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Overexpression of a key regulator of lipid homeostasis, Scap, promotes respiration in prostate cancer cells

Anika Vinayak Prabhu [1], James Robert Krycer *,[1], Andrew John Brown

School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney 2052, Australia

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ABSTRACT

Prostate metabolism is unique, characterised by cholesterol accumulation and reduced respiration. Are these related? We modulated cholesterol levels and despite changes in mitochondrial cholesterol content, we saw no effects on lactate production or respiration. Instead, these features may be related via sterol regulatory element-binding protein 2 (SREBP-2), the master transcriptional regulator of cholesterol synthesis. SREBP-2 diverts acetyl-CoA into cholesterol synthesis and may thus reduce respiration. We examined LNCaP cells overexpressing the SREBP-2 regulator, Scap: although having higher SREBP-2 activity, these cells displayed higher respiration. This striking observation warrants further investigation. Given that SREBP-2 and Scap are regulated by factors driving prostate growth, exploring this observation further could shed light on prostate carcinogenesis.
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1. Introduction

In developed countries, prostate cancer (PCa) is the most common cancer in men and has the second highest rate of cancer-related mortality [1]. With no cure for the advanced ('castration-resistant') stages of PCa, there is an urgent need to better understand the mechanisms driving this disease. There has been recent interest in cancer metabolism, with metabolic changes being considered a hallmark of cancer [2] and a target for cancer therapy [3].

In this vein, there are two metabolic features unique to prostate epithelial cells. Firstly, normal prostate cells have higher cholesterol synthesis than the liver, and elevated cholesterol levels compared to the surrounding tissue [4]. This cholesterol accumulation increases further in the ageing prostate and PCa [4]. Secondly, the prostate displays high glycolytic activity and reduced respiration [5,6]. Here, we consider whether these features are related.

For instance, cholesterol is hydrophobic, allowing it to embed within the cellular membrane and decrease membrane fluidity [7]. This can pose a physical barrier to prevent oxygen diffusion [7,8], as well as reducing passive proton permeability in mitochondria [9]. High mitochondrial membrane-cholesterol content may also disrupt mitochondrial proteins (e.g., [10,11]), which can impair respiration [12]. Normally, the mitochondrial membranes are cholesterol-poor [13,14], but become enriched with cholesterol in cancers, thus influencing cancer metabolism. One well-studied example are hepatomas [9,15–17], whereby enriching non-cancerous liver mitochondrial membranes with cholesterol produced the impaired mitochondrial activity observed in hepatomas [18]. Conversely, depleting cholesterol from hepatoma mitochondria improved respiration efficiency [19]. Beyond the hepatoma setting, heart mitochondrial membrane cholesterol levels increase during ischaemia, leading to reduced State 3 respiration [20]. Given that these two features (high cholesterol, reduced respiration) are present in the prostate, we hypothesised a similar relationship may occur in the prostate setting; a link that has not been examined previously.

Another unexplored relationship is between respiration and cholesterol synthesis. To generate cholesterol, an intermediate of the citric acid cycle, citrate, is shuttled out of the mitochondria to provide acetyl-CoA for cholesterol synthesis. Indeed, high citrate

Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FASN, fatty acid synthase; FCS, foetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGCR, HMG-CoA reductase; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; MT-ND1, nicotinamide adenine dinucleotide dehydrogenase 1; NCS, newborn calf serum; NCLPDS, lipoprotein-deficient NCS; PCa, prostate cancer; qRT-PCR, quantitative real-time PCR; SRD, sterol-regulation deficient; SREBP, sterol regulatory element-binding protein

* Corresponding author. Address: School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney 2052, New South Wales, Australia.

E-mail address: j.krycer@unsw.edu.au (J.R. Krycer).

[1] These authors contributed equally to this work.

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levels have been detected in the prostate [5,21,22]; hence, citrate flux out of the citric acid cycle could impair respiration whilst fuelling cholesterol synthesis.

A key regulator of cholesterol synthesis is the sterol regulatory element-binding protein 2 (SREBP-2). This cholesterogenic transcription factor upregulates the protein involved in cholesterol uptake, the low-density lipoprotein receptor (LDLR), and the majority of the enzymes in cholesterol biosynthesis, including the flux-controlling enzyme HMG-CoA reductase (HMGCR) [23,24]. In turn, SREBP-2 activity is regulated by cholesterol via an elegant feedback mechanism [25]: when cholesterol levels are low, SREBP-2 is escorted by Scap from the endoplasmic reticulum (ER) to the Golgi, where SREBP-2 is cleaved. This generates the mature transcription factor, which upregulates cholesterol synthesis and uptake. As cholesterol levels rise, Scap is bound by the retention protein, Insig, in the ER. This prevents the activation of SREBP-2. We and others have shown that the activation of SREBP-2 is enhanced by growth-promoting factors, with Akt kinase promoting ER-to-Golgi transport of SREBP-2 [26,27] and androgens upregulating *Scap* expression [28,29]. Both of these factors are important for the PCa setting [30,31], implicating SREBP-2 in PCa development [31].

Overall, there is evidence supporting a relationship between cholesterol accumulation and diminished respiration in the prostate setting. Thus, we aim to test two hypotheses: firstly, that increasing cellular cholesterol levels impairs respiration, and secondly, that increasing SREBP-2 activity also impairs respiration. Together, this could contribute to the metabolic phenotype observed in prostate cells.

2. Materials and methods

2.1. Materials

Foetal calf serum (FCS) was obtained from Bovogen (Vic, AU), newborn calf serum (NCS), zeocin and penicillin/streptomycin from Life Technologies (Vic, AU), and all other media components from Sigma–Aldrich (NSW, AU). Lipoprotein-deficient NCS (NCLPDS) was prepared from NCS, as described previously [32]. Methyl-β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, oligomycin, 2,4-dinitrophenol, rotenone, and antimycin A were obtained from Sigma–Aldrich. Cholesterol was obtained from Steraloids (USA) and cholesterol/methyl-β-cyclodextrin complexes were prepared as described previously [33].

2.2. Cell culture

The PCa cell-line, LNCaP, was a gift from Dr. Pamela Russell (Australian Prostate Cancer Research Center, AU), and maintained in Medium A (RPMI 1640, supplemented with 10% (v/v) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin). Stable LNCaP cell-lines, transfected with empty vector (LNCaP-EV), wild-type Scap (LNCaP-Scap-WT), or Y234A-mutant Scap (LNCaP-Scap-mut), were generated previously [34] and maintained in Medium B (Medium A, supplemented with 250 μg/ml zeocin). Before plating LNCaP sub-lines, plates and dishes were treated with polyethyleneimine (Sigma–Aldrich) to enhance cellular adhesion as described previously [34].

The Chinese hamster ovary (CHO) cell-lines, CHO-7 and sterol-regulation deficient sub-line 1 (SRD-1), were a gift from Dr. Michael Brown and Dr. Joseph Goldstein (University of Texas Southwestern Medical Center, USA). SRD-15 cells were a gift from Dr. Russell DeBose-Boyd (University of Texas Southwestern Medical Center, USA). CHO-7 cells were maintained in Medium C (DF12, supplemented with 5% (v/v) NCLPDS, 100 U/ml penicillin, and 100 μg/ml streptomycin). SRD-1 and SRD-15 cells were maintained in Medium C, supplemented with 1 μg/ml 25-HC.

2.3. Cholesterol modulation and cholesterol assay

When modulating cholesterol levels, treatment conditions (time and concentration) were optimised to produce the maximum effect without compromising cell viability (data not shown). Following treatment, cells were harvested and cellular cholesterol levels were determined as described previously [29].

2.4. Lactate assay

Following treatment, cells were incubated with or without 100 ng/ml oligomycin for 4 h. Oligomycin inhibits ATP synthase and thus respiration, maximising glucose flux into lactate production.

The media was centrifuged at 2000×*g* to remove detached cells and the supernatant was assayed for lactate content in a microtitre plate format: 10 μl of supernatant was diluted in 90 μl water in duplicate wells. To one set of wells, 100 μl of assay buffer (4 mM NAD^+, 1 M glycine pH 9.2, 0.4 M hydrazine in 1.2 M NaOH, 2.5 mM EDTA), final pH adjusted to 9.2 with NaOH) was added to each well. The second set of wells received 100 μl of assay buffer, supplemented with 2 U/ml lactate dehydrogenase (LDH). The first set of wells determines background NAD^+ reduction, whilst the latter determines lactate-specific NAD^+ reduction. After incubation for 2 h at room temperature, the absorbance of NADH at 340 nm was measured using the Spectra Max 340 plate-reader (Molecular Devices, USA). Comparison with a lactate standard determined media-lactate content.

Cells were harvested for protein with RIPA buffer (1% (v/v) NP-40, 0.1% (w/v) sodium dodecylsulfate, 1 mM Na_3VO_4, 150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 0.5% (w/v) sodium-deoxycholate). Protein content was determined using the Pierce BCA assay kit (ThermoScientific, Vic, AU), using bovine serum albumin standard. Media lactate levels were normalised to protein content.

2.5. Oxygen consumption analysis

Cellular oxygen consumption was measured using the Oxygraph-2k (Oroboros Instruments, AT). Before each run, the instrument was calibrated with Medium A, according to the manufacturer's instructions. Following treatment, cells were ~60–70% confluent, although higher confluences had little impact upon oxygen consumption (Fig. S1A). Cells were trypsinised and resuspended in Medium A (1×10^6 cells/ml), from which 2 ml were added to each chamber.

After allowing the oxygen consumption rate to stabilise (Rate A), cells were treated sequentially with 500 ng/ml oligomycin (Rate B), 100 μM 2,4-dinitrophenol (Rate C), 0.25 μM rotenone, and 12.5 μM antimycin A (Rate D). Oxygen consumption after each treatment was calculated using DatLab (Oroboros), and the following parameters calculated: basal respiration rate = A–D, leak respiration rate = B–D, maximal respiration rate = C–D [35]. The biological significance of these parameters is explained in Section 3 and Fig. 2B.

2.6. Mitochondrial isolation

LNCaP cells (10×10^6 cells) were seeded in a 100 mm dish in Medium A. Following treatment, mitochondria were harvested using the anti-TOM22 mitochondrial isolation kit (Miltenyi Biotec, NSW, AU), according to the manufacturer's instructions.

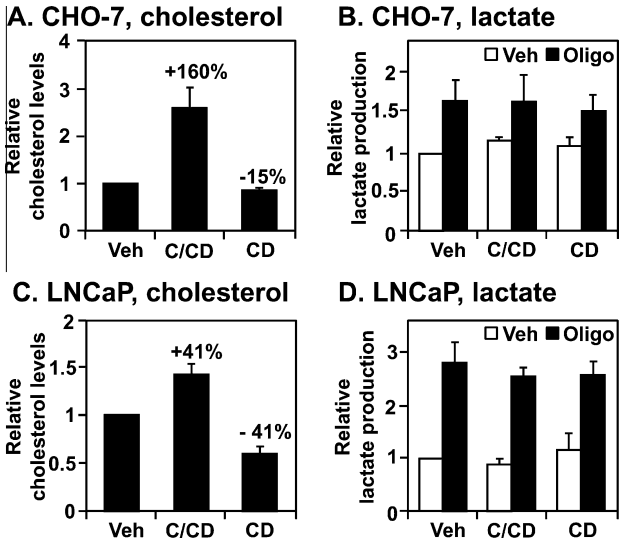

Fig. 1. Cholesterol modulation does not influence lactate production. (A and B) CHO-7 cells were treated with cholesterol/methyl-β-cyclodextrin (C/CD, 20 μg/ml) for 4 h, or with 2-hydroxypropyl)-β-cyclodextrin (CD, 0.5% w/v) for 2 h, all in Medium C. Cells were then assayed for (A) cholesterol content or (B) lactate production in Medium C, with or without oligomycin (oligo, 100 ng/ml). (C and D) LNCaP cells were treated with C/CD (20 μg/ml) or methyl-β-cyclodextrin (CD, 0.25% w/v) for 2 h in Medium A. Cells were then assayed for (C) cholesterol content or (D) lactate production in Medium A, with or without oligomycin (oligo, 100 ng/ml). All data presented as mean + S.E., relative to the vehicle (Veh) condition, from at least three separate experiments, each performed with triplicate wells per condition.

2.7. mRNA expression analysis

Following culturing, RNA was harvested and reverse-transcribed, and mRNA levels determined by quantitative real-time PCR (qRT-PCR) as described previously [29]. All primers used have been described previously [29].

2.8. Mitochondrial copy number analysis

Following culturing, cells were harvested for DNA as described previously [36], except that a non-shaking waterbath was used for overnight digestion. Furthermore, following extraction with phenol:chloroform:isoamyl alcohol, an additional extraction with 1 vol of 24:1 (v/v) chloroform:isoamyl alcohol was performed prior to precipitating DNA.

For mitochondrial copy number analysis, 25 ng DNA was used as a template for qRT-PCR (described above). The mitochondrial gene amplified was nicotinamide adenine dinucleotide dehydrogenase 1 (*MT-ND1*), with the following primers [37]: MT-ND1-F: 5′-ACGCCATAAAACTCTTCACCAAAG-3′ and MT-ND1-R: 5′-GGGTTCATAGTAGAAGAGCGATGG-3′. The genomic reference gene amplified was glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), with the following primers [38]: gGAPDH-F: 5′-AACGTGTCAG TGGTGGACCTG-3′ and gGAPDH-R: 5′-AGTGGGTGTCGCTGTTGAAGT-3′. These genes have been used previously in a PCa setting [37,38]. Relative mitochondrial copy number was calculated from *MT-ND-1* levels using the $\Delta\Delta C_t$ method, normalised to *GAPDH*.

3. Results

First, we tested the effect of cellular cholesterol levels on aerobic metabolism. We conducted pilot experiments using CHO cells, since they have been used previously in this context [8], and are commonly-used to study cholesterol homeostasis (e.g., [33,34]). To modulate cholesterol levels, we used cholesterol–cyclodextrin for cholesterol-loading and cyclodextrin alone for cholesterol-depletion (Fig. 1A). However, neither treatment had any effect on either basal or stimulated lactate production (Fig. 1B) – a change in lactate production would have implied an opposite change in respiration. Repeating these conditions in PCa (LNCaP) cells yielded a similar result (Fig. 1C and D). Interestingly, oligomycin had a greater effect on lactate production in LNCaP cells, with a ~150% increase (Fig. 1D) versus ~50% increase in CHO-7 cells (Fig. 1B). This implies CHO-7 cells heavily favour lactate production, reducing our potential effect size. Combined with our focus on the prostate setting, we restricted subsequent experiments to LNCaP cells.

To assess whether these cyclodextrin treatments were influencing the mitochondria, we isolated mitochondria from treated LNCaP cells. We found that mitochondrial-cholesterol content increased markedly with cholesterol-loading (Fig. 2A). To examine any downstream effects on respiration, we measured cellular oxygen consumption, focusing on several parameters: (1) *basal respiration*, (2) *leak respiration*, whereby ATP synthase is inhibited by oligomycin yet oxygen is still consumed due to proton leak, and (3) *maximal respiration*, whereby the proton gradient is dissipated by the uncoupler 2,4-dinitrophenol (Fig. 2B). Furthermore, Complexes I and III were inhibited by rotenone and antimycin A respectively to obtain the *non-mitochondrial respiration rate*, which provided the background value to calculate the basal, leak, and maximal respiration rates (Fig. 2B, [35]). We found that cholesterol modulation had no effect on oxygen consumption (Fig. 2C). Overall, this shows that cellular cholesterol levels have little effect on aerobic metabolism in PCa cells.

Next, we tested the impact of SREBP-2 on aerobic metabolism. Although previous studies have used CHO mutants with impaired SREBP-2 activity for this purpose [8], our experiments with other CHO mutants did not support a relationship between SREBP-2 activity and aerobic metabolism (Fig. S2). To test this relationship

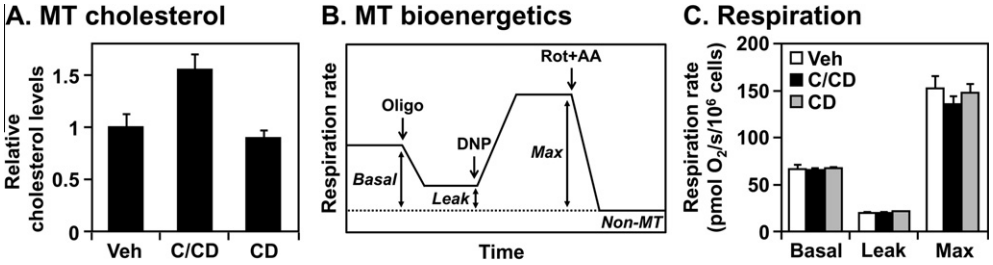

Fig. 2. Cholesterol modulation does not influence respiration. (A) LNCaP cells were treated with cholesterol/methyl-β-cyclodextrin (C/CD, 20 μg/ml) or methyl-β-cyclodextrin (CD, 0.25% w/v) as described in Fig. 1. Following treatment, mitochondria (MT) were isolated and assayed for cholesterol content. Data presented as mean + S.D., from triplicate determinations. Veh, vehicle. (B) Schematic showing the parameters of MT bioenergetics. Details in the text. DNP, 2,4-dinitrophenol; Rot, rotenone; AA, antimycin A; Max, maximal; Non-MT, non-mitochondrial (C) LNCaP cells were treated with C/CD and CD as described in (A), and assayed for oxygen consumption. Data presented as mean + S.E., from four separate experiments.

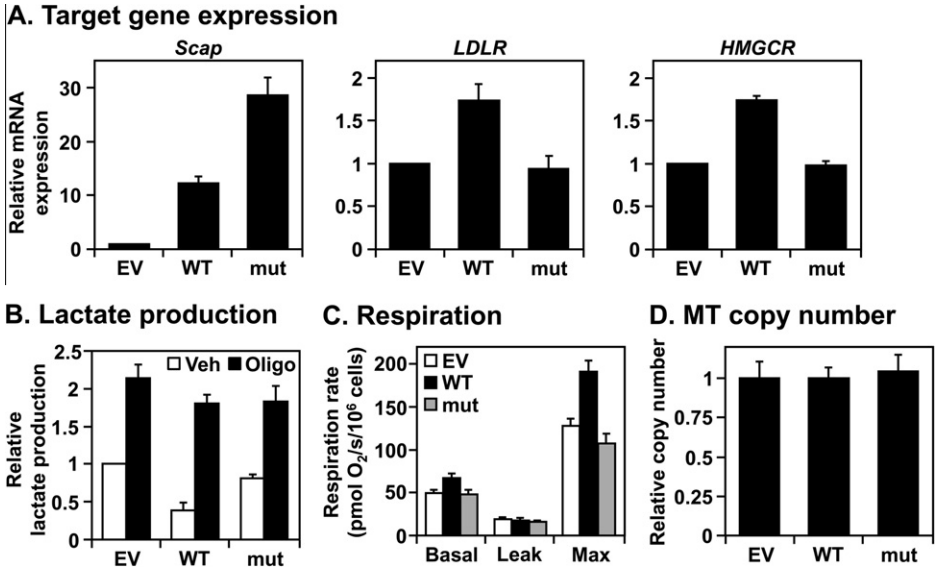

Fig. 3. Overexpression of Scap promotes respiration. (A–D) LNCaP-EV ('EV'), LNCaP-Scap-WT ('WT'), and LNCaP-Scap-mut ('mut') cells were grown in Medium B. (A) Cells were harvested for RNA and assayed for mRNA expression, relative to the EV cells. (B) Cells were then assayed for lactate production in Medium A, with or without oligomycin (oligo, 100 ng/ml). This was made relative to the EV/vehicle (Veh) condition. (C) Cells were assayed for oxygen consumption. Max, maximal. (D) Cells were harvested for DNA and assayed for mitochondrial copy number, made relative to one batch of EV cells. (A–D) Data presented as mean + S.E., from at least three separate experiments, with (A–B) performed with triplicate wells per condition.

in a prostate setting, we used LNCaP-Scap-WT cells [34], LNCaP cells which stably express the SREBP-2 regulator, Scap. We compared these cells to LNCaP cells stably transfected with the empty vector (LNCaP-EV) or a mutant form of Scap (LNCaP-Scap-mut). Confirming previous findings [34], LNCaP-Scap-WT and LNCaP-Scap-mut have higher *Scap* expression than LNCaP-EV cells (Fig. 3A, *left panel*). Although LNCaP-Scap-mut has higher *Scap* expression than LNCaP-Scap-WT, this Scap is mutated so it cannot activate SREBP-2. Consequently, only LNCaP-Scap-WT had higher expression of SREBP-2 target genes, *HMGCR* and *LDLR* (Fig. 3A, *middle and right panels*), demonstrating it has higher SREBP-2 activity than LNCaP-EV or LNCaP-Scap-mut cells.

Consequently, we expected LNCaP-Scap cells to have lower respiration. Surprisingly, we observed the reverse, with LNCaP-Scap cells having ~48% lower lactate production (Fig. 3B, vehicle condition) and ~36% higher basal respiration (Fig. 3C) than LNCaP-EV or LNCaP-Scap-mut cells. Cell number was similar when preparing the cells for these assays (Fig. S1B), implying that this is not due to a difference in growth-rates. These stable cell-lines responded similarly to the metabolic inhibitors, having similar lactate production with oligomyin treatment (Fig. 3B), leak respiration rates (Fig. 3C), minimal respiration rates and maximum respiration:basal respiration ratios (data not shown, $P > 0.05$, paired *t*-test between each pair of cell-lines). In addition, these cell-lines had a similar mitochondrial copy number (Fig. 3D). Thus, these differences in aerobic metabolism are not due to mitochondrial dysfunction, but likely because of Scap overexpression itself.

4. Discussion

In this study, we explored the interplay between cholesterol and aerobic metabolism, using both CHO and prostate cells. Although modulating cholesterol levels had little effect (Figs. 1 and 2), we found that overexpression of Scap promoted respiration and reduced lactate production (Fig. 3).

Although we observed no clear effects with CHO cells (Figs. 1, S2), a previous study by Khan et al. [8] used CHO cells to show that cholesterol modulation with cyclodextrin and mutant sublines with perturbed SREBP-2 activity influence respiration: increased cellular cholesterol levels reduced respiration due to impaired oxygen diffusion across the cholesterol-enriched plasma membrane. We did not observe these findings here for several possible reasons. Firstly, we assessed aerobic metabolism by lactate production instead of respiration, because the lactate assay is higher-throughput and there were differences in mitochondrial number between the CHO-7 mutants (Fig. S2D). Secondly, we used CHO-7 cells whilst other CHO cells were used previously [8] – CHO-7 are adapted to low-sterol conditions (NCLPDS) compared to other CHO cells (grown in FCS [8]), and thus would respond differently to cholesterol modulations. Furthermore, we found the CHO-7 cells heavily favour lactate production (Fig. 1B), which would reduce any apparent effect size for cholesterol enrichment or enhanced SREBP-2 activity. Thirdly, Khan et al. achieved up to ~300% cholesterol enrichment [8], but exceeding our ~160% enrichment (Fig. 1A) compromises CHO-7 cell viability (data not shown). Thus, our conflicting findings can be reconciled.

We similarly found that cholesterol modulation had no effect on lactate production or respiration in LNCaP cells (Figs. 1D and 2C), even though mitochondrial cholesterol levels were affected (Fig. 2D). By contrast, in the hepatoma setting, increased mitochondrial cholesterol levels impair respiratory activity (e.g., [18]). In addition, one group found that cholesterol enrichment promotes the export of citrate out of the mitochondria, truncating the citric acid cycle [39,40]. However, it was later demonstrated that cholesterol-loading liver mitochondria physiologically (via diet) did not truncate the citric acid cycle or affect respiration [41]. Thus, supraphysiological cholesterol enrichment may be required to influence respiration in a hepatic setting. However, the prostate synthesises more cholesterol than the liver [4] and thus may be more susceptible to cholesterol enrichment. Hence, future investigations should compare mitochondria between prostate epithelial cells and the surrounding tissue. This would determine if there is a higher mitochondrial cholesterol content in prostate epithelial cells, whether this impairs respiration and contributes to the citrate accumulation in the prostate [5,21,22], and if these parameters change during progression to PCa.

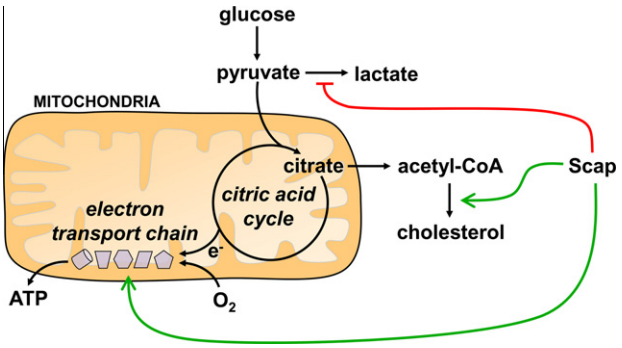

Fig. 4. A key regulator of lipid homeostasis, Scap, promotes respiration and inhibits lactate production. Details provided in the main text.

Nevertheless, a striking observation was that Scap overexpression promoted respiration and reduced lactate production (Fig. 3). In contrast, this effect was not seen when the overexpressed Scap was mutated specifically to prevent it from facilitating SREBP-2 transport (LNCaP-Scap-mut, Fig. 3). To explore this further, we attempted to generate LNCaP cells stably-expressing mature SREBP-2, but were not successful (data not shown). Furthermore, Scap not only activates SREBP-2, but the other SREBP isoforms, SREBP-1a and 1c, which are also involved in fatty acid metabolism. Consequently, we found that overexpressing wild-type Scap increased the expression of fatty acid synthase (*FASN*) by 2.3 ± 0.2-fold ($n = 3$), whereas mutant Scap had no effect on *FASN* expression (0.9 ± 0.1-fold, $n = 3$). We hypothesised that like SREBP-2, this would have diverted acetyl-CoA to lipid synthesis, thus impairing respiration, but our Scap-overexpression experiments would suggest otherwise (Fig. 3). A recent study found that SREBP-2 target gene expression is higher in normal prostate epithelial cells than PCa cells *in vitro* [42], whilst we found no change as PCa cells progress to advanced (castration-resistant) PCa [43]. In line with our results here, this would suggest that the highly glycolytic nature of prostate epithelial cells is enhanced during carcinogenesis. It would be interesting to examine if this is the case in a more physiological setting, such as with tissue slices as used in past studies (e.g., [4]).

Whilst the relationship between SREBP-2 activity and aerobic metabolism has not been deeply explored, studies have shown that overexpression of SREBP-1 isoforms in fibroblasts or hepatoma cells influences the expression of mitochondrial proteins (e.g., [44,45]), also seen by ChIP with endogenous SREBP-1 (e.g., [46]). Furthermore, overexpressing either mature SREBP-1a or -1c increased the expression of *GLUT4*, involved in glucose uptake, and enhanced glucose oxidation and glycogen synthesis [44]. Although it is unclear whether this is due to an overall increase in glucose metabolism or specific changes to glucose flux, the SREBP isoforms could be similarly overexpressed in prostate cells to dissect isoform-specific effects on glucose metabolism.

Another important catabolite is glutamine, which transformed cells utilise as an alternate carbon source [47] – our findings could be explained by increased glucose flux through the pentose phosphate pathway and glutamine flux through aerobic respiration. Although a previous study showed that SREBP-1c overexpression did not affect glutamate dehydrogenase expression [48], assays comparing glutamine metabolism should be considered in future SREBP-overexpression studies. Flux-rate analysis of the glycolysis–citric acid cycle network and microarray analysis for genome-wide, SREBP-dependent changes in gene expression could identify the relevant SREBP targets mediating any potential differences in glucose and glutamine metabolism. This has implications beyond prostate metabolism, given that Scap has been considered a target for modulating SREBPs in metabolic disease [49,50] and recent interest in targeting carbon metabolism in cancer therapy [2,3], including PCa [31].

Overall, we have shown that the overexpression of a key regulator of lipid homeostasis, Scap, promotes respiration in prostate cells (Fig. 4). Although modulating cell cholesterol levels had little effect on respiration, it is possible that Scap influences glucose flux. Given that the SREBP isoforms are regulated by growth-promoting factors [26,28,29,34], particularly by androgens in a prostate setting [28,29], future investigations should explore this work in order to better understand the metabolic phenotype of the prostate.

Conflicts of interest

We declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.02.040.

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