

Original Paper

Targeting Glutamine Synthesis Inhibits Stem Cell Adipogenesis *in Vitro*

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Key Words

Glutamine • Adipogenesis • Lipogenesis • Methionine sulfoximine

Abstract

Background/Aims: Glutamine is the most abundant amino acid in the body and has a metabolic role as a precursor for protein, amino sugar and nucleotide synthesis. After glucose, glutamine is the main source of energy in cells and has recently been shown to be an important carbon source for *de novo* lipogenesis. Glutamine is synthesized by the enzyme glutamine synthetase, a mitochondrial enzyme that is active during adipocyte differentiation suggesting a regulatory role in this process. The aim of our study was therefore to investigate whether glutamine status impacts on the differentiation of adipocytes and lipid droplet accumulation. **Methods:** Mouse mesenchymal stem cells (MSCs) were submitted to glutamine deprivation (i.e. glutamine-free adipogenic medium in conjunction with irreversible glutamine synthetase inhibitor, methionine sulfoximine – MSO) during differentiation and their response was compared with MSCs differentiated in glutamine-supplemented medium (5, 10 and 20 mM). Differentiated MSCs were assessed for lipid content using Oil Red O (ORO) staining and gene expression was analysed by qPCR. Intracellular glutamine levels were determined using a colorimetric assay, while extracellular glutamine was measured using liquid chromatography-mass spectrometry (LC-MS). **Results:** Glutamine deprivation largely abolished adipogenic differentiation and lipid droplet formation. This was accompanied with a reduction in intracellular glutamine concentration, and downregulation of gene expression for classical adipogenic markers including PPAR γ . Furthermore, glutamine restriction suppressed isocitrate dehydrogenase 1 (IDH1) gene expression, an enzyme which produces citrate for lipid

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synthesis. In contrast, glutamine supplementation promoted adipogenic differentiation in a dose-dependent manner. **Conclusion:** These results suggest that the glutamine pathway may have a previously over-looked role in adipogenesis. The underlying mechanism involved the glutamine-IDH1 pathway and could represent a potential therapeutic strategy to treat excessive lipid accumulation and thus obesity.

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Introduction

Glutamine is the most abundant extracellular amino acid in the body, that is primarily derived from skeletal muscle, from where it is transported to target tissues [1, 2]. The importance of glutamine for cell proliferation, survival and immune response is well recognized, especially in rapidly dividing cells [3-6]. Once inside the cell, glutamine can be converted into glutamate by the glutaminase enzyme and enter the tricarboxylic acid (TCA) cycle to maintain oxidative phosphorylation, where in addition to glucose, it is an important carbon source for *de novo* lipogenesis [7-9]. This process involves reverse transfer through the TCA cycle and reductive carboxylation by isocitrate dehydrogenases (IDHs) to generate acetyl-CoA [10]. In addition to being an important precursor for proteins, amino sugars, purines and pyrimidines, glutamine can also regulate gene expression for some transcriptional factors, enzymes, channels and receptors [11-13]. Furthermore, low glutamine availability has been reported to increase the expression of pro-inflammatory genes in white adipose tissue [14], while a number of benefits have been reported in human clinical studies with supplemental glutamine [2], particularly on weight loss [15] and insulin plasma concentration [16] in obese people and for the maintenance for optimal health [2].

A primary function of adipose tissue is to store excess energy in the form of lipids [17]. There are three main types of adipocytes, white, brown and beige [18], which despite their different morphological and functional characteristics, are all mesenchymal in origin with lipid droplet(s) formation, a hallmark of adipogenesis [19, 20]. Unlike white fat, brown and beige adipocytes can dissipate energy in the form of heat through a unique property of the mitochondrial uncoupling protein 1 (UCP1) which uncouples respiration from ATP synthesis [21]. The metabolic requirements of adipocytes for glutamine are mainly dependent on glutamine synthetase (GS), a mitochondrial enzyme that catalyses the reaction between ammonia and glutamate [22, 23]. GS activity increases during adipocyte differentiation [24, 25], pointing its possible role in adipocyte maturation, although effects of manipulating glutamine availability during adipogenesis are not known. The present study therefore aimed to examine whether glutamine has a regulatory role in adipose tissue development by examining the impact of glutamine deprivation or supplementation in the adipogenic differentiation of mesenchymal stem cells (MSCs), an established model of adipogenesis [26, 27].

Materials and Methods

All reagents were purchased from ThermoFisher Scientific (UK) unless otherwise stated.

Cell culture

Mouse MSCs characterised as beige cells [26, 27] were grown and maintained routinely in a standard (ST) medium containing low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non-essential amino acids and 5 mM glutamine. When cells reached confluency, adipogenic differentiation was started using adipogenic (AD) medium. For this purpose, glutamine-free standard medium was used, adding 1 μ M dexamethasone (Cayman Chemicals, USA), 100 μ M isobutylmethylxanthine (IBMX) (Sigma-Aldrich, UK), 1 μ M rosiglitazone (Cayman Chemicals, USA), 10 μ g/ml insulin (Sigma-Aldrich, UK), and 1 nM triiodothyronine (T3) (Sigma-Aldrich, UK). All supplements were prepared according to the manufacturer's guidelines. Adipo-

genic cultures were divided in two groups (Supplementary Fig. 1 – for all supplementary material see www.cellphysiolbiochem.com): either treated with L-methionine sulfoximine (MSO, 3 mM) (Acros Organics, UK), an irreversible glutamine synthetase inhibitor [28-31], or with low (5 mM) medium (10 mM) and high (20 mM) glutamine supplementation. Parallel MSCs kept in ST medium were used as undifferentiated controls (Ctrl). The analyses performed in this study were performed on 3 batches of cells (each one done with three technical replicates), cells were treated for 7 days prior analysis.

Cell Viability Assay and Oil Red O staining

Both cell viability and the presence of lipid droplets were determined by Presto Blue Viability assay and Oil Red O staining (ORO), respectively, as previously described [26, 27].

Intracellular glutamine concentration

The intracellular concentration of glutamine in sonicated cells extracts was determined using a colorimetric EnzyChrom™ Glutamine Assay Kit (EGLN-100; BioAssay Systems, UK) [32-34] according to the manufacturer's instructions.

Gene expression analysis

Extraction of total RNA and quantitative PCR were performed as previously described [26]. Gene expression was determined using the GeNorm normalization algorithm against two selected reference genes (stability value $M = 0.46$), TATA sequence binding protein (TBP) and acidic ribosomal protein subunit P0 (RPLP0) using GeNorm software (version 3.5; Primer Design Ltd). The following murine-specific oligonucleotide primers (Eurofins) were used. For Adiponectin: forward 5'-AACTTGTGCAGGTTGGATGGC-3' and reverse 5'-TTCTCTCCCTTCTCTCCAGGA-3'. For FABP4: forward 5'-TGGAAGCTTGTCTCCAGTGA-3' and reverse 5'-AATCCCATTTACGCTGATG-3'. For PPAR γ : forward 5'-GCCGAGTCTGTGGGATAAA-3' and reverse 5'-GTGAGACATCCACAGCAA-3'. For TBP: forward 5'-CCTTGTACCCTTACCAATGAC-3' and reverse 5'-ACAGCCAAGATTCACGGTAGA-3'. For RPLP0: forward 5'-CTGGAGAACTGCTGCCTCA-3' and reverse 5'-AGTGTCTGAGCTGGCACA-3'. For IDH1: forward 5'-ATGCAAGGAGATGAAATGACACG-3' and reverse 5'-GCATCAGATTCTCTATGCCTAA-3'. For IDH2: forward 5'-ATCAAGGAGAAGCTCATCCTGC-3' and reverse 5'-TCTGTGGCCTTGTACTGGTCG-3'. For IDH3: forward 5'-CCTCCTGCTTAGTGCTGTGA-3' and reverse 5'-CGTTGCCTCCAGATCTTT-3'.

Extracellular glutamine extraction and LC-MS analysis

Extracellular glutamine was measured using liquid chromatography-mass spectrometry (LC-MS). For this purpose, MSCs were cultured in ST medium, while adipogenic cells were differentiated in AD medium supplemented with 5 mM glutamine over 10 days. Medium samples from untreated, MSCs control (ST medium), AD-treated cultures, and AD blank media (no cells) were collected at day 0, 5 and 10 of treatment. Glutamine was extracted from 250 μ L of each culture medium sample by adding 750 μ L of cold methanol in a ratio of 1 : 4 for protein precipitation. Samples were then mixed vigorously and incubated at -20°C for 20 min. After the incubation period, samples were centrifuged at 13,200 g at 4°C for 10 min and transferred to pre-cooled tubes at 4°C and then stored at -80°C prior to LC-MS analysis. The relative quantification of glutamine was performed on LC-MS as previously described [35]. Briefly, extracts of spent culture media were analysed on an Accela system coupled to an Exactive MS (Thermo Fisher Scientific, Hemel Hempstead, UK). Spectral data was acquired in full scan ion mode (m/z 70-1400, resolution 50 000) and chromatographic separation was achieved using a ZIC-pHILIC (150 mm x 4.6 mm, 5 μ m column, Merck Sequant) maintained at 45°C with a flow rate of 0.3 ml/min. Datasets were then processed with XCMS for untargeted peaks and analysed using mzMatch [36, 37]. Identification of glutamine was based on matching the accurate mass and retention time of the detected glutamine peak with those of the authentic standard of glutamine which was co-analysed with the samples under identical experimental conditions. Univariate one-way ANOVA testing was performed to determine significant compounds changes in the level of glutamine consumed and excreted by MSCs (both in untreated and AD treatment) compared to their medium-only blanks, over time.

Statistical analysis

All datasets were analysed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test, unless otherwise stated. Statistical significance was accepted at $p < 0.05$, with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars plotted on graphs represented the mean \pm SEM. Data were analysed using Graph Pad Prism Software (<https://www.graphpad.com>).

Results

The effect of glucose and/or glutamine restriction on adipocyte differentiation

Before analysing the effect of glutamine supplementation, it was necessary to determine whether glucose, as a source of carbon atoms, might compensate for glutamine during adipogenesis [38] and to examine possible effects of different glucose concentrations on differentiation. To this end, the lipid content was measured in cultures differentiated under 3 medium conditions: (i) glucose-free medium, (ii) low glucose (5.5 mM) medium, and (iii) high glucose (25 mM) medium (Fig. 1). None of the conditions triggered lipid accumulation in the absence of adipogenic supplements on the control medium. In glucose-free AD medium, MSCs were unable to differentiate into adipocytes and accumulate lipids, indicating glucose was required for adipogenic differentiation, even when glutamine was added. In the presence of glucose, at both low and high concentrations, AD medium containing glutamine did support differentiation as seen by the significant increase in lipid content compared to the undifferentiated group, confirming adipocyte differentiation. However, when glutamine synthesis was blocked using MSO treatment, cells were not able to differentiate in response to AD medium even in the presence of glucose supplementation at low or high concentration, showing that adipogenic differentiation is glutamine-dependent.

Effects of glutamine availability on cell viability and adipogenic differentiation

Measurement of the intracellular glutamine concentration (Fig. 2) showed a significant increase in conditions where cells were exposed to medium with glutamine supplementation, while it remained close to undetectable with MSO exposure. Cell viability was not significantly altered in any of the treatment groups (Supplementary Fig. 2), however ORO staining of accumulated lipids confirmed the presence of numerous differentiated cells with lipid droplets in glutamine-treated groups, in a dose dependent manner, which was blocked with MSO treatment (Fig. 3).

Fig. 1. Adipogenic differentiation affected by glucose and glutamine content. Quantitative ORO staining of lipid content in adipocytes differentiated either in medium with no glucose (0 mM), low glucose (5.5 mM) or high glucose (25 mM). Ctrl (untreated cells in ST medium); AD+Glutamine (adipogenic medium supplemented with 5 mM glutamine); AD+MSO (glutamine-free adipogenic medium in conjunction with irreversible GS inhibitor methionine sulfoximine (MSO)). Data expressed with arbitrary units analysed using one-way ANOVA, followed by Tukey's post-hoc test and shown as mean \pm SEM. Statistical significance set at $p < 0.05$, $n = 3$.

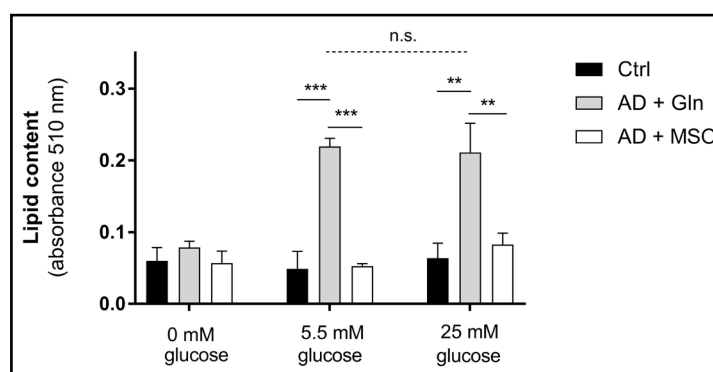


Fig. 2. Intracellular glutamine concentration assay in MSCs-derived adipocytes after 7 days of culture. Cells were treated with MSO or supplemented with 5 mM or 20 mM glutamine. Data analysed using one-way ANOVA, followed by Tukey's post-hoc test and shown as mean \pm SEM. Statistical significance was set at $p < 0.05$, $n = 3$.

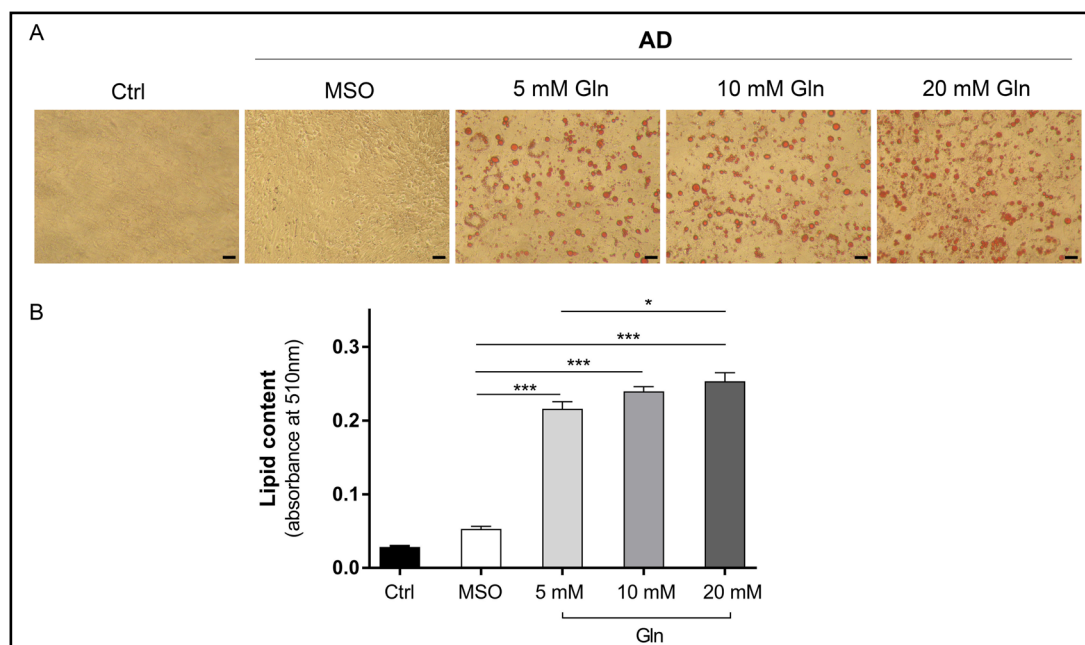
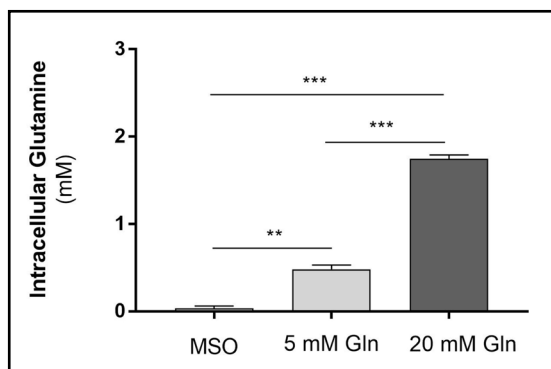


Fig. 3. Adipogenic response in cells treated with different levels of glutamine for 7 days. (A) Representative images of ORO stained cells after 7 days of differentiation treatment with MSO or supplementation with 5, 10, 20 mM glutamine. (B) ORO staining quantitation (arbitrary units). Data analysed using one-way ANOVA, followed by Tukey's post-hoc test and shown as mean \pm SEM. Although statistically significant, the difference between control (Ctrl) and glutamine-supplemented AD groups is not presented in graph. Statistical significance was set at $p < 0.05$, $n = 3$. Gln: glutamine.

Glutamine concentration affects gene expression of common adipogenic markers and isocitrate dehydrogenase enzymes

Gene expression of the adipogenic markers PPAR γ , adiponectin and FABP4 was decreased with exposure to MSO, whereas glutamine supplementation increased gene expression of PPAR γ and adiponectin, while FABP4 was unchanged (Fig. 4A). Exposure to MSO decreased gene expression of IDH1 and IDH3, but not IDH2 (Fig. 4B). Glutamine supplementation to 20 mM (high) had no significant effect on IDH1-3 expression levels.

Changes in glutamine concentration in differentiating culture

The level of extracellular glutamine concentration was measured over 10 days culture in order to evaluate glutamine uptake from the medium containing 5 mM glutamine (Fig. 5). The level of glutamine detected in medium for control cultures (MSCs) was observed to decline over time in undifferentiated cultures, however this decrease was significantly attenuated when cells were treated in AD conditions.

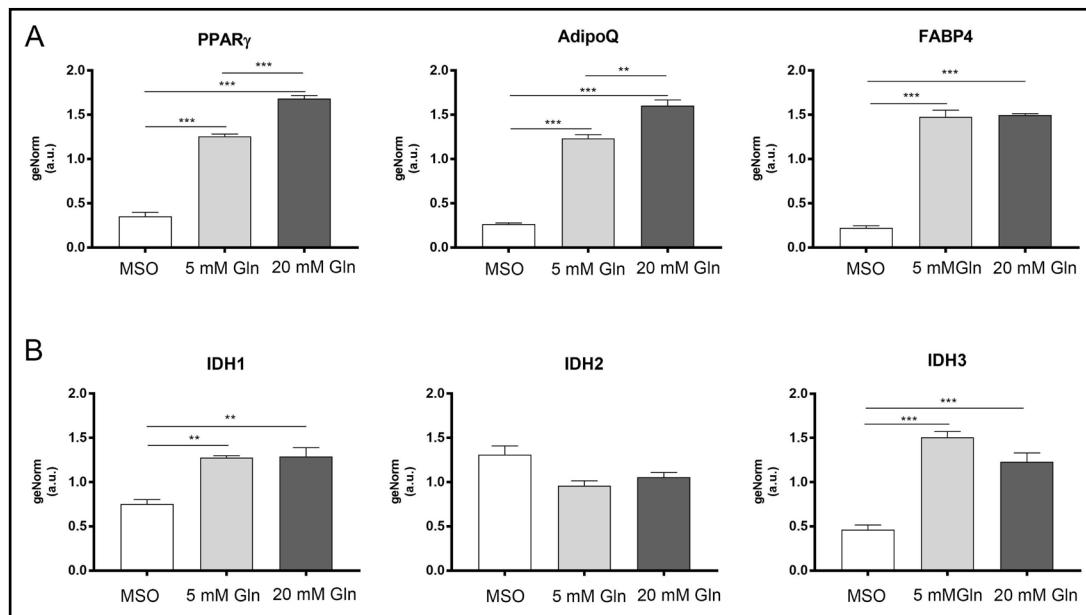
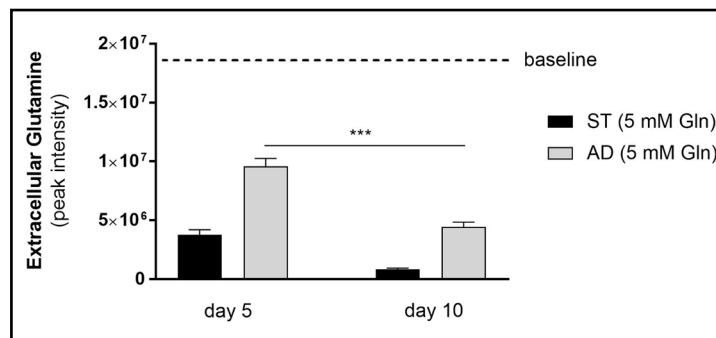


Fig. 4. Relative gene expression of adipogenic markers PPAR γ , AdipoQ and FABP4 (A) and IDHs (B) in AD groups treated with MSO or supplemented with glutamine (5 and 20 mM, respectively). Data represent the mean \pm SEM of three replicates; * p < 0.05; ** p < 0.01; ***p < 0.001.

Fig. 5. Changes in glutamine concentration in the ST and AD culture medium. Effect of treatment time-point (day 5 and 10) on glutamine concentration measured in ST medium, AD medium, and Blank AD medium (no cells) measured as baseline (dashed line). All conditions supplemented with 5 mM glutamine (**p < 0.001, ** p < 0.01, * p < 0.05; n = 6).



Discussion

The differentiation of MSCs into mature adipocytes is a complex process accompanied by diverse morphological changes, with lipogenesis providing the primary structural lipid component [20, 39]. The recognition that glutamine contributes carbon to lipogenic acetyl-CoA [8, 40-42] raises the question of its importance in adipogenic development.

The present study provides new insights on the role of glutamine in adipogenic differentiation of a mesenchymal progenitor cell line. Glutamine deprivation mediated by the irreversible GS inhibitor MSO [30, 31] was observed to prevent adipogenic differentiation, whereas the presence of exogenous glutamine had the opposite effect and increased lipid content. Glucose restriction abolished adipogenesis in a process that could not be compensated by glutamine supplementation to provide acetyl-CoA for lipogenesis. Furthermore, glucose supplementation could not compensate for the glutamine deprivation by MSO treatment, as differentiation was also suppressed, confirming the necessity for both glucose and glutamine in lipogenesis. These findings are thus in accord with Yoo et al, who highlighted distinct roles for glucose and glutamine in lipid synthesis during differentiation in brown adipocyte cultures [38]. They reported that although glucose, rather than glutamine, was the sole carbon source of the glycerol backbone used for the *de novo* lipogenesis, the contribu-

tion of glutamine to lipogenic acetyl-CoA was unexpectedly greater than that of glucose [38]. As shown here, glutamine is necessary for AD differentiation and lipogenesis, with lipid accumulation showing a dose-dependent increase. This was accompanied by increased gene expression of common adipogenic markers PPAR γ and AdipoQ, suggesting a regulatory role of glutamine in beige adipocyte differentiation. Further experiments looking at different adipogenic subtypes could determine whether the role of glutamine differs in brown and white lineages. Glutamine could therefore promote PPAR γ transcription in adipocytes as shown in intestinal epithelial cells [11, 43]. Since PPAR γ and AdipoQ are not only involved in differentiation [20] but also in glucose and lipid metabolism [44, 45], mitochondriogenesis [46] and thermogenic function [47], glutamine may also affect other aspects of adipocyte function. The study of glutaminase knockout/inhibitor models could provide additional information on the contribution of glutamine to adipogenesis, in light of recent studies showing a role for glutaminase in skeletal stem cell differentiation to the adipogenic lineage [48].

The importance of glutamine in AD differentiation was further supported by measurements of both intracellular and extracellular glutamine concentrations. A decrease in extracellular levels as differentiation progresses suggests adipogenic cells could take up extracellular glutamine during adipogenesis via glutamine transporters as identified in rodent and human adipocytes [49, 50]. Furthermore, based on current literature, it seems that the effects of MSO on intra- and extracellular amino acids/metabolites might differ depending on the cell and/or organ type [31, 51-54], which should be considered in future studies.

Although all three IDH isoforms catalyse the same essential conversion of isocitrate to α -ketoglutarate [55], each has a unique role in maintaining cellular redox and energetic status [56]. Our results indicate that IDH1 may play a central role in lipid metabolism, extending earlier studies showing both IDH1 and IDH2 contribute to lipid synthesis [7, 57]. IDH1 was found to be the dominant regulator in lipid synthesis in both non-malignant and malignant cells [58-60]. In contrast, IDH3 is a mitochondrial enzyme that plays a central role in the TCA cycle, promoting oxidative phosphorylation to generate ATP [55]. Cells with IDH3 mutations exhibit reduced maximal and reserve capacity [61], which are direct measures of mitochondrial function and ability to adapt to respiratory stress [61, 62]. Based on the present IDH3 gene expression results, we hypothesise that glutamine deprivation has adverse effects on mitochondrial function, while the upregulation of both IDH1 and IDH3 gene expression may be indicative of glutamine supporting both lipogenesis and ATP production during AD differentiation. Further experiments are now required to determine the contribution of glutamine to both pathways and other aspects of adipogenesis. Analysis of intra- and extracellular metabolites, in particular glutamate, α -ketoglutarate and isocitrate could provide useful information on the link between glutamine and the processes of energy production and lipid synthesis during adipogenesis.

Conclusion

In summary, these latest results provide first evidence that glutamine restriction abolishes adipocyte differentiation *in vitro* and is thus essential for this process. Future studies will examine whether glutamine can contribute to uncoupled respiration and could evaluate whether manipulating glutamine metabolism may represent a possible therapeutic strategy to reduce obesity.

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Author contributions

KV, HALL, MS and VS designed the experiments. KV and HALL carried out cellular and molecular analyses. AS and DHK performed the experimental study on extracellular metabolite detection. KV, HALL, MS and VS finalized the manuscript with input from HS, AS and DHK. All authors contributed to the scientific review and discussion of the study.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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