Altered Endosome Biogenesis in Prostate Cancer Has Biomarker Potential

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Abstract

Prostate cancer is the second most common form of cancer in males, affecting one in eight men by the time they reach the age of 70 years. Current diagnostic tests for prostate cancer have significant problems with both false negatives and false positives, necessitating the search for new molecular markers. A recent investigation of endosomal and lysosomal proteins revealed that the critical process of endosomal biogenesis might be altered in prostate cancer. Here, a panel of endosomal markers was evaluated in prostate cancer and nonmalignant cells and a significant increase in gene and protein expression was found for early, but not late endosomal proteins. There was also a differential distribution of early endosomes, and altered endosomal traffic and signaling of the transferrin receptors (TFRC and TFR2) in prostate cancer cells. These findings support the concept that endosome biogenesis and function are altered in prostate cancer. Microarray analysis of a clinical cohort confirmed the altered endosomal gene expression observed in cultured prostate cancer cells. Furthermore, in prostate cancer patient tissue specimens, the early endosomal marker and adaptor protein APPL1 showed consistently altered basement membrane histology in the vicinity of tumors and concentrated staining within tumor masses. These novel observations on altered early endosome biogenesis provide a new avenue for prostate cancer biomarker investigation and suggest new methods for the early diagnosis and accurate prognosis of prostate cancer.

Implications: This discovery of altered endosome biogenesis in prostate cancer may lead to novel biomarkers for more precise cancer detection and patient prognosis. Mol Cancer Res; 12(12); 1851–62. ©2014 AACR.

Introduction

Prostate cancer is the most common form of cancer in males from developed countries, and the incidence of this disease is predicted to double globally by 2030 (World Cancer Research Fund prostate cancer statistics; http://globocan.iarc.fr). Prostate cancer affects approximately 1 in 8 men globally by the time they reach the age of 70 years (1). The prostate-specific antigen test is currently used for prostate cancer screening; however, this assay suffers from a high percentage of false-positive results (see for example ref. 2), and recently, there have been recommendations to abandon this procedure, particularly in older men (3). In addition, the digital rectal examination, which manually checks the prostate for abnormalities, is limited by the inability to assess the entire gland and to some degree the size of the tumor. Understanding the cell biology of prostate cancer is important to develop new biomarkers for the early diagnosis and accurate prognosis of prostate cancer.

There is mounting evidence for a central role of endosome–lysosome compartments in cancer cell biology (see refs. 4–6). Endosomes and lysosomes are directly involved in the critical processes of energy metabolism (7), cell division (8) and intracellular signaling (see for example ref. 9) and would therefore have a direct role in cancer pathogenesis. The endosome–lysosome system has a specific capacity to respond to environmental change, acting as an indicator of cellular function and will consequently be altered in cancer (10). Moreover, the endosome–lysosome system has a critical role in controlling the secretion of proteins into extracellular fluids (see for example ref. 11), making it an ideal system to identify new biomarkers that are released from cancer cells. Cumulative evidence from patient data and cell lines suggested that the process of lysosomal biogenesis might be altered in prostate cancer (see for example refs. 12, 13). However, we recently demonstrated that a panel of lysosomal proteins was unable to effectively discriminate
between a set of nonmalignant and prostate cancer cells (14). In contrast, the endosomal-related proteins cathepsin B and acid ceramidase displayed increased gene and protein expression in prostate cancer cells and demonstrated some discriminatory capacity when compared to nonmalignant cells. Acid ceramidase was previously shown to be upregulated in prostate cancer tissues, and the overexpression of this enzyme has been implicated in advanced and chemoresistant prostate cancer (15). Importantly, we also showed that LIMP-2, a critical regulator of endosome biogenesis (16), had increased gene and protein expression in prostate cancer cells (14), leading us to postulate that endosome biogenesis is altered in prostate cancer.

Endosome biogenesis involves the synthesis and organization of structural elements of the endosome system to form an integrated set of functional organelles that eventually interact with lysosomes (see for example ref. 17). There are two main endosomal pathways: first, from the biosynthetic compartments (endoplasmic reticulum and Golgi apparatus) via specific vesicular traffic toward distal elements of the endosomal network, including early endosomes, late endosomes, and multivesicular bodies; and from the cell surface through early endosomes to either recycling endosomes or toward late endosomes. In each case, the formation and movement of these dynamic vesicular compartments is controlled by specific targeting signals and trafficking machinery (see for example refs. 17, 18). This vesicular machinery can be used to define individual compartments; including, for example, the small GTPase Rab5 on early endosomes and the small GTPase Rab7 on late endosomes (18, 19). Here, we have investigated the gene expression, amount of protein, and intracellular distribution of a panel of endosomal proteins in prostate cancer and nonmalignant cell lines, to determine whether endosome biogenesis is altered in prostate cancer cells and to identify potential new biomarkers.

Materials and Methods

Antibody reagents

A LIMP-2 sheep polyclonal antibody was generated against the peptide sequence CKKLDDFVETGER (Mimotopes Pty. Ltd.). Rabbit polyclonal antibodies (Abcam PLC) were against Appl1 (0.4 μg/mL), Appl2 (0.4 μg/mL), Rab6 (1 μg/mL), TGN46 (10 μg/mL), TIR1 (1 μg/mL), and TIR2 (1 μg/mL). Akt (1:1,000) and phospho-Akt (Thr308; 1:1,000) from Cell Signaling Technology Inc., and horseradish peroxidase (HRP)–conjugated anti-GAPDH (1:20,000; Sigma-Aldrich Pty. Ltd.). Goat polyclonal antibodies (Santa Cruz Biotechnology) were against Rab5 (1 μg/mL), Rab7 (1 μg/mL), and EEA1 (1 μg/mL). A LIMP-1 (1 μg/mL) mouse monoclonal BB6 was provided by Prof. Sven Carlsson (Umeå University, Umeå, Sweden). HRP-conjugated secondary antibodies for Western blot analysis included anti-goat/sheep (1:2,000; Merck Millipore Pty. Ltd.), anti-rabbit (1:2,000), and anti-mouse (1:2,000; Sigma-Aldrich). The secondary and other antibody-conjugated fluorophores that were used included Alexa Fluor 488 (1:250), Alexa Fluor 633 (1:250), Transferrin-633 (1:1,000), Phalloidin-488 (1:100), and Lyso-Tracker (5 μmol/L); all from Life Technologies Pty. Ltd.

Cell lines and culture conditions

The nonmalignant cell lines PNT1a and PNT2 and prostate cancer cell lines 22RV1 and LNCaP (clone FCG) were obtained from the European Collection of Cell Cultures via CellBank Australia (Children’s Medical Research Institute, Westmead, NSW, Australia). These cell lines were absent from the list of cross-contaminated or misidentified cell lines, version 6.8 (March 9, 2012; ref. 20).

Cell lines were cultured in 75-cm² tissue culture flasks and maintained in RPMI-1640 culture medium (Life Technologies Pty. Ltd.) supplemented with 10% fetal calf serum (In Vitro Technologies Pty. Ltd.) and 2 mmol/L L-glutamine (Sigma-Aldrich Pty. Ltd.). Cells were incubated at 37°C with 5% CO₂ in a Sanyo MCO-17AI humidified incubator (Sanyo Electric Biomedical Co., Ltd.). Cells were cultured to approximately 90% confluence before passage, by washing with sterile PBS (Sigma-Aldrich), trypsin treatment (Trypsin–EDTA solution containing 0.12% trypsin, 0.02% EDTA; SAFC; Sigma-Aldrich) to dissociate the cells from the culture surface and then resuspension in supplemented culture medium.

Preparation of cell extracts and conditioned culture media for protein detection

The culture medium was aspirated from cultures at 80% to 90% confluence, the cells washed once with PBS, and then incubated with 800 μL of a 20 mmol/L Tris (pH 7.0) containing 500 mmol/L sodium chloride and 2% (w/v) SDS. Cells were harvested and an extract prepared by heating at 95°C and sonication for 1 minute. The lysate was then passaged six times through a 25-gauge needle. Total protein in the cell extracts was quantified using a bicinchoninic acid assay according to the manufacturer’s instructions (Micro BCA Kit; Pierce). Samples were quantified using a Wallac Victor optical plate-reader and Work software v2.0 (PerkinElmer Pty. Ltd.), using a five-point parameter standard curve. Cell extracts were stored at −20°C.

Protein was recovered from conditioned culture media, collected at the time of cell harvesting, using trichloroacetic acid precipitation. Briefly, cell debris was removed from the culture media by centrifugation (1,000 × g for 10 minutes), sodium deoxycholate (Sigma-Aldrich) added to a final concentration of 0.02% (v/v) and incubated on ice for 30 minutes. Trichloroacetic acid (Sigma-Aldrich) was then added to a final concentration of 15% (v/v) and incubated on ice for 2 hours. Protein was collected by centrifugation at 4°C (5,500 × g for 30 minutes), washed twice with ice-cold acetone and resuspended in SDS-sample buffer/PBS solution, and stored at −20°C.

Gene expression

Relative amounts of mRNA from nonmalignant and prostate cancer cell lines were defined by quantitative PCR.
(qPCR). Briefly, cells were lysed with TRI reagent (Applied Biosystems, Life Technologies) and RNA extraction performed using RNaseasy (Qiagen Pty. Ltd.) according to the manufacturer’s instructions. Two micrometers of total RNA was reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Life Technologies) following the manufacturer’s instructions. qPCR was performed with 2±μL of a 1:25 dilution of cDNA in 10μL of reaction mixture; containing 5μL Power SYBR Green PCR Master Mix (Life Technologies) and 0.5μL of both 10nmol/L forward and reverse primer. qPCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies). Each target was assessed in triplicate on a single plate and quantified relative to GAPDH endogenous control for each plate, with triplicate biologic replicates run independently. Oligonucleotides (Gene- endogenous control for each plate, with triplicate biologic replicates run independently. Oligonucleotides (Gene-
of patients treated by radical prostatectomy at the Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY; ref. 22), profiling 150 prostate cancer and 29 nonmalignant tissue samples that was performed using Affymetrix Human Exon 1.0 ST arrays. Statistical analysis of microarray gene expression was performed using a two-tailed unpaired t test with Welch correction using GraphPad Prism 5.03 (GraphPad Software Inc.).

Results
Increased endosome-related gene and protein expression in prostate cancer cells

The expression of endosome- and lysosome-related genes was quantified by qPCR in control and prostate cancer cells and normalized to the expression of GAPDH mRNA. The amounts of LIMP2, APPL1, APPL2, RAB5A, EEA1, and RAB4 mRNA were significantly increased in prostate cancer when compared with nonmalignant control cell lines (P ≤ 0.05; Fig. 1). In each case, there was an approximately 2- to 3-fold increase in mRNA expression. There was no significant difference in the amount of either RAB7 or LAMP-1 mRNA detected in prostate cancer cells compared with nonmalignant controls. Western blot analysis (Fig. 2A) demonstrated significant increases in the amount of LIMP-2, Appl1, Appl2, EEA1, and Rab4 protein in extracts from prostate cancer cells when compared with nonmalignant control cell lines (P ≤ 0.05; Fig. 2B). Moreover, for both LIMP-2 and Rab4, the increase was approximately 2- to 4-fold for prostate cancer compared with nonmalignant cells (Fig. 2B). There was no significant difference in the amount of Rab5, Rab7, and LAMP-1 protein detected in nonmalignant compared with prostate cancer cells (Fig. 2B).

Altered distribution of endosomes and lysosomes in prostate cancer cells

Representative confocal images for the distribution of endosomes and lysosome proteins (Fig. 3) show evidence of increased staining and altered distribution in prostate cancer compared with the nonmalignant controls. In nonmalignant control cells, LIMP-2 was concentrated in the perinuclear region, with some punctate vesicular staining in the remainder of the cytoplasm. In contrast, prostate cancer cells displayed relatively smaller LIMP-2 compartments, which had an even distribution throughout the cytoplasm. Appl1-positive endosomes were detected throughout the cell cytoplasm of nonmalignant control cells, whereas in prostate cancer cells, these compartments were more concentrated at the cell periphery, particularly near the plasma membrane in cellular extensions/pseudopodia. In nonmalignant control cells, both Rab5 and its effector EEA1 were concentrated in the perinuclear region, whereas in prostate cancer cells, these endosomal compartments were found throughout the cytoplasm, with some compartments located toward the cell periphery in cellular extensions. Rab7-positive endosomes were located mainly in the perinuclear region of both nonmalignant control and prostate cancer cells. In nonmalignant control cells, LAMP-1 compartments were concentrated in the perinuclear region, whereas in prostate cancer cells the LAMP-1 compartments were distributed away from the perinuclear region and concentrated in cellular extensions. Consistent with the LAMP-1 staining, LysoTracker positive acidic compartments were concentrated mainly in the perinuclear region of nonmalignant control cells, whereas in prostate cancer cells, these compartments were detected in both the perinuclear region and in cytoplasmic extensions (Fig. 3).

Altered distribution of endocytosed transferrin in prostate cancer cells

Previous studies have reported increased uptake of transferrin in prostate cancer cells, prompting the investigation of receptor expression and transferrin endocytosis in relation to the observed increase in endosome protein expression and...
altered endosome distribution. In nonmalignant control cells, endocytosed transferrin was observed in punctate intracellular structures after 5 minutes and in the perinuclear region at 15 and 30 minutes (Fig. 4). The prostate cancer cells endocytosed relatively more transferrin than the non-malignant control cell lines within the first 5 minutes of incubation and at the 30-minute incubation point. In nonmalignant cells at 30 (Fig. 4) and 20 minutes (Fig. 5), this transferrin was tightly concentrated in close proximity to the nucleus. After 15 minutes of incubation, the internalized
transferrin was not as concentrated in the perinuclear region of prostate cancer cells, with more transferrin-labeled compartments in the cell periphery and distributed throughout the cytoplasm when compared with the nonmalignant cells (Fig. 4). There was also a marked reduction in actin staining for the prostate cancer compared with the nonmalignant control cell lines (Fig. 4). In the nonmalignant control cells, transferrin was clustered mainly in LIMP-2- and Rab7-positive endosomes localized in the perinuclear region (Fig. 5). Although the prostate cancer cells had some LIMP-2- and transferring-positive staining in the perinuclear region and some colocalization of transferrin with the Golgi marker TGN46 (yellow colocalization), the majority of transferrin was localized in different endosomal compartments (i.e., Appl1, Rab5, and EEA1) distributed throughout the cytoplasm and in cellular extensions (Fig. 5). The Rab4 recycling endosomes and LAMP-1-positive lysosomes had similar patterns of transferrin staining for the prostate cancer and nonmalignant control cell lines (Fig. 5). Further analysis of the transferrin receptors revealed variable gene and protein expression for TfR1 (TFRC) and TfR2 (TFR2; Fig. 6A and B). There was a significant increase in TFR2 gene expression (P ≤ 0.05) in prostate cancer cells when compared with nonmalignant controls (Fig. 6A), but only a qualitative increase in TR1 protein in the prostate cancer cell line 22RV1 and not for LNCaP (Fig. 6B). Although there was significantly more TR2 protein detected in prostate cancer cells when compared with the nonmalignant cells (P ≤ 0.05), there was only an increase in TFR2 gene expression in the LNCaP cancer cell line (Fig. 6A). Colocalization of TR1 and transferrin was observed in all cell lines, and was in a perinuclear location in nonmalignant cell lines PNT1a and PNT2 compared with a broader cytoplasmic distribution in the cancer cell lines 22RV1 and LNCaP. Conversely, there appeared to be no colocalization of transferrin with TR2 in nonmalignant cells and limited colocalization of transferrin with TR2 compartments in the prostate cancer cells (Fig. 6C).

**Altered Akt signaling in prostate cancer cells**

The total amount of Akt protein detected in nonmalignant control cells was similar to that detected in prostate cancer cells (Fig. 6D and E). There were, however, differences in the amount of phosphorylated Akt in the prostate cancer lines, with 22RV1 showing a marked reduction in the amount of phosphorylated Akt, whereas LNCaP had an increased amount of phosphorylated Akt (Fig. 6D), a phenomenon previously observed by Shukla and colleagues (23) and related to mutations of PTEN in LNCaP. More importantly, following the addition of transferrin, there was a significant increase in the amount of phosphorylated Akt in the nonmalignant control cell lines, but no change in the amount of phosphorylated Akt in either of the cancer cells (Fig. 6E).

**LAMP-1 and APPL1 mRNA expression in a prostate cancer microarray cohort and distribution of LAMP-1 and APPL1 in prostate tissue**

To support the hypothesis of altered endosome biogenesis in prostate cancer, the percentage change of mRNA...
expression for LAMP-1 and APPL1 was analyzed from the Taylor microarray cohort (Fig. 7A). LAMP-1 gene expression was significantly decreased ($P \leq 0.01$) in prostate cancer tissue compared with nonmalignant prostate tissue. APPL1 gene expression was significantly increased ($P \leq 0.05$) in prostate cancer tissue compared with nonmalignant tissue. Immunohistochemistry was used to investigate the distribution of LAMP-1 and Appl1 in prostate cancer patient tissue samples (Fig. 7B). The lysosomal marker LAMP-1 showed tumor-specific staining in some patient samples, but consistent with previous studies, there were variable results with some patient samples having little or no LAMP-1 staining (data not shown for the latter). In nonmalignant tissue, Appl1 clearly delineated basement membranes, whereas in the malignant tissue there was no evidence of basement membrane staining (Fig. 7B). In addition, Appl1 specifically delineated the cancer margins and showed increased staining within the tumor mass (Fig. 7B).

Discussion

Prostate cancer is one of the most frequently diagnosed cancers in men and a leading cause of cancer-related deaths worldwide, particularly, in the United States and Australian populations (24, 25). The prostate-specific antigen is still commonly used to detect prostate cancer, but has significant problems in terms of miss-diagnosis and prognostic prediction (see for example ref. 26). Some promising adjunct tests have recently been developed, including prostate cancer antigen 3 (PCA3; ref. 27), the analysis of cholesterol sulfate (28), and a novel sequence of the gene protein kinase C-zeta (PRKCZ), which is translated to the protein PRKC-ζ (29). However, these biomarkers do not provide early and accurate detection of prostate cancer, which is needed to enable the selection of the most appropriate therapeutic intervention and to avoid potential overtreatment (2). On the basis of our observations of altered LIMP-2 expression (14), we investigated altered endosomal biogenesis in prostate cancer to help provide more sensitive and specific markers for early detection and disease prediction.

There have been extensive protein and proteomic studies undertaken to identify potential new prostate cancer biomarkers (see for example ref. 26); however, the ideal marker with appropriate sensitivity and specificity is yet to be established. Interestingly, many of the early biomarkers investigated, and some of the recent proteins identified in proteomic studies, are either lysosomal hydrolases (e.g., lysosomal cathepsins, acid ceramidase, and acid phosphatase), lysosomal membrane proteins (e.g., LAMP-1-3 also called CD107a, b, and CD63) or proteins that are delivered from the cell surface into the endosome–lysosome system (e.g., sialomucin/CD164, CD1, CD47, and CD75). Additional evidence supports the concept that endosomal–lysosomal biogenesis is altered in prostate cancer, including the altered distribution of lysosomes that has been reported in prostate cancer cells (30). Despite these indications on lysosomal biogenesis, a set of optimal prostate cancer biomarkers have yet to be defined. In a recent study of endosome and lysosome markers in prostate cancer cell lines, we also found that lysosomal markers were unable to discriminate prostate cancer from nonmalignant cell lines, but there was evidence suggesting that endosome biogenesis may be altered in prostate cancer cells (14).

Here, we observed altered distribution of specific endosome subsets and lysosomes into the cellular periphery of prostate cancer cells, which could have important implications for cancer cell biomarker release and intracellular signaling. Acidic extracellular pH has been shown to enhance organelle redistribution, stimulating the traffic of endosome–lysosome–related organelles to the periphery of cancer cells (10, 31). This altered endosome–lysosome traffic has been linked with the release of cathepsin B and tumor invasiveness (32), presumably due to the hydrolysis of extracellular matrix after the exocytosis of this enzyme (33). However, cathepsin B has been reported to be more...
enriched in endosomes (34) rather than lysosomes, whereas the reverse is true for another proposed prostate cancer biomarker cathepsin D (35). The movement of lysosomal-related vesicles to the periphery of prostate cancer cells has been shown to be dependent on GTPases (e.g., RhoA), microtubules, the molecular motor protein KIF5b, and to involve PI3K, Akt/Erk1/2 phosphorylation, and MAPK signaling (32). Moreover, a component of the MAPK signaling pathway, the endosome-localized MAPK/Erk kinase (MEK1) p14–MP1 scaffolding complex, has been shown to specifically interact with and regulate the distribution of endosomes via ERK signaling (36). Increases in Na⁺/H⁺ exchange activity (acidification), RhoA GTPase activity, and PI3K activation have been shown to result in exocytosis from prostate cancer cells (31). The increased endosomal-associated gene and protein expression observed here, together with the previously observed cathepsin B release, suggested that endosome-related proteins may provide an important new focus for prostate cancer disease biomarker studies.

Increased expression of the endosomal protein LIMP-2 has been shown in oral squamous cell carcinoma and was

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**Figure 5.** Transferrin and endosome/lysosome marker coloimmunofluorescence. Confocal micrographs and enlargements showing transferrin (red; endocytosed for 20 minutes) and endosome/lysosome marker (green) in nonmalignant control cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP. Colocalization of markers is depicted by yellow fluorescence.
LIMP-2 has been reported to have a role in endosome biogenesis and its overexpression evoked the enlargement of both early and late endosomes. We observed increased gene and protein expression of the endosomal protein LIMP-2 in prostate cancer cell lines, prompting us to investigate other endosomal proteins in prostate cancer cells. The early endosome-associated proteins Appl1, Appl2, EEA1, and recycling endosome protein Rab4 were significantly upregulated (gene and protein) in prostate cancer cells, supporting the hypothesis of altered endosome biogenesis in prostate cancer. APPL1 expression was significantly increased in the Taylor prostate cancer tissue microarray, supporting the expression profiles observed in cell lines. Furthermore, the each EEA1 and Appl1 endosome subpopulations displayed altered intracellular distribution consistent with altered endosome traffic and potentially function. Interestingly, while Rab7 expression was unaltered, Rab7-positive compartments displayed differential distribution in prostate cancer compared with nonmalignant cells. Changes in subcellular localization may affect signaling in a similar manner to that which transpires through downregulated gene/protein expression that affects prostate cancer progression through enhanced signaling. Thus, the analysis of compartment distribution may distinguish cancer cell phenotypes independently of altered gene and protein expression.

Figure 6. Analysis of transferrin receptor expression and localization with transferrin.

A, quantification of transferrin receptor 1 (TFR1) and transferrin receptor 2 (TFR2) gene expression in nonmalignant and prostate cancer cell lines. B, Western blot analysis and protein quantification of transferrin receptor 1 (TIR1) and transferrin receptor 2 (TIR2). Quantification of gene and protein expression was relative to GAPDH gene and protein, respectively.

C, confocal micrographs and enlargements showing transferrin (red) and transferrin receptor (green) in nonmalignant cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP. Colocalization of transferrin receptor and transferrin is represented by yellow fluorescence. D, Western blot analysis and quantification (E) of AKT phosphorylation relative to total AKT, prior and subsequent to treatment of nonmalignant (PNT1a and PNT2) and prostate cancer cells (22RV1 and LNCaP) with transferrin for 20 minutes.

\(^*, P < 0.05\)
A box-and-whisker graphs were plotted with Tukey outliers (black points). Statistical significance is represented by an asterisk (*, P < 0.05; **, P < 0.01). A, LAMP-1 and APPL1 mRNA expression in normal (n = 29) and prostate cancer tissue (n = 150) from metanalysis of the cohort by Taylor and colleagues (22).

The significant changes that we observed in endosome-associated gene expression and protein expression, together with the altered distribution of endosome populations prompted us to investigate transferrin receptor expression together with transferrin endocytosis, sorting, and Akt signaling as measures of endosome function. Significant increases in the amount of transferrin receptor have previously been reported in prostate cancer cells (39), and this has been linked to c-Myc activation, which alters proliferation and tumorigenesis (40). Akt signaling is also essential for regulating cell growth and survival; and this controls the cell surface expression of transferrin and growth factor receptors (41). The transferrin receptor has previously been observed to colocalize with Rab5 and the motor protein myosin VI; the latter of which is involved in retrograde transport to the plasma membrane (42). This was consistent with our observations of endosome populations containing with labeled transferrin in the cellular periphery of prostate cancer cells. There also appeared to be a deregulation of Akt signaling in prostate cancer cells, with control cells being responsive to transfection endocytosis, but prostate cancer cells being unresponsive, despite having variable high or low amounts of Phospho-Akt/Akt. This altered signaling may be related to the intracellular location of the transferrin receptor that can be disturbed through changes in localization or depletion of PtdIns3P (43) or affected through variable internalization resulting from altered Appl1 or Rab5 expression (as is the case with epidermal growth factor receptor; ref. (44), affecting receptor trafficking and signal modulation.

Appl1 has been shown to be directly involved in insulin signaling and the translocation of the glucose transporter GLUT-4, which is mediated by direct binding of Appl1 to PI3K and Akt (45), inducing endosome relocalization. In prostate cancer cells, Appl1-potentiated Akt activity has also been shown to suppress androgen receptor transactivation (46). The increased gene and protein expression of Appl1 that we observed in prostate cancer cells might be expected to cause increased glucose uptake, due to its effect on GLUT-4 and this could have implications for energy metabolism in these cancer cells. Indeed, Appl1 also regulates other aspects of both lipid and glucose metabolism, activating AMP-activated kinase, p38 MAPK, and PPARα (see for example ref. 45). Appl2 has been shown to function as a negative regulator of adiponectin signaling, by competitive binding with Appl1 for interaction with the adiponectin receptor, again regulating energy metabolism. The increased expression of both Appl1 and Appl2 could therefore impact heavily on prostate cancer cell metabolism with direct significance for increased energy utilization and prostate cancer cell survival. The altered Appl1 expression and effect on Akt signaling in prostate cancer cells would be expected to also have significant consequence for other aspects of prostate cancer biology, due to the importance of the Appl1/PI3K/Akt signaling pathway in leading cell adhesion and cell migration (47). Notably, Appl1 also acts as a mediator of other signaling pathways, by interaction with the cytosolic face of integral or membrane-associated proteins either at the cell surface or in the endosome pathway; where it is directly involved in endosome traffic.

Rab GTPases are integrally involved in the control of endosome traffic, cycling between the cytoplasmic GDP-bound state and the active membrane–associated GTP-bound state. Rab5 and Rab7 respectively define early- and late-endosome compartments and during endosome maturation Rab5 recruits the HOPS complex as a mechanism to activate and be replaced by Rab7. Although mVps39 is known to be a guanine nucleotide exchange factor (GEF) that promotes the GTP-bound state on endosomal Rabs, TBC-2/TBC1D2 is a Rab GTPase-activating protein (GAP).
that promotes the GDP-bound state; and in combination is used to regulate the membrane localization of Rab proteins. TBC-2/TBC1D2 is therefore thought to act as a regulator of endosome to lysosome traffic and is required to maintain the correct size and distribution of endosomes (48). The altered distribution of endosome populations that we observed in prostate cancer cells suggests that TBC-2/TBC1D2 (GAP) and or mVps39 (GEF) might be functionally impaired; particularly, as the early endosomes were routed mainly toward the cell periphery, whereas late endosomes remained in a perinuclear location. Interestingly, microarray analysis has detected increased expression of TBC-2/TBC1D2 and reduced Vps39 mRNA in relation to altered endosomal–lysosomal traffic (49). This altered GEF and GAP function has been shown to be critical for endosomal traffic of integrins and there have been direct links established between altered Rab GTPase activity and cancer progression (50).

The expression of endosome markers has not previously been investigated thoroughly in prostate cancer, although some lysosomal-related cell surface CD (cell differentiation) markers and LAMP-1/LAMP-2 have been used in tissue biopsy analysis. The Gleason grading system is used to define histologic differentiation in conjunction with marker analysis to predict the course of disease in patients with prostate cancer. The lysosomal membrane proteins LAMP-1-3 and CD markers CD164, CD1, CD47, and CD75 are often evident in primary and metastatic cancer biopsies (51), but their consistency and predictive capacity for disease progression is limited. We observed increased amounts of Appl1 protein in malignant tissue from biopsies of patients with prostate cancer, confirming the increased gene and protein expression of Appl1 in prostate cancer cell lines. Appl1 appeared more concentrated in the basement membranes in nonmalignant tissue, whereas in the malignant tissue, there was no basement membrane staining, indicating diagnostic/prognostic potential for this biomarker. Further immunohistochemical and patient tissue analysis of Appl1 and other endosomal proteins is required to establish the validity and predictive value of these proteins as prognostic biomarkers in prostate cancer.

In summary, we have demonstrated increased expression of early endosome markers and altered localization of endosome and lysosome compartments in prostate cancer cells, which was associated with altered endocytosis and recycling of the transferrin receptor and aberrant Akt signaling. The alterations to the endocytic machinery that we have observed here, may increase the amount of endocytosis in prostate cancer cells, which could increase nutrient uptake/availability, provide additional membrane for cell division (9), and alter intracellular signaling (10); which are all hallmarks of cancer cell biology. There appeared to be a specific disconnect between the cellular location of early endosomes (and lysosomes) in the cell periphery and late endosomes in the perinuclear region, which could affect degradative and signaling processes in prostate cancer cells. We concluded that endosome biogenesis and function is altered in prostate cancer cells, opening a potentially new avenue to investigate biomarkers that aid in the diagnosis and prognosis of prostate cancer. Endosomes are directly involved in the processes of cellular secretion and exosome release, making these newly identified endosomal proteins potentially available for detection in patient samples, such as blood and urine.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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