Targeting ASCT2-mediated glutamine uptake blocks prostate cancer growth and tumour development

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Abstract

Glutamine is conditionally essential in cancer cells, being utilized as a carbon and nitrogen source for macromolecule production, as well as for anaplerotic reactions fuelling the tricarboxylic acid (TCA) cycle. In this study, we demonstrated that the glutamine transporter ASCT2 (SLC1A5) is highly expressed in prostate cancer patient samples. Using LNCaP and PC-3 prostate cancer cell lines, we showed that chemical or shRNA-mediated inhibition of ASCT2 function in vitro decreases glutamine uptake, cell cycle progression through E2F transcription factors, mTORC1 pathway activation and cell growth. Chemical inhibition also reduces basal oxygen consumption and fatty acid synthesis, showing that downstream metabolic function is reliant on ASCT2-mediated glutamine uptake. Furthermore, shRNA knockdown of ASCT2 in PC-3 cell xenografts significantly inhibits tumour growth and metastasis in vivo, associated with the down-regulation of E2F cell cycle pathway proteins. In conclusion, ASCT2-mediated glutamine uptake is essential for multiple pathways regulating the cell cycle and cell growth, and is therefore a putative therapeutic target in prostate cancer.

Keywords: ASCT2; SLC1A5; glutamine; cell cycle; metabolism; prostate cancer

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Introduction

Glutamine is the most abundant amino acid in plasma, functioning as a critical source of nitrogen and carbon in cells. The metabolic shift in cancer, however, imparts a new ability to the cells to utilize glutamine as an alternative fuel source to glucose for the tricarboxylic acid (TCA) cycle, and as a source of fatty acid production through reductive carboxylation [1–3]. Many of these changes in glutamine metabolism result directly from oncogenic transformation, such as Myc amplification [4–6], commonly seen in prostate cancer [7].

Another important role for amino acids is to control signalling through the nutrient sensor mTORC1. The tumour suppressor gene, PTEN, is commonly mutated or deleted in prostate cancer [8], leading to up-regulation of the PI3K–Akt–mTORC1 signalling pathway. The amount of intracellular amino acids, such as leucine, determines the activity of mTORC1 through Rag complexes on the lysosome surface [9–13]. In combination with leucine, glutamine has also been shown to be critical for mTORC1 signalling by enhancing glutaminolysis and α-ketoglutarate production [14]. This link is further supported by the role of glutamine in facilitating leucine transport [15] and the feedback generated by activation of mTORC1, which promotes glutaminolysis in cancer [3].

The major glutamine transporter in cancer cells is alanine–serine–cysteine transporter-2 (ASCT2; SLC1A5) [26–28]. ASCT2 is a Na+-dependent, broad-scope neutral amino acid exchanger that belongs to solute carrier (SLC) family-1, which mediates the
obligatory exchange of substrate amino acids, including alanine, serine, cysteine, threonine, glutamine and asparagine [16]. ASCT2 is expressed in the normal prostate and in prostate cancer [17]. Previous studies have shown that ASCT2 function is important for cancer cell growth in melanoma [18], acute myeloid leukaemia [19], lung cancer [20], neuroblastoma [21] and pancreatic ductal carcinoma [22].

In this study we showed that ASCT2 expression is increased in prostate cancer patient samples, and that knockdown inhibits cell cycle progression, prostate cancer growth and spontaneous metastasis in vivo. ASCT2 chemical inhibitors also suppressed glutamine transport, cell growth and glutamine metabolism in vitro. Our study suggests that compounds targeting ASCT2 may provide novel therapeutics for prostate cancer.

Materials and methods

For additional materials and methods, please see the online Supplementary material.

Patient specimens

Prostate cancer specimens from radical prostatectomy (n = 194) were obtained from the Vancouver Prostate Centre Tissue Bank (http://www.prostatecentre.com/our-research/core-facilities/biorepository). This project was approved by the institutional review boards at the University of British Columbia (Vancouver, Canada) and the CHUQ Research Centre (Québec, Canada). Written informed consent was obtained from all participants. The haematoxylin and eosin (H&E)-stained slides were reviewed and desired areas were identified on paraffin blocks. Tissue microarrays (TMAs) were manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm for each sample, with quantitative analysis calculated from individual cores (individual Gleason scores) or the average of duplicate cores (NHT TMA analysis).

TMA immunohistochemistry

TMA staining was conducted using a Ventana autostainer, model Discover XT™, with an enzyme-labelled biotin–streptavidin system and solvent-resistant DAB Map kit, using a 1:4000 dilution of rabbit anti-SLC1A5 (HPA035240). Staining was graded by a pathologist (LF), using a semi-quantitative, four-point scale, in which 0 represents no staining, 1 faint or focal staining, 2 moderate intensity in at least a quarter of neoplastic cells, and 3 intense staining in the majority of neoplastic cells. Examples of the staining are shown in Figure S1B (see supplementary material).

Cell culture and uptake assay

Human prostate cancer cell lines LNCaP-FGC, PC-3 and DU145 were purchased from ATCC (Rockville, MD, USA). LNCaP cells had been passaged directly from original low-passage stocks (2009), and we confirmed PC-3, LNCaP and DU145 cell identity by STR profiling in 2010 and 2014 (Cellbank, Sydney). Cells were cultured in RPMI 1640 medium containing 10% v/v fetal bovine serum (FBS), penicillin–streptomycin solution and 1 mM sodium pyruvate. Cells were maintained at 37°C in an atmosphere containing 5% CO2. Chemicals were diluted as follows, with control wells treated with the appropriate vehicle controls: H-Ser(Bzl)-OH (BenSer; Bachem; diluted in H2O); l-γ-glutamyl-p-nitroanilide (GPNPA; MP Biochemicals; diluted in H2O); bicalutamide (Astra Zeneca; diluted in DMSO). The [3H]-l-leucine and [3H]-l-glutamine uptake assays were performed as detailed previously [18,32].

Knockdown of ASCT2

ASCT2 shRNA knockdown was performed as previously described [18]. Two different shRNAs for ASCT2 were used in this study (Sigma): shASCT2#1 (CCGGGCTGTCTATCCGCTTCTTCAACTCGAGTTT GAAGAAGCGGATAAGCAGCTTTTGG) and shASC T2#2 (CCGGCTGGATTATGAGAAGATCGGATCTCG A GTATCCATTCTCATAATCCAGGTG)

Gene expression analysis

PC-3 cells were incubated in the presence or absence of GPNA or BenSer for 48 h, the cells were harvested and total RNA was extracted from them using Trizol. RNA quality was confirmed (RIN values 9.8–10), using RNA 6000 Nano Chips on an Agilent 2100 Bioanalyser (Agilent Technologies). Libraries were prepared using TruSeq Stranded Total RNA kit (Illumina, San Diego, CA, USA) and paired-end sequencing was performed on an Illumina HiSeq 2500 at the Kinghorn Cancer Centre (Sydney). Paired-end RNA-sequencing reads were trimmed and mapped to annotated human genome (hg19/GRCh37.p13), using TopHat2 with default settings. For each gene, the number of reads were counted and normalized to the library size. The threshold for expression was set at five reads in at least one experimental group. Exact tests were applied to compare differences in the means of read-counts from four replicates between the treated and untreated groups. The gene expression levels were estimated counts/million reads (CPM). The National Center for Biotechnology Information Gene Expression Omnibus number for mRNA sequencing datasets described in this study is GSE65112.

PC-3–luc xenografts and bioluminescence imaging

Athymic (nu/nu) male nude mice (Animal Resource Centre, Perth, Australia), aged 6–8 weeks, were housed in a specific pathogen-free facility, in accordance with the University of Sydney animal ethics committee guidelines. Mice were anaesthetized via 2% isoflurane inhalation and received subcutaneous (s.c.) injections of
1 × 10⁶ PC-3–luc cells resuspended in 100 ml Hanks’ balanced salt solution (HBSS). Xenografts were transplanted in both the right and left ventral flanks of mice, as detailed previously [30]. Tumour growth was monitored via bioluminescence imaging performed 48 h following cell implantation and biweekly thereafter for 32 days. During the experiments, two shASCT2 mice were dead 4 days after injection, due to fighting; they were not included in the analyses. Anaesthetized mice received intraperitoneal (i.p.) injections of D-luciferin substrate (150 mg/kg in DPBS; Gold Biotechnology) and images were acquired after a 15 min interval, using the Xenogen in vivo imaging system (IVIS) Lumina II (Caliper Life Science, MA, USA). Regions of interest were determined using Living Image software (Caliper Life Science) and quantified in photons/s (p/s). After 32 days, the animals were sacrificed following the final imaging time point. Livers and lungs were removed for IVIS-Lumina II analysis to detect spontaneous metastases. After being imaged and weighed, tumours were collected in either Trizol for RNA analysis or in PBS for protein expression analysis. Sections were stained with haematoxylin and eosin (H&E) for histological examination. Immunohistochemistry (IHC) was performed on H&E sections of paraffin-embedded tissue to assess ASCT2 expression, as detailed previously [30]. Immunohistochemical staining was also performed to assess leucine transporter 1 (LAT1) and amino acid transporter 5 (SLC7A5) expression, which are involved in glutamine transport. After 3 days of treatment with bicalutamide, cell growth was significantly decreased in LNCaP, PC-3 and DU145 cells (Figure 2D). We have previously shown that LAT1 inhibition affects the cell cycle through E2F-regulated cell cycle proteins CDK1, CDC20 and UBE2C [23]. These three cell cycle-regulatory proteins are also significantly increased in prostate cancer metastasis [23]. CDK1, CDC20 and UBE2C exhibited decreased expression after BenSer treatment (Figure 2E).

Inhibition of ASCT2 suppresses prostate cancer cell growth

Analysis of ASCT2 protein showed high expression in both LNCaP and PC-3 cells, with lower levels detected in DU145 cells (Figure 2A). Addition of the ASCT2 competitive inhibitor benzylserine (BenSer; see supplementary material, Figure S2A) [25] significantly reduced both glutamine and leucine uptake in LNCaP, PC-3 and DU145 cells (Figure 2B, C). Leucine uptake inhibition occurs due to exchange of glutamine for leucine by L-type amino acid transporter 1 (LAT1; SLC7A5), but may also be through direct LAT1 inhibition [15]. Inhibition of androgen receptor signalling by bicalutamide also significantly reduced glutamine uptake in androgen-sensitive LNCaP cells, but not androgen-insensitive PC-3 cells (see supplementary material, Figure S2B, C), confirming that AR signalling regulates ASCT2 levels [23] and ASCT2-mediated glutamine transport. After 3 days of treatment with BenSer, cell growth was significantly decreased in LNCaP, PC-3 and DU145 cells (Figure 2D). We have previously shown that LAT1 inhibition affects the cell cycle through E2F-regulated cell cycle proteins CDK1, CDC20 and UBE2C [23]. These three cell cycle-regulatory proteins are also significantly increased in prostate cancer metastasis [23]. CDK1, CDC20 and UBE2C exhibited decreased expression after BenSer treatment (Figure 2E).

Inhibition of ASCT2 suppresses glutamine metabolism in prostate cancer

As leucine and glutamine are involved in the activation of mTORC1 signalling [14,26,27], we examined
Figure 1. ASCT2 is androgen receptor-regulated and expressed in prostate cancer patient samples and xenografts. (A) ASCT2 mRNA expression in matched prostate cancer samples compared to adjacent normal prostate from the TCGA dataset (data are mean ± SEM; paired t-test; n = 36). (B) Representative images of ASCT2 protein expression in prostate cancer patient samples from Gleason grades 3, 4 and 5 and after neoadjuvant hormone therapy (NHT) treatment following an interval of 1–6 months, 7–12 months and in recurrent cancer; scale bar = 100 μm. (C) Immunohistochemical scoring of ASCT2 expression in patient cohorts before (n = 46) and after NHT treatment for 1–6 months (n = 54), 7–12 months (n = 76) and recurrent cancer (n = 32); data are mean ± SEM; Mann–Whitney U-test. (D) Microarray analysis of ASCT2 mRNA expression from LNCaP xenograft tumours harvested from non-castrated mice (intact; n = 10), post-castration regressing tumours (regressing; n = 6), at PSA nadir after castration (nadir; n = 10), after recurrent prostate cancer had developed (recurrent; n = 6) and castration-resistant prostate cancer (CRPC; n = 13); data are mean ± SEM; Mann–Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001

Table 1. SLC1A5/ASCT2 expression in Oncomine datasets

<table>
<thead>
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<th>Dataset</th>
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<td>26</td>
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<td>2·087</td>
<td>7·44E-04</td>
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<td>69</td>
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<td>Magee prostate</td>
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<td>0·018</td>
<td>8</td>
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<tr>
<td>Bittner multi-cancer</td>
<td>1·459</td>
<td>3·64E-12</td>
<td>59</td>
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<tr>
<td>Welsh prostate</td>
<td>1·399</td>
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T389 phosphorylation of p70S6K, a downstream target of mTORC1. BenSer reduced the phosphorylation of p70S6K in PC-3 cells, but not in LNCaP cells (Figure 2F). Utilizing another ASCT2 inhibitor, l-γ-glutamyl-p-nitroanilide (GPNA; see supplementary material, Figure S2D)[28], confirmed that glutamine deprivation inhibits the mTORC1 pathway in PC-3 cells, but not LNCaP cells (Figure 2F). These data suggest that mechanisms other than mTORC1 must also contribute to regulate cell growth in prostate cancer.

The majority of intracellular glutamine is converted into glutamate, which can be utilized by the TCA cycle for ATP and fatty acid production, or exported from the cell by xCT (SLC7A11) in exchange for cysteine, thereby contributing to glutathione production and protection against oxidative stress. Alternatively, glutamine acts as a nitrogen and carbon donor for macromolecular synthesis, including nucleotides and non-essential amino acids/proteins. We therefore set out to determine how changes in intracellular glutamine affect these downstream pathways. Using the Seahorse flux analyser, BenSer or GPNA treatment resulted in a reduction in basal oxygen consumption rates (OCRs) compared to control in LNCaP and PC-3 cells (see supplementary material, Figure S2E, F). GPNA, but not BenSer, significantly reduced basal OCR in both LNCaP and PC-3 cells (Figure 3A, B). However, this reduction in OCR did not appear to result directly from glutamine usage, as there was no significant reduction in oxidized 14C-labelled glutamine in either cell line after BenSer or GPNA (Figure 3C, D), despite lower 14C-glutamine uptake (see supplementary material, Figure S2G, H). Lipid synthesis from glutamine, however, was significantly decreased in LNCaP cells (Figure 3E), but not in PC-3 cells (Figure 3F). These data suggest that LNCaP cells directly utilize glutamine for fatty acid synthesis, while PC-3 cells may utilize...
glutamine for other pathways, including exchange for
leucine (Figure 2C) to activate mTORC1 signalling
(Figure 2F), which indirectly affects mitochondrial
respiration.

To further investigate lipid homeostasis, we mea-
sured the levels of cellular phospholipids and neutral
lipids. Lipid content analysis of LNCaP cells revealed
that GPNA significantly reduced the cellular level of
phospholipids (Figure 3G) and BenSer and GPNA
reduced the neutral lipids (Figure 3I) as well as lipid
droplet number (Figure 3K). Notably, the magnitude
of inhibition by BenSer and GPNA was similar to
that of 5-(tetradecyloxy)-2-furoic acid (TOFA), which
inhibits the rate-limiting enzyme of fatty acid synthesis,
acyetyl-CoA carboxylase (Figure 3G, I, K). A significant
decrease of phospholipids was also observed in PC-3
cells (Figure 3H); however, there were no significant
decreases in neutral lipids (Figure 3I) or lipid droplet
numbers (Figure 3L). Taken together, pharmacological
inhibition of ASCT2 provoked cell line-specific effects
on the flux of glutamine as a fuel source, yet ultimately
led to reduced OCR and cellular lipid levels in both
prostate cancer cell lines. However, neither BenSer nor
GPNA led to increased levels of reactive oxygen species
(ROS; see supplementary material, Figure S2I).

Global effects of BenSer or GPNA inhibition in PC-3
cells
To determine the global effects of ASCT2 inhibition,
we used next-generation sequencing to determine
mRNA expression changes in PC-3 cells treated with
BenSer or GPNA for 48 h. Analysis of genes signifi-
cantly (p < 0.05) up- or down-regulated by GPNA
(see supplementary material, Table S1) compared to
control showed a substantial overlap (up-regulated,
45.1%; down-regulated, 49.7%) with BenSer gene
expression, highlighting common targets of ASCT2
(Figure 4A). BenSer appears to have additional
non-ASCT2-mediated effects, with more genes showing
significant up- and down-regulation (see supplementary
material, Table S2) compared to GPNA (Figure 4A).
This is not surprising, since BenSer has been shown to
inhibit both ASCT2 and LAT1 function in oocytes [18].

Gene set enrichment analysis (GSEA) using Gene
Ontology categories showed significant enrichment in
control compared to GPNA for RNA and DNA pro-
cessing and metabolism, as well as cell cycle processes
(Figure 4B; see also supplementary material, Table S3).
Similar categories were also enriched in control com-
pared to BenSer (Figure 4C; see also supplementary
material, Table S4). Analysis of motif enrichment by
GSEA showed a significant association with E2F transcription factor motifs for control compared to either GPNA or BenSer (Figure 4D, E; see also supplementary material, Tables S5, S6). We have previously shown that inhibition of leucine uptake in LNCaP cells shows similar E2F-mediated cell cycle inhibition, with a subset of 122 genes down-regulated by the leucine uptake inhibitor BCH [23]. Analysis of this 122-gene signature in control versus GPNA and BenSer showed enrichment in the control group (Figure 4F, G), suggesting that there is a common mechanism regulating gene expression after leucine starvation and glutamine starvation in prostate cancer.

Amino acid starvation leads to up-regulation of the stress-response transcription factor ATF4, which in turn leads to increased expression of diverse amino acid transporters, including ASCT2 and LAT1 [23]. ATF4 was the first ($p=1.10E-11$) and sixteenth ($p=4.21E-21$) most significantly up-regulated gene in GPNA- or BenSer-treated cells, respectively (see supplementary material, Tables S1, S2), which was verified by RT-qPCR (see supplementary material, Figure S3A, B). ATF4 has previously been shown to directly transcriptionally regulate the expression of transporters including ASCT2, ASCT1, xCT and SLC3A2 [23,29]. Analysis of gene sets up-regulated after GPNA
Figure 4. RNA-seq analysis of PC-3 cells treated with BenSer or GPNA. (A) Venn diagram of genes up- or down-regulated in both BenSer- and GPNA-treated groups. (B, C) Gene set enrichment analysis (GSEA) plot of Gene Ontology categories RNA Processing and Cell Cycle Process in control versus GPNA (1 mM; B) or BenSer (10 mM; C) treatment. (D, E) GSEA plot of E2F transcription factor motif gene set in control versus GPNA (1 mM; D) or BenSer (10 mM; E) treatment. (F, G) GSEA plot of BCH down-regulated gene set (from [23]) in control versus GPNA (1 mM; F) or BenSer (10 mM; G) treatment.
inhibition showed significant enrichment for Gene Ontology categories, including amino acid transport (see supplementary material, Figure S3C), with significant up-regulation of transporters, including SLC1A5 (ASCT2, \( p = 0.020 \)), SLC1A4 (ASCT1; \( p = 7.72E-5 \)), SLC7A11 (xCT; \( p = 7.02E-8 \)), SLC3A1 (SNAT1; \( p = 3.51E-5 \)), SLC7A8 (LAT2; \( p = 5.63E-5 \)), SLC3A2 (4F2hc; \( p = 0.041 \)) and SLC3A1 (\( p = 0.005 \)). Another critical gene significantly increased by both GPNA (\( p = 0.009 \)) and BenSer (\( p = 0.0002 \)) was EIF2AK3 (PERK), which can phosphorylate eIF2α, leading to global protein synthesis suppression. A set of genes that belong to eukaryotic translation initiation factor are significantly decreased after GPNA treatment, including EIF3B (\( p = 0.0003 \)), EIF2A (\( p = 0.003 \)), EIF4H (\( p = 0.005 \)), EIF5A (\( p = 0.009 \)), EIF2B3 (\( p = 0.034 \)), EIF4G1 (\( p = 0.039 \)), EIF2B4 (\( p = 0.040 \)) and EIF2B5 (\( p = 0.048 \)).

**Knockdown of ASCT2 suppresses growth in prostate cancer cells**

We used lentiviral shRNA-mediated knockdown of ASCT2 to substantiate the direct ASCT2 effects of BenSer and GPNA in LNCaP and PC-3 cells. Two different shRNAs decreased ASCT2 expression and glutamine transport in LNCaP and PC-3 cells compared to a non-targeting shRNA control (Figure 5A, B; see also supplementary material, Figure S3D). Inhibition using shASCT2#2 also significantly decreased leucine uptake in both LNCaP and PC-3 cells (Figure 5C), suggesting that ASCT2-transported glutamine is utilized for leucine uptake. LNCaP and PC-3 cells expressing shASCT2#2 showed a significant decrease in cell viability compared to shControl cells (Figure 5D, E). Analysis of BrdU incorporation (Figure 5F) and apoptosis (see supplementary material, Figure S3E) in shASCT2#2 expressing PC-3 cells confirmed that ASCT2 knockdown inhibits the cell cycle rather than apoptosis. Similar results for glutamine uptake, BrdU incorporation and apoptosis were also observed in shASCT2#1 PC-3 cells (see supplementary material, Figure S3D, F, G). Finally, we examined mTORC1 pathway activation by western blotting, showing reduced phosphorylation of p70S6K in PC-3 cells expressing shASCT2#2 (Figure 5G).

**Knockdown of ASCT2 suppresses tumour growth in prostate cancer xenografts**

To determine whether ASCT2 function is critical for tumour growth *in vivo*, PC-3 cells expressing shControl or shASCT2#2 were transduced with a lentiviral vector co-expressing eGFP and firefly luciferase [30]. Cells were enriched to high purity on a narrow band of high GFP expression, resulting in similar GFP/luciferase expression in each shControl and shASCT2 cell line (see supplementary material, Figure S4A). PC-3–luc cells were subcutaneously injected into nude mice, with similar luciferase expression in shControl and shASCT2 tumours confirmed after 48 h (Figure 6A). Bioluminescence was analysed twice weekly for 32 days, showing a significant decrease in shASCT2 tumour size by day 25 (Figure 6B).

Mice were euthanized after 32 days, due to the size of the shControl tumours. The tumours were isolated, photographed and weighed, with shControl tumours being significantly larger than shASCT2 tumours (Figure 6C, D; \( p < 0.001 \)). Western blots of xenograft tumours showed that phosphorylation of p70S6K was decreased in shASCT2 tumours compared to shControl tumours (Figure 6E). Analysis of E2F2, CDK1, CDC20, UBE2C and the proliferation biomarker Ki67 in xenograft sections showed consistently lower levels in the shASCT2 tumours (Figure 6F). There were no changes in cleaved caspase 3 levels (see supplementary material, Figure S4B).

Micrometastases were detected by isolation and *ex vivo* bioluminescence analysis of organs immediately after euthanasia (see supplementary material, Figure S4C). Analysis of shControl mice showed that nine of 10 had metastases in the liver and four of 10 in the lung, while shASCT2 mice showed that four of nine had metastases in the liver, with no lung metastases (\( p = 0.004 \); Figure 6G). Metastatic bioluminescence burden in the liver and lung also showed a significant decrease in shASCT2 xenografted mice (Figure 6H).

**Discussion**

ASCT2 can be regulated by multiple transcription factors, including Myc [6], Rb/E2F [31], androgen receptor [23] and ATF4 [21,23]. This permits similar ASCT2 protein expression in both androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cell lines, and facilitates sufficient glutamine for cell growth. Our patient and LNCaP xenograft data confirmed that androgen receptor regulation of ASCT2 contributes to its expression in untreated/primary cancer and that under androgen-deprivation therapy, ASCT2 levels decrease. Similar to other androgen receptor- and ATF4-regulated genes [23], re-expression of ASCT2 was observed in recurrent disease. Importantly, ATF4 levels are significantly increased in androgen-independent prostate cancer metastasis compared to primary cancer (2.25-fold; \( p = 3.82E-7 \)) [23], suggesting that activation of ATF4 transcriptional targets is important in either metastasis or maintenance of the metastatic tumour. Interestingly, ASCT2 knockdown tumours showed decreased metastasis in the liver and lung, although this was confounded by the smaller primary tumours.

Blocking ASCT2 using either chemical or genetic means reduced cell proliferation and cell cycle in both LNCaP and PC-3 cell lines. This coincided not only with reduced glutamine levels but also with a reduction in the essential amino acid leucine, likely through LAT1-mediated exchange for leucine. Although knockdown of ASCT2 resulted in similar glutamine uptake
inhibition in LNCaP and PC-3 cells, leucine uptake was inhibited at a higher level in PC-3 cells, coincident with the higher LAT1 expression compared to LNCaP cells [32], further supporting the ASCT2–LAT1 exchange hypothesis, which subsequently leads to altered mTORC1 signalling [15,33]. ASCT2 inhibition did not directly alter glutamine metabolism in PC-3 cells, suggesting that the effects on basal OCR may be due to combined glutamine/leucine inhibition and subsequent shut-down of energy-consuming processes, such as protein, lipid, RNA and DNA synthesis. Previous studies have shown that mTORC1 controls mitochondrial activity through a 4EBP-dependent translation regulation [34] and mitochondrial gene expression [35]. This mechanism is further supported by the GPNA-mediated down-regulation of GO categories for RNA and DNA Processing and Mitochondrial Genesets in PC-3 cells. Analysis of glutamine metabolism in LNCaP cells, however, showed that inhibition of ASCT2 suppresses basal oxygen consumption, as well as TCA cycle glutaminolysis through complete oxidation and conversion to lipids. LNCaP cells have low expression of LAT1 and high expression of the leucine uniporter LAT3, and thereby are not as reliant on glutamine exchange to maintain leucine levels. They appear more reliant on glutamine anaplerosis than PC-3 cells.

Analysis of gene expression in PC-3 cells treated with BenSer or GPNA showed an adaptive response to glutamine deprivation through ATF4-mediated transcription, resulting in reduced cell cycle and RNA processing, and up-regulation of amino acid transporters in an attempt to restore glutamine levels. Previous studies have suggested that targeting this ATF4-mediated stress response may provide an effective cancer therapy [36]. However, in glioblastoma, the ATF4 response to glutamine deprivation directs apoptosis through NOXA and PUMA [37]. Unlike glioblastoma, we did not observe an increase in apoptosis in PC-3 cells after ASCT2 inhibition. These chemicals, or ASCT2 shRNA knockdown, do not induce complete glutamine deprivation, suggesting that perhaps the magnitude of glutamine deprivation, and subsequent ATF4 activation, may be critical in driving cell fate decisions for survival or apoptosis.

High expression of ASCT2 has previously been reported in the normal prostate [17] and many rapidly dividing normal cells rely on ASCT2 expression for glutamine uptake. Despite this important function in normal cells, the ASCT2 knockout mice do not show any obvious abnormalities in growth or survival, making ASCT2 an attractive therapeutic target [40]. Compared to the immunosuppressive effects of directly inhibiting mTORC1, the ASCT2 knockout mice have a relatively mild immune phenotype involving Th1 and Th17 lineage differentiation [40]. Despite this mild phenotype, it will still be important to monitor immune cells and effects on other normal cells when developing ASCT2-targeted therapies.

Our in vivo knockdown data support the development of ASCT2 as a therapeutic target in prostate cancer. Since ASCT2 is androgen-regulated, one could envisage targeting ASCT2 in either primary or advanced prostate cancer. Importantly, our data showed that targeting ASCT2 not only inhibited known pathways, such as mTORC1, but also regulated the critical metastasis-expressed, E2F-regulated, cell cycle genes CDK1, CDC20 and UBE2C, thereby resulting
Figure 6. ASCT2 is required for tumour growth in vivo. (A) PC-3-luc cells stably expressing shControl or shASCT2#2 were injected subcutaneously into the right and left dorsal flanks of male nude mice; bioluminescent images are shown at days 2 and 32. (B) Tumour growth was measured twice weekly by bioluminescence in shControl (n = 10) and shASCT2 (n = 8) mice; data are mean ± SEM; significance was assessed using two-way ANOVA; **** < 0.001. (C, D) Tumours (shControl, n = 20; shASCT2#2, n = 15) were harvested after 32 days, imaged (C) and weighed (D). (E) Phosphorylated p70S6K was measured after shASCT2 knockdown in PC-3 xenograft. (F) Sections from shControl and shASCT2 tumours were stained for CDK1, CDC20, UBE2C and Ki67 expression; representative images; scale bar = 100 μm. (G, H) Number (G) and size (H) of spontaneous metastases in liver and lung were measured by bioluminescence. (B) Data are mean ± SEM; significance was assessed using two-way ANOVA. (D, H) Data are mean ± SEM; Mann–Whitney U-test was used to analyse data. (G) Number of spontaneous metastases at day 32 in shControl and shASCT2 mice was assessed using two-tailed Fisher exact test; *p < 0.05, **p < 0.01, ***p < 0.001.

In summary, we have shown that ASCT2 is the key glutamine transporter that regulates prostate cancer proliferation and metabolism. We have identified critical cell cycle and metabolic pathways, which may provide
new avenues for therapeutic intervention in prostate cancer.

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Conception and design, JH and QW; development of methodology, JH, QW, CGB and WR; acquisition of data, QW, RH, AJH, MvG, MG, LF, JLLW, SB, MS, RN, CM, NP and NO; analysis and interpretation of data, JH, QW, RH, AJH, MvG, DG, WR, LF, ML, CCN and JEJR; writing, review and/or revision of the manuscript, JH, QW, JEJR and CGB; and study supervision, JH.

References

Regulation of glutamine transport in prostate cancer


SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Supplementary materials and methods

Figure S1. ASCT2 IHC scoring and Gleason expression.

Figure S2. Inhibition of glutamine uptake and metabolism.

Figure S3. ATF4 activation and ASCT2 shRNA inhibition.

Figure S4. shASCT2 in vivo apoptosis and metastasis.

Table S1. Genes upregulated and downregulated by GPNA.

Table S2. Genes upregulated and downregulated by BenSer.

Table S3. Gene ontology upregulated in control vs GPNA.

Table S4. Gene ontology upregulated in control vs BenSer.

Table S5. Motif upregulated in control vs GPNA.

Table S6. Motif upregulated in control vs BenSer.

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