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Evidence for Down-Regulation of Calcium Signaling Proteins in Advanced Mouse Adenocarcinoma

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ABSTRACT

The abundance of several important proteins in prostate tissue was compared between wild-type mouse dorsal prostate and TRAMP mouse dorsal prostates with well-differentiated adenocarcinoma, and between wild-type mouse dorsal prostate and poorly-differentiated TRAMP mouse tumor tissue. In TRAMP dorsal prostates with well-differentiated adenocarcinoma, there were few significant changes in the protein abundances compared to wild-type dorsal prostates, with the exception of increases in proliferating cell nuclear antigen (PCNA) and beta tubulin, two proteins implicated in cell proliferation, and a more than 2-fold increase in Hsp60, a protein involved in the suppression of apoptosis. In the poorly-differentiated tumors, the changes in protein abundance were substantial. While some of those changes could be related to the disappearance of stromal tissue or the appearance of epithelial tissue, other changes in protein abundance were more significant to the cancer development itself. Most notable was the overall decrease in calcium homeostasis proteins with a ten-fold decrease in calreticulin and Hsp70 and a forty-fold decrease in creatine kinase bb in the cancerous tissue.

Prostate cancer (PCa) is currently the second leading cancer related death in men in the United States (*1*), with a staggering 10 % of men being diagnosed with PCa in their life time. A significant problem in PCa diagnosis is the lack of physical symptoms until late into the development of the cancer. Some tumor markers exist, prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) (*2, 3*), however, they can be problematic. PSA fails to distinguish between neoplasia and hyperplasia at a range of PSA levels between 4-20 ng PSA/mL serum *(3-5)* and some very aggressive forms of PCa do not cause elevated PSA levels at all. PAP is only diagnostic in advanced cancer (*6, 7*). Furthermore, no reliable prognosis can be made in the early stages of the disease as to whether the cancer is likely to progress to an aggressive, androgen-independent form. Additionally, there is mounting evidence that androgen ablation may select for androgen-independent cells since both cell phenotypes are present, actually enhancing the growth of those tumor cells (*8-11*). Therefore, determining the biological factors that control the transition from predominantly androgen-dependent to predominantly androgen-independent cancer is critical for developing appropriate treatment.

The early stage of PCa (androgen-dependent) and advanced stage of PCa (androgen-independent) are distinguishable by tissue differentiation, and cell-phenotype. Normal prostate tissue consists of glands containing epithelial tissue surrounded by stroma tissue; the stroma contains smooth muscle and fibroblast cells necessary to contract the glands and force the release of the prostatic fluid. In the progression of adenocarcinoma, the stroma gradually disappears, and growth of both exocrine and neuroendocrine cells of the prostate epithelia is over-stimulated until only epithelial tissue remains (Figure 1) (*12*). Neuroendocrine cells represent an androgen independent cell phenotype and evidence

exists that it is particularly increased in abundance in human prostate cancer (*13-15*). Not surprisingly, proteins located in the stromal tissue (actin, tropomyosin, prolyl-4-hydroxylase, and others) have been repeatedly shown to decrease as the disease progresses *(16, 17)*. Eventually, the cancer metastasizes into the bones of the pelvis, lumbar spine and other distal bony sites (*18*).

The loss of tissue differentiation within the prostate is associated with androgen-dependent PCa, however, metastasis into other sites is associated with androgen-independent PCa, to which approximately 25% of patients progress *(19).* While androgen-dependent cancer growth depends on increased cell proliferation and is thus treatable with androgen ablation, the onset of androgen-independence is associated with a different cell phenotype, which is characterized by inhibited apoptosis and a complex restructuring of the entire calcium homeostasis, rather than enhanced cell proliferation *(20-22).* There are currently no chemical therapies available for the treatment of advanced, androgen-independent PCa (*23, 24*).

Calcium is a signaling molecule in a variety of processes in the cell, including cell proliferation and apoptosis (*22, 25, 26*) and the calcium dependent signaling pathways can alter during the progression of the cancer (*27*). There is evidence that the calcium concentration in cancerous tissue increases and enhances the proliferative phenotype of some cancers (*26, 28-30*). Calcium in high concentrations has been repeatedly shown to induce apoptosis in healthy and cancerous cell lines, and some evidence indicates that the intracellular calcium concentration in advanced cancer is elevated (*31-33*). Vanoverberghe et. al. have shown that calcium homoestasis is involved in the apoptotic resistance of neuroendocrine prostate cells (*34*). Calcium induced apoptosis has become a

focus of drug therapies for androgen-independent PCa, and highlight the need of understanding calcium homeostasis throughout the progression of the cancer disease (*29*).

Possible therapy targets regulating calcium signaling in apoptosis include calcium channel blockers and calcium chaperones (*30, 35, 36*). Batra et al. showed that the calcium channel blocker, Verapamil, inhibits cell proliferation in the human cell line DU 145 while, paradoxically, increasing the calcium concentration (*37*). Jan et al. demonstrated that Fendilin, an anti-anginal drug that also inhibits L-type calcium channels, elevates intracellular calcium in PC3 cell lines (androgen-independent) (*38*). Ordinarily, one would expect that calcium channel blockers would lead to a lower calcium concentration in the cell, however, the opposite was observed. A proposed explanation to this conundrum is that calcium in the ER is released into the cell to compensate for the lowered influx of calcium, while depletion of calcium stores opens alternative calcium channels in androgen-independent cell lines (*39*). The role of calcium in androgen-dependent and independent cancer cell lines was further investigated by Zhu and coworkers, who studied the regulation of the calcium chaperone, calreticulin. They found that calreticulin is an androgen response gene in androgen-independent cancer and increase in androgen increases the calreticulin expression and thus decreases the sensitivity of cells against apoptosis from free cytosolic calcium *(36, 40)*. However, they also found that down-regulation of calreticulin alone is not sufficient to induce apoptosis, suggesting other partners in the apoptosis process *(36)*.

In the current investigation, we compare cell lysates of cancerous and non-cancerous mouse prostate tissue, in order to identify additional proteins that are involved in calcium homeostasis in PCa. Previously, proteomic methods have been used on human prostate

cell lines and animal models in order to find proteins that serve as prognostic biomarkers for aggressive adenocarcinoma (*16, 41-43*). Investigators have studied human prostate tissue (*16*), urine (*44*), prostatic fluid (*45*) and have found abnormally expressed proteins involved in cell proliferation, such as PCNA, inhibition of apoptosis, such as heat shock proteins, and loss of stroma, such as actin (*16, 46, 47*). While these studies underline the usefulness of proteomics to detect a remarkable number of proteins abnormally expressed, an investigation of changes in protein abundance at different stages in the development PCa has not been attempted (*41, 48-51*). We therefore compared protein abundances in a transgenic mouse model of prostate cancer (TRAMP) at two stages of progression (well-differentiated and poorly-differentiated), with 2D-gel electrophoresis, to find proteins that are indicative of progression from androgen-dependent to androgen-independent adenocarcinoma.

MATERIALS AND METHODS

Samples. Prostate tissues from TRAMP [C57BL/6TRAMPxFVB]F1) mice (TG) and and wild-type mice (WT) were used in this study (*52*). The TRAMP mouse prostates were harvested at two different stages of progression: well-differentiated adenocarcinoma (from mice approximately 14 weeks of age) and poorly-differentiated tumors (from mice approximately 25 weeks of age). The well-differentiated dorsal prostate tissue was compared with dorsal normal prostate tissue. The prostates from poorly-differentiated prostate tissue, were not dissected, since in this advanced stage it is not possible to try to differentiate the lobes. Tissues were divided and either flash frozen or fixed in 10% buffered formalin. Histological grade was confirmed by analysis of H&E stained sections from each sample.

Sample preparation. The prostate tissue (13-50 mgs in 30 mM Tris base, 7 M Urea, 2 M thiourea, 4% CHAPS, 0.5% triton X-100, all adjusted to pH = 8.5.) were treated with the Plus One Grinding Kit (Amersham Bioscience) and the Plus One Clean Up Kit (Amersham Bioscience) according to manufactures protocol. The cleaned cell lysate was subsequently re-suspended in the same lysis buffer as above. A total of four aliquots per sample were quantified with the Plus One 2D Quant kit (Amersham Bioscience) at 480 nm. In each experiment, between 8 and 10 samples, 50 μg each, were prepared. Half of these tissue samples were cancerous and half wild type.

Labeling. The pH of all samples were adjusted to a pH of 8.5 with NaOH, after which all samples were labeled with 400 pmoles of Cy5 per 50 μg of protein. The labeling of the proteins is carried out by amine reactive dyes (CyDyes Amersham) (*53*). The amount of dye added was the limiting factor in the reaction and therefore each protein is

minimally labeled (approximately 1-2 % of the lysine residues of each protein are labeled). Additionally, 400 μg of a 50:50 mixture of WT and TG samples were labeled with Cy3 as an internal standard, to reduce experimental variability (*54*). Labeling proceeded for 30 minutes on ice in the dark, before the reaction was quenched by addition of 1 μL of 10 mM Lysine for 10 minutes in the dark on ice. At this point 50 μg of the internal standard were added to each of the 8 samples.

Re-hydration. The labeling of each sample was followed by addition of re-hydration buffer (8 M urea, 4 % CHAPS, 13 mM DTT, 2 % IPG {pH = 4-7}, 1 % w/v bromophenol blue) to a volume of 450 μL per sample. In addition to the analytical gels, overloaded gels, with protein concentrations of 500 μg were run, however, these lysates were not labeled. All Immobilized pH Gradient strips (IPG) were 4-7 pH, 24 cm, and were re-hydrated over night in the dark in a swelling tray.

1st Dimension. The isoelectric focusing was carried out with a Multiphor II apparatus (Amersham Bioscience) at 20 ˚C. Prior to focusing, the cathode electrode pad was soaked in 13 mM DTT, and the anode electrode pad was soaked in Millipor water. The first dimension was run at 500 V for 60 minutes followed by an increase in 500 V every hour, until 3000 V were reached. The strips were then run for another 18-20 hours at 3000 V.

2nd Dimension. Each strip was equilibrated first in a 0.5 % solution of DTT (10 minutes), then in 4.5 % iodoacetamide (10 minutes). Both DTT and iodoacetamide were dissolved in equilibration buffer (50 mM Tris base, pH = 8.8, 6 M urea, 30 % glycerol, 2 % SDS, trace of bromophenol blue). The plates used with the 12.5 % acrylamide gels were made of low-fluorescent glass. One hour prior to pouring the gels, the non-spacer plates were treated with 2 mL of bind silane (8 mL ethanol, 200 μL glacial acetic acid, 5

μL gamma-methacryloxypropyltrimethoxysilane). After equilibration, the strips were washed in 2 % SDS running buffer (25 mM Tris, 192 mM glycine, 0.2 % SDS) and subsequently inserted into the top of the gel layered with agrose gel (0.5 % low melt agrose, trace amounts of bromophenol blue dissolved in 100 mL of 2 % SDS running buffer) layered on top of the gel. The gels were run at 25 ˚C in a DALT12 gel runner (Amersham) at 2.5 watts per gel for 30 minutes or until the bromophenol blue front was visible in the gels. At this point the wattage was increased to 12 watts per gel. When the blue dye migrated off the gel a quick scan was performed to insure that the proteins had migrated to the bottom of the gel. Otherwise, they were returned to the gel runner for additional running time.

Scanning. The top glass plate of the gels was removed, and the gels were fixed in 30% EtOH, 10% acetic acid over night. All gels were scanned with a resolution of 100 microns, 550 photomultiplier voltage (pmt voltage) with the Typhoon 9400 Variable mode Imager (Amersham Bioscience). All Cy3 images were scanned with a 532 nm Laser and an emission filter of 580 BP (band path) 30. All Cy5 images were scanned with a 633 nm Laser and an emission filter of 670 BP 30. After scanning, all images were cropped to the same size with ImageQuant 5.2 (Amersham Bioscience). After fixing, the overloaded gels were stained with Sypro Ruby Dye (Molecular Probes), followed by de-staining in 10 % MeOH, 7 % glacial acetic acid. The overloaded gels were scanned with a 532 laser and an emission filter of 610 nm BP 30, followed by cropping. After scanning, the overloaded gels were further Coomassie stained, for visual inspection.

Analysis. Gel analysis was carried out with the DeCyder 5.1 (Amersham Bioscience) (*53*). Protein detection was carried out with the batch processor, based on differential in-gel analysis (DIA).

Further analysis was conducted with the Biological Variation Analysis (BVA) software. Each gel contains a pooled standard/sample gel image (WT or TG). The parameter that expresses the change in abundance from WT to TG is called the volume ratio. A negative volume ratio indicates that the abundance has decreased from WT to TG and a positive volume ratio indicates an increase. The change in the volume ratio is normalized to the total amount of protein; the statistical analysis is carried out under the assumption of equal amounts of protein in each gel.

Digestion. Proteins of interest were excised from the gel with the Ettan Protein Picker (Amersham Bioscience) and put in a 96 well plate with 200 μL of water. They were stored at -20 ˚C until digestion. For digestion, the water covering the plugs was removed, followed by three 20 minute washes in 100 μL 50 mM ammonium bicarbonate / 50 % MeOH and one 20 minute wash in 100 % acetonitrile. The plugs were completely dried under nitrogen, before addition of 25 μL of an 8 ng / μL trypsin dissolved in 20 mM ammonium bicarbonate. The digest proceeded overnight, at which point it was quenched with 3 μL of 1 % trifluoroacetic acid (TFA). The liquid was subsequently removed to a new 96 well plate. The plugs were washed three additional times, twenty minutes each, with 20 μL of 75 % acetonitrile / 0.1 % TFA and all liquids combined. The liquid was evaporated as before.

MALDI-ToF analysis. The proteins were analyzed using an Ettan MALDI-ToF/Pro (Amersham Bioscience). The digested proteins were reconstituted in 5 μL of 50 %

acetonitrile, 0.5 % TFA and 0.4 μL of sample was applied per target on the slide. After evaporation of the liquid, the same target was covered with 0.4 μL solution of saturated α-cyano-hydroxycinnamic acid (dissolved in 50 % acetonitrile / 0.5 % TFA) and dried again. The MALDI-ToF was calibrated before each use with angiotensin III (MW 897.52, Sigma) and adrenocorticotropic hormone fragment 18-39 (MW 2465.7, Sigma). The proteins were identified by peptide mass fingerprinting using sequencing database searching with the internal Amersham Bioscience software and database.

Western Blot analysis. To compare specific protein abundance independently by Western Blot analysis, equal amounts of protein (3 μg per well for actin, desmin, and calreticulin and 20 μg per well for Hsp70) from 4 samples, (TG and WT), were run on 12 % acrylamide gels in 2 % SDS running buffer. The proteins were transferred from the gels to PVDF-Plus Transfer Membranes (Osmotic, Inc.) with a Mini-Protean II Cell (Bio-Rad) submerged in transfer buffer (2.9 g glycine, 5.8 g tris base, 0.37 g SDS and 200 mL ethanol, per liter, pH = 8.3). After transfer, the blots were immersed in 5 % non-fat milk in TBS-T (137 mM sodium chloride, 2.7 mM potassium chloride, 25 mM tris base, 0.05 % Tween 20, pH = 7.4) overnight at 4 ˚C for blocking. The blots were then incubated with the primary antibody (desmin, actin, calreticulin and Hsp70 from Stressgen, from a rabbit host in all cases) diluted in TBS-T/5% non-fat milk. After one hour of incubation at room temperature, the membranes were washed three times (one time for 15 minutes, twice for 5 minutes) in TBS-T. The blots were subsequently incubated with a secondary antibody (goat-anti-Rabbit IgG, peroxidase conjugate, Sigma) for one hour at room temperature. The membranes were then washed as before, with an additional wash with TBS (no Tween) to prevent interference with the detection method. The antigens were

detected with Amersham Bioscience ECL Plus via chemi-fluorescence and visualized with the Typhoon 9400. Quantitative analysis was performed with ImageQuant 5.2.

RESULTS

Representative images of the histopathology of the tissues examined are presented in Figure 1. The tissues examined were from normal dorsal, well-differentiated dorsal, poorly-differentiated tissue, as indicated in Table 1.

Difference in Protein Abundance in 2D-gel electrophoresis (poorly-differentiated tissue). A total of 400 spots detected in gels generated using poorly-differentiated tumors were proteins. Protein matching was carried out by different researchers for the same experiment to insure objective matching (*53*). Approximately 1/3 of these proteins decreased, 1/3 increased and 1/3 remained the same as compared to WT DP. Due to the large number of changes, the cut-off values for the statistics were set at a change of +/- 2.00 (100 % change), with a t-test value of 0.05 or less. We found 96 proteins matched in both WT and TG samples, with t-test values under 0.05. Of these proteins 60 decreased and 36 increased in abundance when compared to normal tissue. We have Identified 24 proteins using MALDI-ToF and of these proteins 16 decreased, 8 increased, and 3 proteins that remained the same (Table 2). All proteins were digested and identified at least twice from separate gels with MALDI-ToF. An expectation value of zero denotes a perfect match; an expectation value of 0.01 indicates a 1% chance that the identification is random. All reported identifications have an expectation value of ≤ 0.01 (Table 2). The coverage ranged from 11 % to 41 %, while at least 50 % of detected peptides were used for the ID (Table 3). A Sypro-Ruby stained gel, indicating the location of some proteins, is shown on Figure 2. We found differentially expressed proteins involved in tissue differentiation, cell proliferation, inhibition of apoptosis, and calcium homeostasis.

Difference in Protein Abundance in Western blots (poorly-differentiated tissue). Western blots were carried out with the antibodies of desmin, actin, Hsp70, and calreticulin. Desmin decreased 6-fold, actin decreased 3-fold, Hsp70 and calreticulin decreased 10-fold (Table 4). The Western blots were repeated at least twice. In all cases the results of the Western blots coincide with the results of the proteomics experiments (Table 4).

Difference in Protein Abundance in 2D-gel electrophoresis (well-differentiated tissue). In the well-differentiated tissue, approximately the same number of proteins was detected as in the poorly-differentiated tissue for DP (~ 400). Only ~ 60 increased and ~40 decreased in DP. Due to the much lower number of spots that changed intensity, the cut-off values for the change of statistics were set at of +/- 1.50 (50 % change), with a t-test value of 0.1 or less. We found proteins involved in cell proliferation (tubulin beta and proliferating cell nuclear antigen), and proteins involved in calcium homeostasis (phospholipase C and Hsp70). Additionally, we specifically looked for changes in proteins that had been found to be abnormally expressed in poorly-differentiated tissue.

Difference in Protein Abundance in Western blots (well-differentiated tissue). Western blots were carried out with the antibodies of desmin, actin, and calreticulin. Desmin, actin, and calreticulin did not change in DP. The Western blots were repeated at least twice. In all cases the results of the Western blots coincide with the results of the proteomics experiments (Table 4).

DISCUSSION

Cell lines, human tissue, and fluids have been explored previously for differentially expressed proteins as possible biomarkers of PCa (*16, 43-45*), however, little is known about potential biomarkers which indicate the transition from androgen-dependent to androgen-independent PCa. Samples derived from human tissue or fluids are only of limited use for proteomics due to patient variability. For example, when correlating protein expression with Gleason scores, which are based on tissue differentiation and thus do not necessarily indicate the virulence of the cancer, the researchers found little correlation between abnormally expressed proteins and gleason scores (*47*). Cell lines exist for both, the androgen-dependent and -independent cancer, however, they are derived from different hosts with potentially different forms of PCa. Animal models for prostate cancer in mice, however, provide an excellent model for proteomics and the development of PCa because of their reproducible disease progression (*8, 55*). Human and mouse prostates differ anatomically in that the mouse prostate actually consists of four lobes, while the human prostate is made up of one glandular organ that can be subdivided into at least 3 distinct zones (*56*). The mouse dorsal prostate has been reported to be most similar to the human peripheral zone (from which most human cancers arise), although this contention has recently been challenged (Ref. Cancer Res. 2004 Mar 15;64(6):2270-305). More importantly though, previous research showed that PCa in TRAMP mice and humans progresses similarly in terms of the onset of androgen-independent adenocarcinoma and metastasis (*8, 55*). In addition, there is some evidence

that TRAMP, in agreement with some data in human prostate cancer, exhibit increased abundance in the neuroendocrine phenotype (*56*). For these reasons we have investigated the TRAMP mice model to follow the progression of the disease with proteomics.

In order to identify proteins indicating the onset of androgen-independent adenocarcinoma, we studied tissue from 14 weeks old (well-differentiated, predominantly androgen-dependent cell phenotype) and 25 weeks old (poorly-differentiated, predominantly androgen-independent cell phenotype) TRAMP mice. Using 2D-gel electrophoresis, differentially expressed proteins between normal and cancerous tissue were determined. In poorly-differentiated adenocarcinoma (~25 weeks), all cells had progressed to the androgen-independent stage, associated with very pronounced changes in protein abundances. Differentially expressed proteins identified from poorly-differentiated adenocarcinoma can be associated with four processes in cancer development; these processes include tissue differentiation, cell proliferation, apoptosis, and calcium-dependent signaling pathways. In well-differentiated adenocarcinoma (~14 weeks), the progression of adenocarcinoma is early in the transition from the androgen-dependent to the androgen-independent stage, and the changes in protein abundances of the four processes mentioned are much less distinct.

Changes in protein expression corresponding to tissue differentiation were clearly found in poorly-differentiated prostate tissue. Actin, desmin, prolyl-4-hydroxylase, and tropomyosin, all proteins found in the muscle tissue of the stroma, decrease significantly (Table 2). The change in protein abundances based on cell structure has been observed previously and corresponds to the decrease in stroma tissue (*46, 47, 50, 57*). However, most of the changes in protein abundance associated with the decrease of stroma tissue

could not be observed in well-differentiated adenocarcinoma. The second group of proteins identified are involved in cell proliferation. Beta tubulin was up-regulated 4-fold and proliferating cell nuclear antigen (PCNA) was up-regulated 2.4-fold in poorly-differentiated prostate tissue. PCNA is an auxiliary protein to DNA polymerase and beta tubulin is necessary for mitosis (*58-60*). Both proteins are essential for cell proliferation and are primarily located in the epithelium. An increased expression is thus not surprising and has been documented before for advanced adenocarcinoma (*46, 61*). Since the main mode of cancer growth in androgen-independent PCa is inhibition of apoptosis rather than increased cell proliferation, and since the increase in epithelial tissue at this stage is significant, it is reasonable to assume that the increased expression of PCNA and beta tubulin in poorly-differentiated tissue is mainly due to tissue differentiation. The same proteins are also up-regulated in the dorsal prostate tissue of the well-differentiated prostate tissue. While the main reason for up-regulation in poorly-differentiated tissue is the increase in epithelial cells, the increase in well-differentiated tissue is mainly based on enhanced cell proliferation. This finding is consistent with the fact that the main mode of PCa in the androgen-dependent cell phenotype is increased cell proliferation (*8-11*)

Apoptosis inhibiting proteins were also differentially expressed in poorly-differentiated tissue. Hsp60, a heat shock protein, was up-regulated 3-fold, in accordance with existing literature (*16, 62*). The expression level of ERP44, a protein induced by apoptosis related stress, was increased 10-fold in poorly-differentiated tissue. ERP44 is a protein of the thioredoxin family involved in protein folding and is located in the endoplasmic reticulum. When proteins unfold due to stress induced apoptosis, ERP44 serves to refold proteins, thereby preventing the advancement of apoptosis (*63*). Since inhibition of

apoptosis is the main mode of cancer progression in androgen-independent cancer but not in androgen-dependent cancer, it is consistent that the apoptosis inhibiting proteins were only up-regulated in advanced cancer.

In addition to well documented differential expression of proteins involved in tissue differentiation, cell proliferation, and apoptosis (*16, 46, 47*), we also found a significant change in abundance of four proteins involved in calcium homeostasis in poorly-differentiated adenocarcinoma; calreticulin (10-fold decrease), Hsp70 (11-fold decrease), creatine kinase brain (40-fold decrease), and phospholipase C (4-fold increase). Calreticulin, Hsp70 and creatine kinase brain are all documented to increase in abundance in cancerous tissue of the prostate (*64-68*). Calreticulin and Hsp70 suppress apoptosis in part by calcium buffering (*69*). Calreticulin is regulated by androgen, a process that is preserved between mouse and human (*8*) and has been to be involved in buffering cytotoxic calcium (*70, 71*). HspP70 is a stress induced protein that binds two calcium ions per enzyme (*72*) and protects cells against apoptosis induced by TNF- and TNF- (*73*). Creatine kinase brain is a membrane protein, that in close conjunction with calcium-pumps, plays a crucial role in the energetics of calcium-homeostasis (*74*). While some literature cites an increase in expression in cancerous prostate tissue of these three proteins (*16, 66, 75*)**,**our data indicates a decrease in expression which implies that these three proteins may be biomarkers for the severity of the cancer. This is consistent with Wang et al. who found that a 75 % decrease in calreticulin serves as a biomarker for aggressive cancer (*76*) and others who showed a down regulation of Hsp70 in advanced PCa (*62, 77*).

Phospholipase C is different from these three proteins in that it increased in expression level in the poorly-differentiated adenocarcinoma. Phospholipase C is located in the epithelium (Genatlas). Therefore the evidence for up-regulation in advanced cancer cells from our current data is likely to be due to an increase in epithelial tissue. However, we also found a 2-fold up-regulation of phospholipase C in the well-differentiated tissue. While there is evidence that phospholipase C is down-regulated in some invasive prostate cancer, a recent investigation has shown phospholipase C to initiate oncogenesis in breast cancer cell lines (*78, 79*). Activation of phospholipase C triggers the release of stored calcium into the cytosol by mobilizing the production and release of inositol 1,4,5-trisphosphate (*78, 80*). Therefore, it is conceivable that the two-fold increase in phospholipase C expression serves to release additional Ca^{2+} and thus enhances proliferation in the well-differentiated tissue.

In contrast to the poorly-differentiated adenocarcinoma, the well-differentiated adenocarcinoma did not manifest an abundance change of calreticulin or creatine kinase bb, however, Hsp70 exhibited a 2-fold increase, opposite to what was found in poorly-differentiated tissue. This may be related to the necessity of Hsp70 for the survival of tumorgenic cells in the early stage, while other apoptosis inhibiting mechanisms take over at the later, androgen-independent stage (*77*). This cell phenotype dependent protein expression has also been observed for another calcium chaperone, Bcl-2, which is up-regulated in androgen-dependent cancer cell lines, whereas in androgen-independent cell lines Bcl-2 is down-regulated (*81*).

In conclusion, we were able to demonstrate several important points regarding the progression of PCa in mice. First, the differential protein expression between normal,

well-differentiated and poorly-differentiated tissue is due to a combination of the loss of tissue differentiation, enhancement of cell proliferation and inhibition of apoptosis in PCa in TRAMP mice. Second, proteins known to inhibit apoptosis are only increased in abundance in the androgen-independent cancer in accordance with their cell phenotype, however, there are potentially low abundance proteins beyond our detection limits. This is consistent with the tumor deregulated proteins in humans and is further proof that TRAMP mice are an excellent model for proteomics research. Third and most importantly, we found three proteins involved in calcium homeostasis which have depressed expression in the androgen-independent PCa. Therefore, differentially expressed proteins involved in calcium homeostasis may be excellent biomarkers for androgen-independent cancer cells, since calcium signaling clearly changes significantly from the androgen-dependent to the androgen-independent cell phenotype. This differentiation between the two cell types could help determine whether androgen ablation is a reasonable therapy for a given patient and further investigations into the role of calcium regulated proteins in PCa are currently in progress.

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