Androgen Receptor and Nutrient Signaling Pathways Coordinate the Demand for Increased Amino Acid Transport during Prostate Cancer Progression

Qian Wang1,2, Charles G. Bailey2,4, Cynthia Ng2, Jessamy Tiffen1,2, Annora Thoeng2, Vineet Minhas1,2, Melanie L. Lehman1,2, Stephen C. Hendy1,2, Grant Buchanan5, Colleen C. Nelson6,7, John E.J. Rasko2,3,4, and Jeff Holst1,2,4

Abstract
L-type amino acid transporters such as LAT1 and LAT3 mediate the uptake of essential amino acids. Here, we report that prostate cancer cells coordinate the expression of LAT1 and LAT3 to maintain sufficient levels of leucine needed for mTORC1 signaling and cell growth. Inhibiting LAT function was sufficient to decrease cell growth and mTORC1 signaling in prostate cancer cells. These cells maintained levels of amino acid flux through androgen receptor–mediated regulation of LAT3 expression and ATF4 regulation of LAT1 expression after amino acid deprivation. These responses remained intact in primary prostate cancer, as indicated by high levels of LAT3 in primary disease, and by increased levels of LAT1 after hormone ablation and in metastatic lesions. Taken together, our results show how prostate cancer cells respond to demands for increased essential amino acids by coordinately activating amino acid transporter pathways vital for tumor outgrowth. Cancer Res; 71(24); 1–12.
©2011 AACR.

Introduction
Prostate cancer is initially regulated by androgens through androgen receptor signaling (1). Androgen receptor is a ligand-dependent transcription factor that translocates into the nucleus and binds to androgen response elements (ARE; ref. 2). Androgen receptor mediates expression of many critical factors required for proliferation and viability of prostate cancer growth, as well as kallikreins (KLK) such as prostate-specific antigen (PSA; KLK3).

In addition to androgen receptor signaling, the PI3K/PTEN/Akt pathway is critical in prostate cancer. Mutation of PTEN, which commonly occurs in prostate cancer (3), leads to phosphorylation and activation of Akt (4, 5) leading to activation of mTORC1 signaling (6). The mTORC1 comprising mTOR, raptor, mLST8, and PRAS40 (7) requires intracellular amino acids such as leucine for its activity (8, 9). Recently, inositol polyphosphate multikinase (IPMK) has been shown to function as a physiologic mTOR cofactor to stabilize the interaction of mTORC1 and raptor, facilitating activation of mTORC1 by Rag GTPases in the presence of amino acids (10). Rag GTPases, together with the class III phosphoinositide 3-kinase (PI3K) Vps34, both mediate this amino acid-mTORC1 signaling (11–13). Alternatively, amino acid deprivation may activate GCN2-eIF2α-ATF4 which triggers gene expression via amino acid response elements (AARE; ref. 14).

Amino acid transporters regulate intracellular amino acid levels; thus, changes in transporter expression or activity may alter mTORC1 signaling. Prostate cancer–overexpressed gene 1 (POV1/LAT3/SLC43A1), first described as a gene overexpressed in human prostate cancer (15, 16), is a member of the L-type amino acid transporter (LAT) family (17). Members of the LAT family mediate the Na+/independent transport of large neutral amino acids. LAT1 (SLC7A5) has been detected in tumors of the lung (18) and its expression correlates with high-grade prostate cancer (19).

In this study, we show that signaling via the androgen receptor and ATF4 pathways regulates expression of the amino acid transporters LAT1 and LAT3, thereby coordinating their increased expression in prostate cancer cells. This new connection linking these pathways provides the necessary essential amino acids to promote mTORC1 signaling and cell growth in prostate cancer.
Materials and Methods

Cell culture

Human prostate cancer cell lines LNCaP-FGC, PC-3, and DU145 were purchased from American Type Culture Collection. Human C4-2B prostate cancer cells were obtained from Virotech Laboratories Inc. Human MDA-PCA-2b prostate cancer cells were a kind gift from A/Prof. Qihan Dong at the University of Sydney, Sydney, NSW, Australia. LNCaP cells have been passaged directly from original low-passage stocks (2009), and we confirmed PC-3 and DU145 cell identity by short tandem repeat profiling in 2010 (CellBank). Cells were cultured in RPMI-1640 medium (Invitrogen) containing 10% (v/v) FBS or charcoal-stripped serum, which has been described previously (20). Chemicals were diluted as follows, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (Sigma), and dextran-coated charcoal-stripped serum, which has been described previously (20). Chemicals were diluted as follows, with control wells treated with the appropriate vehicle controls: 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, Sigma; in PBS), bicalutamide (dimethyl sulfoxide, AstraZeneca), dihydrotestosterone (DHT, Sigma; in ethanol), and 1 mM sodium pyruvate (Invitrogen). Cells were maintained at 37°C in an atmosphere containing 5% CO2. MDAPCa-2b cells were cultured in Ham's F-12K medium with dextran-coated charcoal-stripped serum, which has been described previously (20). Chemicals were diluted as follows, with control wells treated with the appropriate vehicle controls: 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, Sigma; in PBS), bicalutamide (dimethyl sulfoxide, AstraZeneca), dihydrotestosterone (DHT, Sigma; in ethanol), and R1881 (ethanol; DuPont).

Leucine uptake assay

The [3H]-L-leucine uptake was conducted as detailed previously (21). Further details are available in Supplementary Materials and Methods.

Cell viability assay

MTT (Millipore) assays were conducted as per the manufacturer's instructions and as described previously (22). Further details are available in Supplementary Materials and Methods.

Antibodies for Western blotting, immunofluorescence, and chromatin immunoprecipitation

Full details are available in Supplementary Materials and Methods. Antibodies used were LAT3 [a kind gift from Dr. Kunimasu Yan, Kyorin University, Japan (ref. 23)]; phospho-mTOR (Ser2448), mTOR, phospho-p70S6K (Thr389), p70S6K, p15, p21, p27, cyclin D1, and cyclin D3 (all from Cell Signaling Technology); LAT1 (Cosmo Bio); ATF4, androgen receptor, α-tubulin, control rabbit IgG (all from Santa Cruz Biotechnology); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam); and horseradish peroxidase–conjugated donkey anti-mouse IgG, donkey anti-rabbit IgG, and goat anti-mouse IgM secondary antibodies (Millipore).

Knockdown and overexpression constructs for LAT1 and LAT3

Gene knockdown was achieved by stable transduction with lentivirus, using the plKO.1-puro vector system (Sigma) containing a LAT1- or LAT3-specific short hairpin RNA (shLAT1 and shLAT3; Supplementary Table S1). Control plasmid consisted of a plant microRNA sequence (miR159a; shControl; Supplementary Table S1), which has no specific targets in mammalian cells.

LAT1 and LAT3 cDNAs were inserted into an IRES-eGFP cassette from pIRE2-eGFP (Clontech) and subcloned into a tat-dependent lentiviral vector pHIV-1SDmCMVpre (22, 24). Further details are available in Supplementary Materials and Methods.

Statistical analysis

The data are expressed as mean ± SEM. The Student t test was used to compare differences among groups.

Results

Prostate cancer cell growth and mTORC1 signaling are inhibited following blockade of LAT transport

To determine whether LATs are responsible for leucine uptake in LNCaP and PC-3 cells, we treated cells with the leucine analogue BCH, which is a specific inhibitor of LATs (25). Incubation of LNCaP and PC-3 cells in the presence of BCH significantly decreased the radiolabeled leucine uptake (Fig. 1A). Leucine uptake was inhibited by 79% and 61% of control in LNCaP and PC-3 cells, respectively, upon BCH treatment, suggesting that the majority of leucine transport in these cells is dependent on the LAT family.

As leucine is an essential amino acid known to be important for protein synthesis, we next examined cell growth using the MTT assay. BCH-treated LNCaP and PC-3 cells showed a significant decrease in cell growth and viability over 3 days, compared with controls (Fig. 1B and C). The inhibition was consistent with the reduced level of leucine uptake by BCH, whereas removal of leucine from the culture media resulted in complete growth arrest (Fig. 1B and C). To directly test the regulation of cell proliferation by the LAT family, we used a clonogenicity assay to examine the effect of BCH on cell growth. In the presence of BCH, there was a significant decrease in colony formation in LNCaP cells (74% inhibition) and in PC-3 cells (21% inhibition; Fig. 1D; Supplementary Fig. S1A). BCH treatment also inhibited leucine uptake (Supplementary Fig. S1B) and decreased cell growth (Supplementary Fig. S1C) in DU145 cells, which contain wild-type PTEN.

In the control LNCaP cells, 75% of cells accumulated in the G0-G1 phase, 13% in S-phase, and 11% in G2-M phase (Fig. 1E; Supplementary Fig. S1D). However, in the presence of BCH, significantly more cells accumulated in the G0-G1 phase (88%), with only 7% in S-phase and 4% in G2-M phase (Fig. 1E; Supplementary Fig. S1D). However, in PC-3 cells, there was no significant change in the percentage of cells in each cell-cycle stage (Fig. 1F; Supplementary Fig. S1E).

To further examine cell proliferation, we used a bromodeoxyuridine (BrdUrd) incorporation assay. When LNCaP cells were treated with BCH, BrdUrd incorporation decreased significantly from 37% to 16% compared with untreated control cells (Fig. 1G; Supplementary Fig. S1F). In PC-3 cells, BrdUrd incorporation decreased from 45% to 28% (Fig. 1G; Supplementary Fig. S1F). These results suggest that the rate of prostate cancer cells entering the cell cycle is decreased when LATs are blocked.
Figure 1. Blocking LATs represses leucine uptake and cell growth. A, LNCaP and PC-3 cells were cultured in the presence or absence of BCH (10 mmol/L) for 1 hour prior to incubation with [3H]-L-leucine to assess uptake. LNCaP (B) and PC-3 (C) cells were cultured in the presence or absence of BCH or leucine (Leu) for 3 days. MTT assays were conducted every 24 hours to assess cell growth. D, LNCaP and PC-3 colonies were cultured in the presence or absence of BCH and scored after 10 (PC-3) or 12 (LNCaP) days. Cell cycle for LNCaP (E) and PC-3 (F) cells were analyzed by flow cytometry. G, LNCaP and PC-3 cells were cultured in the presence or absence of BCH for 18 hours, followed by BrdUrd and BCH for 6 hours. BrdUrd incorporation was analyzed by fluorescence microscopy. Cell-cycle regulators p27, p21 and p15 (H), or cyclin D3 and cyclin D1 (I) were examined by Western blotting in LNCaP cells. Time course of LNCaP (J) or PC-3 (K) cells incubated in the presence or absence of BCH. Total and phosphorylated (p-) mTOR and p70S6K signaling proteins were detected by Western blotting. Data in A–G represent mean ± SEM, n = 3 experiments. Data in H–K are representative blots of 3 independent experiments, with α-tubulin used as a loading control.
To determine whether known cell-cycle regulators were responsible for the changes observed in LNCaP cell cycle following LAT blockade, we examined the expression of the p21 and p27 family and the p15 family by Western blotting. BCH treatment led to increased expression of the cell-cycle inhibitor p27 (1.8-fold) after 24 hours, with no change in p21 or p15 levels (Fig. 1H). We also observed a decrease in the expression of cyclin D3 (0.6-fold), with no change in cyclin D1 expression (Fig. 1I). These results suggest that blocking LAT-mediated leucine transport affected cell-cycle progression through G1 phase checkpoints.

As mTORC1 signaling is regulated by leucine, we examined the activation of mTOR and its downstream target p70S6K by phosphorylation using Western blotting. In the presence of BCH, phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K) levels were decreased in LNCaP cells and to a lesser extent in PC-3 cells (Fig. 1J and K).

To examine whether BCH inhibition of LATs induced apoptosis, we used Annexin V/propidium iodide (PI) staining and measured caspase 3/7 activation. Blocking leucine transport did not substantially increase the amount of apoptosis in LNCaP or PC-3 cells (Supplementary Fig. S2A–S2C).

LAT3 regulates LNCaP and PC-3 cell growth
Because BCH inhibits all members of the LAT family, we next set out to determine which specific transporters were mediating cell growth in prostate cancer. Both LAT3 (16) and LAT1 (19) are expressed in prostate cancer tissues. Analysis of microarray data (GDS1699; ref. 26) showed that LAT1 and LAT3 exhibit higher expression than other LAT family members in a variety of prostate cancer cell lines (Supplementary Fig. S3A–S3D). To confirm these data, we used Western blotting of LNCaP and PC-3 cell lines to show LAT3 protein expression (Fig. 2A). Furthermore, we confirmed the correct distribution of LAT3 protein on the plasma membrane in both cell lines by immunofluorescence (Fig. 2B).

To determine whether LAT3 mediates leucine uptake in LNCaP and PC-3 cells, we used lentiviral vectors to stably express shRNAs for a nontargeting control (plant miR159a; shControl) or against LAT3 (shLAT3). Western blot analyses showed that shLAT3 could effectively reduce LAT3 expression by more than 50% in both LNCaP and PC-3 cells (Fig. 2C). Knockdown of LAT3 expression significantly reduced the uptake of leucine in LNCaP (61%) and PC-3 cells (40%) compared with shControl cells (Fig. 2D) and decreased p-p70S6K activation (Supplementary Fig. S4A). The expression of shLAT3 led to a significant decrease in cell growth and clonogenicity in LNCaP, and to a less extent PC-3 cells, similar to that observed in BCH-treated control cells (Fig. 2E–G; Supplementary Fig. S1A). These results suggest that LAT3 is the major LAT family member regulating cell growth in the androgen-sensitive LNCaP cell line.

To further examine the function of LAT3 in prostate cancer cell lines, we ectopically expressed LAT3 in LNCaP and PC-3 cells. While Western blot analyses showed that LAT3 was marginally overexpressed (Supplementary Fig. S4B), there was a significant increase in leucine transport in LNCaP cells (4-fold; Fig. 2H). This did not affect cell growth (Supplementary Fig. S4C) but resulted in a significant increase in clonogenicity (4-fold of control; Fig. 2I). Cell-cycle analysis also showed that LAT3 overexpression significantly increased cell-cycle progression (Supplementary Fig. S4E). However, no change was observed on PC-3 cell growth after LAT3 overexpression (Fig. 2I; Supplementary Fig. S4D and S4F).

LAT1 regulates PC-3 cell growth
We next examined LAT1 expression in prostate cancer cells. Western blotting showed low levels of LAT1 in LNCaP cells, with high expression in PC-3 cells (Fig. 3A). We confirmed that LAT1 is correctly localized to the plasma membrane (Fig. 3B), with strong expression in the lamellipodia of PC-3 cells (Fig. 3B arrows), suggesting that LAT1 may also have a function in regulating cell migration. To examine the function of LAT1, we used lentiviral shRNA knockdown of LAT1 (shLAT1) in LNCaP and PC-3 cells, which decreased LAT1 expression by more than 50% in both LNCaP and PC-3 cells (Fig. 3C). shLAT1 expression significantly reduced leucine uptake in LNCaP (22%) and PC-3 (63%) cells (Fig. 3D) and decreased p-p70S6K activation (Supplementary Fig. S5A). While inhibition of LAT1 expression had no effect on cell growth or clonogenicity in LNCaP cells (Fig. 3E and G; Supplementary Fig. S1A), there was a significant reduction in PC-3 cell growth and clonogenicity (Fig. 3F and G; Supplementary Fig. S1A). These results suggested that LAT1 is the major leucine transporter regulating cell growth in the androgen-insensitive PC-3 cell line.

To further examine the function of LAT1 in prostate cancer cell lines, we ectopically expressed LAT1 in LNCaP and PC-3 cells and confirmed this by Western blotting (Supplementary Fig. S5B). LAT1 overexpression led to significantly increased leucine transport in both LNCaP and PC-3 cells (Fig. 3H); however, there was no effect on cell growth or cell-cycle progression in either cell line (Supplementary Fig. S5C–S5F). A significant increase in the clonogenicity of LNCaP cells was observed after LAT1 overexpression (2-fold of control; Fig. 3I).

LAT3 expression is regulated by androgen receptor binding
Elevated LAT3 expression in prostate cancer compared with normal prostatic tissue has been previously reported (15, 16, 27); however, the pathway leading to altered LAT3 expression is unknown. The presence of high levels of LAT3 in primary prostate cancer, prostate development (27), and androgen-sensitive LNCaP cells (Supplementary Fig. S3C) suggested to us that the androgen receptor may regulate its expression. Indeed, the synthetic androgen agonist R1881 strongly elevated LAT3 mRNA expression in LNCaP cells by quantitative reverse transcriptase PCR (qRT-PCR; Supplementary Fig. S6A), confirming previous microarray data (28). Stimulation of LNCaP cells with various concentrations of DHT led to a dose-dependent increase in LAT3 expression shown by Western blot analysis (Fig. 4A). LAT3 expression was also regulated by DHT in androgen-sensitive cell lines MDA-PCa-2b and C4-2B (Supplementary Fig. S6B). This effect was inhibited by the androgen receptor inhibitor bicalutamide in LNCaP cells (Fig. 4A), suggesting that LAT3 expression is regulated by androgen receptor signaling.
Figure 2. LAT3 is required for LNCaP and PC-3 cell growth. A, LAT3 protein expression was detected in LNCaP and PC-3 cells by Western blotting, with α-tubulin used as a loading control. B, localization of LAT3 protein was determined by immunofluorescent staining and confocal microscopy, with nuclei visualized using 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 30 μm. C, expression of LAT3 was inhibited using a specific shRNA (shLAT3), with a nontargeting miRNA (shControl) used as a control. LAT3 expression was detected by Western blotting to confirm knockdown in LNCaP and PC-3 cells. D, LNCaP and PC-3 cells expressing shControl or shLAT3 were cultured in leucine-free media with [3H]-L-leucine for 15 minutes to assess uptake. Data represent mean ± SEM, n = 4. LNCaP (E) and PC-3 (F) cells expressing shControl or shLAT3 were cultured in the presence or absence of BCH for 3 days. MTT assay was conducted every 24 hours to determine changes in cell growth. Data represent mean ± SEM, LNCaP, n = 3; PC-3, n = 8. G, LNCaP and PC-3 colonies expressing shControl or shLAT3 were scored after 10 (PC-3) or 12 (LNCaP) days in culture. Data represent mean ± SEM, n = 3. H, LNCaP and PC-3 cells containing empty vector or LAT3 expression vector were cultured in leucine-free media with [3H]-L-leucine for 15 minutes to assess uptake. Data represent mean ± SEM, n = 3. I, LNCaP and PC-3 colonies containing empty vector or LAT3 expression vector were scored after 10 (PC-3) or 12 (LNCaP) days. Data represent mean ± SEM, n = 3.

To determine whether LAT3 expression is directly regulated by androgen receptor, we analyzed androgen receptor chromatin immunoprecipitation (ChIP)-Seq data from LNCaP cells grown in CSS medium in the presence or absence of R1881 (our unpublished data; refs. 29–31). This global survey revealed two putative androgen receptor binding sites 12.5 kb downstream from the transcription start site (TSS) of LAT3 within the second intron [LAT3(+12.5)]; Supplementary Fig. S6C]. Analysis of transcription factor–binding sites in this region revealed two predicted AREs 42 bp apart (Fig. 4B) on the basis of the canonical class 1 nuclear receptor consensus AGAA-CANmTGTTC. To verify this result, ChIP was conducted using an androgen receptor antibody on LNCaP cells grown in CSS medium in the presence or absence of 10 nmol/L DHT for 4 hours. Using qPCR, we observed a 5-fold enrichment of androgen receptor binding to ARE-III upstream of the KLK3 promoter after the addition of DHT, similar to previous reports (ref. 32; Fig. 4C). No androgen receptor binding was observed proximal to the LAT3 TSS (−0.4 kb and +0.6 kb) or to an intronic region in LAT1 containing an unrelated enhancer element (control; Fig. 4C). A 6-fold enrichment of androgen receptor binding to the LAT3(+12.5) enhancer was observed in the presence of DHT (Fig. 4C). For further confirmation of LAT3 regulation by androgen receptor, we constructed luciferase reporter plasmids containing wild-type and mutant versions of this putative enhancer region. LNCaP cells were transfected with the constructs and cultured in CSS medium in the presence or absence of DHT for 18 hours. Minimal luciferase expression was observed in the KLK3 ARE-III control or LAT3 ARE enhancer region transfections in the absence of DHT; however, a 36- and 63-fold induction in luciferase expression was observed upon the addition of DHT, respectively, confirming that both regions were androgen receptor responsive. Mutagenesis of LAT3 ARE-I or ARE-II separately, or in concert, resulted in the abrogation of androgen receptor regulation (Fig. 4D).
LAT1 is required for PC-3 cell growth. A, LAT1 protein expression was detected in LNCaP and PC-3 cells by Western blotting, with α-tubulin used as a loading control. B, localization of LAT1 protein was determined by immunofluorescent staining and confocal microscopy, with nuclei visualized using 4′,6-diamidino-2-phenylindole (DAPI). Arrows show that LAT1 is expressed in the lamellipodia of PC-3 cells. Scale bar, 30 μm. C, expression of LAT1 was inhibited using a specific shRNA (shLAT1), compared with a nontargeting miRNA (shControl). LAT1 expression was detected by Western blotting. D, LNCaP and PC-3 cells expressing shControl or shLAT1 were cultured in leucine-free media with [3H]-L-leucine for 15 minutes to assess uptake. Data represent mean ± SEM, n = 4. LNCaP (E) and PC-3 (F) cells expressing shControl or shLAT1 were cultured in the presence or absence of BCH for 3 days. MTT assay was conducted every 24 hours to determine changes in cell growth. Data represent mean ± SEM, LNCaP, n = 5; PC-3, n = 5. G, LNCaP and PC-3 colonies expressing shControl or shLAT1 were scored after 10 (PC-3) or 12 (LNCaP) days in culture. Data represent mean ± SEM, n = 3. H, LNCaP and PC-3 cells containing empty vector or LAT1 expression vector were cultured in leucine-free media with [3H]-L-leucine for 15 minutes to assess uptake. Data represent mean ± SEM, n = 3. I, LNCaP and PC-3 colonies containing empty vector or LAT1 expression vector were scored after 10 (PC-3) or 12 (LNCaP) days in culture. Data represent mean ± SEM, n = 3.

LAT1 expression is regulated by AARE signaling

To date, no single regulatory element has been identified in the LAT1 gene that accounts for its regulation in normal tissues and cancer. We observed that LAT1 expression was increased 2.4-fold in LNCaP cells and 1.5-fold in PC-3 cells after 3-day culture in leucine-free media, suggesting that it may be regulated by amino acid starvation (Fig. 5A). Blocking leucine transport by BCH also upregulated ATF4 expression in both LNCaP and PC-3 cells (Supplementary Fig. S6D). Because LAT1 was shown to have reduced expression in Atf4−/− embryonic fibroblasts (33), we analyzed ATF4 protein expression after 6 hours of culture in leucine-free media (Fig. 5A). ATF4 controls an important transcriptional program initiated by cellular stress due to amino acid limitation (14), and the substantial increase in ATF4 expression suggested that LAT1 expression may indeed be regulated by ATF4. We also observed that both LAT1 (1.9-fold) and ATF4 (3.4-fold) expression were increased in LNCaP cells grown in CSS (Fig. 5A), suggesting that androgen deprivation may stimulate ATF4 translation, leading to increased LAT1 expression.

Our analysis of the LAT1 promoter region shows that it is poorly conserved amongst higher eukaryotes. Hence, we analyzed conserved regions within the LAT1 locus based on conservation scores and alignments of 28 vertebrate genomes. We identified a conserved AARE within the first intron of LAT1, 15.7 kb downstream from the TSS [LAT1(15.7)], consisting of the sequence TGATGCAAT (Fig. 5B). The consensus sequence TGATGnAAn is a composite C/EBP-ATF site bound by the transcription factor ATF4 (14). We conducted ATF4 ChIP on
PC-3 cells, grown in leucine-containing or leucine-free medium for 6 hours. We observed a 4.5-fold enrichment of ATF4 binding to LAT1 (+15.7) when the cells were grown in leucine-free medium (Fig. 5C). We observed similar enrichment for binding of ATF4 to known AARE sequences within CHOP, 4EBP1, and xCT gene loci (ref. 14; Fig. 5C). ATF4 binding was not observed to the LAT3 (+12.5) enhancer as expected (Fig. 5C), and leucine deprivation did not increase LAT3 levels (Supplementary Fig. S6E). To further validate these results, we constructed luciferase reporter constructs containing a single copy of wild-type (wt) or mutant (mut) LAT3 (+12.5) enhancer and transduced into LNCaP cells grown in the presence or absence of DHT to assay enhancer expression of luciferase. KLK3 ARE-III was used as a positive control. Normalized luminescence values are presented as a ratio of the enhancer containing construct to empty vector. Schematic of luciferase reporter constructs are shown. Data represent mean ± SEM, n = 3.

**LAT expression is coordinately regulated during prostate cancer progression**

We hypothesized that transcriptional regulation of LAT3 and LAT1 expression might explain why LAT1 is strongly expressed in androgen-insensitive PC-3 cells, whereas LAT3 is strongly expressed in androgen-sensitive LNCaP cells. Also, these data suggested that microenvironment changes, such as nutrition deprivation or hormone ablation, may stimulate cancer development and affect LAT3 and LAT1 expression. We analyzed LAT1 and LAT3 expression in silico using publically available microarray data from GDS1439 (34), GDS2545 (35, 36), and Holzbeierlein and colleagues (37). Gene expression of both LAT1 and LAT3 was low in benign or normal prostate tissue (Fig. 6). In primary prostate cancer tissue, however, LAT3 expression decreased (Fig. 6A and D), whereas LAT1 expression remained at low levels (Fig. 6B and E). In castrate-resistant metastatic cancer tissue, LAT3 expression decreased (Fig. 6A and D), whereas LAT1 is significantly increased (Fig. 6B and E). After hormone ablation therapy,
LAT3 expression decreases (Fig. 6G), whereas LAT1 expression was increased (Fig. 6H). These results suggest that decreased androgen receptor signaling may lower LAT3 expression and consequently increase LAT1 expression. Analysis of these two genes together separated primary and metastatic tumors into distinct groups in all three data sets (Fig. 6C, F, and I).

To confirm this inverse relationship between LAT1 and LAT3 during prostate cancer development, we conducted LNCaP xenografts in nude mice, followed by castration. Tumors were harvested from noncastrated mice (intact), 15 days after castration (Nadir), and about 4 weeks after castration once castrate resistance had developed (castration-resistant prostate cancer). Analysis of LAT3 and LAT1 expression by qRT-PCR in these samples showed that LAT3 is high in intact tumors and decreases after castration (Fig. 6J), whereas LAT1 is low in intact tumors and increases after castration (Fig. 6K). This is similar to that seen in human samples after hormone ablation therapy (Fig. 6G and H). Unlike the human microarray data, however, upon castration resistance, LAT3 is reactivated and LAT1 decreases (Fig. 6J and K).

**Discussion**

In this study, we examined the molecular mechanisms that lead to increased amino acid import in prostate cancer. We have identified a unique regulatory system that comprises a combination of androgen response and amino acid stress pathways working in concert to maintain adequate intracellular amino acid levels throughout prostate cancer progression. When LAT transport was inhibited in LNCaP and PC-3 cells, we observed a decrease in cell growth, coincident with a decrease in leucine uptake, and decreased mTORC1 pathway activation. Our results raised the possibility that members of the LAT family may be increased in prostate cancer to ensure that adequate leucine is available for activation of the mTORC1 pathway. Direct inhibition of LAT1 or LAT3 in both LNCaP and PC-3 cells further showed that the decrease we observed in cell growth was predominantly due to LAT transport activity. The higher levels of LAT3 in LNCaP cells coincided with a more dramatic inhibition of leucine uptake and decrease in cell growth.
growth in LNCaP cells subjected to LAT3 knockdown. Furthermore, knockdown of LAT1 led to a smaller decrease of leucine uptake in LNCaP cells consistent with the predominance of LAT3 in androgen-sensitive LNCaP cells. In contrast, leucine uptake and cell growth in androgen-insensitive PC-3 cells subjected to LAT3 knockdown was less pronounced. This is likely due to the predominance of LAT1 in PC-3 cells, as LAT1 knockdown led to a significant decrease in leucine uptake, cell growth, and clonogenicity.

The regulation of LAT3 by androgen receptor adds to an increasing number of links between the androgen receptor and PI3K/Akt/mTOR pathways. Indeed, the combination of a PI3K and androgen receptor inhibitor has recently been shown to be effective in PTEN-deficient mouse models (38). Androgen receptor signaling and the PI3K pathway have also been shown to regulate each other by reciprocal negative feedback (39, 40). Inhibition of PI3K pathway in PTEN-deficient prostate cancer results in activation of androgen receptor signaling, which may induce LAT3 expression and further activate mTOR signaling.

We have also shown that LAT1 is regulated by the amino acid stress pathway and ATF4-mediated transcription. Therefore, when androgen receptor signaling is compromised, for example, after hormone ablation therapy, the levels of LAT3 would decrease, leading to decreased amino acid levels and rapid ATF4 transcription. Atf4 knockout mice have decreased levels of LAT1, 4F2hc, 4EBP1, and ASCT2 (slc1A5; ref. 33). We showed that LAT1 and 4EBP1 are regulated by ATF4 binding to AARE sites present in their introns, with 4EBP1 playing a role in suppressing global protein translation in the absence of amino acids. LAT1/4F2hc and ASCT2 have been shown to cooperatively regulate the influx of leucine into cells and play a role in a number of cancers (41, 42). Their increased expression because of ATF4-mediated transcription and translation would restore the balance of amino acids, allowing cell growth to continue through the mTORC1 pathway, in the absence of androgen receptor–mediated LAT3 expression.

Our studies suggest that prostate cancer cells have strict regulation of amino acid transporters to maintain intracellular levels of leucine. In this model, androgen receptor signaling activates LAT3 transcription in primary prostate cancer (Fig. 7A), increasing LAT3 membrane expression, intracellular leucine, and ultimately stimulating mTORC1 signaling to drive
cell growth. After hormone ablation therapy in patients, LAT3 expression is decreased. Low intracellular leucine levels will activate ATF4 translation, which initiates the transcription of LAT1 and 4EBP1. Once LAT1 protein levels are increased, intracellular leucine levels are restored and mTORC1 signaling can again drive cell growth (Fig. 7B). The differences seen in castrate-resistant metastatic cancer in humans, compared with castrate-resistant tumors in mice may reflect either the androgen-sensitive nature of LNCaP cells or the differences between cells at the primary cancer site compared with metastatic sites.

At this stage, it remains unclear whether LAT1 can regulate progression to metastasis; however, increased localization of LAT1 on the lamellipodia of PC-3 cells suggests a possible role. LAT1 forms a disulfide-linked heterodimeric complex with 4F2hc, which is required for plasma membrane localization (43). 4F2hc has been shown to interact with β1 integrin (44), which may play an important role in cell adhesion in metastasis (45). Furthermore, the inverse expression of LAT1 and LAT3 in prostate cancer may not apply for androgen-independent cancers that remain androgen receptor sensitive. For example, the C4-2B cell line (derived from LNCaPs) expresses androgen receptor but is androgen independent (46) and expresses high levels of both LAT3 and LAT1 (Supplementary Fig. S6F). As such, there may be mechanisms other than ATF4 that can increase the expression of LAT1 in prostate cancer.

We have shown that inhibition of either LAT1 or LAT3 can lead to decreased growth of prostate cancer cells. As such, LATs may be a viable target for therapeutic intervention in primary prostate cancer (LAT3) and late-stage prostate cancer (LAT1). Both LAT1 and LAT3 have overlapping substrate specificities but distinct protein-coding sequences, making them attractive candidates for drug design. While the addition of simple competitive inhibitors such as BCH alone does not kill prostate cancer cells, anti-nutrient transporter therapy may slow cell growth. For slow growing cancers like prostate cancer, this may be used in combination with "watch-and-wait" type approaches or current therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Kunimasa Yan (Kyorin University) for providing the LAT3 antibody; A/Prof. Qihan Dong (University of Sydney) for providing human

Figure 7. Model of LAT3 and LAT1 regulation in prostate cancer. A, androgen receptor (AR) signaling activates LAT3 transcription in primary prostate cancer, leading to leucine uptake, mTORC1 signaling, and cell growth. B, decreased androgen signaling and LAT3 expression results from hormone ablation therapy leading to ATF4 translation, which initiates the transcription of LAT1. LAT1 expression can subsequently mediate leucine uptake, mTORC1 signaling, and cell growth.
M. Amino Acid Transport in Prostate Cancer

MDA-PCa-2b prostate cancer cells (Dr. Renae Ryan (University of Sydney) for assistance with amino acid uptake assays; and Dr. William Ritchie for assistance with analysis of 4,6-diamidino-2-phenylindole (DAPI) cell images).

Grant Support

The study was supported by Prostate Cancer Foundation of Australia, Cancer Institute NSW, Ramaciotti Foundation, Rebecca L Cooper Medical Research Foundation (all to J.J. Holst); Cure the Future (J.E.J. Rasko); anonymous foundation (J.E.J. Rasko); and the Australian-Canadian Prostate Cancer Research Alliance (C. C. Nelson).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 27, 2011; revised August 30, 2011; accepted October 4, 2011; published OnlineFirst October 17, 2011.

References


Androgen Receptor and Nutrient Signaling Pathways Coordinate the Demand for Increased Amino Acid Transport during Prostate Cancer Progression

Qian Wang, Charles G. Bailey, Cynthia Ng, et al.

Cancer Res  Published OnlineFirst October 17, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1821

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/10/17/0008-5472.CAN-11-1821.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.