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Isolation and analysis of discrete human prostate cellular populations

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Abstract

The use of lineage tracing in transgenic mouse models has revealed an abundance of subcellular phenotypes responsible for maintaining prostate homeostasis. The ability to use fresh human tissues to examine the hypotheses generated by these mouse experiments has been greatly enhanced by technical advances in tissue processing, flow cytometry and cell culture. We describe in detail the optimization of protocols for each of these areas to facilitate research on solving human prostate diseases through the analysis of human tissue.

Introduction

More than forty years ago it was discovered that embryonic mesenchyme orchestrated epithelial morphogenesis and differentiation in prostate (Cunha, 1972). There is now a greater appreciation of the diverse subpopulations of adult stroma and epithelia in prostate development and disease. This review will cover the technical advances in flow cytometry, organoid culture, and tissue regeneration with human cells that are used to address questions regarding the functional roles of epithelial and stromal subpopulations in prostate development and disease.

Mouse vs. Human Prostate

The human prostate is a major cause of both morbidity and mortality. Unfortunately, there are no good experimental animal models that mimic the human organ structure and disease profile. Chimpanzees have a prostate anatomy similar to that of humans, and indeed suffer from human-like benign prostatic hyperplasia (BPH) (Steiner et al, 1999). However in chimpanzees the disease progression is slow and stochastic, which, aside from the ethical issues incumbent to working with primates, makes the model impractical to use

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experimentally. The only other commonly encountered species that suffers from prostate cancer and hyperplasia is the dog, although the canine prostate structure and pathobiology have significant differences from humans (Berry et al, 1986, Isaacs, 1984, Teske et al, 2002). Again, disease occurrence is sporadic and is associated with aging, making the model impractical for most purposes. The prostatic structure and disease profile in the most common experimental animal models (rats and mice) is very different from humans, with a lobular rather than zonal anatomy, a very different stromal to epithelial cell ratio (with much more prominent stroma in the human), and important differences in the ratio of basal to luminal epithelial cells (El-Alfy et al, 2000, Price, 1963, Price and Williams-Ashman, 1961, Sugimura et al, 1986). Furthermore, stem cell surface marker profiles are distinctly found in the basal cells of human prostate epithelium while in mice they are also expressed in a subset of luminal epithelia (Leong et al, 2008, Missol-Kolka et al, 2011). The *ex vivo* culture conditions for mouse versus human epithelial cells also reveals different nutritional requirements (Hofner et al, 2015, Karthaus et al, 2014). Finally, rats can be induced by various means, notably by hormonal carcinogens to undergo malignant transformation in the prostate (Noble, 1977, Wang and Wong, 1998) while the mouse prostate is relatively resistant to malignant transformation (Shappell et al, 2003).

Human prostate diseases

Nodular expansion of the prostate transition zone resulting in benign prostatic hyperplasia (BPH) is the most common symptomatic condition in aging men. The typical human prostate transition zone expands from 15 grams, in men in their 40's, to 45 grams in men in their 60's (Roehrborn, 2005). Over the course of decades, the transition zone slowly expands with a doubling time estimated at 4.5 years in men 51 to 70 years old (Berry et al, 1984). The histological incidence of BPH and associated lower urinary tract symptoms increase dramatically with age (Platz et al, 2012). Patients are treated with α -adrenergic blockers and/or 5 α -reductase inhibitors (5ARI) that respectively relax and shrink the gland. Baseline prostate volume and PSA levels are the best predictors of patients who will fail medical therapy, and almost 250,000 men per year undergo some form of surgery for benign prostatic hyperplasia/lower urinary tract symptoms (BPH/LUTS) (Roehrborn et al, 1999, Wei et al, 2008). The predominant medical therapy for an enlarged prostate is a 5-alpha-reductase inhibitor (5ARI), which reduces total prostate volume through apoptosis of both stroma and epithelium (1993). The 5ARI, Finasteride reduces the risk of symptomatic progression of BPH by only 34%, and the drug must be taken continuously with undesirable side effects (McConnell et al, 2003). The cost of treating BPH has been steadily growing commensurate with our aging population and in 2005 was estimated at nearly \$4 billion per year (Saigal and Joyce, 2005). In summary, the development of personalized medical interventions that reliably prevent prostatic enlargement or better reduce prostate volume with fewer side effects is needed.

Prostate cancer is the most common non-skin malignancy in Western males and the second leading cause of cancer-related mortality averaging more the 27,000 deaths per year in the U.S. (Siegel et al, 2015). The widespread use of PSA testing means that prostate cancer is overdiagnosed, while our inability to predict which tumors will remain indolent and which will progress to lethal disease means that patients are steered towards more aggressive

approaches. This combination gives rise to an epidemic of overtreatment (Klotz, 2013, Vickers et al, 2014). Our current treatment paradigm of depriving aggressive tumors of androgens typically extends lifespan by 1–3 years, but ultimately fails due to a number of tumor compensatory mechanisms (Antonarakis et al, 2014, de Bono et al, 2011, Scher et al, 2012). Accordingly, the most important clinical problems in prostate cancer treatment include the development of biomarkers of lethal disease and the treatment of castration-resistant tumors. The evolution of castration-resistant tumors and 5ARI-resistant BPH is the result of dynamic multicellular interactions among epithelia, stroma and inflammation; therefore, the development of novel therapeutic strategies requires a more detailed understanding of the functional contribution of each cell type to disease progression.

In addition to BPH and cancer, the prostate is also the center for prostatitis, inflammation resulting from chronic or acute infections, or from other irritants in the organ. Prostatitis has been linked to chronic pelvic pain syndrome, but this major clinical problem does not seem to involve novel prostatic cells and is outside of the scope of this review.

Prostate cellular hierarchy

The necessity of tissue interactions for organ development and function also extends to the patterning of tissues into cellular hierarchies. Similar to other organs, the prostate is organized with mesenchymal support of parenchymal epithelial tissue. A detailed view of the mechanisms governing these interactions in development and disease is emerging (Strand et al, 2010). In particular, identification of stem cell markers and the use of cellular lineage tracing have uncovered a cellular hierarchy of epithelium during development and in response to injury (Bonkhoff and Remberger, 1996, Choi et al, 2012, Chua et al, 2014, Collins et al, 2001, Garraway et al, 2010, Goldstein et al, 2010, Goldstein et al, 2008, Karthaus et al, 2014, Leong et al, 2008, Luo et al, 2013, Ousset et al, 2012, Richardson et al, 2004, Stoyanova et al, 2013, Vander Griend et al, 2008, Wang et al, 2014a, Wang et al, 2009, Wang et al, 2014b, Wang et al, 2013, Xin et al, 2005, Xin et al, 2003, Xin et al, 2007). A number of distinct stromal cell lineages have also been identified (Peng et al, 2013). The hope is that a detailed characterization of how these stromal and epithelial subpopulations are altered in disease will aid our understanding of the cell(s) of origin in the pathogenesis of prostate diseases for the rational design of targeted therapeutics.

The hierarchy of prostate epithelium has been extensively reviewed (Shen and Abate-Shen, 2010, Wang et al, 2001). In brief, there are three main epithelial lineages: neuroendocrine, basal and luminal. The emergence of a neuroendocrine phenotype in advanced prostate cancer necessitates a deeper understanding of what is normally a tiny subpopulation of epithelium (Beltran et al, 2014). The basal and luminal epithelial lineages have recently been subdivided into multiple subpopulations suggesting these positional terms simply reflect their orientation in a pseudo stratified cellular bilayer. Finally, a transitory intermediate epithelial lineage with characteristics of both basal and luminal cells is observed only in development, regeneration following castration and subsequent androgen replacement, disease and cellular culture (Hudson et al, 2001, van Leenders and Schalken, 2003, van Leenders et al, 2003). Human prostate stroma is normally predominantly smooth muscle with occasional fibroblastic cells, blood vessels, nerves and infiltrating immune/

inflammatory cells. In BPH and cancer the form of the stroma can become more reactive with increased levels of collagen, less well differentiated smooth muscle, increased angiogenesis and often increases in leukocytic infiltrate. The cellular arrangement of the prostate is shown stylistically in Figure 1.

Cellular hierarchy in human prostate diseases

There is currently no consensus on the cell of origin in either benign or malignant prostate disease. In benign disease, both stromal and epithelial tissues expand, but the majority of proliferation occurs in the basal epithelium (Bonkhoff et al, 1994, Dermer, 1978). The lack of phenotypic progression, and the nodular nature of the lesions, means that human BPH is defined at a gross rather than microscopic level, and that biopsies are unhelpful in the diagnosis; therefore, measurements of prostate volume and voiding dysfunction are utilized. It should be noted that this clearly separates human BPH from most models of “hyperplasia” in experimental animals such as mice where phenotypes such as epithelial hyperplasia are obvious on microscopic samples. Human prostate cancer is characterized pathologically by Gleason score (Gleason, 1992, Humphrey, 2004) and stromal composition (Ayala et al, 2003), but the predictive power of these scoring systems is limited, resulting in vast overtreatment (Klotz, 2013, Vickers et al, 2014). The ultimate failure of androgen blockade in both benign and malignant prostate tumors suggests the need for a deeper understanding of intrinsic and extrinsic control of cellular proliferation.

The basal and luminal epithelial compartments of the normal adult prostate house androgen-independent progenitors capable of perpetually repopulating the epithelium after androgen withdrawal and re-administration (English et al, 1987, Wang et al, 2009, Wang et al, 2014b, Wang et al, 2013). All advanced prostate cancers become resistant to anti-androgen therapy, but it is not yet clear whether the prostate cancers are organized hierarchically into castration-resistant stem cell niches, or whether clonal evolution selects for castration-resistant phenotypes (Wang and Shen, 2011).

There has been healthy debate over whether the cell of origin in prostate cancer is a basal, luminal or intermediate epithelial cell. Lineage tracing in transgenic animals shows that driving the same prostate oncogenes in either basal or luminal cells can produce tumors (Choi et al, 2012), but mouse prostate tumors originating in luminal cells more closely mimic human prostate tumors (Wang et al, 2014b, Wang et al, 2013). While cancers in some organs commonly show multiple cellular phenotypes, the vast majority of prostate tumors present as luminal adenocarcinomas, albeit with distinct genomic alterations (Robinson et al, 2015). However, the ability of benign human basal cells transformed with oncogenes to propagate phenotypically-luminal tumors after tissue regeneration with inductive mesenchyme suggests that a simple differentiation event separates the cell of origin in basal or luminal lineages (Goldstein et al, 2010, Stoyanova et al, 2013). This suggests that perhaps we should now focus on the cellular mechanisms of transformation and castration resistance more than the original cell phenotype.

To better understand the castration-resistant cell types capable of regenerating prostate epithelium, a number of cell surface markers have recently been discovered that enrich for prostate stem cell activity including $\alpha 2\beta 1$ integrin, CD49f, CD44, TROP2, CD133, CD117,

CD338, and Sca-1 (mice only) (Garraway et al, 2010, Goldstein et al, 2010, Goldstein et al, 2008, Guo et al, 2012, Lawson et al, 2007, Lawson et al, 2010, Leong et al, 2008, Lukacs et al, 2010, Mulholland et al, 2009, Richardson et al, 2004, Shi et al, 2014, Xin et al, 2005, Xin et al, 2007). Fractions with these markers are enriched for cells that display increased clonogenicity, serial passaging as spheroids, and tissue regenerative capacity as xenografted recombinants with inductive urogenital sinus mesenchyme. Evidence for the cancer stem cell hypothesis shows that cells with these features are enriched in castration-resistant tumors (Yang et al, 2014). It is not clear whether any of these cell phenotypes are enriched in 5ARI-resistant benign tumors of the transition zone.

There is abundant evidence that the stromal microenvironment also plays a role in benign and malignant tumor progression (Franco et al, 2010), and evidence is now mounting that a specific subcellular mix of carcinoma-associated fibroblasts can select for more aggressive tumor phenotypes (Franco et al, 2011), which may actually be cancer stem cells (Liao et al, 2010). It is not yet clear whether carcinoma-associated fibroblasts can be targeted therapeutically to eradicate or stabilize tumors. The immune system is also an influential component of both benign and malignant tumors. Inflammation is a predictor of symptomatic progression in BPH (Nickel et al, 2007) and also of prostate cancer progression (Sfanos and De Marzo, 2012). However, it is still unclear what the functional contribution of inflammation is to tumor progression, or whether it can be used as a therapeutic target.

The differences between mouse and human prostate tissue mean that mouse models fail to fully recapitulate human prostate diseases (Ittmann et al, 2013, Shappell et al, 2004). Therefore, questions regarding the cellular etiology and whole tissue evolution of benign and malignant tumors make use of fresh tissue as well as primary and immortalized cell lines. The subsequent sections will cover current technical advances and challenges for addressing the most pressing questions in prostate cellular biology using primary human tissues and immortalized cell lines in models developed to answer select questions.

Methods

1. Human tissue collection
2. Human tissue processing
3. Cell Identification and Isolation
 - a. Cell Sorting
 - b. Immunohistochemistry
 - c. Cell culture
4. Generation of cell lines

I. Human prostate tissue collection

There is no indication for the removal of normal prostate tissue from young adults, although the ideal control for many experiments is low volume, age-matched tissue. Some groups have had success in collecting tissue from traffic accident victims, but the legal restrictions

relating to this approach vary by jurisdiction and are normally time consuming so this resource is of limited value to most investigators. There is also no routine excision of tissues for early stage BPH or prostatitis. As such, prostate tissue is primarily available from three major sources: prostate cancer patients undergoing prostatectomy as a primary therapy for their cancer, bladder cancer patients undergoing cystoprostatectomy for bladder cancer, and patients undergoing various surgical procedures for advanced BPH. In addition there is limited access to tissues collected through a warm autopsy service. This is especially valuable for studies of prostate cancer metastases and can be accessed through the Prostate Cancer Biorepository network: <http://www.prostatebiorepository.org>

For investigators interested in prostate cancer, radical prostatectomy tissue is the principle source. Tumor size at surgery has been reduced (with associated downgrading of tumors) since the initiation of widespread PSA testing. As a result many of the tumors that are now excised are small and difficult for pathologists to identify by gross examination. For this reason it is essential that a small amount of any sample is fixed or frozen for histological confirmation of the source material. Our experience over a number of years is that tumor is present in approximately 50% of samples labeled “tumor” based upon gross examination. A decision must also be made as to the minimum area occupied by tumor that constitutes a useable sample since many tumors are mostly surrounded by benign areas. A further complication is that the move to robotic laparoscopic prostatectomy, in which tissues are deprived of a blood supply at body temperature for prolonged periods, compromises the viability of tumor cells that are subsequently harvested compared to open surgeries. As a result, viable normal epithelial cells and stromal cells from such samples are relatively easy to isolate while cancer epithelial cells are much more likely to be compromised by the prolonged period of hypoxia between dissection of the blood supply to the prostate and removal of the gland from the body of the patient.

The downsizing and downgrading of prostate tumors means that there are many glands excised in which the tumors are small and localized to the peripheral zone. These patients display a wide range of BPH related symptoms, making them good sources of transition zone (TZ) tissue with which to examine the genesis of BPH (Bauman et al, 2014a, Bauman et al, 2014b, Lin-Tsai et al, 2014, Ma et al, 2012, Nicholson et al, 2013). We typically collect 1–3 grams of TZ from these patients, and upon histological evaluation, approximately 50% of the tissues are viable, cancer-free specimens. Assessment of International Prostate Symptom Score (IPSS) and drug use, as well as the gross appearance of TZ nodules in these patients reveals a full range of BPH, from patients who have little to no benign disease to some with advanced disease. We attempt to reduce the risk of a field effect from the peripheral zone tumor by screening the samples histologically; however, it is not possible to exclude the possibility that underlying molecular changes might have already occurred prior to histological manifestation, which may alter the nature of detectable differences in early compared with advanced BPH specimens. There are a number of additional sources of tissue from patients with advanced BPH symptoms, including Holmium laser enucleation (HoLEP) and simple prostatectomy both of which provide large amounts (typically 20–30 grams) of good quality tissue. Samples from transurethral resection of the prostate (TURP) can also be used with anywhere from 1 to 20 grams harvested, but investigators need to be particularly aware of cautery artifact in these samples.

Peripheral zone tumor samples are typically small (0.5–2 grams) given the aforementioned downgrading of tumors and the fact that much of the tissue must be retained for diagnostic pathology. Centers have a varying incidence of large more advanced tumors, so while these are much less common than they were 20 years ago, large high-grade tumors are still occasionally available. Given their relative scarcity it is important to consider the processing options available for cancer samples to maximize their utility.

Collection of tissues needs to be well coordinated between urology, pathology and the individual investigator under the auspices of appropriate regulatory authorities. Samples must be treated according to the desired endpoint(s). Freshly resected tissues should be kept in ice-cold saline or HBSS where possible. Logistical issues need to be worked through at individual institutions to ensure optimal tissue recovery in terms of both quantity and quality. The timing of the release of fresh tissue from surgery and pathology is somewhat unpredictable and the processing can be lengthy depending on the downstream application. First, most tissue must go through a procurement group that is allowed to enter surgery and pathology suites. IRB approval for the individual investigator and project must be obtained to request tissues from the procurement group, which coordinates with physicians and clinical staff to consent the patients and enter clinical data. Patient confidentiality must be maintained when transporting tissue and through secured communications and databases. For the optimization of tissue viability, it is essential that surgeons and pathologists immediately and continuously keep fresh tissue in cold saline, which may not necessarily be routine as most tissues are normally processed for formalin fixation only. There may also be different requirements for tissues excised for different reasons, with less intervention in cases where malignancy has already been excluded, and surgery is purely to relieve mechanical obstruction. For example, in some institutions by arrangement with the pathology department, open simple prostatectomy tissue can be immediately placed in ice-cold saline and sent directly to the laboratory avoiding tissue procurement. In contrast, tissue from laparoscopic robotic prostate cancer surgeries must go through tissue procurement, which can delay the pickup of tissue by several hours. Our experience is that the overall viability of cells derived from tissue obtained through laparoscopic surgery is approximately half of that seen in samples derived from simple prostatectomy. This is unfortunate from a research perspective, but reflects procedures to provide the best diagnosis and care for patients.

II. Fresh tissue processing

A number of options are available for processing fresh human tissue depending on the intended downstream analyses. Whether generating genomic, proteomic and lipidomic analyses on whole tissue or cellular subpopulations, coordinating the first steps in obtaining high quality tissue with the surgeon and pathologist is crucial. Typically, flash freezing or heat inactivation is sufficient for processing whole tissue for most downstream applications. However, if the desired output of the fresh tissue is single cells, then mechanical and enzymatic digestion is necessary.

There are a wide variety of options based on the length of the protocol. First, tissue must be mechanically minced into smaller pieces, which can be accomplished with autoclaved

scissors and tweezers or a benchtop tissue dissociator. The time-saving utility (and consequent increased cellular viability) of the semi-automated tissue dissociator cannot be underestimated especially when trying to dissociate large volumes such as with simple prostatectomies, which can be 10–20 grams of tissue. Manual dissociation with scissors or razors must be done in ice-cold PBS and can take 1–2 hours potentially causing variation in viability depending on the size of the tissue. However, a significant investment must be made to purchase benchtop dissociators, and great care must be taken to keep the tissue from overheating. Typically, once mechanically dissociated tissue can be passed through a 10-ml pipette, it is ready to be enzymatically digested into single cells.

The digestion of tissue chunks into single cells can be performed at 37°C overnight or for 4 hours with a 5-fold increased enzyme concentration, depending on what time of day the fresh tissue was collected. We have found that increased enzyme concentration facilitates faster digestion without sacrificing viability; however, the post-digestion single cell purification procedure detailed below, and illustrated in figure 2, requires an extra 2 hours making for an impractically long day if the fresh tissue is not received until the afternoon. Typically fresh tissue is unavailable in most centers until after 11 am, so an overnight digest that includes a controlled freezing step in high serum and DMSO allowing delayed analysis by flow cytometry is a reasonable compromise.

We recently directly compared a number of different enzyme formulations on individual tissue samples to determine the best conditions for the highest cellular viability for flow cytometry. Per previous protocols (Goldstein et al, 2011, Hofner et al, 2015), we began by digesting with 1mg/ml collagenase I, II or IV overnight. Crude collagenases, especially type I and II, contain both variable collagenase activity and variable amounts of other enzymes active in the digestive process. This means that batch testing and then purchasing a large supply of suitable batches of these reagents is desirable. Given the batch-to-batch variability in units of enzymatic activity with traditional sources of collagenase, we tested the commercially available formulations of Liberase enzymes (Roche), which have batch-to-batch consistency and higher specific activity due to an optimized purification process. We found that Liberase TH reproducibly resulted in 10% higher viability vs. the other formulations of Liberase (DL, DH, TL, TM) or traditional collagenases I, II or IV. It should be mentioned that the choice of enzyme can also affect cell surface proteins, negatively impacting downstream applications such as flow cytometry. Figures 3C and D demonstrate a severe loss of cell surface detection for CD49f with Liberase, and many other antigens were similarly negatively affected. Therefore, the balance of viability must be considered in concert with downstream detection methods so we suggest collagenase digestion for FACS. In addition, if primary cells are to be grown out of the fresh tissue, 10µM ROCK inhibitor will decrease dissociation-induced apoptosis (Watanabe et al, 2007). Finally, 100U/mL Penicillin, 100µg/mL Streptomycin, 2.75µg/mL Amphotericin B are added to reduce contamination. All media and tools must also be free of contaminants. Table 1 details the step-by-step protocol for human tissue digestion (benign or cancer) while Table 2 provides details of reagents.

The last step of the digestion protocol is to freeze cells down for later analysis. The difficulty of coordinating the reservation of flow cytometer core time with the unpredictable timing of

fresh tissue collection is often problematic. Therefore, we adapted a freeze-thaw protocol, used by others (Deon Doxie and Jonathan Irish personal communication) to allow a break in the protocol. The viability of cells does not change even after >6 months storage in liquid nitrogen.

iv. Identification and Isolation of discrete cell populations

There are two major approaches to examining and identifying specific cellular subtypes in prostate tissue. These are immunohistochemical localization and FACS.

Immunohistochemistry provides spatial data, showing the positional relationships between cells. FACS provides a more rigid analysis of cell numbers at the cost of knowing the position of the cells and also the loss of 50% of total cells to cell death, the majority being luminal epithelia (Table 5). It is also possible to examine the colocalization of a much larger number of markers simultaneously with FACS, without the use of exotic and expensive approaches. In contrast, large numbers of stains used simultaneously for multispectral imaging are problematic for immunohistochemical or immunofluorescent analysis.

Isolation of cell types can also be approached in two ways, cell culture and FACS sorting. Cell culture approaches have been used for several decades to isolate both epithelial and fibroblast populations, which can either be cultured as primary or low passage cultures or further developed into cell lines (Franco et al, 2011, Hayward et al, 1987, Jiang et al, 2010, Olumi et al, 1999).

Cell Sorting

The epithelial and stromal cell hierarchy of the prostate has been delineated beautifully with lineage tracing and castration/regeneration in mouse models (Ousset et al, 2012, Peng et al, 2013). The normal prostate is an immunocompetent organ and contains small numbers of resident T and B lymphocytes (Steiner et al, 1994). Here we provide protocols for the identification and isolation of inflammatory, epithelial and stromal cells, however, there are many options available at each step. For instance, although we detail a protocol for fluorescence-activated cell sorting (FACS) on a BD FACS Aria III cell sorter, magnetic bead-based sorting (MACS) is quicker, less expensive, and preserves better cellular viability with the caveat that the signal is either positive or negative, rather than a continuum as with FACS. For instance, MACS would be appropriate for the isolation of total epithelium based on CD326, but not for basal epithelium with CD49f where the signal is high or low. Also, both epithelial stem cells (Collins et al, 2001) and stromal cells (Olumi et al, 1999) are capable of *ex vivo* enrichment by attachment to matrix-coated culture plates or Percoll gradients, the advantages of which should be judged by the downstream application.

The step-by-step flow cytometry protocol and antibody information for identifying various epithelial and inflammatory cellular subtypes are detailed in Tables 3 and 4. Figure 3 shows a gating schema for identification of cellular lineages after standard forward and side-scatter gating (FSC-A vs. SSC-A, FSC-H vs. FSC-W and SSC-H vs. SSC-W) to remove apoptotic cells and cell multiplets. Amine reactive viability dyes are very useful when sorting dissociated prostate tissues, as cell viability tends to be around 40 to 60%, and the non-viable cells often bind antibodies non-specifically. The viable cells can be sorted using

antibodies for CD326 and CD45. Epithelia stain CD326⁺/CD45⁻, while leukocytes stain CD326⁻/CD45⁺. The CD326⁻/CD45⁻ fraction is the stromal compartment and include all non-epithelia and non-leukocyte cells, including endothelial cells. This compartment can be further sorted as needed, for example gating out CD31⁺ cells to remove endothelial cells. It is important to note that the protocol used to dissociate the cells from the tissue can affect some of the epitopes used for flow cytometry. Figure 3C shows epithelial cells plotted with CD49f and CD26 to separate basal (CD49f^{HI}/CD26⁻) and luminal (CD49f^{LO}/CD26⁺) fractions from a tissue sample that was dissociated with 1mg/mL collagenase I overnight. Figure 3D, in comparison, was the same tissue dissociated in 37.5ng/mL Liberase TH overnight, and shows a loss in CD49f reactivity from the surface of basal cells. Liberase would only affect some cell surface epitopes and should be determined if this dissociation protocol is compatible with the desired antibody panel. Liberase appears to cleave CD49f, CD163, CD4, CD8 and CD16 [(Hagman et al, 2012) and Strand unpublished]. Separating the CD326⁺ epithelia into basal (CD49f^{HI}/CD26⁻) and luminal (CD49f^{LO}/CD26⁺) fractions, we were able to further determine that luminal epithelia were particularly sensitive to the digestion protocol showing an average of around 50% viability compared to around 90% for basal epithelium, CD45⁺ leukocytes and stromal cells. This results in an underrepresentation of the proportion luminal epithelium (Figure 3B). Antibody fluorophore combinations and average viabilities for each subcellular fraction are shown in Tables 4 and 5.

The immune/inflammatory cell system has been extensively studied using FACS. However identification of stromal cell subtypes is less well understood. In particular the identification of fibroblast subpopulations and the significance of these in prostate biology is presently an underdeveloped area. Several markers have been proposed for the isolation of fibroblasts from normal and cancer tissues using FACS technology. The spatial expression of CD markers in prostate cancer patients with GS 6 (3+3) has been characterized by immunohistochemical analysis (Liu and True, 2002). In this study fibromuscular stroma was enriched for CD49a, CD49e, CD61, CD81 and w131, and endothelial cells were enriched for CD31, CD34, CD39, CD62E, CD62P, CD105 and LMP-1. It has also been shown that there is an increased population of CD90^{Hi} expressing fibroblasts in the stroma of prostate cancer tissues (Pascal et al, 2011). CD90, CD73, and CD105 triple positive mesenchymal stem cells (MSC) have been shown to be present in the tumor stroma. These MSCs can differentiate into osteoblasts, chondrocytes, and adipocytes, and their presence may have implications during carcinogenesis (Brennen et al, 2013). PDGFR-alpha (CD140a) and -beta (CD140b) have also been proposed to be markers of fibroblast populations in animal models (Erez et al, 2010, Pietras and Ostman, 2010). Fibroblasts from prostate tissues can be enriched using a depletion method by negatively gating out leukocytes (CD45⁺), epithelia (CD326⁺) and endothelia (CD31⁺) (see Figure 4). The remaining cells can be selected for these cell surface markers. However these cell populations require further testing using an in vivo system of tumor promotion to understand the functional consequences of their presence during carcinogenesis (Olumi et al, 1999).

The digestion protocol provided here preserves about 85% viability of CD45⁺ cells (Figure 4, Table 5). While immunohistochemistry of tissue blocks can provide an estimate of individual infiltrates, multi-color flow cytometry offers the opportunity to identify multiple leukocytic populations simultaneously, some of which require multiple markers for proper

identification (e.g., M2 macrophages in Figure 4B). Flow cytometry of larger pieces of whole fresh tissue is superior to tiny tissue cores, which may not provide adequate coverage of focally located cell types such as clustering B cells. However, unbiased slide-scanning computerized quantitation of various inflammatory cell types can be used on archived blocks where fresh tissue is not obtainable (Figure 4E).

Immunohistochemistry/Immunofluorescence

Immunocytochemistry and immunofluorescence can be used to identify, localize and quantitate cell populations based upon either cell surface or intracellular marker expression. Traditional approaches to quantitating immunohistochemical data have involved the generation of in house approaches to attempt to rationalize data sets. However immunohistochemistry is not a strictly quantitative approach as staining intensity is dependent upon a number of variables that are not always under the control of individual investigators, such as the length of fixation of specific samples and the inter-sample variability of this criterion.

The development of automated slide scanning and data capture has improved the ability to quantitatively capture data of this nature, although, as always, some caveats apply. Slide scanning can be performed following immunohistochemistry (Table 6) to identify location and quantity of specific cell populations, for example inflammatory cells (Table 7). Slide scanners, for example, the Leica SCN400 (Leica Biosystems) system allow for immunostained tissue slides to be imaged at various magnifications. Cells can then be identified utilizing standard analysis scripts provided by software such as Ariol (Leica). These analysis scripts utilize algorithms, which set upper and lower thresholds for color, saturation, intensity, cell size, roundness and axis length for both blue Hematoxylin-stained nuclei and brown DAB-stained positive cells (Figure 4E). Therefore positive cells can easily be distinguished from negative cells. The advantages of using this method are (1) the process can be carried out using paraffin-embedded tissue when fresh tissue is not available for FACS analysis, (2) the areas of interest in a tissue sample or tissue microarray can be isolated once scanned into the computer software and (3) the computer software analyzes the sample in a more accurate and unbiased fashion compared to analyzing the sample manually. Furthermore, using this technology allows one to determine mathematically by area analysis, if there is clustering of positively stained cells and if the positive cells are more abundant in or adjacent to the epithelial or stromal compartment. The disadvantages of utilizing this technology include the lack of application in live cells and the technical difficulty involved in staining for multiple cell populations at once.

Cell culture

For cell culture, tissues are collected and processed as described above (Table 1). Selection of epithelial cells may be performed both mechanically and by plating onto plastic or collagen gel in selective medium formulations (specifically relatively low serum, the presence of EGF, insulin, and cholera toxin as well as the use of bovine pituitary extract – see Table 8). To enrich specifically for epithelial cells, tissues can be digested to a point at which the stromal cells have dissociated from the epithelium but the epithelial organoid

structures are still visible. These can be separated from the stromal cell isolates using unit gravity sedimentation in conical bottomed 50ml plastic tubes, serially removing the supernatant rewashing and resuspending the structures until the epithelium appears clean by visual and low magnification microscopic examination. This allows a highly enriched epithelial starting population, however this process can never exclude all fibroblasts, and so strategies to eliminate residual fibroblasts using appropriate medium are necessary. In general epithelial cells grow better in the presence of relatively low levels of serum (0.5–2.5%) while fibroblastic cells proliferate more in higher serum concentrations (5–10%). Various additives including EGF and bovine pituitary extract seem to preferentially benefit the growth of epithelium, while fibroblasts can be inhibited by the presence of cholera toxin in the medium. In addition, epithelial cells will adhere better to some culture surfaces, such as collagen and will grow preferentially on this matrix. If pure primary epithelial cell cultures are required, it is necessary to carefully monitor cultures to visually confirm the absence of contaminating stromal cells. Contaminating fibroblasts can be removed by partial trypsinization as the epithelial cell sheets that grow from benign sources are much more resistant to removal than the fibroblastic cells that will round up and detach more rapidly and can be removed by this method. Trypsin can then be neutralized using serum, and epithelial culture continued in the same flask.

Fibroblasts can be easily cultured from single cell suspensions by simply plating in growth medium containing 10% fetal bovine serum. Fibroblasts will preferentially adhere and aspirating the non-adhered cells after 30–60 minutes can quickly eliminate most contaminating epithelium. Fibroblasts also survive trypsinization better than epithelium, in a reversal of the approach described in the previous paragraph to clean epithelial cell cultures, fibroblasts can be cleared of epithelial contamination by exploiting the same rapid release from plastic surfaces. Any contamination can be easily checked by plating cells onto glass slides and staining with a wide spectrum cytokeratin to identify epithelial cells and VWF to identify any endothelial cells. Plated fibroblasts normally will express vimentin with variable amounts of α -actin, desmin and myosin. This suggests a mixed fibroblastic/myofibroblastic phenotype. Well-differentiated smooth muscle cells, expressing muscle markers but not vimentin, do not grow well (if at all) in culture.

IV. Generation of cell lines

Historically there have been a limited number of human prostate cancer cells available reflecting the difficulty of deriving lines from tissues, and especially the difficulty of deriving tumor cell lines. The preponderance of work performed on LNCaP and its derivatives, PC3 and DU145 (Horoszewicz et al, 1980, Kaighn et al, 1979, Stone et al, 1978, Wu et al, 1994) speaks to the limited resources available. The situation has improved somewhat with more cancer and some benign lines becoming available (Gao et al, 2014, Jiang et al, 2010), but there is a continuing need to work with human cells and tissues to address questions including, but not limited to the cell type of origin for prostate cancer, mechanisms of cellular renewal, metabolism, differentiation and organ maintenance, the presence and role of specific and presently undefined subpopulations of cells within stromal and epithelial tissues, and the interactions between prostatic tissues and the immune/inflammatory system.

Immortalization of primary cell cultures can be performed by a number of routes. Historically cells have been immortalized by indiscriminate methods such as chemical carcinogens (Rhim et al, 1997) or by the introduction of viral oncogenes such as SV40T or HPV E6/7 (Bello et al, 1997, Hayward et al, 1995, Rhim et al, 1994). These techniques are effective but give rise to cell lines with major genetic anomalies, which can occasionally be useful but do not truly represent disease processes as they occur in human prostate. More recent work has tended to utilize less genetically damaging approaches including the use of telomerase (Franco et al, 2011) and carefully nurturing cells through culture crisis to establish “spontaneously” immortalized lines (Jiang et al, 2010). These approaches have been useful in generating cells that can undergo reasonably normal appearing differentiation when given appropriate environmental cues. However it should always be borne in mind that immortalized cell lines always have some significant genetic changes from wild type cells, and, as such, data generated using them must be treated with appropriate caution.

Summary and Future directions

Isolation of individual cell populations gives rise to the ability to genetically modify and functionally test aspects of prostate cell and organ biology. Methods for the genetic modification of cells using retro- and lentiviral vectors have been extensively described elsewhere, and we have applied these to both stromal and epithelial cell populations to explore these effects (Ao et al, 2007, Franco et al, 2011, Williams et al, 2005). Clearly the use of appropriate models is key in biology. Two-dimensional cell culture is well recognized for being extremely limited in its ability to address questions aimed at organ biology (while being well suited to many detailed molecular questions). The developments in cell isolation, three-dimensional culture and co-culture of cell populations as well as the continued use of xenografts and tissue recombination approaches can all utilize human prostatic cells to address questions relating to organ biology and disease. In order to function properly cells need to be able to interact in an appropriate three-dimensional arrangement and to communicate with other cell types. To fully appreciate this, a range of approaches is often needed. For example our experiments identifying carcinoma-associated fibroblasts as promoters of cancer progression depended upon the development and use of an appropriate tissue recombination bioassay. However the use of this in vivo approach required two-dimensional culture to isolate cells as an intermediate technology.

The development of transgenic animals opened new possibilities to develop new models of prostate cancer. These have increased in sophistication over the last few years and now represent quite good approximations to some aspects of human disease (Ittmann et al, 2013, Shappell et al, 2003). However even the best models available do not show all aspects of the human disease, and there is a strong case to be made for the necessity of also working with human cells and tissues.

In contrast to cancer studies, modeling BPH in mice is more problematic since we do not yet understand what pathways are driving nodular growth in humans. Many insults can result in epithelial hyperplasia in the mouse prostate, and in some cases there is an associated stromal expansion. Such models must be treated on their merits, which often means that they provide insights into specific aspects of the disease, even though it is difficult to conclude that any of

the available mice truly mimic the focal nodular human disease let alone the complex environment in which it develops. The limitations of specific models must always be borne in mind, and a range of options is clearly both desirable and necessary. For these reasons there is an ongoing need to dissect the molecular mechanisms of BPH and prostate cancer by studying primary human tissues as well as developing experimental models with human cell lines to replicate specific aspects of human disease. The methods outlined here provide a basis for investigators to identify and isolate human cells from which they can generate basic tools to pursue such studies.

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Figure 1.

Major cell populations found in the prostate. The stroma is a complex mix of smooth muscle, resident fibroblasts and immune/inflammatory infiltrate along with blood vessels and nerves that are not illustrated here. The epithelium is composed of luminal and basal cells. The basal epithelium includes presumptive stem cells. The luminal epithelial cells are predominantly secretory. Neuroendocrine cells are normally rare but can expand in some forms of advanced cancer. Expansion of intermediate epithelial cells is associated with inflammation *in vivo* and with *ex vivo* culturing of primary cells.

Figure 2.

Tissue preparation and analysis workflow. (A, B) Pathologist prepares 20–30 gram transition zone section. Ten grams is digested overnight into ~100 million single cells (C) and frozen down into ten separate 10 million cell aliquots in 90% FBS/10% DMSO.

Figure 3.

Flow cytometry strategy to fractionate stromal, epithelial, and leukocytic cell populations in a simple prostatectomy specimen. (A) Live/Dead stain identifies viable cells already enriched by scatter plots for further analysis. (B) The three major cellular populations in human prostate are fractionated by CD326 (epithelia) and CD45 (leukocytes) with stroma being negative for both. (C, D) Enzymatic digestion with collagenase (C) preserves CD49f antigen for identification of basal epithelia, which is eliminated with Liberase TH digestion (D). CD26 detection of luminal epithelia is unaffected, but other antigens such as those presented by leukocytes are also cleaved by Liberase digestion.

Figure 4.

(A–D) Flow cytometry and immunohistochemistry for chronic inflammatory cells in BPH tissue. Percentages listed are of total CD45⁺ leukocytes in a representative simple prostatectomy sample digested with collagenase. (A) 50% of all viable leukocytes are CD11b⁺ monocytes with 36% also positive for the M2 macrophage marker CD163 (B). Only 0.3% of all CD45⁺ leukocytes were CD4⁺ (C) while 3% were CD8a⁺ (D). (E) Example of a high resolution scanned slide utilizing a Leica SCN400 slide scanner. Cells were immunostained for clustered CD20+ B cells. Screenshot of Ariol software showing a selected area (boxed) of automated quantification of positive cells in red (DAB-stained cells), with blue denoting negative cells (Hematoxylin-stained nuclei).

Table 1

Human tissue digestion protocol – 1–2 hours prep, 12–14 hour digestion.

1	Weigh tissue and transfer to 100mm plate with cold HBSS with 1% BSA.
2	Use autoclaved scissors and forceps to cut tissue into small enough pieces to be passed through a 10ml pipette.
3	Transfer tissue pieces and HBBS to a conical tube.
4	Spin at 1800rpm for 5 min at 4°C.
5	Aspirate supernatant and resuspend in 30mls cold PBS. Repeat wash.
6	Add enzymatic digestion solution at 10ml/g of tissue (Note: fill to less than 90% capacity) <i>0.8–1.5mg/mL Collagenase I (depending on the batch)</i> , <i>10µM ROCKi (optional)</i> , <i>0.2mg/ml DNaseI</i> , <i>10µl/mL Fungizone</i> , <i>10µl/mL Pen/Strep in HBSS</i>
7	Digest overnight at 37°C with rocking on an Adams Nutator mixer.
8	Spin at 1800rpm for 5 min at 4°C. (Note: floating debris is normal and discarded)
9	Wash 2X in 25ml cold PBS.
10	Resuspend cells in 5 ml TrypLE and incubate for 5 min on Nutator mixer at 37°C.
11	Add to TrypLE 15 mls DMEM + 10% FBS with 1mg/ml DNaseI.
12	Spin at 1800rpm for 5 min at 4°C.
13	Resuspend in 10ml DMEM + 10% FBS and pass through 18-G needle/10ml syringe 2–5X. Repeat with 20-G and 22-G needles. (Note: if tissue cannot be passed through 18-G needle, first proceed to step 14, forcing through needles decreases viability)
14	Filter cells/media through 100µm cell strainer and wash conical tube with additional 10 ml DMEM + 10% FCS.
15	Spin at 1800rpm for 5 min at 4°C.
16	Resuspend in 5mls of 1X RBC lysis buffer (10x plus distilled water) for 5 min on ice with occasional shaking.
17	Add 25 ml cold PBS and spin at 1800rpm for 5 min at 4°C.
18	Resuspend pellet in 10 ml DMEM + 10% FBS and filter through 40µm cell strainer. Wash with addition 10 ml DMEM + 10% FBS.
19	Spin at 1800rpm for 5 min at 4°C.
20	Resuspend cell pellet with 10ml DMEM + 10% FBS for counting with trypan blue.
21	Spin down and resuspend 10million cells/ml in 10% DMSO, 90% FBS for freezing in liquid nitrogen.

Table 2

Reagents needed for human tissue digest.

Fresh human prostate tissue
DMEM (GE Healthcare, cat. no. SH30249.01)
PBS (Corning, cat. no. 21-031-CV)
HBSS (GE Healthcare, cat. no. SH30268.01)
FBS (Sigma, cat. no. F2442)
DMSO (Fisher, cat. no. BP237-700)
Trypan Blue (Life Technologies, cat. no. 15250-061)
TrypLE Express (Life Technologies, cat. no. 12605-010)
RBC lysis buffer (BioLegend, cat. no. 420301)
Collagenase type I (Gibco, cat. no. 17100-017)
Penicillin-Streptomycin (Corning, cat. no. 30-004-CI)
Fungizone (Life Technologies, cat. no. 15290-018)
DNase I (Roche, cat. no. 10104159-001)
Cell strainers (Corning, cat. nos. 352360, 352340)
Rock inhibitor (StemRD, cat. no. y-005)
18-, 20-, 22-G needles (BD Becton Dickinson, cat. nos. 305196, 305175, 305155)
10ml syringe (BD Becton Dickinson, cat. no. 309604)

Table 3

Flow cytometry protocol – 2 hour prep, 1.5–4 hour protocol.

1	Thaw cells at 37°C until almost unfrozen and add to DMEM/F12 plus 20% FBS and 100U/mL Penicillin, 100µg/mL Streptomycin, 0.25µg/mL Amphotericin B.
2	Spin and wash twice with PBS.
3	Spin and resuspend in 1ml PBS.
4	Add 1µL (or according to manufacturer's protocol) of amine reactive viability dye and incubate for 30 minutes at 4°C.
5	Spin and wash once with a cell-staining buffer (eg. PBS with 2% FBS and 0.1% sodium azide, or a commercially available alternative).
6	Resuspend in 100µL and add Fc receptor blocking antibody according to manufacturer's instructions for 5min at RT.
7	Split each sample in two (one to be stained and the other to remain unstained, approximately 5 million to 500,000 cells each) and bring to 100µL in cell staining buffer.
8	Either aliquot approximately 100,000 cells or a commercially available compensation bead as recommended by the manufacturer for single antibody compensation.
9	Add all antibodies to the cells set aside to be stained, and each antibody to the single antibody compensation tubes.
10	Incubate in dark on ice for 15–30min with occasional shaking.
11	Spin and wash once in cell staining buffer.
12	Spin and resuspend in 500µL-1mL cell staining buffer and filter through 25–40µm strainer into tube for flow cytometry. <ol style="list-style-type: none"> a. At this point cells can be fixed, permeabilized and stained if antibodies for internal proteins are to be detected.
13	Store on ice and in the dark until flow cytometry.

Table 4

Antibodies for identification by FACS.

Antigen	Conjugate	Cell type
CD45	PE/Cy5	Leukocytes
CD326	PE/Cy7	Epithelial
CD49f	PEBV421	Epithelial, Stromal
CD26	APC	Luminal Epithelial
CD11b	BV650	Monocytes
CD163	PE	M2 macrophages
CD4	PerCP/Cy5.5	T helper cells
CD8	FITC	Cytotoxic T cells
Live/Dead	APC/Cy7	

Table 5

Typical viabilities for each subcellular fraction.

Cell type	Avg. Viability	Percent of total
Basal epithelia	95%	40%
Luminal epithelia	47%	3%
Stroma	93%	33%
Leukocytes	86%	18%

Table 6**General protocol for Immunohistochemistry.**

Day 1 – 3 hour protocol:	
Deparaffinization	
1	Submerge slides in HistoClear two times for 10 minutes.
Rehydration	
2	Submerge slides in a series of graded ethanol washes. <ul style="list-style-type: none"> a. 100% ethanol for 10 minutes. b. 95% ethanol for 10 minutes. c. 70% ethanol for 10 minutes.
3	To block endogenous peroxidase place slides in 0.3% H ₂ O ₂ in MetOH @ RT for 30 minutes.
4	Wash in running distilled water for 5 minutes.
Antigen Retrieval	
5	Dilute 10x Antigen Retrieval Solution to 1x in distilled water.
6	Pre-heat 1x Antigen Retrieval Solution for 5 min in microwave (100% power).
7	Place the tissues into a container with enough 1X target retrieval solution to completely cover slides.
8	Cook slides in microwave for 20 minutes at 30% power – watch for evaporation.
9	Cool to room temperature for 20 minutes.
10	Rinse slides in 1X PBS for 5 minutes.
11	Incubate in 2.5% horse serum (diluted in 1X PBS) for 60 minutes at room temperature.
12	Dilute Primary antibody in 1X PBS + 2.5% horse serum (prepare ≈75 µL per slide).
13	Incubate slides in primary antibody overnight at 4°C.
Day 2 – 5.5 hour protocol:	
14	Wash in 1x PBS two times for 30 minutes at 4°C.
15	Dilute secondary antibody in 1x PBS + 2.5% horse serum (prepare ≈75 µL per slide).
16	Incubate slides in secondary antibody at room temperature for 60 minutes.
17	Wash in 1x PBS two times for 30 minutes at 4°C.
	*Note: after first 30 minutes take out and prepare Vectastain ABC reagent.
18	Incubate with Vectastain ABC reagent at room temperature for 60 minutes.
19	Wash in 1x PBS two times for 30 minutes at 4°C.
20	Develop with DAB at room temperature until stained.
21	Wash in running tap water for 10 minutes.
	*Note: tap water is alkaline and allows for better counterstain.
22	Incubate in hematoxylin at room temperature for 1–2 minutes (time varies depending on time of hematoxylin used).
23	Wash in running tap water for 2–3 minutes.
24	Dip 3 times in 1% acid alcohol
25	Wash in running tap water for 2 min
26	Dip 10 times (or until blue) in 0.3% ammonia water.
27	Wash in running tap water for 1 minute.
28	Dehydrate in 70% EtOH – 2 × 10 dips, 95% EtOH – 2 × 10 dips, 100% EtOH – 2 × 10 dips and HistoClear 3 × 20 dips.

29 Mount coverslips to slides.

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Table 7

Materials and reagents needed for immunohistochemistry:.

Cut slides from paraffin-embedded prostate tissue
100%, 95%, 70% ethanol
30% Hydrogen peroxide (Fisher, cat. no. H325-100)
Methanol
Antigen Retrieval Solution (Dako, cat no. S169984-2)
PBS (Corning, cat. no. 21-031-CV)
Horse Serum (Vector, cat. no. S-2012)
Vectastain universal ABC reagent (Vector, cat. no. PK-6200)
DAB (Fisher, cat. no. 34002)
Harris Hematoxylin (Sigma, cat. no. HHS128-4L) or hematoxylin of choice
1% acid alcohol
0.3% ammonia water
Mounting media such as Cytoseal (Thermo Scientific, cat. no. 8312-4)

Table 8

Media for Primary Culture of Human Epithelium and Fibroblasts.

Epithelium	
	RPMI-1640
	2.5% DCC-FBS (dextran coated charcoal-stripped and heat-inactivated fetal bovine serum)
	Penicillin-Streptomycin (Corning, cat. no. 30-004-CI)
	Fungizone (Life Technologies, cat. no. 15290-018)
	4µl/ml bovine pituitary extract - (Fisher Sci CB-40123B)
	1/100 ITS - insulin-transferrin-selenium (Fisher Sci 50-101-5467)
	5µg/ml Cholera toxin (Sigma Aldrich C9903)
	10ng/ml EGF (Fisher Sci CB-40052)
Fibroblasts	
	RPMI-1640
	10% fetal bovine serum