Minireview

New concepts concerning prostate cancer screening

Rebecca A Fillmore[1], Chinatsu Kojima[2], Chevaun Johnson[2], Georgina Kolcun[2], Lawrence J Dangott[3] and Warren E Zimmer[2,4,5]

[1]Department of Biological Sciences, University of Southern Mississippi Gulf Coast, Long Beach MS 39560, USA; [2]Department of Medical Physiology, College of Medicine, Texas A&M Health Science Center, Texas A&M University, College Station, TX 77843-1114, USA; [3]Department of Biochemistry and Biophysics, College of Agriculture and Life Sciences, Texas A&M University, College of Medicine, TX 77843, USA; [4]Interdisciplinary Faculty of Toxicology, Texas A&M University, College Station, Texas, 77843; [5]Faculty of Genetics, Texas A&M University, College Station, TX 77843, USA

Corresponding author: Warren E. Zimmer. Email: WEZimmer@medicine.tamhsc.edu

Abstract

Prostate Cancer (CaP) is rapidly becoming a worldwide health issue. While CaP mortality has decreased in recent years, coincident with the widespread use of Prostate-Specific Antigen (PSA) screening, it remains the most common solid tumor in men and is the second leading cause of cancer death in the United States. The frequency of CaP is growing not only in western cultures, but also its incidence is dramatically increasing in eastern nations. Recently, examination of data from long-term trials and follow up has cast a shadow on the effectiveness of employing PSA as a primary screening tool for CaP. In this review, we not only summarize opinions from this examination and synthesize recommendations from several groups that suggest strategies for utilizing PSA as a tool, but also call for research into biomarkers for CaP diagnosis and disease progression. We also describe our recent work that identified a smooth muscle contractile protein in prostate epithelia, namely smooth muscle gamma actin, and indicate the potential for this molecule as a new unique footprint and as a CaP marker.

Keywords: Prostate cancer, prostate-specific antigen, Prostate Cancer Antigen 3, TMPRSS2–EGR gene fusion, smooth muscle gamma actin

***Experimental Biology and Medicine* 2014; 239: 793–804. DOI: 10.1177/1535370214539091**

Introduction

The prostate is an accessory gland of the male reproductive system that in adults resides just under the bladder.[1] In humans, it arises from the epithelial budding of the urogenital sinus,[2,3] with glandular epithelium budding into surrounding mesenchyme. It surrounds the urethra which is joined within the glandular tissue by the ejaculatory ducts from the seminal vesicle providing the route for sperm release.[4,5] The gland remains small and relatively inactive until puberty when the surge in circulating androgen leads to its rapid growth and the secretory function of the gland becomes active.[2,3,6–9] While the prostate lacks the architecture normally associated with secretory glandular tissue, the prostate is said to be zonal, containing central, transitional, and peripheral zones[3] surrounded by a fibromuscular stroma. The outermost peripheral zone is the largest of the zones and is the site in which the majority of the CaP arises.[1,10]

Prostate cancer is one of the most pronounced, non-cutaneous malignancies diagnosed in men. In 2012, over 240,000 men were diagnosed with CaP in the United States with approximately 30,000 deaths attributed to the disease (American Cancer Society[11–13]). CaP is considered a disease of the elderly because the incidence of clinically diagnosed cancer is strongly associated with ageing. Rarely is CaP observed in men younger than 40 years and 97% of clinical cases are diagnosed in men 50 years and older.[14,15] However, histopathological analyses have shown initiating lesions in prostate glandular tissue (PIN) can be observed in men as young as 20 and increases in frequency as men age to 50 years.[3] The lifetime risk for a male in the United States to develop CaP is about one in six, while estimates based upon pathological analyses may suggest that all men 50 years and older have initiating lesions.[3,7,16] Aside from age, recent analyses have demonstrated other risk factors for CaP including, race, life style, family history, and genetics.[14] It is interesting that in African-American men, CaP is much more prevalent than other races, and these men are more likely to have an aggressive cancer leading to death.[17–19] While nationality has been considered a risk factor due to the lower rates observed in Asian cultures, more recent application of screening protocols indicate

that this difference may be due to early detection through screening of asymptomatic individuals.[16,20]

Similarly, the effects of life style and diet have been implicated as potential risk factors in CaP. However, the exact role has not been elucidated, and it is possible that these will accompany an increased inflammatory response, both systemic and prostate specific (termed *prostatitis*), which has been positively correlated with the risk of many cancers.[21,22] CaP also has a clear genetic component. If a direct relative (parent, sibling, or offspring) has cancer, there is a higher risk of cancer development, and the more relatives that have cancer the higher the risk factor of cancer development.[23] Several genes and genetic loci have been shown to be associated with CaP risk.[23–28] Some of these are common to other cancers (for example, BRCA2 for breast cancer) although the clinical application of genetic markers has not been fully realized. It is clear that research into molecular markers will further the definition of CaP, and hopefully identify more definitive risk identifier(s) of cancer at the molecular level.

As indicated above, the lesion within prostate tissue termed prostatic intraepithelial neoplasia or PIN is believed to be a precursor for human CaP. PIN is recognized as a continuum of morphologies that describe the events that lead from normal prostate tissue to metastatic cancer.[9] Moreover, advancement of cancer through PIN morphological stages can be correlated with watershed events in CaP progression, therefore, allowing a model or description of cancer initiation.[3,7,9,28] This is evident in genetic analyses of chromosome aberrations in CaP tumors/tissues which have demonstrated losses in heterozygosity at chromosomes 8p, 10q, 13q, and 17p. Further losses have been described for chromosomes 6q, 7q, and 18q.[29–32] Some of these aberrations have been linked to potential tumor suppressor genes including Nkx3-1 on chromosome 8p[6]; PTEN on chromosome 10q[20,33]; and p53 on chromosome 17q.[34] Most frequently, allelic loss is found to result in a reduction of the expression of the associated tumor suppressor genes; however, the finding that there is no singular chromosomal event being causative of CaP leads us to the conclusion that prostate adenocarcinoma requires cumulative chromosomal events to manifest a metastatic state. This accumulation of chromosomal events idea is consistent with recent findings in mouse models for CaP, and might be the basis for the deliberate progression of this cancer in humans.[6,9,35]

Prostate-specific antigen

The use of biomarkers has long been a staple for the detection and management of CaP. Prostate-specific antigen (PSA) was initially identified in the 1970s as a low-molecular weight protein component of the seminal fluid expressed by prostate epithelia.[36–38] It was subsequently demonstrated to be a serine protease of the kallikrein family, encoded by the kallikrein 3 gene (KLK3), that participates in maintaining the fluidity of the seminal fluid for sperm motility.[39] In 1987, Stamey et al.[39] demonstrated that PSA, found in low levels in the serum, increased in serum content, coincident with altered prostate function, and PSA detection was more sensitive than that of prostatic acid phosphatase. These studies led the US Food and Drug Administration to approve the measurement of PSA levels as an adjunctive test to the digital rectal exam for the detection of CaP in men older than 50,[40] being adopted even though the specificity of the assay was unsure. Subsequent validation of PSA as a marker for prostate in large clinical studies, coupled with enhanced clinical techniques in surgery and biopsy, cemented its rise as the diagnostic for CaP. It was initially thought that PSA would be discriminatory between benign and malignant prostate because of its apparent difference in expression levels in these cells compared with normal prostate tissue. With the widespread application of PSA testing as a detector of CaP, there was a large increase in the number of men diagnosed with the disease, peaking in the United States in the early 1990s (1991–1992). The testing of asymptomatic men led to a detection of cancer at a much earlier stage and has led to a change of diagnosing men without metastatic cancer.[41–43] This has brought a paradigm shift in thinking of the best management for men with disease diagnosis. As more men have been identified with CaP, there has been a concomitant rise in clinical procedures with marked increases in biopsies. This increase in invasive procedures has led many to question the validity of PSA testing, specifically there emerged a picture of men with an elevated PSA result that demonstrated no cancer upon biopsy.[41,44] Indeed, estimates of PSA-directed false positives have been estimated to be 25–40% or higher[44–46]; thus, large numbers of patients were undergoing unneeded biopsy, and the side effects accompanying these avoidable procedures were increasing. The large numbers of men those have been PSA tested has allowed for a look at its usefulness with some certainty. It is becoming apparent that PSA testing has limited usefulness for management of cancer, likely due to studies indicating that serum PSA may demonstrate better correlations with the progression of benign prostate hyperplasia (BPH), since BPH nodules are normally present near CaP foci.[47–49] While mortality rates for CaP have decreased since the 1990s, and the introduction of widespread PSA testing has detected cancer at stages much earlier in its progression, it is not clear that the benefits for early detection warrant the aggressive treatments of cancer and has initiated a vigorous debate to elucidate better, perhaps different management protocols.

Many of the clinical procedures and therapies developed for CaP can have negative consequences as side effects. Biopsies can have issues with bleeding, introduction of infections, and possibility of urinary difficulties.[43,50–53] These can lead to an increase in hospitalizations and time incurred in the hospital for these patients.[52,53] Additionally, recognized CaP therapies of radiation and surgery have been associated with sexual dysfunction, altered bowel function and urinary incontinence in significant numbers (40–50%) of patients. Moreover, the psychological stress associated with a diagnosis and worry if therapy will be effective can be debilitating, which is exacerbated by the large numbers of over diagnoses. Since an elevated PSA level can lead to a diagnosis that will likely not manifest in clinical presentation, the aggressive nature of biopsy and treatment have been called into question. Adding to this

idea is the realization of a high false positive rate observed for PSA screening and the fact that this can be a very slowly progressing cancer. Thus, many patients will present with other issues before metastatic cancer. This is borne out in recent data that showed a similar survival benefit for men who were closely monitored, the so called ''watchful waiting,'' in comparison with those who had radical prostatectomy.[54–56] As a result, a strenuous review on the value of PSA as a singular diagnostic and therapy guide was essential to bring clarity to patients and physicians regarding efficient and effective care of CaP.

Presently, watchful waiting is often the prescribed approach for those patients presenting with low Gleason score (Gleason <6) tumors since they are normally considered to be low risk based on clinical parameters. Although the majority of patients harboring the low Gleason score tumors will remain without symptoms for the remainder of their lives, a very small number of those tumors will advance to a more metastatic state. As a result, the need to distinguish among those low Gleason score tumors that are destined to become aggressive versus those that will remain indolent will be an essential clinical tool that can be used to prevent unnecessary overtreatment. As such, previous studies using gene signature enrichment analyses (GSEA) identified distinct gene signatures responsible for not only identifying normal, benign, and metastatic CaP clinical subgroups but also correlated those samples with stage-specific prostate cancer stem cells.[57] Additional GSEA studies were carried out by Ishrad et al.[58,59] comparing a defined gene signature from low Gleason score (Gleason 6) indolent versus aggressive prostate tumors divided into subgroups based on different time frames for biochemical recurrence (BCR) revealed a differential expression of genes associated with ageing and senescence, strongly suggesting a 19-gene indolence signature. Furthermore, decision tree model analyses identified *CDNK1A*, *FGFR1*, and *PMP22* as the specific molecular biomarkers for distinguishing indolent versus aggressive tumors with low Gleason score. Not only was the differential expression of this 3-gene signature verified at the mRNA and protein expression level, but it was also shown to be useful as a prognostic factor in prostate tumor samples assessed.[58,59] The future development of this signature as a diagnostic tool, when combined with other clinical parameters, such as PSA levels and Gleason scores, promises to help thwart some of the overtreatment of CaP seen in recent years while using PSA as one of the sole diagnostics.

Approximately, over the 20-year experience of PSA screening, there have been several studies aimed at evaluating the effectiveness of this diagnostic tool. The two trials of most importance are the Prostate, Lung, Colorectal, and Ovarian (PLCO) trial in the United States,[51] and the European Randomized Study of Screening for CaP (ERSPC).[53] Each was a large cohort of men with significant follow up (~8–11 years) and while the ERSPC found a relatively significant reduction in mortality among men from 55 to 69 years of age, both of these large trials failed to find an overall benefit to PSA screening with regard to patient survival. In reviewing this data, and that of other smaller trials, the US Preventative Task Force (USPSTF) recently published a recommendation that no man be PSA screened unless they exhibited signs of CaP.[60,61] This USPSFT recommendation sparked a major debate concerning PSA screening by many groups including National Comprehensive Cancer Network (NCCN), the American Cancer Society (ACS), and American College of Preventive Medicine (ACPM), among others. Although most agree with the basic tenants of the large trials, there has been much scrutiny of the methods, patient populations, and analyses of the data to explain the nuanced differences and similarities of the analyses. Moreover, the PSA CaP test has become fully integrated into the physician practice. Thus, many are not inclined to change their behavior, as shown by a recent survey.[62] A synthesis of the available data from trials and the estimates of cost/benefit of over diagnoses with concomitant aggressive therapies by the American Society of Clinical Oncology (ASCO) produced a reasoned approach to clinical guidelines. There are four major points of the ASCO guidelines: (1) In men with life expectancy of ≤10 years, general screening for CaP with PSA be discouraged due to harms outweighing the potential benefits; (2) In men with life expectancy ≥10 years, the physicians should discuss fully with patients if PSA testing is appropriate for monitoring cancer progression; (3) Information on the efficacy of PSA testing should be written in concise and clear lay language and be available for the physician to facilitate the discussions of risk/benefit of PSA screening; and (4) More research is needed for the benefit/harm of PSA screening and to initiate analysis of further biomarkers to help guide more effectively CaP therapies. It is also recognized that PSA can be useful in the monitoring of cancer reoccurrence following surgical treatments for patients where such action is warranted.[48,54] Although the implications of these guidelines on CaP care are not yet realized, it surely will have an impact on the usefulness of planned major screens in Asian countries (China and others) as well as the coverage of test costs by insurance in the changing medical practice environment. Clearly, thoughtful debate will result in further biomarker research and provide a framework to move forward in the diagnostics and therapies for CaP.

Prostate cancer biomarker research

The application of molecular techniques to the discovery of new disease biomarkers has revolutionized the pace of bringing these to the clinical setting. Analyses of individual tumor and tissue samples with high throughput techniques including genome-wide sequencing,[26,31] microarray RNA profiling,[63] and mass spectrometry protein profiling[64–66] advance a molecular definition of cancer and defined newer biomarker signatures. These studies have also demonstrated nuances in DNA modifications and epigenetic signatures, which accompany cancer progression.[67–70] For CaP, these analyses have identified several promising loci for predicting cancer risk and response to therapeutic interventions, in addition to the altered expression of known tumor-associated genes.[71] While these studies have led to an advanced genomic sequencing/profiling of

individual cancer tumors to promote an individual therapeutic approach, another emphasis has technologically advanced the concept of noninvasive assays to detect cancer and hopefully determine progression, both are unmet needs in CaP.[72] There will be investigations into developing markers, including miRNAs and circulating tumor cells[7,71,72] to begin meeting these needs, and here we will focus upon the best defined current assays developed around molecules for noninvasive CaP detection: Prostate Cancer Antigen 3 (PCA 3) and TMPRSS2–ERG gene fusion, and then discuss our work to define a new prostate-specific molecular signature, smooth muscle gamma actin (SMGA).

PCA 3

PCA 3 was first identified as a cancer related gene RNA that was identified by PCR assays of cancer tumors compared with normal prostate tissue.[73] It was found at high levels in tumor cells, at significantly lower levels in RNA derived from PIN tissues and undetectable levels in normal prostate epithelium. It appears to be a long noncoding RNA that varies in levels strictly in prostate epithelia. That is, it is not found in normal tissues from several organs (testicle, bladder, kidney, lungs, and others) but increased in tissues with prostate epithelia dysfunction and importantly was not observed in BPH tissues. With this apparent sensitivity and selectivity in mind, a landmark study in 2003 demonstrated that PCA 3 mRNA could be detected from sloughed cells into urine samples and that the detection of PCA 3 was superior to PSA in detecting cancer in patients undergoing biopsy protocols.[74–77] This was quickly followed by several studies examining the discrimination of cancer in various patient populations confirming the initial results and leading to more extensive clinical trials.

Multicenter trials concerning the effectiveness of PCA 3 as a diagnostic were conducted in the United States and in Europe in patients undergoing clinical biopsy protocols.[74,78] Although there were some minor differences in these studies, both concluded that adding a PCA 3 analysis in combination with risk factors such as age, digital rectal examination, and free PSA percentile did enhance the diagnostic value. Based upon these initial results, others have found that adding PCA 3 into the calculation of risk improved the predictive values for cancer.[79,80] Further, application of a PCA 3 test to the risk calculations allowed a reduction in biopsies that were deemed unnecessary and demonstrated to be accurate, potentially allowing for a significant reduction in invasive protocols and fewer hospital complications.[81,82] However, PCA 3 is not without detractions. Although the test is ~10 times more expensive to perform than the PSA exam, this might be offset with a reduction in unneeded invasive procedures. Additionally, it has not been studied in unscreened populations, so the real effectiveness of PCA 3 mass screening of asymptomatic patients has not yet been tested. Weighing the positive aspects of studies indicating a reduction in invasive procedures with PCA 3 testing, the US Food and Drug Agency in 2012 approved the use of a PCA 3 test developed by Gen-Probe, San Diego, California (Progensa test) in patients over 50 who have at least one negative biopsy, and that further biopsy has been indicated based upon the prevailing standard of care. The European Association of Urology has adopted similar guidelines, however, they also note that the cost of PCA 3 testing might be a mitigating factor.[81,82] PCA 3 has been proven to be superior to PSA (both total PSA and free PSA ratios) in detecting cancer with elevated PSA levels. While it shows promise, it remains to be determined if the examination of asymptomatic men with a PCA 3 test to direct clinical CaP therapy will prove efficient.

TMPRSS2–ERG gene fusion

Gene fusions are common genomic rearrangements found in many cancer lesions. This occurs through an altered DNA/genomic maintenance mechanism(s) that results in the joining of DNA molecules from dissimilar genetic loci. This fusion leads to aberrant expression of a molecule under direction of different genetic elements, or through fusing the activities of proteins forming a protein with a new function. If the abnormal expression or activity causes increased cell proliferation, this can be an integral activity driving cancer progression.[83,84] In CaP, there have been several of these gene fusion identified. Recent studies have shown that there is a prominent fusion event between the gene TMPRSS2; a loci located at 21q22.3 which is regulated by androgen,[85–87] and ERG (21q22.2) and ETS-related gene that have previously been shown to be highly expressed (overexpressed) gene products in prostate tumors[88–91] and is proto-oncogenic through activation of C-MYC.[92,93] Genetic studies indicate that TMPRSS2–ERG gene fusions can account for upwards of 85–90% of all fusion events in CaP[86,87,94,95] and appear to be most prominent in prostate and considered specific to CaP.[86,87] TMPRSS2–EGR fusions have been detected in PIN lesions adjacent to cancerous tissue, but are absent in benign tissue.[96–98] These findings have increased the interest in developing a non-invasive test for the TMPRSS2–EGR fusion as a potential diagnostic tool.

Similar to PCA 3 analyses, RNA for TMPRSS2–EGR fusion can be detected in the urine using a PCR based assay.[87,94,96] The detection of TMPRSS2–EGR RNA in the urine is highly specific and demonstrates a high positive predictive value for CaP,[87] which suggests it would be a good candidate for a prognostic test for cancer presence. A recent significant study showed that TMPRSS2–EGR fusion RNA analyses combined with a PCA 3 analysis was better at predicting CaP, outperforming standard PSA screening analyses.[86,87] Further, recent studies have shown a positive correlation with aggressive cancer[86,87]; however, this has not been a universal finding in that others have not seen this association.[99,100] Moreover, some studies have indicated that the TMPRSS2–EGR fusion may be most associated with early onset CaPs and that late onset cancers do not show the androgen-regulated gene fusions.[101,102] It has also been shown that the TMPRSS2–EGR fusion may not be prevalent in Asian populations which limit the usefulness of this assay as a universal diagnostic and screening tool. With these caveats, TMPRSS2–ERG fusion products may be more inviting as therapeutic targets than as a diagnostic.

ETS factors have been targets in other cancers,[103] and recent studies have linked the TMPRSS2 promoter as been regulated by the Nkx 3-1 homeodomain transcription factor, a critical tumor suppressor gene for CaP, which is being evaluated as a mechanism of targeted therapeutics.[104] Thus, the TMPRSS2–EGR fusion may be more useful for targeted therapy instead of a diagnostic, and there remains a need for further identification of biomarkers that provide a unique footprint of CaP progression.

Smooth muscle gamma actin: A unique biomarker for prostate cancer

Nkx 3-1 is an important regulator of prostate development and also is a critical factor in the initiation of CaP. It is the gene residing in the human chromosome 8p21 region that exhibits haploinsufficiency in CaP. The subsequent misregulation of Nkx 3-1 expression is correlated with cancer progression. Many studies of Nkx 3-1 activity in human as well as mouse and other models have defined this homeodomain as a tumor suppressor for CaP.[7,14] Therefore, a fundamental understanding of prostate development and carcinogenesis would be gained from knowledge of Nkx 3-1 regulated genes. We have identified the SMGA gene as a molecular target for Nkx 3-1.[105–107] In our studies, we demonstrated that Nkx 3-1 required the co-expression of serum response factor (SRF) to activate SMGA transcription. These proteins work synergistically to activate SMGA transcription, and required a specific DNA sequence (termed the NK/CArG motif) for transcriptional activation. It is a novel biological target for Nkx 3-1, and the transcriptional synergy of this homeodomain with SRF works through an Nkx 3-1 dependent mechanism.[105,107,108] Although this is consistent with our overall hypothesis that SRF works as a platform for other regulatory proteins, we reasoned that for this to be the case in prostate, SMGA and SRF needed to be expressed in epithelial cells. To test this idea, we examined SMGA, SRF, and Nkx 3-1 mRNA levels in an androgen responsive (LNCaP) and androgen insensitive (PC-3) CaP cell lines. Nkx 3-1 was responsive to treatment of the LNCaP cells with the synthetic androgen R1881, and importantly, this androgen-dependent increase in Nkx 3-1 is followed/paralleled by an increase in SMGA mRNA.[108] Additionally, these data showed that SMGA mRNA is over expressed in the PC-3 cells, cells that do not express Nkx 3-1, indicating that SRF is no longer receiving differentiated signals (via Nkx 3-1), ultimately resulting in an altered gene regulation program leading to altered cell

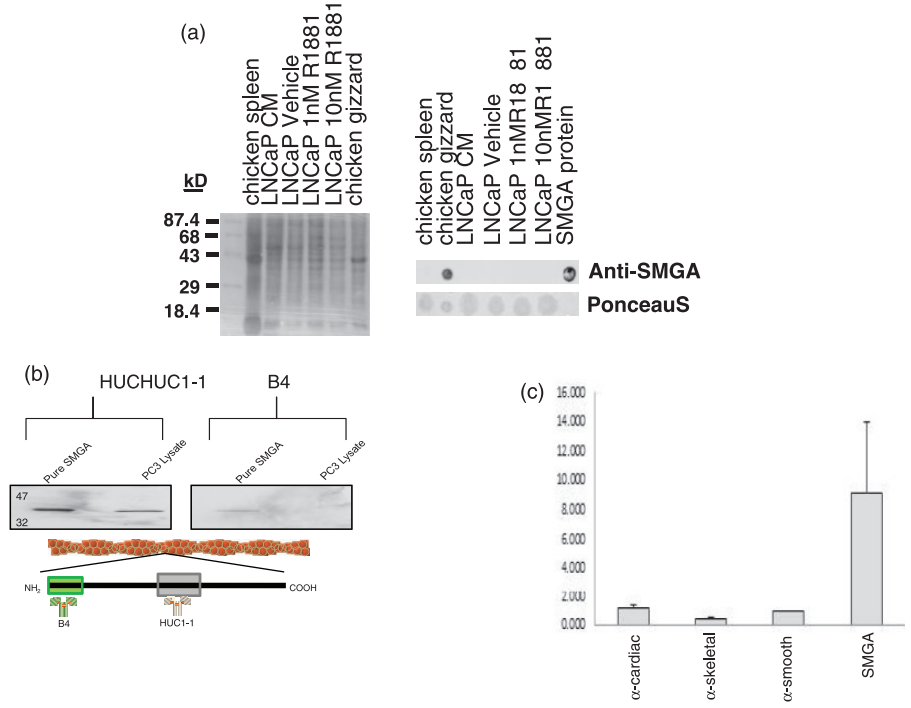

Figure 1 Identification of muscle actin(s) in prostate epithelia. (a) This shows a gel displaying lysates from adult chicken tissues, spleen, and gizzard, as well the human prostate cancer cell line LNCaP. The LNCaP cells were treated with the synthetic androgen R1881 or vehicle only as outlined in Fillmore et al.[108] This shows that prostate lysates contained proteins that were not significantly degraded through the lysis protocol. We next conducted a protein dot blot to display the proteins from the tissues shown on the gel and an amount (15 ng) of actin purified from adult chicken gizzard, which is known to contain ~90% SMGA. The dotted proteins were incubated in the SMGA selective monoclonal antibody; clone B4, which recognized proteins only in the gizzard lysate and the purified SMGA. The Ponceau stain demonstrates that protein of approximately the same content/amount was placed in each dot. (b) Western blots were prepared from gels containing PC-3 prostate cancer cell protein lysates and actin protein purified from adult chicken tissue. The blot on the left was incubated with the monoclonal antibody HUC 1-1, which recognizes a region of the protein common to all muscle actin isoforms. Both the purified SMGA and PC-3 lysates contained immunoreactive material. The blot on the right was incubated with the monoclonal B4, which recognizes SMGA selectively. This antibody epitope maps to the amino terminal 8–10 amino acid of the mature SMGA molecule. Only the purified SMGA demonstrated B4 reactivity. (c) RNA isolated from PC-3 cells was subjected to RT-PCR analysis to quantify the mRNA for the human muscle actin isoforms using primers specific for the various isoforms. Only human SMGA shows significant RNA content in PC-3 cells for the muscle actins tested

growth functions characteristic of late stage CaP. We have further demonstrated that SMGA gene transcription is directly regulated by Nkx 3-1/SRF interactions in prostate epithelia, and this occurs via specific protein:protein and protein:DNA interactions.[105,108] Changes in the transcriptional regulatory partner milieu available, particularly at certain times like the metastatic state, may help provide an explanation for the disparate regulated appearance of SMGA in androgen-responsive versus overexpression in androgen-independent metastatic CaP. For example, *Pten* and *Smad4* (both interacting partners with SRF) inactivation is correlated with tumor cell proliferation and invasion, and has been implicated as part of a metastatic prostate gene signature (see Figure 4). While SRF has been shown to regulate Pten expression via induction of a specific miRNA, it has also been shown that cells depleted for Pten or SRF have altered smooth muscle-specific protein expression.[109] Similarly, studies in which *Smad4* was depleted in mice not only led to embryonic lethality, but also to an alteration in the expression of smooth muscle-specific genes, thus ultimately leading to cellular proliferation and migration.[110] Thus, upsetting the balance in the transcriptional factors essential for expression of smooth muscle proteins such as SMGA in prostate epithelia, likely the reason for its loss of regulated expression in metastatic CaP. As these experiments conclusively show that SMGA mRNA is produced (and regulated) in the differentiated prostate epithelial cell, it begs the interesting question of what function a smooth muscle contractile protein might have in a decidedly non-smooth muscle cell.

With the novel finding of mRNA encoding a smooth muscle contractile protein in prostate epithelia, we next wanted to localize SMGA in these cells as a prelude to understanding function. Using an SMGA-specific monoclonal antibody (Clone B4), we were unable to detect the SMGA polypeptide. This is the same antibody we and others have used for numerous studies[106,108,111] to localize SMGA in smooth muscle tissue. The epitope of this

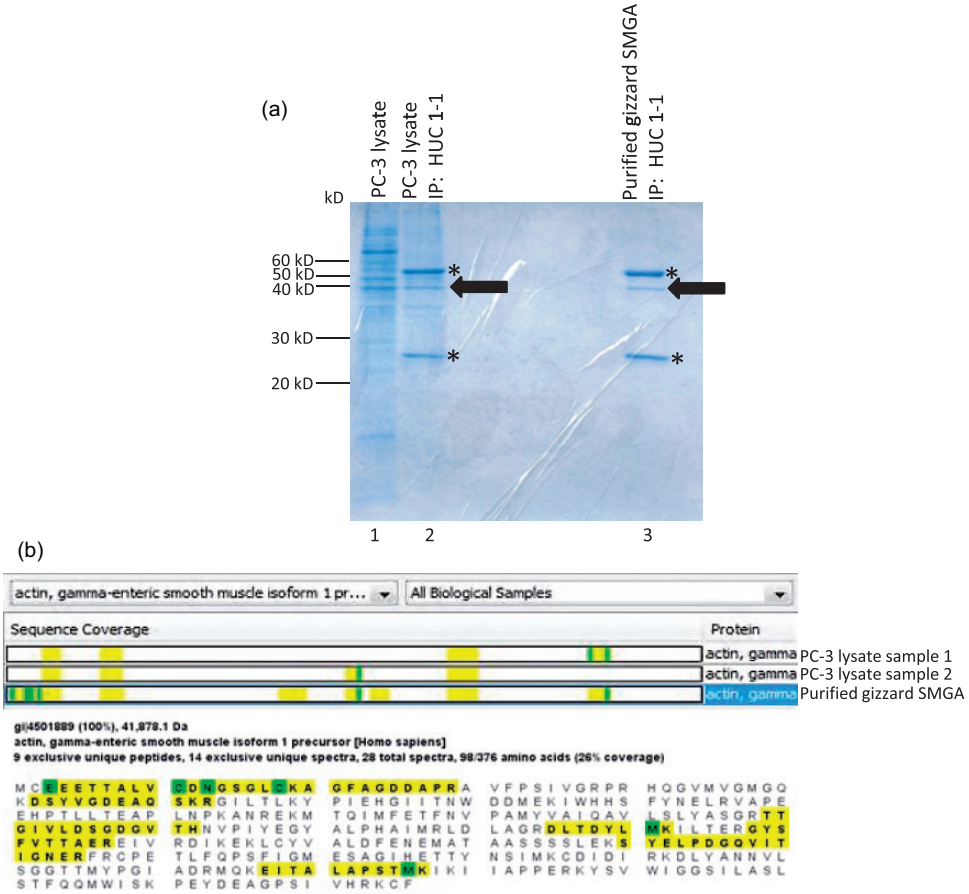

Figure 2 Immunoprecipitation of smooth muscle gamma-actin from PC-3 prostate cancer cell lysates. (a) 12.5% polyacrylamide gel displaying the results from an experiment in which muscle actin was immunoprecipitated from protein lysate derived from PC-3 prostate cancer cells. Lane 1 shows the PC-3 whole protein lysate. Lane 2 shows PC-3 lysate after immunoprecipation with HUC 1-1 actin antibody. The asterisks indicate the migration of the antibody heavy and light chains while the arrow denotes the band that was subsequently cut from the gel and subjected to MS/MS proteomic mass spectrometry. Lane 3 indicates the results of a control immunoprecipitation performed in parallel using ~100 ng of SMGA purified from adult chicken gizzard tissue along with the HUC 1-1 antibody. The asterisks and arrow denote the same proteins indicated above. (b) This shows the results from a representative MS/MS mass spectrometry experiment in which actin bands from two separate PC-3 lysate samples and the SMGA immunoprecipitated from control reactions (as specified above) were subjected to proteomic evaluation. The upper portion of the figure demonstrates the positions of peptides that were sequenced from this analysis; the upper two from the PC-3 lysates and the lower one from the purified chicken gizzard SMGA sample. The yellow bars demonstrate peptides identified as human SMGA sequence shown in the lower portion of the figure, with the green demonstrating amino acids that differ from the sequence. This shows that the majority of the actin precipitated from the PC-3 lysate was SMGA

antibody maps to the first eight amino acids of the native smooth muscle protein. As shown in Figure 1, protein lysates derived from CaP cell lines (PC-3) do not demonstrate immunoreactive material with the amino terminal-specific SMGA monoclonal antibody (clone B4). We then tested a second monoclonal antibody, clone HUC 1-1, which recognizes an epitope shared by muscle actins within the central segment of the protein, but not found on non-muscle actin isoforms.[112–114] This antibody recognizes a 42–43 kDa protein in lysates derived from PC-3 CaP cells that was not identified using the anti-SMGA monoclonal B4 (Figure 1(b)). Our previous studies showed that of the muscle actins, only SMGA mRNA is measurable in CaP PC-3 cells[108] which we confirmed with real time PCR (Figure 1(c)). Thus, we conclude that the HUC 1-1 immunoreactive material is SMGA. Although a reasoned conclusion, at the protein level we acknowledge a conundrum: whereas the HUC1-1 antibody will recognize all of the four muscle actins, one of which is SMGA, the antibody that is specific to SMGA (B4) does not identify protein in prostate samples. Thus, we set out to directly demonstrate SMGA in PC-3 samples using the HUC 1-1 antibody to immunoprecipitate material from lysates and subject the ~43 Kd precipitin to mass spectroscopy sequencing analyses. Figure 2 demonstrates that the HUC 1-1 antibody immunoprecipitates a protein of ~42 Kd in PC-3 cell protein lysates which is similar migration to that of purified SMGA from chicken gizzard. The region of gel containing the PC-3, 42 Kd band and the purified SMGA band was cut from the gel, incubated in protease solution and then subjected to Tandem MS/MS mass spectrometry sequencing. The results of this analysis demonstrated multiple peptides diagnostic of SMGA elucidated from the PC-3 protein lysates (Figure 2(b)), definitively identifying the HUC 1-1 immunoprecipitated actin from the prostate epithelial cells as the smooth muscle gamma isoform of actin. Interestingly, the mature amino-terminal segment from the control SMGA isolated from chicken gizzard was identified, yet the same segment of the protein was not identified in the prostate cell SMGA molecule. We have shown that SMGA mRNA from LNCaP and PC-3 CaP cells is identical to that sequenced from smooth muscle,[108] and no altered SMGA gene structures have been identified to date. Thus, we put forth the hypothesis that SMGA in prostate cells is different from its smooth muscle counter part due to post-translational events which mask the amino terminal epitope for the clone B4 SMGA antibody (Figure 3). The mature SMGA in smooth muscle is cleaved post-translationally to remove the terminal Met-Cys dipeptide. Thus, the prostate SMGA might be alternately cleaved to remove most or all of the amino terminal epitope identified by the clone B4 monoclonal SMGA antibody. It also may be altered by no cleavage and/or the addition of other agents (phosphates, methyl groups, etc.) post-translationally. Either of

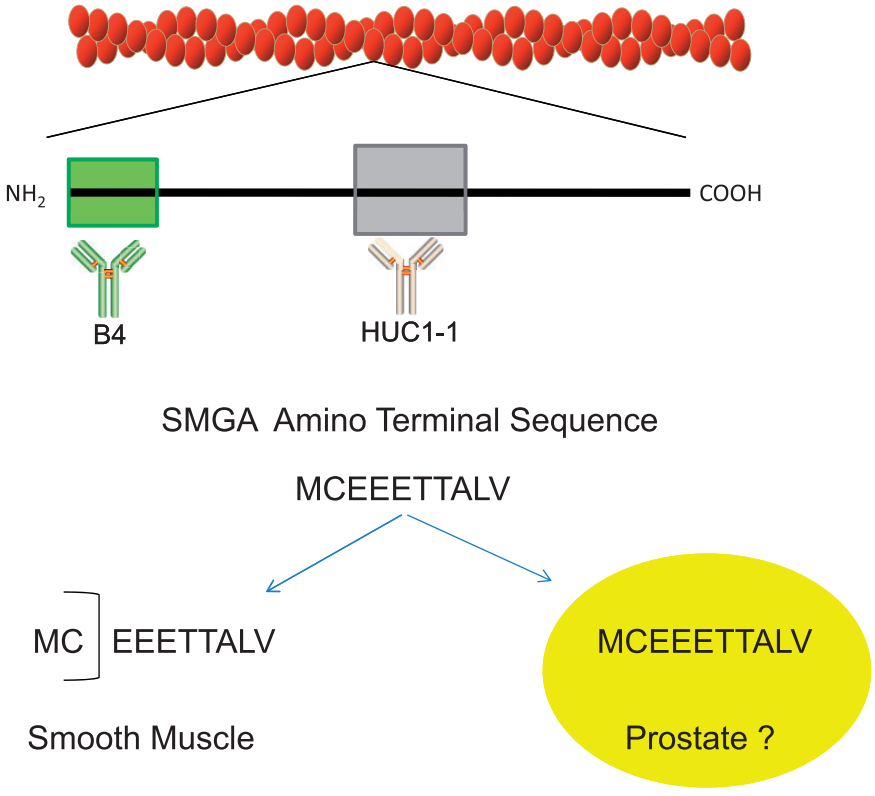

Figure 3 Model of SMGA found in prostate epithelia. This diagram summarizes the results from the HUC1-1 immunoprecipitation experiment of actin from prostate epithelia. The clone B4 does not recognize an actin in prostate, which indicates the amino terminal 8–10 amino acids of the muscle actin in prostate cells is different from the smooth muscle (SMGA) mature protein isoform. It is possible that it is not present due to amino terminal cleavage, similar to the Met-Cys dipeptide that is cleaved in smooth muscle cells. It is also possible that the Met-Cys dipeptide is not cleaved at all in prostate or that there is a blocking of the amino terminal prostate SMGA sequence such that it renders the amino terminus unrecognizable by the B4 amino terminal epitope

these would create a unique prostate-specific smooth muscle actin, which we believe presents a new footprint for the prostate epithelia that can be utilized as a biomarker for normal and diseased tissue.

Summary and conclusion

Presently, the most utilized diagnostic screening test for CaP is analysis of Prostate Serum Antigen (PSA). This is the elevated presence of PSA in blood, which has been correlated with cancer onset and progression. However, the PSA test has many issues, including the frequency of false reports (both positive and negative), leading to much anguish and increased clinical, invasive procedures which elevate cost and can have devastating side effects. Although many improvements have been identified since the original PSA test, the analyses of long term studies have called into question the screening of asymptomatic men due to undesirable cost/benefit issues. This discussion has evolved to the point in which many organizations, including the US Preventative Task Force and American Urologic Association have issued statements to discontinue PSA screening. With some modifications, a general guideline has emerged which includes not screening men under age 40; selective screening of men aged 40–55 (those demonstrating multiple risk factors); sharing in the decision with their physician whether screening would be useful for men aged 55–69; no routine screening of men greater than 70 or with life expectancy less than 5 years; and, finally, support for research to identify more biomarkers of CaP diagnosis. With the application of molecular techniques, many genetic alterations have been identified for CaP. From this research, several new potential markers of CaP progression have emerged. The best of these new markers include PCA 3 and TMPRSS2–EGR fusion, which are presently being evaluated for their potential use in cancer identification and to monitor cancer progression. We have identified a prostate-specific actin that is a post-translationally modified form of the SMGA that is normally predominately found in enteric muscle. We propose that identifying the unique character of this actin will provide a new diagnostic footprint for CaP initiation and progression. From our data that demonstrates increased SMGA expression in aggressive CaP cell lines we have tentatively assigned SMGA in the lineage of cancer progression at the boundary of organ-confined and aggressive/metastatic cancer steps (Figure 4). Going forward, it is likely that future therapies for CaP will be guided by multiplex analyses of markers, one of which will be the prostate-specific SMGA molecule.

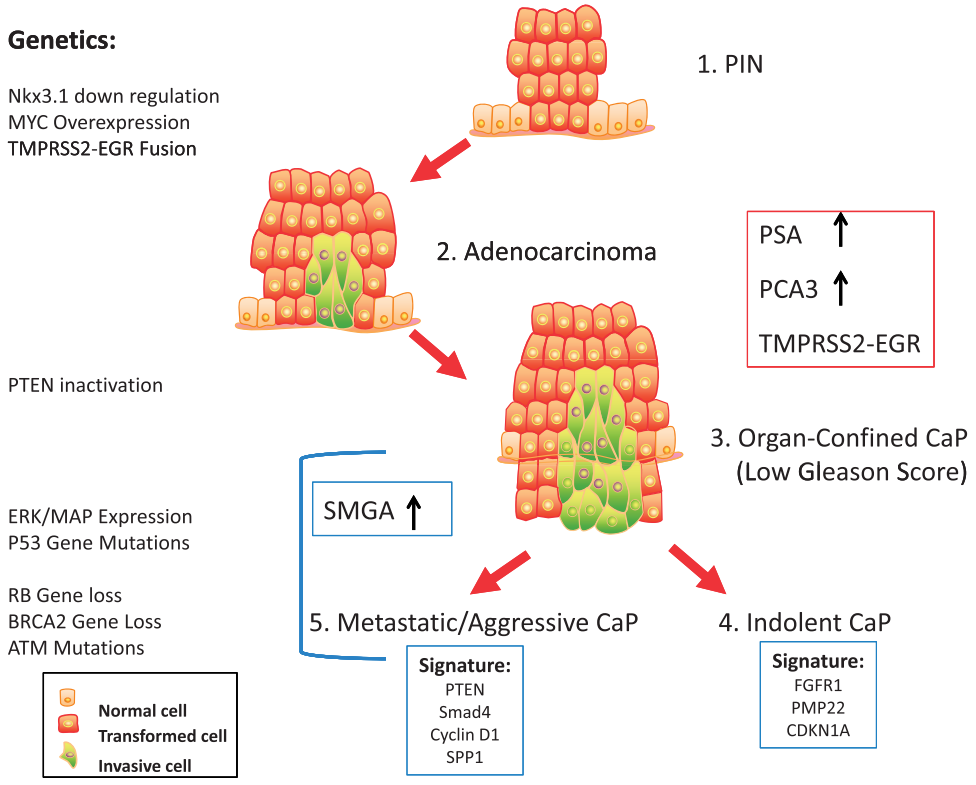

Figure 4 Correlation of SMGA in CaP progression. This diagram shows the progression of prostate cancer from PIN to aggressive cancer and major genetic changes. CaP follows a pathway from initiation of cancer that begins with lesions of replicative cells within the gland (Step 1, PIN) and continues with cells that undergo an epithelial-to-mesenchymal transition (Steps 2 and 3). Leading to either a slow growing indolent cancer (Step 4) or can lead to an aggressive highly metastatic cancer (Step 5). There are several confirmed genetic changes that are diagnostic of this progression, shown on the left margin of the diagram. Recent bioinformatics have allowed the definition of potential signatures of genes that help define the ability of cancer to be either slow growing (Indolent) or aggressive (metastatic). These are in addition to the PSA, PCA3, and TMPRSS2–EGR biomarkers defined to date. We propose that post-translationally modified SMGA will be a marker for the transition of cancer from the Organ-confined to aggressive, metastatic CaP

AUTHOR CONTRIBUTIONS

RF, LD, and WZ were instrumental in experimental design and all authors performed experiments. The manuscript was drafted by WZ and edited by RF and LD. CJ, CK, RF, and WZ were charged with data collection and figure design. All authors have read and approved of the final manuscript.

ACKNOWLEDGEMENTS

The authors acknowledge members of the Zimmer lab that helped with editing, reading, and helpful discussions in the completion of the work. Parts of this work were completed as dissertation projects for RF and CK. The support of the Texas A&M College of Medicine Summer Research Program and the Texas A&M Cancer Research Council was instrumental in completing this work.

REFERENCES

1. Koslin DB, Kenney PJ, Koehler RE, Van Dyke JA. Magnetic resonance imaging of the internal anatomy of the prostate gland. *Invest Radiol* 1987;**22**:947–53
2. Gardner WA, Jr., Bennett BD. The prostate – overview: recent insights and speculations. *Monogr Pathol* 1992:129–48
3. Xia T, Blackburn WR, Gardner WA Jr. Fetal prostate growth and development. *Pediatr Pathol* 1990;**10**:527–37
4. Droes JT. Observations on the musculature of the urinary bladder and the urethra in the human foetus. *Br J Urol* 1974;**46**:179–85
5. Droes JT. The musculature of the bladder and the urethra. *Acta Morphol Neerl Scand* 1971;**8**:238
6. Abate-Shen C, Shen MM, Gelmann E. Integrating differentiation and cancer: The nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis. *Differentiation* 2008;**76**:717–27
7. Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: New prospects for old challenges. *Genes Dev* 2010;**24**:1967–2000
8. Abate-Shen C, Shen MM. Mouse models of prostate carcinogenesis. *Trends Genet* 2002;**18**:S1–5
9. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. *Genes Dev* 2000;**14**:2410–34
10. Allen KS, Kressel HY, Arger PH, Pollack HM. Age-related changes of the prostate: Evaluation by mr imaging. *AJR Am J Roentgenol* 1989;**152**:77–81
11. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in europe: Estimates for 40 countries in 2012. *Eur J Cancer* 2013;**49**:1374–403
12. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;**64**:9–29
13. Siegel R, Naishadham D, Jemal A. Cancer statistics for hispanics/latinos, 2012. *CA Cancer J Clin* 2012;**62**:283–98
14. Gelmann EP. Complexities of prostate-cancer risk. *N Engl J Med* 2008;**358**:961–63
15. Johns LE, Houlston RS. A systematic review and meta-analysis of familial colorectal cancer risk. *Am J Gastroenterol* 2001;**96**:2992–3003
16. Stemmermann GN, Nomura AM, Chyou PH, Yatani R. A prospective comparison of prostate cancer at autopsy and as a clinical event: The hawaii japanese experience. *Cancer Epidemiol Biomarkers Prev* 1992;**1**:189–93
17. Moses KA, Utuama OA, Goodman M, Issa MM. The association of diabetes and positive prostate biopsy in a us veteran population. *Prostate Cancer Prostatic Dis* 2012;**15**:70–4
18. Moses KA, Paciorek AT, Penson DF, Carroll PR, Master VA. Impact of ethnicity on primary treatment choice and mortality in men with prostate cancer: Data from capsure. *J Clin Oncol* 2010;**28**:1069–74
19. Moses KA, Abd TT, Goodman M, Hsiao W, Hall JA, Marshall FF, Petros JA, Issa MM. Increased low density lipoprotein and increased likelihood of positive prostate biopsy in black americans. *J Urol* 2009;**182**:2219–25
20. Zhang L, Yang BX, Zhang HT, Wang JG, Wang HL, Zhao XJ. Prostate cancer: An emerging threat to the health of aging men in asia. *Asian J Androl* 2011;**13**:574–78
21. Bardia A, Platz EA, Yegnasubramanian S, De Marzo AM, Nelson WG. Anti-inflammatory drugs, antioxidants, and prostate cancer prevention. *Curr Opin Pharmacol* 2009;**9**:419–26
22. Blum DL, Koyama T, M'Koma AE, Iturregui JM, Martinez-Ferrer M, Uwamariya C, Smith JA Jr., Clark PE, Bhowmick NA. Chemokine markers predict biochemical recurrence of prostate cancer following prostatectomy. *Clinical Cancer Res* 2008;**14**:7790–97
23. Johns LE, Houlston RS. A systematic review and meta-analysis of familial prostate cancer risk. *BJU Int* 2003;**91**:789–94
24. Huang Q, Whitington T, Gao P, Lindberg JF, Yang Y, Sun J, Vaisanen MR, Szulkin R, Annala M, Yan J, Egevad LA, Zhang K, Lin R, Jolma A, Nykter M, Manninen A, Wiklund F, Vaarala MH, Visakorpi T, Xu J, Taipale J, Wei GH. A prostate cancer susceptibility allele at 6q22 increases rfx6 expression by modulating hoxb13 chromatin binding. *Nat Genet* 2014;**46**:126–35
25. Jin G, Zheng SL, Lilja H, Kim ST, Tao S, Gao Z, Young T, Wiklund F, Feng J, Isaacs WB, Rittmaster RS, Gronberg H, Condreay LD, Sun J, Xu J. Genome-wide association study identifies loci at atf7ip and klk2 associated with percentage of circulating free psa. *Neoplasia* 2013;**15**:95–101
26. Xu J, Mo Z, Ye D, Wang M, Liu F, Jin G, Xu C, Wang X, Shao Q, Chen Z, Tao Z, Qi J, Zhou F, Wang Z, Fu Y, He D, Wei Q, Guo J, Wu D, Gao X, Yuan J, Wang G, Xu Y, Yao H, Dong P, Jiao Y, Shen M, Yang J, Ou-Yang J, Jiang H, Zhu Y, Ren S, Zhang Z, Yin C, Dai B, Hu Z, Yang Y, Wu Q, Chen H, Peng P, Zheng Y, Zheng X, Xiang Y, Long J, Gong J, Na R, Lin X, Yu H, Tao S, Feng J, Sun J, Liu W, Hsing A, Rao J, Ding Q, Wiklund F, Gronberg H, Shu XO, Zheng W, Shen H, Jin L, Shi R, Lu D, Zhang X, Zheng SL, Sun Y. Genome-wide association study in chinese men identifies two new prostate cancer risk loci at 9q31.2 and 19q13.4. *Nat Genet* 2012;**44**:1231–35
27. Berndt SI, Sampson J, Yeager M, Jacobs KB, Wang Z, Hutchinson A, Chung C, Orr N, Wacholder S, Chatterjee N, Yu K, Kraft P, Feigelson HS, Thun MJ, Diver WR, Albanes D, Virtamo J, Weinstein S, Schumacher FR, Cancel-Tassin G, Cussenot O, Valeri A, Andriole GL, Crawford ED, Haiman C, Henderson B, Kolonel L, Le Marchand L, Siddiq A, Riboli E, Travis RC, Kaaks R, Isaacs W, Isaacs S, Wiley KE, Gronberg H, Wiklund F, Stattin P, Xu J, Zheng SL, Sun J, Vatten LJ, Hveem K, Njolstad I, Gerhard DS, Tucker M, Hayes RB, Hoover RN, Fraumeni JF Jr., Hunter DJ, Thomas G, Chanock SJ. Large-scale fine mapping of the hnf1b locus and prostate cancer risk. *Hum Mol Genet* 2011;**20**:3322–29
28. Sun J, Kader AK, Hsu FC, Kim ST, Zhu Y, Turner AR, Jin T, Zhang Z, Adolfsson J, Wiklund F, Zheng SL, Isaacs WB, Gronberg H, Xu J. Inherited genetic markers discovered to date are able to identify a significant number of men at considerably elevated risk for prostate cancer. *Prostate* 2011;**71**:421–30
29. Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev* 2001;**20**:173–93
30. Lapointe J, Li C, Giacomini CP, Salari K, Huang S, Wang P, Ferrari M, Hernandez-Boussard T, Brooks JD, Pollack JR. Genomic profiling reveals alternative genetic pathways of prostate tumorigenesis. *Cancer Res* 2007;**67**:8504–10
31. Zhao H, Kim Y, Wang P, Lapointe J, Tibshirani R, Pollack JR, Brooks JD. Genome-wide characterization of gene expression variations and DNA copy number changes in prostate cancer cell lines. *Prostate* 2005;**63**:187–97
32. Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevad L, Rayford W, Bergerheim U, Ekman P, DeMarzo AM, Tibshirani R, Botstein D, Brown PO, Brooks JD, Pollack JR. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci USA* 2004;**101**:811–16
33. Abate-Shen C, Banach-Petrosky WA, Sun X, Economides KD, Desai N, Gregg JP, Borowsky AD, Cardiff RD, Shen MM. Nkx3.1; pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. *Cancer Res* 2003;**63**:3886–90

34. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Socci ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL, Gerald WL. Integrative genomic profiling of human prostate cancer. *Cancer Cell* 2010;**18**:11–22
35. Gelmann EP, Henshall SM. Clinically relevant prognostic markers for prostate cancer: The search goes on. *Ann Intern Med* 2009;**150**:647–49
36. Kuriyama M, Wang MC, Papsidero LD, Killian CS, Shimano T, Valenzuela L, Nishiura T, Murphy GP, Chu TM. Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. *Cancer Res* 1980;**40**:4658–62
37. Papsidero LD, Kuriyama M, Wang MC, Horoszewicz J, Leong SS, Valenzuela L, Murphy GP, Chu TM. Prostate antigen: A marker for human prostate epithelial cells. *J Natl Cancer Inst* 1981;**66**:37–42
38. Wang MC, Papsidero LD, Chu TM. Prostate-specific antigen, p30, gamma-seminoprotein, and e1. *Prostate* 1994;**24**:107–10
39. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med* 1987;**317**:909–16
40. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, Andriole GL. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N Engl J Med* 1991;**324**:1156–61
41. Potosky AL, Feuer EJ, Levin DL. Impact of screening on incidence and mortality of prostate cancer in the united states. *Epidemiol Rev* 2001;**23**:181–86
42. O'Brien ME, Socinski MA, Popovich AY, Bondarenko IN, Tomova A, Bilynsky BT, Hotko YS, Ganul VL, Kostinsky IY, Eisenfeld AJ, Sandalic L, Oldham FB, Bandstra B, Sandler AB, Singer JW. Randomized phase iii trial comparing single-agent paclitaxel poliglumex (ct-2103, ppx) with single-agent gemcitabine or vinorelbine for the treatment of ps 2 patients with chemotherapy-naive advanced non-small cell lung cancer. *J Thorac Oncol* 2008;**3**:728–34
43. Sanda MG, Dunn RL, Michalski J, Sandler HM, Northouse L, Hembroff L, Lin X, Greenfield TK, Litwin MS, Saigal CS, Mahadevan A, Klein E, Kibel A, Pisters LL, Kuban D, Kaplan I, Wood D, Ciezki J, Shah N, Wei JT. Quality of life and satisfaction with outcome among prostate-cancer survivors. *New Engl J Med* 2008;**358**:1250–61
44. Evans R, Edwards AG, Elwyn G, Watson E, Grol R, Brett J, Austoker J. ''It's a maybe test": Men's experiences of prostate specific antigen testing in primary care. *Br J Gen Pract* 2007;**57**:303–10
45. Draisma G, Boer R, Otto SJ, van der Cruijsen IW, Damhuis RA, Schroder FH, de Koning HJ. Lead times and overdetection due to prostate-specific antigen screening: Estimates from the european randomized study of screening for prostate cancer. *J Natl Cancer Inst* 2003;**95**:868–78
46. Killian CS, Yang N, Emrich LJ, Vargas FP, Kuriyama M, Wang MC, Slack NH, Papsidero LD, Murphy GP, Chu TM. Prognostic importance of prostate-specific antigen for monitoring patients with stages b2 to d1 prostate cancer. *Cancer Res* 1985;**45**:886–91
47. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. *J Clin Oncol* 2003;**21**:383–91
48. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: Prediction, detection and monitoring. *Nat Rev Cancer* 2008;**8**:268–78
49. Ulmert D, Cronin AM, Bjork T, O'Brien MF, Scardino PT, Eastham JA, Becker C, Berglund G, Vickers AJ, Lilja H. Prostate-specific antigen at or before age 50 as a predictor of advanced prostate cancer diagnosed up to 25 years later: A case-control study. *BMC Med* 2008;**6**:6
50. Fowler FJ Jr., Barry MJ, Walker-Corkery B, Caubet JF, Bates DW, Lee JM, Hauser A, McNaughton-Collins M. The impact of a suspicious prostate biopsy on patients' psychological, socio-behavioral, and medical care outcomes. *J Gen Intern Med* 2006;**21**:715–21
51. Andriole GL, Crawford ED, Grubb RL 3rd, Buys SS, Chia D, Church TR, Fouad MN, Gelmann EP, Kvale PA, Reding DJ, Weissfeld JL, Yokochi LA, O'Brien B, Clapp JD, Rathmell JM, Riley TL, Hayes RB, Kramer BS, Izmirlian G, Miller AB, Pinsky PF, Prorok PC, Gohagan JK, Berg CD. Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med* 2009;**360**:1310–19
52. Roobol MJ, Kerkhof M, Schroder FH, Cuzick J, Sasieni P, Hakama M, Stenman UH, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M, Denis L, Recker F, Berenguer A, Ruutu M, Kujala P, Bangma CH, Aus G, Tammela TL, Villers A, Rebillard X, Moss SM, de Koning HJ, Hugosson J, Auvinen A. Prostate cancer mortality reduction by prostate-specific antigen-based screening adjusted for nonattendance and contamination in the european randomised study of screening for prostate cancer (erspc). *Eur Urol* 2009;**56**:584–91
53. Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M, Denis LJ, Recker F, Berenguer A, Maattanen L, Bangma CH, Aus G, Villers A, Rebillard X, van der Kwast T, Blijenberg BG, Moss SM, de Koning HJ, Auvinen A. Screening and prostate-cancer mortality in a randomized european study. *N Engl J Med* 2009;**360**:1320–28
54. Venderbosch S, de Wilt JH, Teerenstra S, Loosveld OJ, van Bochove A, Sinnige HA, Creemers GJ, Tesselaar ME, Mol L, Punt CJ, Koopman M. Prognostic value of resection of primary tumor in patients with stage iv colorectal cancer: Retrospective analysis of two randomized studies and a review of the literature. *Ann Surg Oncol* 2011;**18**:3252–60
55. Venderbos LD, Roobol MJ. Psa-based prostate cancer screening: The role of active surveillance and informed and shared decision making. *Asian J Androl* 2011;**13**:219–24
56. Venderbos LD, Bokhorst LP, Bangma CH, Roobol MJ. Active surveillance: Oncologic outcome. *Curr Opin Urol* 2013;**23**:268–72
57. Mazzoleni S, Jachetti E, Morosini S, Grioni M, Piras IS, Pala M, Bulfone A, Freschi M, Bellone M, Galli R. Gene signatures distinguish stage-specific prostate cancer stem cells isolated from transgenic adenocarcinoma of the mouse prostate lesions and predict the malignancy of human tumors. *Stem Cells Transl Med* 2013;**2**:678–89
58. Irshad S, Bansal M, Castillo-Martin M, Zheng T, Aytes A, Wenske S, Le Magnen C, Guarnieri P, Sumazin P, Benson MC, Shen MM, Califano A, Abate-Shen C. A molecular signature predictive of indolent prostate cancer. *Sci Transl Med* 2013;**5**:202ra122
59. Irshad S, Abate-Shen C. Modeling prostate cancer in mice: Something old, something new, something premalignant, something metastatic. *Cancer Metastasis Rev* 2013;**32**:109–22
60. Chou R, Croswell JM, Dana T, Bougatsos C, Blazina I, Fu R, Gleitsmann K, Koenig HC, Lam C, Maltz A, Rugge JB, Lin K. Screening for prostate cancer: A review of the evidence for the U.S. *Preventive services task force.* Ann Intern Med 2011;**155**:762–71
61. Chou R, Arora B, Dana T, Fu R, Walker M, Humphrey L. Screening asymptomatic adults with resting or exercise electrocardiography: A review of the evidence for the U.S. *Preventive services task force.* Ann Intern Med 2011;**155**:375–85
62. Jaramillo E, Tan A, Yang L, Kuo YF, Goodwin JS. Variation among primary care physicians in prostate-specific antigen screening of older men. *JAMA* 2013;**310**:1622–24
63. Cooper CS, Campbell C, Jhavar S. Mechanisms of disease: Biomarkers and molecular targets from microarray gene expression studies in prostate cancer. *Nat Clin Pract Urol* 2007;**4**:677–87
64. Gao X, Pang J, Li LY, Liu WP, Di JM, Sun QP, Fang YQ, Liu XP, Pu XY, He D, Li MT, Su ZL, Li BY. Expression profiling identifies new function of collapsin response mediator protein 4 as a metastasis-suppressor in prostate cancer. *Oncogene* 2010;**29**:4555–66
65. Pang J, Liu WP, Liu XP, Li LY, Fang YQ, Sun QP, Liu SJ, Li MT, Su ZL, Gao X. Profiling protein markers associated with lymph node metastasis in prostate cancer by dige-based proteomics analysis. *J Proteome Res* 2010;**9**:216–26
66. LeRoy B, Painter A, Sheppard H, Popiolek L, Samuel-Foo M, Andacht TM. Protein expression profiling of normal and neoplastic canine prostate and bladder tissue. *Vet Comp Oncol* 2007;**5**:119–30
67. Bjorkman M, Rantala J, Nees M, Kallioniemi O. Epigenetics of prostate cancer and the prospect of identification of novel drug targets by rnai screening of epigenetic enzymes. *Epigenomics* 2010;**2**:683–89
68. Cooper CS, Foster CS. Concepts of epigenetics in prostate cancer development. *Br J Cancer* 2009;**100**:240–45

69. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM. Abnormal DNA methylation, epigenetics, and prostate cancer. *Front Biosci* 2007;**12**:4254–66
70. Diaw L, Woodson K, Gillespie JW. Prostate cancer epigenetics: A review on gene regulation. *Gene Regul Syst Bio* 2007;**1**:313–25
71. Choudhury AD, Eeles R, Freedland SJ, Isaacs WB, Pomerantz MM, Schalken JA, Tammela TL, Visakorpi T. The role of genetic markers in the management of prostate cancer. *European urology* 2012;**62**:577–87
72. Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. Beyond PSA: The next generation of prostate cancer biomarkers. *Sci Transl Med* 2012;**4**:127rv123
73. de Kok JB, Verhaegh GW, Roelofs RW, Hessels D, Kiemeney LA, Aalders TW, Swinkels DW, Schalken JA. Dd3(pca3), a very sensitive and specific marker to detect prostate tumors. *Cancer Res* 2002;**62**:2695–98
74. Hansen J, Auprich M, Ahyai SA, de la Taille A, van Poppel H, Marberger M, Stenzl A, Mulders PF, Huland H, Fisch M, Abbou CC, Schalken JA, Fradet Y, Marks LS, Ellis W, Partin AW, Pummer K, Graefen M, Haese A, Walz J, Briganti A, Shariat SF, Chun FK. Initial prostate biopsy: Development and internal validation of a biopsy-specific nomogram based on the prostate cancer antigen 3 assay. *Eur Urol* 2013;**63**:201–09
75. Chun FK, de la Taille A, van Poppel H, Marberger M, Stenzl A, Mulders PF, Huland H, Abbou CC, Stillebroer AB, van Gils MP, Schalken JA, Fradet Y, Marks LS, Ellis W, Partin AW, Haese A. Prostate cancer gene 3 (pca3): Development and internal validation of a novel biopsy nomogram. *Eur Urol* 2009;**56**:659–67
76. Deras IL, Aubin SM, Blase A, Day JR, Koo S, Partin AW, Ellis WJ, Marks LS, Fradet Y, Rittenhouse H, Groskopf J. PCA3: A molecular urine assay for predicting prostate biopsy outcome. *J Urol* 2008;**179**:1587–92
77. Marks LS, Fradet Y, Deras IL, Blase A, Mathis J, Aubin SM, Cancio AT, Desaulniers M, Ellis WJ, Rittenhouse H, Groskopf J. Pca3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. *Urology* 2007;**69**:532–35
78. Perdona S, Cavadas V, Di Lorenzo G, Damiano R, Chiappetta G, Del Prete P, Franco R, Azzarito G, Scala S, Arra C, De Sio M, Autorino R. Prostate cancer detection in the ''grey area'' of prostate-specific antigen below 10 ng/ml: Head-to-head comparison of the updated pcpt calculator and chun's nomogram, two risk estimators incorporating prostate cancer antigen 3. *Eur Urol* 2011;**59**:81–7
79. Auprich M, Bjartell A, Chun FK, de la Taille A, Freedland SJ, Haese A, Schalken J, Stenzl A, Tombal B, van der Poel H. Contemporary role of prostate cancer antigen 3 in the management of prostate cancer. *Eur Urol* 2011;**60**:1045–54
80. Bjartell A. Genetic markers and the risk of developing prostate cancer. *Eur Urol* 2011;**60**:29–31
81. Roobol MJ, Schroder FH, van Leenders GL, Hessels D, van den Bergh RC, Wolters T, van Leeuwen PJ. Performance of prostate cancer antigen 3 (pca3) and prostate-specific antigen in prescreened men: Reproducibility and detection characteristics for prostate cancer patients with high pca3 scores (>/= 100). *Eur Urol* 2010;**58**:893–99
82. Schroder FH, Roobol MJ. Erspc and plco prostate cancer screening studies: What are the differences? *Eur Urol* 2010;**58**:46–52
83. Sonnenschein C, Soto AM. The aging of the 2000 and 2011 hallmarks of cancer reviews: A critique. *J Biosci* 2013;**38**:651–63
84. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011;**144**:646–74
85. Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, Nelson PS. Prostate-localized and androgen-regulated expression of the membrane-bound serine protease tmprss2. *Cancer Res* 1999;**59**:4180–84
86. Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaiyan AM. Tmprss2:Etv4 gene fusions define a third molecular subtype of prostate cancer. *Cancer Res* 2006;**66**:3396–400
87. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. Recurrent fusion of tmprss2 and ets transcription factor genes in prostate cancer. *Science* 2005;**310**:644–48
88. Reddy ES, Rao VN, Papas TS. The erg gene: A human gene related to the ets oncogene. *Proc Natl Acad Sci USA* 1987;**84**:6131–35
89. Rao VN, Papas TS, Reddy ES. Erg, a human ets-related gene on chromosome 21: Alternative splicing, polyadenylation, and translation. *Science* 1987;**237**:635–39
90. Tan SH, Furusato B, Fang X, He F, Mohamed AA, Griner NB, Sood K, Saxena S, Katta S, Young D, Chen Y, Sreenath T, Petrovics G, Dobi A, McLeod DG, Sesterhenn IA, Srivastava S. Evaluation of erg responsive proteome in prostate cancer. *Prostate* 2014;**74**:70–89
91. Furusato B, Tan SH, Young D, Dobi A, Sun C, Mohamed AA, Thangapazham R, Chen Y, McMaster G, Sreenath T, Petrovics G, McLeod DG, Srivastava S, Sesterhenn IA. Erg oncoprotein expression in prostate cancer: Clonal progression of erg-positive tumor cells and potential for erg-based stratification. *Prostate Cancer Prostatic Dis* 2010;**13**:228–37
92. Chen H, Nandi AK, Li X, Bieberich CJ. Nkx-3.1 interacts with prostate-derived ets factor and regulates the activity of the psa promoter. *Cancer Res* 2002;**62**:338–40
93. Petrovics G, Liu A, Shaheduzzaman S, Furusato B, Sun C, Chen Y, Nau M, Ravindranath L, Dobi A, Srikantan V, Sesterhenn IA, McLeod DG, Vahey M, Moul JW, Srivastava S. Frequent overexpression of ets-related gene-1 (erg1) in prostate cancer transcriptome. *Oncogene* 2005;**24**:3847–52
94. Hessels D, Schalken JA. Recurrent gene fusions in prostate cancer: Their clinical implications and uses. *Curr Urol Rep* 2013;**14**:214–22
95. Leyten GH, Hessels D, Jannink SA, Smit FP, de Jong H, Cornel EB, de Reijke TM, Vergunst H, Kil P, Knipscheer BC, van Oort IM, Mulders PF, Hulsbergen-van de Kaa CA, Schalken JA. Prospective multicentre evaluation of pca3 and tmprss2-erg gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer. *Eur Urol* 2014;**65**:534–42
96. Hessels D, Smit FP, Verhaegh GW, Witjes JA, Cornel EB, Schalken JA. Detection of tmprss2-erg fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. *Clin Cancer Res* 2007;**13**:5103–08
97. Xiao WZ, Han DH, Wang F, Wang YQ, Zhu YH, Wu YF, Liu NT, Sun JY. Relationships between pten gene mutations and prognosis in glioma: A meta-analysis. *Tumour Biol* 2014;PMID:24705863, Epub 8 April
98. Han B, Mehra R, Lonigro RJ, Wang L, Suleman K, Menon A, Palanisamy N, Tomlins SA, Chinnaiyan AM, Shah RB. Fluorescence in situ hybridization study shows association of pten deletion with erg rearrangement during prostate cancer progression. *Mod Pathol* 2009;**22**:1083–93
99. Fine SW, Gopalan A, Leversha MA, Al-Ahmadie HA, Tickoo SK, Zhou Q, Satagopan JM, Scardino PT, Gerald WL, Reuter VE. Tmprss2-erg gene fusion is associated with low gleason scores and not with high-grade morphological features. *Mod Pathol* 2010;**23**:1325–33
100. Gopalan A, Leversha MA, Satagopan JM, Zhou Q, Al-Ahmadie HA, Fine SW, Eastham JA, Scardino PT, Scher HI, Tickoo SK, Reuter VE, Gerald WL. Tmprss2-erg gene fusion is not associated with outcome in patients treated by prostatectomy. *Cancer Res* 2009;**69**:1400–06
101. Hu Y, Dobi A, Sreenath T, Cook C, Tadase AY, Ravindranath L, Cullen J, Furusato B, Chen Y, Thangapazham RL, Mohamed A, Sun C, Sesterhenn IA, McLeod DG, Petrovics G, Srivastava S. Delineation of tmprss2-erg splice variants in prostate cancer. *Clin Cancer Res* 2008;**14**:4719–25
102. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan SH, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, Patel V, Vahey M, Gutkind JS, Sreenath T, Petrovics G, Sesterhenn IA, McLeod DG, Srivastava S. Tmprss2-erg fusion, a common genomic alteration in prostate cancer activates c-myc and abrogates prostate epithelial differentiation. *Oncogene* 2008;**27**:5348–53
103. Rahim S, Uren A. Emergence of ets transcription factors as diagnostic tools and therapeutic targets in prostate cancer. *Am J Transl Res* 2013;**5**:254–68
104. Thangapazham R, Saenz F, Katta S, Mohamed AA, Tan SH, Petrovics G, Srivastava S, Dobi A. Loss of the nkx3.1 tumorsuppressor

promotes the tmprss2-erg fusion gene expression in prostate cancer. *BMC Cancer* 2014;**14**:16
105. Zhang Y, Fillmore RA, Zimmer WE. Structural and functional analysis of domains mediating interaction between the bagpipe homologue, nkx3.1 and serum response factor. *Exp Biol Med (Maywood)* 2008;**233**:297–309
106. Carson JA, Culberson DE, Thompson RW, Fillmore RA, Zimmer W. Smooth muscle gamma-actin promoter regulation by rhoa and serum response factor signaling. *Biochim Biophys Acta* 2003;**1628**:133–39
107. Carson JA, Fillmore RA, Schwartz RJ, Zimmer WE. The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mnkx3-1, and serum response factor. *J Biol Chem* 2000;**275**:39061–72
108. Fillmore RA, Dean DA, Zimmer WE. The smooth muscle gamma-actin gene is androgen responsive in prostate epithelia. *Gene Expr* 2002;**10**:201–211
109. Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, Zhang J, Perry SR, Labrot ES, Wu X, Lis R, Hoshida Y, Hiller D, Hu B, Jiang S, Zheng H, Stegh AH, Scott KL, Signoretti S, Bardeesy N, Wang YA, Hill DE, Golub TR, Stampfer MJ, Wong WH, Loda M, Mucci L, Chin L, DePinho RA. Smad4-dependent barrier constrains prostate cancer growth and metastatic progression. *Nature* 2011;**470**:269–73
110. Mao X, Debenedittis P, Sun Y, Chen J, Yuan K, Jiao K, Chen Y. Vascular smooth muscle cell smad4 gene is important for mouse vascular development. *Arterioscler Thromb Vasc Biol* 2012;**32**:2171–77
111. Browning CL, Culberson DE, Aragon IV, Fillmore RA, Croissant JD, Schwartz RJ, Zimmer WE. The developmentally regulated expression of serum response factor plays a key role in the control of smooth muscle-specific genes. *Dev Biol* 1998;**194**:18–37
112. Oko R, Hermo L, Hecht NB. Distribution of actin isoforms within cells of the seminiferous epithelium of the rat testis: Evidence for a muscle form of actin in spermatids. *Anat Rec* 1991;**231**:63–81
113. Sawtell NM, Hartman AL, Lessard JL. Conserved tissue-restricted expression of huc 1-1 actin phenotype among eumetazoan organisms. *J Exp Zool* 1990;**256**:54–62
114. Hartman AL, Sawtell NM, Lessard JL. Expression of actin isoforms in developing rat intestinal epithelium. *J Histochem Cytochem* 1989;**37**:1225–33