract TPS 4590.
- 33.** Higano C, **Armstrong AJ**, Cooperberg MR, Kantoff PW, Bailen J, Concepcion RS, Kassabian, Dakhil SR, Finkelstein SE, Vacirca JL, Rifkin RM, Sandler A, McCoy C, Whitmore JB, Tyler RC, Sartor AO. Impact of prior docetaxel (D) on sipuleucel-T (sip-T) product parameters in PROCEED patients (pts). Proc Am Soc Clin Oncol 2013, abstract 5034.
- 34.** Scher HI, Fizazi K, Saad F, Chi KN, Taplin ME, Sternberg CN, **Armstrong AJ**, Hirmand M, Forer D, de Bono JS. Impact of on-study corticosteroid use on efficacy and safety in the phase III AFFIRM study of enzalutamide (ENZA), an androgen receptor inhibitor. Proc Am Soc Clin Oncol 2013, abstract 6.
- 35.** TM Beer, **AJ Armstrong**, CN Sternberg, C Higano, P Iversen, Y Lortot, DE Rathkopf, S Bhattacharya, J Carles, J de Bono, CP Evans, AM Joshua, C Kim, G Kimura, P Mainwaring, H Mansbach, K Miller, SB Noonberg, P Venner, B Tombal. Enzalutamide in Men with Chemotherapy-naïve Metastatic Prostate Cancer (mCRPC): Results of the Phase 3 PREVAIL Study. ASCO GU Symposium, San Francisco 2014, LBA1 abstract.
- 36. Armstrong AJ**, Rhonda L Bitting, Gabor Kemeny, Daniel J George. Evidence for Circulating Tumor Cell (CTC) Alkaline Phosphatase (AP) Expression in Men with Bone-Metastatic CRPC During Abiraterone Acetate Treatment Response. ASCO GU Symposium, San Francisco, 2014, abstract 178.
- 37. Armstrong AJ**, Beaver J, Li J, Bitting RL, Gregory S. Genomic Analysis of Circulating Tumor Cells (CTCs) from Men with Metastatic Castration Resistant Prostate Cancer (mCRPC) in the Context of Enzalutamide Therapy. ASCO GU Symposium, San Francisco, 2014, abstract 65.
- 38. Armstrong AJ**, Halabi S, Healy P, Lee WR, Koontz BF, Moul JW, Mundy K, Creel P, Yenser Wood S, Davis K, Reimer B, Nguyen M, Spitz AN, Bratt E, Kim S, Tran PT, Stein MN, Carducci MA, George DJ. A phase 2 multimodality trial of docetaxel/prednisone with sunitinib followed by salvage radiation therapy (RT) in men with PSA recurrent prostate cancer (PC) after radical prostatectomy (RP). J Clin Oncol 33, 2015 (suppl 7; abstr 35).
- 39.** Ware KE, Schaeffer D, Somarelli J, Zhang T, Foo W, Li J, Garcia-Blanco MA, **Armstrong AJ**. Snail regulates androgen receptor biology and enzalutamide resistance. Presented at AACR 2015 poster presentation and PCF Retreat 2015, Washington DC.

c: Editorials, position, and background papers

1. **Armstrong AJ**, Eisenberger M. Commentary on: The risk of clinical fractures after gonadotropin-releasing hormone agonist therapy for prostate cancer. *Nature Clin Pract Urol* 2006; 3: 246-7.
2. **Armstrong AJ**, Garrett-Mayer ES, Eisenberger MA. Adaptive therapy for androgen-independent prostate cancer. *J Natl Cancer Inst* 2008;100:681-3.
3. **Armstrong AJ**, Moul JW, George DJ. What to order from the prostate cancer treatment menu? *Oncology*. 2012; 26:87-88.
4. Sonpavde G, **Armstrong AJ**. Objective evaluation of bone metastases in prostate cancer: to what end? *Eur Urol Epub* July 2012.
5. **Armstrong AJ**. The STAMPEDE trial and celecoxib: how to adapt? *Lancet Oncol* 2012 Epub May 2012.
6. **Armstrong AJ**. Docetaxel for men with prostate cancer: how early should we start? *Lancet Oncol* 2015 (in press)
- 7.

And see above for additional titles.

Consultant and Speaker's Bureau appointments:

Pfizer Pharmaceuticals, research support
Sanofi Aventis, research support
Dendreon (Speaker, advisory)
Active Biotech/Ipsen, steering committee, consultant, research support
Bristol-Myers-Squibb, research support
Medivation/Astellas, consultant, research support
Bayer (advisor, consultant)
Janssen (advisor, consultant)

Industry Research funding (clinical trials, see support documentation):

Imclone
Bristol-Myers-Squibb
Active Biotech
Sanofi-aventis
Novartis
Pfizer-Wyeth
Medivation/Astellas
Dendreon
Kanglaite
Bayer

Editorial Board Positions

2014-present	Journal of Clinical Oncology,
2014-present	Prostate Cancer and Prostatic Diseases

Technology Development:

1. **Nomogram application (app) software.** First smartphone-based software application for iphone or android for CRPC prognostic assessments, based on two Clin Cancer Res publications (2007, 2010) relevant to the pre- and post-docetaxel disease states. Launch: October 2013.
2. **Patent for development of novel technology for circulating tumor cell capture based on EMT**

biology (application number PCT/US10/50223), patent pending.

National/International Reviewer/Editorial Positions:

2014-present	Editorial board, Journal of Clinical Oncology
2014-present	Editorial board, Prostate Cancer and Prostatic Diseases
2013-present	Reviewer, New England Journal of Medicine
2013-present	Peer reviewer, American Urologic Association (AUA) guidelines
2011-present	Reviewer, Cancer Discovery
2011-present	Reviewer, PLoS One
2011-present	Prostate Cancer Foundation YIA Reviewer
2010-present	Reviewer, Lancet and Lancet Oncology
2010-present	Reviewer, Journal of Clinical Oncology
2010-present	Reviewer, BMC Cancer
2010-10-18	Ad hoc Reviewer, Investigational New Drugs
2009-present	Scientific Editor, Prostate Cancer Foundation patient education webpage
2007-present	Reviewer, Cancer Investigation
2008-present	Reviewer, Clinical Advances in Hematology and Oncology
2007-present	Ad hoc reviewer, European Journal of Urology
2006-present	Reviewer, Prostate Cancer and Prostatic Diseases, ed. Judd Moul
2006-present	Reviewer, Clinical Cancer Research, Cancer Research, and Molecular Cancer Therapy (AACR journals)
2005-6	Faculty Reviewer, First Aid for the Boards, McGraw-Hill

Professional awards, National Committees, and Special Recognitions:

2015	Outstanding Postdoc Mentor Award Nomination, Duke University
2015-present	Panel member, NCCN Guidelines for Prostate Cancer Resource Stratification
2015	Course Director, Duke Urologic Assembly, Orlando Florida
2014-present	ALLIANCE A031201 Correlative Science Chair: PHASE III TRIAL OF ENZALUTAMIDE VERSUS ENZALUTAMIDE, ABIRATERONE AND PREDNISONE FOR CASTRATION RESISTANT METASTATIC PROSTATE CANCER
2014-present	Duke Cancer Institute Shared Resources Oversight Committee member
2014-present	NCCN Vice Chair of the Prostate Cancer Guideline Committee
2014-present	Prostate Cancer Foundation-Movember Global Treatment Sciences Challenge Award
2014-present	Prostate Cancer Foundation Global Research Council member
2012-	Fellow of the American College of Physicians (FACP)
2012-	Associate Director, Clinical and Translational Research in Genitourinary Oncology, Duke Cancer Institute
2012-	ALLIANCE Cooperative Group, GU Correlative Science Committee member
2012-present	Co-Director, Duke Scholars in Molecular Medicine, Oncology

2011	Medical Director, Duke Prostate Center Symposium
2010-	NCCN Prostate Cancer Expert Panel Member (national guidelines)
2010-15	Department of Defense Physician Research Training Award, PCRP
2009-	Genitourinary Oncology co-Program Leader, Duke University
2009	Prostate Cancer Foundation Top Performing Young Investigator
2008-11	Prostate Cancer Foundation Young Investigator Award
2007-9	National Comprehensive Cancer Network (NCCN)-mTOR inhibition in Solid Tumors Task Force
2007-9	Gold Star Service Champion, Duke University Medical Center, 9300 inpatient service
2006-8	American Society of Clinical Oncology (ASCO) Young Investigator Award (YIA)
2006-9	American Association for Cancer Research (AACR) Clinical/Translational Research Fellowship
2006-9	Duke Comprehensive Cancer Center K12 Award
2006	Cancer Education Consortium Grant Recipient, Amelia Island, Florida
2005-6	Ad hoc reviewer, Urology (The Gold Journal), editor Alan W. Partin, MD PhD, Johns Hopkins Hospital, Baltimore MD.
2004	K12 NIH Training Grant, Graduate Training Program in Clinical Investigation
2003	National Medical Jeopardy Contestant, ACP National Convention, San Diego
2000	Mulholland Society Teaching Award, University of Virginia
1999	Alpha Omega Alpha
1996	Tau Beta Pi National Engineering Honor Society
1996	Graduation with Distinction in BME, <i>magna cum laude</i>
1994-5	Summer research fellow at the National Cancer Institute's Advanced Biosciences Laboratory (NCI-ABL) under Drs. George F. Vande Woude and Kenji Fukasawa
1995	Vice President, Duke University School of Engineering Student Body
1994	Golden Key National Honor Society
1993	Phi Eta Sigma National Honor Society
1992-6	National Science Foundation Scholarship Award

Organizations and participation: (Offices held, committee assignments, etc.)

2013-present	ALLIANCE Genitourinary Oncology Committee and Correlative Sciences Subcommittee
2012-present	Society for Urologic Oncology (SUO) member
2008-present	Duke University Institutional Review Board
2008-present	Scientific Editor, Duke Prostate Center News
2007-present	Duke Comprehensive Cancer Center Editorial Advisory Committee
2007-present	American Association for the Advancement of Science
2007-present	NCCN mTOR inhibition in Solid Tumors Task Force
2006-present	Duke Comprehensive Cancer Center Cancer Protocol Committee
2006-present	Duke Clinical Research Institute Faculty Member
2006-present	Cancer and Leukemia Group B, Active Member GU Committee
2005-present	American Association for Cancer Research (AACR), Active Member
2003-present	American Society of Clinical Oncology (ASCO), Active Member
2003-present	American Society of Hematology (ASH), Active Member
2000-present	American College of Physicians (ACP), Member
1996-present	American Medical Association, Member
1995-6	Vice President, Duke University School of Engineering Student Body

Teaching and lecturing responsibilities including continuing medical education (CME):

March 2015	Systemic Therapy and Sequencing for Metastatic Prostate Cancer, Duke Urologic Assembly, Orlando FL (co-course director)
March 2015	Risk Adapted Therapy for Renal Cell Carcinoma, Duke Urologic Assembly, Orlando FL
March 2015	New Treatment Options in Castration-Resistant Prostate Cancer. National Comprehensive Cancer Network annual meeting, Hollywood FL.
Feb 2015	Docetaxel for Metastatic Castrate-Sensitive Prostate Cancer: UnCHAARTED Waters. Research to Practice Symposium, Orlando FL.
2015	Oncology Fellows Lecture Series, Prostate Cancer 101, 201.
Jan 2014	Invited lecture, ASCO GU Symposium 2014: "Beyond Enzalutamide and Abiraterone: What's Next in Hormonal Therapy?"
2013-14	Multiple grand rounds on CRPC, Updates in Therapy
Oct 2013	Prognostic, Predictive, and Surrogate Biomarkers in CRPC, Labroots talk (CME)
Sept 2013	Update in CRPC, Dayton OH and separate grand rounds talk in Columbia SC
April 2013	Prostate Cancer lecture, Duke Medicine Housestaff
Feb/April 2013	Medical Oncology Lectures on Prostate Cancer (2)
January 2013	Geriatrics Grand Rounds, lecture on Prostate Cancer in the Elderly
April 2013	Prostate Cancer lecture, Duke Medicine Housestaff
October 2012	Lecture on Epithelial Plasticity in Prostate Cancer, Prostate Cancer Foundation Retreat, Carlsbad, CA
October 2012	Prognostic and Predictive Biomarkers in CRPC, SUO Symposium

Fukuoka, Japan

7/20/2012	Best of ASCO Highlights, Cary NC
2010-12	Lunch and learn series: topics in GU Oncology (monthly lectures to research staff)
5.2012	Updates in CRPC; William J Smith Memorial Oncology Conference, Asheville, NC
4.2012	Oncology Grand Rounds: Duke Debate
4.2012	Updates in CRPC Podcast, CancerNetwork
2.2012	Novel therapies for CRPC: EAU Invited Lecture, Paris France
2012	RCC CME Program, France Foundation (Duke CME Program): includes podcasts, presentations, interviews, development of slide deck
01.2011	2011 Testicular Cancer Lecture and 2011 Prostate Cancer Lecture
11.2010	Talk entitled: "CRPC: What Else is Out There?" for the UK Cancer Convention, Royal Institute of British Architects (RIBA), London
9. 2010	Oncology Care Live 2010 Virtual Oncology Congress, speaker
2010	Medical Oncology Grand Rounds: A randomized multicenter international phase II study of tasquinimod in chemotherapy naïve patients with metastatic castrate-resistant prostate cancer (CRPC).
2009	Medical Oncology Grand Rounds: "Epithelial Plasticity in Prostate Cancer: A Biomarker for the Lethal Phenotype"
1.2010	CALGB Duke/Duke Oncology Network CRA Workshop
4.2008	Duke Prostate Center and Duke Urologic Assembly, Prostate Cancer Update: "Complications of Androgen Deprivation Therapy"
2009	Duke Oncology Network ASCO Updates
2008	Duke Tuesdays in Urology: "Update in Castration Resistant Prostate Cancer"
10.2007	"Current Directions in Advanced Prostate Cancer Therapy", Fall Oncology Conference, Birmingham, AL
5.2007	Updates in Prostate Cancer, 2007. Nash General Hospital, Rocky Mount, NC
3.2007	Update in Advanced Prostate Cancer. Duke Prostate Center Symposium, Durham, NC
2006-8	Attending and Teaching physician, 9300 solid tumor inpatient unit, Duke University Hospital
2007-pres.	Fellow lecture series, medical oncology, Duke University
2006-7	Teaching physician, mock tumor board, Duke University School of Medicine
2006	Attending and Teaching Physician, 9300 Inpatient Service in Medical Oncology

- 2003-6** Fellow in oncology and hematology with teaching responsibilities for Johns Hopkins medical housestaff: inpatient services and consultative services
- 2000** Mulholland Society Teaching Award, University of Virginia School of Medicine

Mentoring Responsibilities:

1. Primary clinical and translational mentor for **Jing Li, MD PhD**, 2nd year post-doctoral medical oncology fellow. Project: "Novel mesenchymal capture and genomic characterization of CTCs in men with mCRPC".
2. Primary clinical and translational mentor for **Tian Zhang, MD**, 2nd year medical oncology post-doctoral fellow, project entitled "The role of c-met in promoting AR independent prostate cancer growth", and "Development of a novel CTC capture method based on c-met expression."
3. **Kathryn Ware, PhD**. Post-doctoral fellow. Clinical and translational co-mentor along with Mariano Garcia-Blanco. Project: "Association between AR variants and epithelial plasticity in CRPC."
4. **Rhonda Bitting, MD** (Oncology Fellow). Project is around developing methods for the analysis of RNA expression profiles in circulating tumor cells from men with metastatic CRPC. Co mentor is Mariano Garcia-Blanco. 2010-present
5. **Abhinav ETTYREDDY** (Duke Undergraduate). Third year project for thesis work dedicated to development of FGFR2 isotype specific antibodies for use in tissue and circulating tumor cell research. Co mentor is Mariano Garcia-Blanco. 2010-present
6. Clinical/translational mentor for post-doctoral T32 fellows in Mariano Garcia-Blanco laboratory: **Daneen Schaeffer PhD, Matthew Marengo PhD, Jason Somarelli PhD**, 2010-present
7. Clinic Mentor for medical oncology fellows in GU. **Prateek Mendiratta and Franklin Chen**. 2008-2009.
8. Clinic mentor for several undergraduate students: **Sarah Wang, Geoffrey Houtz**, 2012-present
9. Clinical/translational mentor for Molecular Medicine Scholars in Oncology, 2011-present
10. Clinical Mentor, Michael Humeniuk MD, medical oncology fellow 2015-17

Areas of research interests (basic and applied) - list:

1. Predictors of sensitivity and clinical efficacy of targeted therapies in advanced prostate cancer (PI3K,

mTOR inhibitors, other pathways)

2. Novel designs of clinical trials and pharmacodynamic/translational studies in GU malignancies
3. Pre-operative models for drug development of novel agents in human testing in prostate cancer
4. Novel therapies and drug development for prostate, renal, and bladder cancer
5. Developing prognostic models for progression and survival in metastatic castration resistant prostate cancer
6. Examining surrogate markers of mortality in metastatic castration-resistant prostate cancer
7. Development of circulating molecular predictors of systemic therapy benefit in men with mCRPC
8. Non-clear cell renal cell carcinoma novel therapeutic strategies
9. Circulating tumor cell biology and genomics/genetics for personalized medicine approaches to CRPC
10. Optimizing systemic approached to men with CRPC

Current Projects and Studies

See other support page

External support - gifts, grants, and contracts: see other support page for details

<u>PI</u>	<u>% Effort</u>	<u>Purpose</u>	<u>Approximate Amount</u>	<u>Duration</u>
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Past:

Johns Hopkins K12 (Institutional)	Ross Donehower	n/a	GTPCI Training Grant (Fellowship)	\$60,000/year	2004-6
Duke University K12 (Institutional K12, DCCC)	H. Kim Lyerly	75%	Salary Support	\$115,000/yr	2006-9
GCRC (Johns Hopkins)	Carducci, MA Armstrong AJ	n/a	Rapamycin Study	\$15,000	2006-7
ASCO YIA (Young Investigator Award)	Armstrong AJ	n/a	Rapamycin Study	\$36,000	2006-8
AACR (Clinical and Translational Fellowship Grant)	Armstrong AJ	n/a	Rapamycin Study and Salary Support	\$40,000	2006-8

Present (see other support page):

PCF-Movember	Armstrong AJ	10%	Global Treatment Sciences Challenge	\$1,400,000	2014-17
Prostate Cancer Foundation	Armstrong AJ	1-5%	Epithelial Plasticity in Prostate Cancer	\$75,000/yr	2008-11
DOD PRTA	Armstrong	53%	Epithelial Plasticity In CRPC	\$685,172	2010-2015
DOD NIA	Armstrong	10%	Polymersomes	\$291,000	2012-15
NIH R01	Garcia-Blanco	5%	Epithelial plasticity	see OS	2008-14

Multiple clinical trial awards (see other support page)

Department of Defense Prostate Cancer Clinical Trials Consortium Grant (DOD PCCTC), sub-investigator, 5%, 5 years 2009-2016.

Clinical activity - type of practice and estimate of time commitment:

- 2006-2012 Assistant Professor, Duke University Department of Medicine, Division of Medical Oncology, 33% effort for direct patient care
- 2012-present Associate Professor of Medicine and Surgery, Duke Cancer Institute. 33% effort for patient care.
Recognition as an international leader in experimental therapeutics in prostate cancer, biomarker development in GU malignancies, prognostic and predictive biomarkers. Practice includes one physician assistant, Kristen Davis PA, who follows patients with me during systemic treatments for GU cancers.
- Currently spend one half day per month additional clinic session in the Duke Multidisciplinary Prostate Cancer Clinic for newly diagnosed men, 2010-present.
- 2013-present Associate Director for Clinical Research, Genitourinary Program, Duke Cancer Institute, 200 hours/year
Duties: 1) oversee staff of 8 research coordinators, 3 regulatory coordinators, 3 data managers, 2 clinical trial assistants, finance (pre and post-award) personnel; 2) hold regular weekly data and safety monitoring meetings related to clinical trial patient care; 3) lead weekly new protocol meetings to develop new ideas, grants, processes, database studies, clinical trials
- 2013-present Member, Prostate Cancer Strategy Group, Duke Cancer Institute, monthly meetings
Goal: To work with multidisciplinary team to set strategic goals around prostate cancer screening, detection, diagnosis, risk stratification, management, and to develop recruitment priorities for the DCI.
- Developed prostate cancer screening MAESTRO template for use in all Duke primary care clinics.
- 2013-present Director of the Prostate Cancer Forum

Participation in academic and administrative activities of the University and Medical Center:

- 2014-present Duke Cancer Institute Shared Resources Oversight Committee
- 2013-present Duke Cancer Institute Prostate Cancer Strategy Group
- 2013-present Associate Director for Clinical Research, GU Program, DCI
- 2010-present Duke Comprehensive Cancer Center Oncology Trials Shared Resource, co-GU Program Lead
- 2009-present Duke University Department of Medicine Residency Interviewing Committee
- 2010-present Duke Fellowship Advisory Committee
- 2008-present Member, Duke University Institutional Review Board
- 2008-present Internal Medicine residency interviewing committee
- 2007-2010 Duke Comprehensive Cancer Center Editorial Advisory Committee
- 2007-present Senior Scientific Editor, Duke Prostate Center News (periodical)
- 2006-present Fellowship recruitment and interviewing committee, Duke University Department of Medicine
- 2006-present Cancer Protocol Committee (CPC), Duke Comprehensive Cancer Center

Research Support

Completed

AACR Clinical/Translational Fellowship (Armstrong)

07/01/2006 - 06/30/2008

AACR

A Pharmacodynamic Study of Pre-Prostatectomy Rapamycin in Men with Advanced Localized Prostate Cancer.

Goal: Project was to determine the safety and optimal target dose of the oral mTOR inhibitor rapamycin when administered daily as a single agent to men with localized high-risk prostate cancer prior to undergoing radical prostatectomy and to identify predictors of pharmacodynamic response to rapamycin using tissue-based mechanistic studies in locally advanced prostate cancer.

American Society of Clinical Oncology Young Investigator Award (YIA)

7/01/06-6/30/2008

ASCO: PI Andrew J. Armstrong, MD

A Pharmacodynamic Study of Pre-Prostatectomy Rapamycin in Men with Advanced Localized Prostate Cancer.

Goal: This grant funds some of the correlative science work for this clinical trial, including pharmacokinetics and pathologic/immunohistochemical assessments.

NIH 5K12 CA100639 (Lyerly, H.)

08/04/2004 – 07/31/2009

NIH Clinical Oncology Research Career Development Program (Armstrong)

Goal: Dr. Armstrong was supported over a 3-year term to conduct a trial which includes the following specific aims: (1) to evaluate the safety and tolerability of the oral mTOR inhibitor rapamycin when administered daily as a single agent to men with localized intermediate and high-risk prostate cancer prior to undergoing radical prostatectomy; (2) to determine the POD of rapamycin in men with newly diagnosed, intermediate and high-risk prostate cancer using prostatic tissue collected at the time of prostatectomy; and (3) to identify predictors of biologic response to rapamycin using tissue-based mechanistic studies in locally advanced prostate cancer.

(Armstrong)

08/01/08 - 07/31/11

Bristol-Myers Squibb

Phase I/II Study of Dasatinib and Docetaxel in Metastatic Hormone Refractory Prostate Cancer

Goal: Phase II portion of this clinical research study is to learn how the study drugs (dasatinib, docetaxel, and prednisone) affect each other in the body

07TASQ08 (Armstrong)

01/02/08 - 01/02/11

Active Biotech AB

Phase II Randomized Double Blind Placebo-Controlled Study to Determine Efficacy of ABR-215050

Goal: Evaluate the efficacy of ABR-215050 vs. placebo in asymptomatic patients with metastatic CRPC, as measured by the proportion of patients who have not progressed at 6 months and to evaluate the effect of ABR-215050 vs. placebo in asymptomatic patients with metastatic CRPC

(Armstrong)

10/22/08 - 09/30/11

ImClone Systems, Inc.

A Phase 2, Multicenter, Randomized Study of Metastatic Androgen-Independent Prostate Cancer (AIPC) Following Disease Progression on Docetaxel-Based Chemotherapy

Goal: The purpose of this study is to evaluate the effects of IMC-A12 or IMC-1121B combined with Mitoxantrone and Prednisone in patients with metastatic prostate cancer on progression free survival.

Current/Ongoing/Active**Department of Defense W81XWH-10-1-0483 (Armstrong)**

07/01/10 - 07/31/15

Epithelial Plasticity in Castration-Resistant Prostate Cancer: Biology of the Lethal Phenotype

Goal: Investigate the prevalence of epithelial plasticity and stem cell biomarkers on CTCs; identify oncogenic

pathways through RNA expression profiling that are activated in CTCs compared to matched leukocytes and metastatic tumor samples; investigate the clonality of prostate cancer metastases through analysis of DNA copy number changes in matched CTCs and metastatic sites.

Department of Defense W81XWH-12-1-0253 (Armstrong)

09/10/12 - 09/09/14

Development of a Novel Method to Detect Prostate Cancer Circulating Tumor Cells (CTCs)

Goal: Develop and optimize a novel polymersome-based CTC capture method using NIR-EPs bearing conjugated antibodies to EpCAM, N and O-cadherins, and PSMA; Assessment of CTC capture using novel antibody-targeted NIR-EPs in men with mCRPC; long term goal of this DOD IDA/NIA is to develop a noninvasive strategy for detection and characterization of non-epithelial CTCs to improve upon and complement existing epithelialbased CTC detection technology, and identify novel CTC populations and thus therapeutic targets to prevent or delay metastatic progression in men with PC.

National Institutes of Health 5R01-CA127727-05 (Garcia-Blanco)

12/01/08 - 11/30/13

Alternative Splicing and Epithelial-mesenchymal Plasticity in Prostate Tumors

Goal: Investigate the mechanisms involved with the alternative splicing of FGFR2 in prostate cancers as they transition from an epithelial phenotype to a mesenchymal phenotype.

National Institutes of Health

5R01-CA155296-03 (Halabi)

07/06/11 - 05/31/15

Prognostic Models of Clinical Outcomes In Men With Castration Resistant Prostate Cancer

Goal: Develop a prognostic model that will predict overall survival in men with CRPC who failed first line chemotherapy. The model will be validated for predictive accuracy using an independent dataset; develop a prognostic model that will predict progression-free survival in CRPC men who failed first line chemotherapy.

Prostate Cancer Foundation (Armstrong)

09/01/08 - 08/31/13

Epithelial Plasticity in Advanced Prostate Cancer: A Biomarker for Lethal Disease

Goal: Identify CTCs in patients with PC with this aggressive mesenchymal/pro-metastatic phenotype that are not currently identified in existing assays.

INDUSTRY SPONSORED PROJECTS

Aggregated Effort

2.16 calendar

Duke University lists aggregated effort assigned to the following eligible industry-sponsored clinical trial projects. Each of these individual projects has a varying need of effort depending on the type of activity currently in progress such as protocol development, start-up, patient recruitment, enrollment, follow-up, monitoring, data analysis, publication, and closeout. Faculty determines each project's need and adjust their effort between projects within the total aggregated effort assigned to the clinical projects.

Novartis Pharmaceuticals Corporation (Armstrong)

11/05/09 - 02/29/16

A Randomized Phase II Study of Afinitor (RAD0001) vs. Sutent (Sunitinib) in Patients with Metastatic Non-Clear Cell Renal Carcinoma (ASPEN)

Goal: Compare the anti-tumor activity of RAD001 and sunitinib in subjects with metastatic renal cell carcinoma (mRCC) with non-clear cell pathology, as measured by progression free survival (PFS) following treatment initiation.

Bristol-Myers Squibb (Armstrong)

10/21/08 - 04/30/14

A Randomized Double-Blind Phase III Trial Comparing Docetaxel Combined with Placebo in Castration-resistant Prostate Cancer

Goal: Compare overall survival for dasatinib plus docetaxel and prednisone versus placebo plus docetaxel and prednisone in subjects with metastatic castration-resistant prostate cancer; compare the rate of change from baseline in urinary N-telopeptide between the 2 treatment arms; compare the time to first skeletal related event between the 2 treatment arms; compare the rate of change from baseline in pain intensity between the 2 treatment arms; compare the time to PSA progression between the 2 treatment arms.

Active Biotech AB (Armstrong)

03/23/11 - 03/31/14

A Phase III Randomized Double-blinded Placebo Controlled Study of Tasquinimod in Men with Metastatic

Castrate-resistant Prostate Cancer

Goal: To confirm the effect of tasquinimod on delaying disease progression compared with placebo.

Medivation, Inc. MDV3100-09 (Armstrong)

01/10/2013 - 12/31/14

STRIVE: A Multicenter Phase II, Randomized, Double-Blind, Efficacy and Safety

Goal: Determine the benefit of enzalutamide (formerly MDV3100) as compared to bicalutamide as assessed by progression-free survival (PFS).

Novartis Pharmaceuticals Corporation (Armstrong)

01/01/11 - 12/31/13

A Randomized Phase II Study of BKM120 in Men with Castration-resistant Metastatic Prostate Cancer

Goal: Evaluate the effects of the study drug BKM120 and changes in response to BKM120.

Pfizer, Inc. (Armstrong)

09/10/09 - 12/31/13

A Randomized Phase II Study of Afinitor (RAD001) vs. Sutent (Sunitinib) in Patients with Metastatic Non-Clear Cell Renal Cell Carcinoma

Goal: Compare the anti-tumor activity of RAD001 and sunitinib in subjects with metastatic renal cell carcinoma (mRCC) with non-clear cell pathology, as measured by progression free survival (PFS) following treatment initiation.

Pfizer, Inc (Armstrong)

12/01/08 - 11/30/13

Multimodality therapy for recurrent high-risk prostate cancer, a phase 2 trial

Goal: To determine the rate of progression free survival (PFS) at 24 months in men with PSA recurrent non-metastatic prostate cancer following radical prostatectomy who received multimodality therapy.

Medivation, Inc. (Armstrong)

10/05/10 - 11/11/13

A Multinational Phase 3, Randomized, Double-blind, Placebo-Controlled Efficacy and Safety Study of Oral MDV3100 in Chemo-Naïve Men with mCRPC

Goal: Determine the benefit of MDV3100 as compared to placebo as assessed by overall survival and to determine the benefit of MDV3100 as compared to placebo as assessed by progression-free survival (PFS).

KangLaiTe USA Inc (Armstrong)

08/01/11 - 09/30/13

Efficacy and Safety of oral Kanglaite (KLTc) Gelcap in Men with Prostate Cancer

Goal: Evaluate the effects and safety of two different doses (3 or 6 capsules, four times daily) of KLTc on prostate specific antigen doubling time (PSADT) in men who have rising PSA after initial local therapy for localized prostate cancer during 12 months of study period.

Dendreon Corporation (Armstrong)

09/18/09 - 08/31/13

An Open-Label Study of Sipuleucel-T in Men with Metastatic Castrate resistant Prostate Cancer

Goal: Provide sipuleucel-T to men with metastatic castrate resistant prostate cancer (CRPC), while marketing approval is being pursued; obtain safety data, to evaluate the magnitude of immune responses to treatment with sipuleucel-T, and to further characterize the cellular components of sipuleucel-T.

Dendreon Corporation Protocol P10-3 (Armstrong)

07/11/11 - 08/01/13

A Registry of Sipuleucel-T Therapy in Men with Advanced Prostate Cancer

Goal: This is strictly an observational study. The patient will receive sipuleucel-T and the study doctor will observe and collect information about the patient.