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https://doi.org/10.1007/s11427-024-2761-y

RESEARCH PAPER

Multi-omics analysis revealed the addiction to glutamine and susceptibility to *de novo* lipogenesis of endometrial neoplasm

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Received 25 January 2025; Accepted 20 April 2025; Published online 24 July 2025

The endometrium is a proliferative tissue controlled by the menstrual cycle. Endometrial hyperplasia (EH) is a type of neoplastic disease that may develop into endometrial hyperplasia with atypia (EHA) or endometrial adenocarcinoma (EA). We performed a multi-omics analysis of a collection of endometrial tissues with four different proliferative statuses from two independent cohorts of patients. A positive association between the level of glutamine and malignancy, as well as addiction of EHA/EA neoplasms to glutamine, was identified. Further investigation revealed the dual mechanism by which glutamine influences the development of endometrial neoplasms. On one hand, glutamine regulates the level of c-MYC by controlling its translational process. On the other hand, glutamine is a major source of energy for endometrial neoplasms. Reprogramming the glutamine metabolism towards *de novo* lipogenesis affects the growth of endometrial adenocarcinoma *in vitro*, *ex vivo* and *in vivo*. Our study revealed the importance of maintaining metabolic homeostasis in endometrial tissues. The enhancement of *de novo* lipogenesis is a promising therapeutic strategy for treating endometrial adenocarcinoma.

endometrial cancer | glutamine | lipogenesis

INTRODUCTION

The endometrium is a proliferative tissue controlled by the menstrual cycle. Endometrial hyperplasia (EH) is a type of neoplastic disease in women and is characterized by abnormal thickening of the endometrium in the uterus. It is a pathological condition that is characterized by abnormal proliferation compared with that in the proliferative phase or after oestrogen stimulation. This condition usually manifests as abnormal vaginal bleeding in patients. The diagnosis of EH is based on histological changes, including a crowded proliferative endometrium and an abnormally high gland-to-stroma ratio, with or without gland crowding and distortion (Auclair et al., 2019; Ring et al., 2022; Sanderson et al., 2017; Sobczuk and Sobczuk, 2017). Specifically, abnormal proliferation of glandular cells and the presence of atypia cells in patients with EH are typical signs of endometrial hyperplasia with atypia (EHA). Atypia cells are characterized by substantial nuclear enlargement and prominent basophilic nucleoli. The progression of EHA to endometrial adenocarcinoma (EA) has been widely observed in patients (Campbell and Barter, 1961; Kurman et al., 1985). In many situations, patients have a mixture of EHA and EA pathology simultaneously (Matsuo et al., 2015; Nees et al., 2022; Sorosky, 2012). Surgical removal of the uterus used to be the recommended therapy for EHA/EA. In recent decades, several efforts have been made to improve clinical practices. For example, much milder therapies such as progestin treatment have been developed to preserve the reproductive ability of patients (Armstrong et al., 2012; Chandra et al., 2016; De Rocco et al., 2022; Reed et al., 2009). However, the molecular mechanisms underlying the pathogenesis of EHA/EA remain unknown.

c-MYC is recognized as one of the most important proteins for the development of neoplasms (Dang, 2012; Dhanasekaran et al., 2022). Regulating the level of c-MYC, transcriptionally or post-transcriptionally, has been demonstrated to be a promising way to prevent the development of cancer (Delmore et al., 2011; Hung et al., 2021). It has been reported that the inhibition of c-MYC prevents the proliferation of endometrial adenocarcinoma, although comprehensive studies on its mechanism are rather limited (Pang et al., 2022; Qiu et al., 2016). Although the expression of c-MYC in EH was also investigated a few decades ago (Bai et al., 1994; Bircan et al., 2005), scientists have not

Citation: Zhu, Y., Zheng, Y., Zhang, Q., Chen, L., Ouyang, Y., Song, Z., Zhao, B., Gu, C., Kong, B., Huang, H., et al. (2025). Multi-omics analysis revealed the addiction to glutamine and susceptibility to *de novo* lipogenesis of endometrial neoplasm. Sci China Life Sci 68, 2923–2933. https://doi.org/10.1007/s11427-024-2761-y

reached a conclusion regarding its role in the development of EHA/EA.

The correlation between metabolic disorders and endometrial neoplasms has been widely observed in various clinical studies (Liu et al., 2021; Mooney and Sumithran, 2023; Onstad et al., 2016; Yang and Wang, 2019; Zhu et al., 2024). In particular, several studies reported that weight loss induced by various factors, such as bariatric surgery, was associated with prolonged survival in patients with EHA/EA (Lee et al., 2022; Upala and Anawin, 2015), although such an association has not been reported in other studies (Mahvi et al., 2021). These close correlations indicate an important role of metabolism in the development of endometrial neoplasms. However, there is a lack of mechanistic studies on how metabolic disorders regulate the development of endometrial neoplasms.

The development of multi-omics assays, including next-generation sequencing, metabolomics and lipidomics assays, has dramatically accelerated the progress of biomedical research, including studies of diseases affecting women (Guo et al., 2023; Lu et al., 2022b). Several biomarkers for the diagnosis and prognosis of endometrial neoplasms have been identified on the basis of these technologies (Jha et al., 2017; Li et al., 2022; Sone et al., 2021). However, there is a lack of in-depth and comprehensive analyses of the endometrium under various physiological or pathological conditions, which may increase our understanding of the biological properties of endometrial neoplasms in a high-throughput and unbiased manner.

Through multi-omics analysis of endometrial samples from two independent cohorts of patients and cellular experiments, we identified the pivotal role of glutamine/glutamate in the development of endometrial neoplasms. Reshaping the glutamine metabolism towards *de novo* lipogenesis affects the growth of endometrial adenocarcinoma cells *in vitro* and *in vivo*. Our study suggests that reprogramming glutamine metabolism is a promising therapeutic strategy for endometrial neoplasms.

RESULTS

Elevation of glutamine/glutamate in endometrial neoplasms

Endometrial samples in different proliferative statuses, including normal endometrium in the proliferative phase (NE), estrogen stimulated endometrium (EE), EH and EHA/EA, were analyzed via targeted metabolomics (Figure 1A; Table S1). Two-way ANOVA test identified 95 differentially abundant metabolites (Table S2 and Figure S1A). Partial least squares discriminant analysis (PLS-DA) revealed dramatic differences in cellular metabolism among the groups (Figure 1B), as did the top 10 metabolites in the clustering analysis (Figure S1B). The top-50 differentially abundant metabolites are presented in a heatmap (Figure 1C; Table S3).

Functional enrichment analysis revealed the enrichment of metabolites related to amino acid metabolism in the EH and EHA/EA samples (Figure S1C). Notably, specific increases in glutamine and glutamate levels were detected in the EH and EHA/EA samples (Figure 1D). However, the relative abundance of serine and aspartate, two important amino acids involved in glutamine-dependent cancer development (Zhang et al., 2017), did not change (Figure S1D and E). In advance, we observed the elevated expression of proteins for glutamine transportation in the EHA/EA samples compared with the normal endometrial sample, based on the results of proteomics analysis from public databases (Geffen et al., 2023; Li et al., 2023) (Figure 1E).

We further investigated the correlation between glutamine/glutamate levels and the malignancy of endometrial neoplasms in a larger independent cohort of 52 patients with EHA/EA (Figure 1F). The relative abundances of multiple metabolites in endometrial tissues were measured via targeted metabolomics. We observed that the higher mean relative abundances of glutamine and glutamate in the endometrial samples (referred to as GLUTs) were associated with a higher Ki67-positive rate, more advanced stage and diffusion of EHA/EA (Figure 1G–I). In contrast, such correlations between malignancy and relative abundance were absent for serine or aspartate (Figure S1F and G). These data indicate that the level of glutamine/glutamate was positively correlated with the developmental process and malignancy of endometrial neoplasms.

Glutamine regulates the synthesis of c-MYC

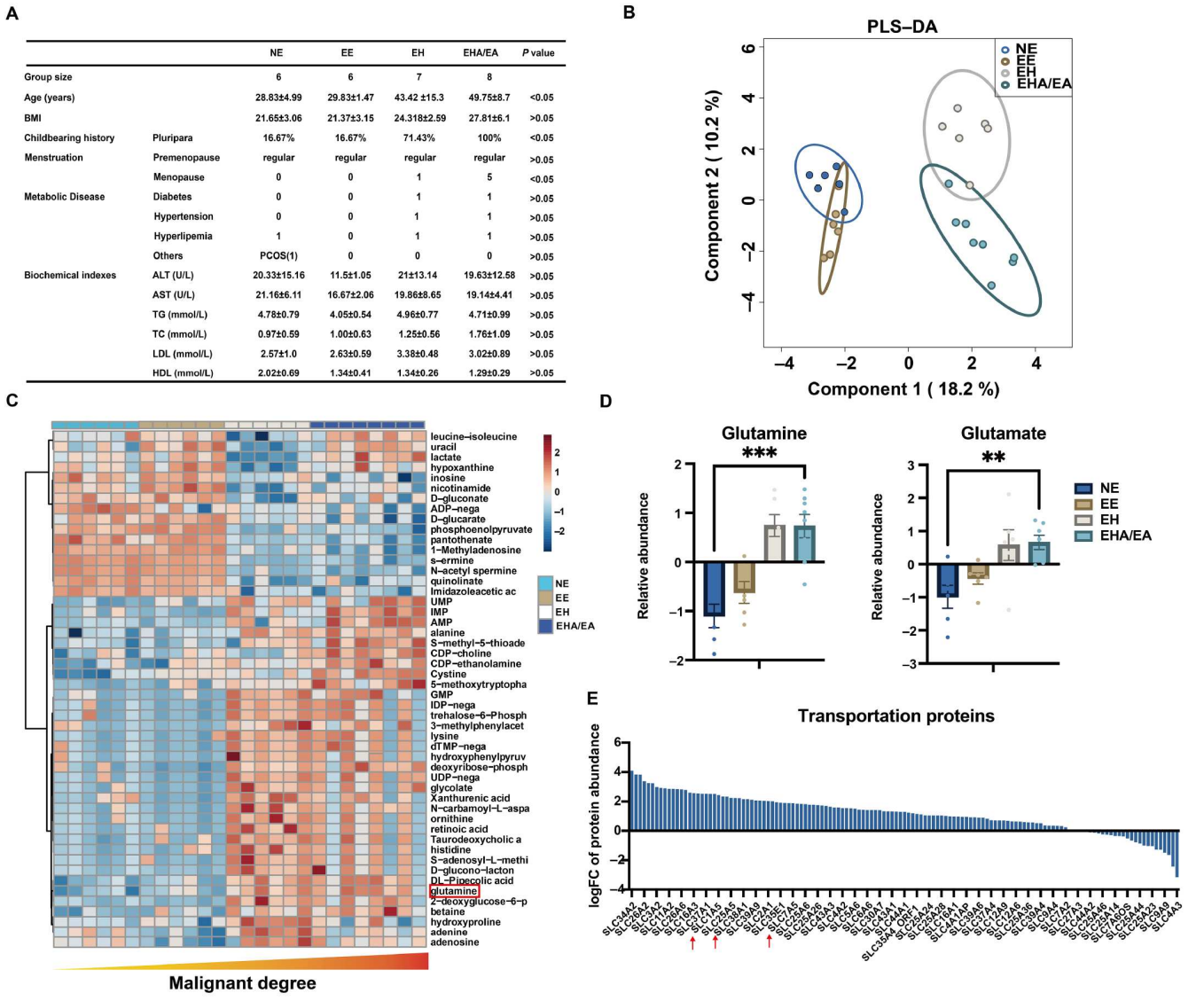
The metabolic status of cells affects their transcriptional status, as metabolites interact with transcription factors (Bishop and Ferguson, 2015; Hardy and Tollefsbol, 2011; Stine et al., 2015). We therefore investigated the transcriptional features of endometrial samples in four different proliferative stages via an RNA-seq assay. Principal component analysis (PCA) revealed dramatic differences in their transcriptomes (Figure 2A; Table S4). Differential expression analysis (DEA) was performed for EE *vs*. NE, EH *vs*. NE and EHA/EA *vs*. EH. Various numbers of genes whose expression significantly changed were identified (Figure 2B; Figure S2A–C). The top-50 differentially expressed genes were presented in a heatmap (Figure S2D–F). We observed a trend towards increased expression of genes encoding proteins involved in glutamine transportation in the EHA/EA samples (Figure S2G). In particular, Gene set enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) revealed that genes whose expression was upregulated in the EH or EHA/EA samples, compared with the NE samples, were involved in the translational process (Figure S2H and I).

It is widely accepted that the metabolism of amino acids, including asparagine and serine/glycine, has important impacts on the translational process and may control the development of cancer (Gheller et al., 2021; Pavlova et al., 2018). We therefore explored the specific protein(s) whose translation may be regulated by glutamine metabolism in endometrial neoplasms. GSEA revealed the upregulation of c-MYC-targeted genes in the EHA/EA samples (Figure 2C). Consistently, the protein expression of c-MYC was upregulated in higher-grade EHA/EA samples, as shown by the results of proteomics analysis using public databases (Geffen et al., 2023; Li et al., 2023) (Figure S3A). In addition, upregulation of the c-MYC protein was detected in the EHA/EA samples compared with the NE samples via Western blot analysis (Figure 2D). However, the mRNA expression of *MYC* was not elevated in the EHA/EA samples (Figure S3B). GSEA of RNA-seq data from glutamine-treated Ishikawa cells, an EA cell line, also revealed the upregulation of genes related to ribosomes (Figure S3C and Table S5). These results indicate the enhancement of translation by the glutamine supplement.

We then validated the effects of glutamine on c-MYC protein abundance via a cellular assay. Glutamine supplement increased the c-MYC protein level in Ishikawa cells, which was abolished by

A

		NE	EE	EH	EHA/EA	*P* value
Group size		6	6	7	8	
Age (years)		28.83±4.99	29.83±1.47	43.42 ±15.3	49.75±8.7	<0.05
BMI		21.65±3.06	21.37±3.15	24.318±2.59	27.81±6.1	>0.05
Childbearing history	Pluripara	16.67%	16.67%	71.43%	100%	<0.05
Menstruation	Premenopause	regular	regular	regular	regular	>0.05
	Menopause	0	0	1	5	<0.05
Metabolic Disease	Diabetes	0	0	1	1	>0.05
	Hypertension	0	0	1	1	>0.05
	Hyperlipemia	1	0	1	1	>0.05
	Others	PCOS(1)	0	0	0	>0.05
Biochemical indexes	ALT (U/L)	20.33±15.16	11.5±1.05	21±13.14	19.63±12.58	>0.05
	AST (U/L)	21.16±6.11	16.67±2.06	19.86±8.65	19.14±4.41	>0.05
	TG (mmol/L)	4.78±0.79	4.05±0.54	4.96±0.77	4.71±0.99	>0.05
	TC (mmol/L)	0.97±0.59	1.00±0.63	1.25±0.56	1.76±1.09	>0.05
	LDL (mmol/L)	2.57±1.0	2.63±0.59	3.38±0.48	3.02±0.89	>0.05
	HDL (mmol/L)	2.02±0.69	1.34±0.41	1.34±0.26	1.29±0.29	>0.05



F

		Ki–67<50%	Ki–67≥50%	*P* value
Group size		29	23	
Age (years)		56.14±10.21	59.17±11.84	>0.05
Menstruation	irregular	2	2	>0.05
Metabolic Disease	Diabetes	3	2	>0.05
	Hypertension	3	8	0.0439
	CHD	1	0	>0.05
Stage	I	23	14	>0.05
	II	1	4	
	III	4	5	
	IV	1	0	
Grade	Low	2	6	0.088
	Middle	3	8	
	High	33	9	
Type	Regional	11	5	>0.05
	Diffuse	5	10	

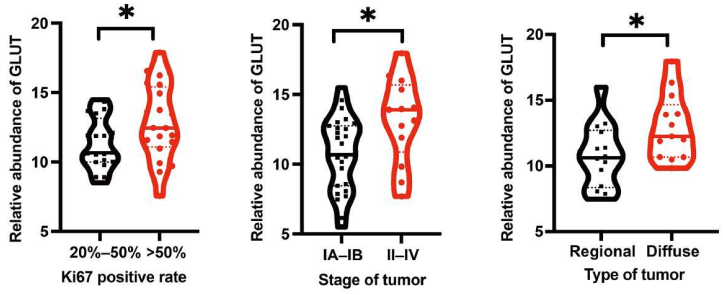
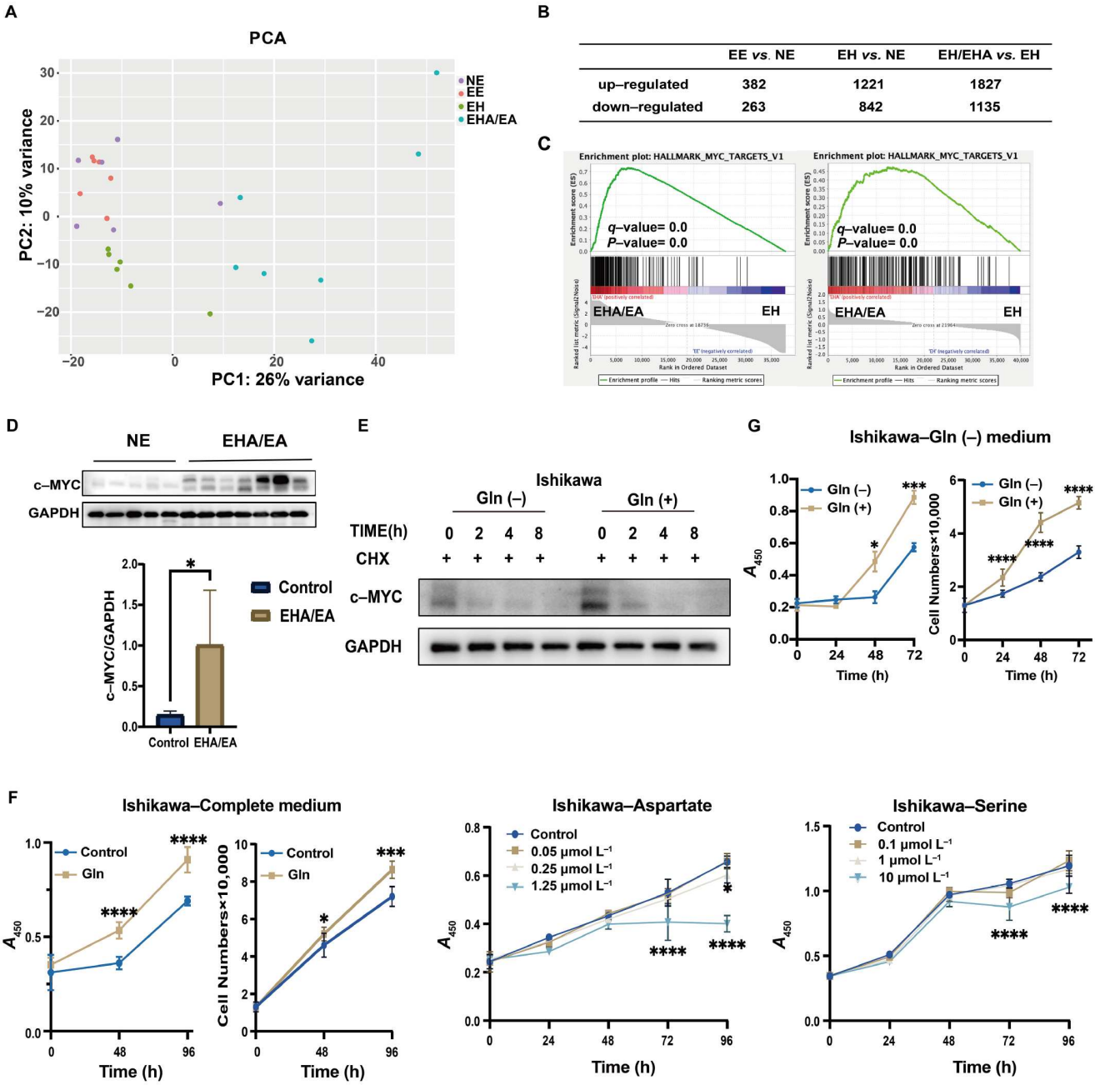


Figure 1. The increase of glutamine/glutamate levels in endometrial neoplasms. A, Basic information of the patients in the four-stage cohort. B, PLS-DA of the targeted metabolomics data. C, Heatmap of the top-50 differentially abundant metabolites. D, Relative abundances of glutamine and glutamate in various samples. n=6–8 (NE, n=6; EE, n=6; EH, n=6; HEA/EA, n=8). E, Expression of glutamine transporters (highlighted in red) in the EHA/EA samples compared with the normal samples. F, Basic information of the patients in the EHA/EA cohort. G–I, Relative abundance of GLUTs (mean of glutamine and glutamate) in the EHA/EA samples with different Ki-67-positive rates (G, n=20), stages (H, n=13–27) or types (I, n=13–14). *, P<0.05; **, P<0.01; ***, P<0.001.

treatment with the translation inhibitor cycloheximide (CHX) but not the proteasome or calpain inhibitor MG132 (Figure S3D and E). As the upregulation of c-MYC can be prevented by CHX, translation is likely the key factor contributing to the regulation



	EE *vs.* NE	EH *vs.* NE	EH/EHA *vs.* EH
up–regulated	382	1221	1827
down–regulated	263	842	1135

Figure 2. Glutamine regulates the translational process of c-MYC. A, PCA of transcriptome data from various types of samples. B, Summary of differentially expressed genes among various types of samples. C, The results of GSEA of the RNA-seq data. D, Levels of c-MYC protein in NE and EHA/EA samples. E, Effects of glutamine supplementation, with or without CHX treatment for various durations, on the level of c-MYC protein in Ishikawa cells. F, Growth curve of Ishikawa cells with glutamine (measured by CCK-8 assay or counting the number of cells), serine, or aspartate supplementation. n=6. G, Growth curve of Ishikawa cells deprived of glutamine, as measured by a CCK-8 assay or by counting the number of cells. n=6. *, P<0.05; ***, P<0.001; ****, P<0.0001.

of c-MYC protein expression by glutamine supplementation. In comparison, glutamine deprivation decreased the protein level of c-MYC (Figure S3F). The effects of CHX on the translation of glutamine-regulated c-MYC translation were time-dependent in both Ishikawa cells and AN3CA cells, another EA cell line (Figure 2E; Figure S3G). Moreover, glutamine supplementation had no obvious effect on the mRNA expression of *c-MYC* in Ishikawa cells (Figure S3H). These data suggest that glutamine enhances the translation of c-MYC. Functionally, supplementation with glutamine, but not serine or aspartate, promoted the growth of Ishikawa cells and AN3CA cells (Figure 2F; Figure S3I). However, glutamine deprivation inhibited the growth of Ishikawa cells and AN3CA cells (Figure 2G; Figure S3J). These results suggest that glutamine controls the translation of c-MYC and cell proliferation in EHA/EA.

Glutamine is a major source of energy metabolism for endometrial neoplasms

Amino acids usually have diverse functions in cells. Driven by

https://doi.org/10.1007/s11427-024-2761-y

curiosity, we further explored the mechanism by which glutamine regulates the development of endometrial neoplasms in addition to affecting c-MYC translation. By employing a targeted metabolomics assay, we discovered that both glutamine deprivation and the inhibition of c-MYC by JQ1 treatment led to dramatic changes in cellular metabolism (Figure S4A, B and Tables S6 and S7). The top-50 differentially abundant metabolites are presented in heatmaps (Figure S4C and D). The metabolites whose expression significantly changed by at least 1.25-fold are presented in volcano plots (Figure S4E and F). The enrichment analysis revealed that the metabolites altered by glutamine deprivation, but not by JQ1 treatment, were associated with the TCA cycle (Figure S4G and H).

We then specifically investigated the changes in metabolites related to energy metabolism. We found that inhibition of c-MYC by glutamine deprivation, but not the inhibition of c-MYC by the treatment of JQ1, significantly decreased the abundance of TCA cycle-related metabolites and increased the abundance of lactate (Figure S5A and B). As changes in energy metabolism may have considerable effects on cell proliferation, these results indicate the complex role of glutamine, in addition to its effects on c-MYC translation, in the development of endometrial neoplasms.

In addition to serving as signaling factors, amino acids are also important sources of energy via their catabolism to TCA-related metabolites. Interestingly, the chemical inhibition of glutaminolysis decreased the proliferation rate and the levels of TCA-related metabolites in Ishikawa cells (Figure 3A and B; Table S8). In addition, the GSEA of the RNA-seq data revealed increased expression of genes related to oxidative phosphorylation in the EHA/EA samples compared with the EH samples (Figure 3C). Notably, glutamine supplementation led to a dramatic increase in the metabolic rate (Figure 3D) in Ishikawa cells compared with glucose (Figure S5C) and lactate (Figure S5D) supplementation. These data highlight the unique roles of glutamine in energy metabolism.

We further investigated the effects of glutamine on energy metabolism with a metabolic labeling assay based on fully C13-labelled metabolites and targeted metabolomics (Figure 3E). Most of the C13-labelled glucose was converted into lactate but not α-ketoglutarate (Figure S5E and Table S9), indicating that a large part of the glucose did not directly participate in the TCA cycle. Similarly, we detected a low conversion rate of C13-labelled lactate to either pyruvate or α-ketoglutarate (Figure S5F and Table S10), suggesting that glucose or lactate may not be a major contributor to the TCA cycle in Ishikawa cells. Consistently, neither glucose nor lactate supplementation promoted the proliferation of Ishikawa cells (Figure S5G and H). In contrast, the conversion rates of C13-labelled glutamine to α-ketoglutarate and malate were remarkably higher (Figure 3F; Table S11). Consistently, we observed a positive correlation between the abundances of GLUT and α-ketoglutarate in the EHA/EA samples, which was much greater than the correlation between the abundances of glucose-6-phosphate or lactate and α-ketoglutarate (Figure 3G). These results suggest that glutamine is an important source of energy for EHA/EA.

Reshaping glutamine metabolism towards *de novo* lipogenesis prevents the development of endometrial neoplasms

Considering the specific role of glutamine in the development of endometrial neoplasms, we manipulated the intracellular abundance of glutamine/glutamate by targeting its transporter. As a proof of concept, we targeted the *SLC1A5* gene, which is highly expressed in Ishikawa cells according to RNA-seq analysis (Figure S6A) and encodes a glutamine transporter. Encouragingly, the application of a SLC1A5 inhibitor (Figure S6B) or genetic depletion of *SLC1A5* (Figure S6C and D) dramatically prevented the proliferation of Ishikawa cells. Owing to the important role of glutamine in normal tissue homeostasis and the difficulties associated with the tumour-specific delivery of SLC1A5 inhibitors, it is unrealistic to apply this strategy in clinical settings.

Amino acids can also be catabolized and then synthesized into lipids via *de novo* lipogenesis. Interestingly, untargeted lipidomic analysis revealed decreases in the relative abundance of triglyceride (TG) and diglyceride (DG) in the EHA/EA samples compared with the EH samples (Figure 4A; Table S12). We therefore tested whether the intracellular lipid content had any effects on the proliferation of endometrial adenocarcinoma. The intracellular lipid content can be affected by exogenous lipid uptake or endogenous *de novo* lipogenesis. Directly manipulating the intracellular lipid level via supplementation with oleic acid (OA) or inhibition of DGAT1/2 (Qi et al., 2012) did not affect the proliferation of Ishikawa cells (Figure S6E and F).

Inhibition of the liver X receptor (LXR) has been proposed as an important regulator of *de novo* lipogenesis in a tissue-specific manner (Korach-André et al., 2011; Wang and Tontonoz, 2018). Treatment with the LXR antagonist SR9243 promoted the proliferation of Ishikawa cells (Figure S7A), although combination treatment with SR9243 and EGCG had effects similar to those of EGCG treatment alone (Figure S7B). The relationship between LXR inhibition and glutaminolysis needs to be studied in the future. In contrast, treatment with the LXR agonist LXR623, which increased the abundance of intracellular fatty acids (Figure 4B; Figure S7C), decreased the proliferation of Ishikawa cells and AN3CA cells (Figure 4C; Figure S7D). We observed changes in the expression of classic *de novo* lipogenesis-associated genes in Ishikawa cells and AN3CA cells treated with SR9243 or LXR623 (Figure S7E and F). In contrast, these *de novo* lipogenesis-associated genes were upregulated by LXR623 treatment (Figure S7E and F). These data indicated that the activation of LXR in EHA/EA cells promoted *de novo* lipogenesis and prevented their proliferation.

Targeted metabolomics analysis revealed that LXR623 treatment dramatically changed the metabolism of Ishikawa cells (Figure S7G and Table S13). The top-50 differentially abundant metabolites are presented in a heatmap (Figure S7H). Specifically, the enhancement of *de novo* lipogenesis caused by LXR623 treatment decreased the levels of TCA-related metabolites (Figure 4D). Interestingly, we discovered a trend toward increased expression of multiple genes related to lipogenesis in the EHA/EA samples (Figure S7I). These data show that the enhancement of *de novo* lipogenesis can reprogram cellular metabolism and prevent the growth of EHA/EA.

We further validated our hypothesis *ex vivo* with patient-derived organoids for endometrial adenocarcinoma, which were established in a published study (Ren et al., 2022). The organoids from two patients were used for the analysis. The establishment and validation of these organoids have been described previously (Ren et al., 2022). The basic information of these patients is provided in Figure S8A. Glutamine supplementation promoted

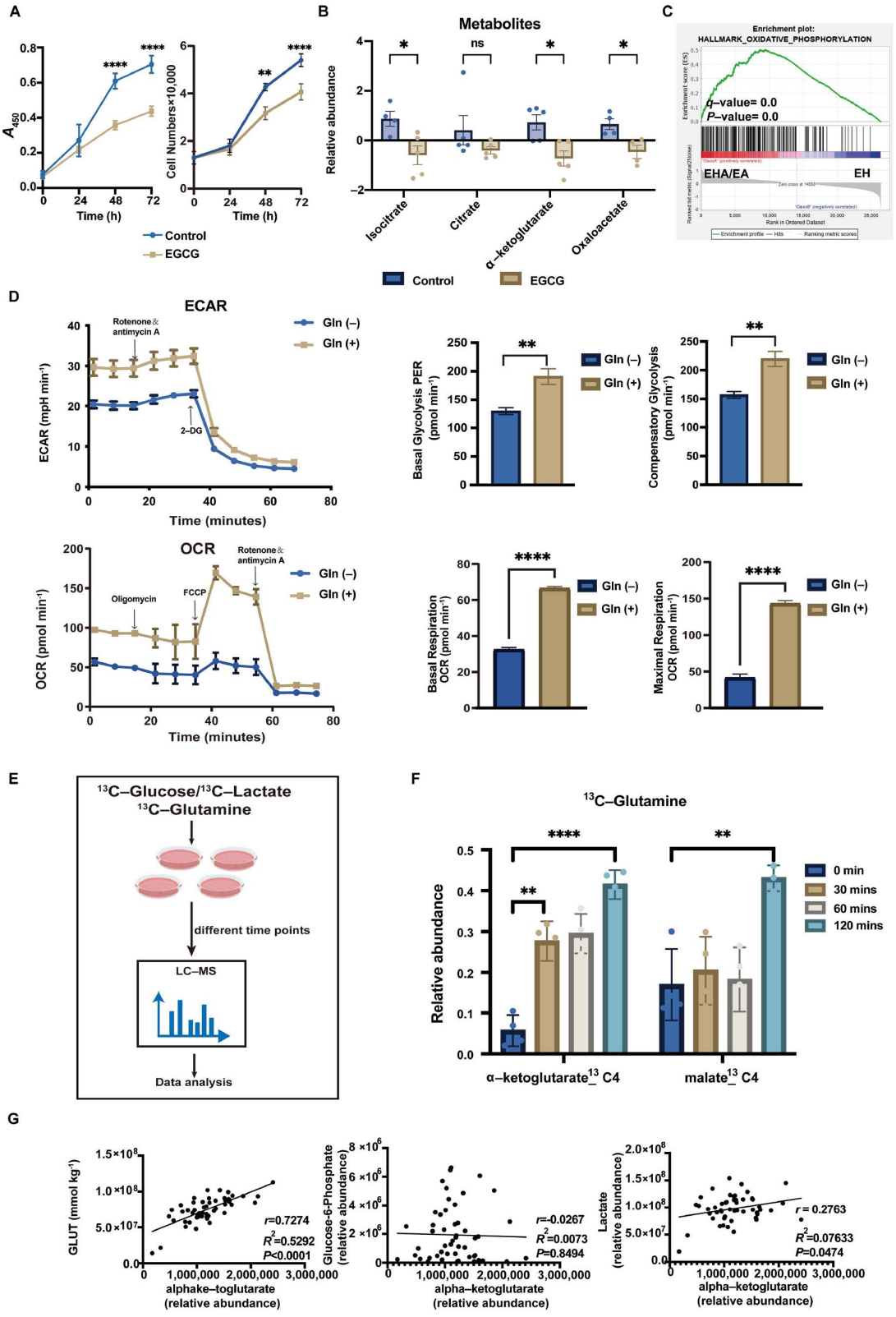


Figure 3. Glutamine is a major energy supplement for endometrial neoplasms. A, Growth curves of Ishikawa cells with or without treatment with the glutaminolysis inhibitor EGCG. *n*=6. B, The relative abundance of TCA-related metabolites with or without treatment with the glutaminolysis inhibitor EGCG. *n*=6. C, GSEA of the RNA-seq data revealed enrichment of genes enriched in the EHA/EA for oxidative phosphorylation. D, Effects of glutamine on Ishikawa cell metabolic rate for ECAR and OCR measured by the Seahorse assay. *n*=3. E, Illustration of the metabolic flux assay based on stable isotope-labelled metabolites. F, Labelling rates of glutamine for α-ketoglutarate and malate in Ishikawa cells. *n*=3–4. G, The correlation of the relative abundance of GLUT (the mean relative abundance of glutamine and glutamate), glucose-6-phosphate, or lactate and α-ketoglutarate in the EHA/EA cohort. *n*=52. *, *P*<0.05; **, *P*<0.01; ****, *P*<0.0001; ns: not significant.

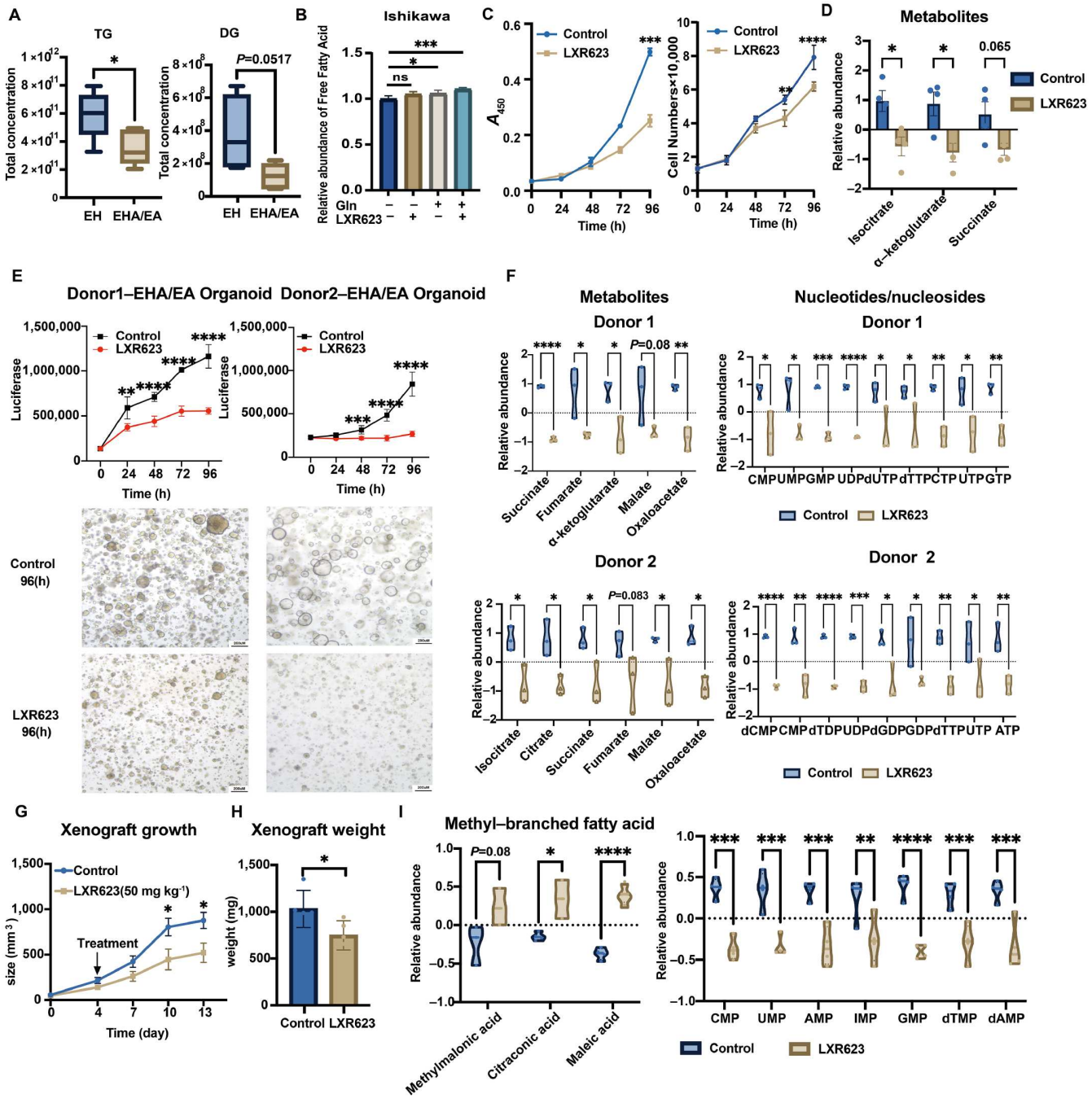


Figure 4. Enhancing *de novo* lipogenesis prevents the development of endometrial neoplasms. A, The relative abundance of TG or DG in the EH and EHA/EA samples of the four-stage cohort. EH, n=6; EHA, n=8. B, The level of intracellular free fatty acids in Ishikawa cells treated with or without the LXR agonist LXR623. n=3. C, Growth curves of Ishikawa cells treated with or without the LXR agonist LXR623 treatment, measured by CCK-8 assay or by counting the number of cells. n=6. D, The relative abundance of TCA-related metabolites in Ishikawa cells treated with LXR623. n=5. E, The growth curve and representative images of EHA/EA organoids with or without LXR623 treatment. Scale bar, 200 μm. n=3. F, Relative abundance of TCA-related metabolites in organoids treated with LXR623. n=3. G and H, The size and weight of Ishikawa cell-based xenografts with or without LXR623 treatment. n=5. I, Increases in the abundance of methyl-branched fatty acids and decreases in the abundance of nucleotide-related metabolites in LXR623-treated xenografts. Control, n=4; LXR 623, n=5. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

the growth of the organoids, but treatment with ECGC prevented their growth (Figure S8B and C). These data confirmed the addiction of EHA/EA to glutamine *ex vivo*. In particular, treatment with LXR623 dramatically prevented the growth of the organoids (Figure 4E; Figure S8D). The results of targeted metabolomics analysis indicated that treatment with LXR623 led to dramatic changes in cellular metabolism (Figure S8E). The top-50 differentially abundant metabolites are presented in a heatmap (Figure S8F). In particular, treatment with LXR623 decreased the relative abundance of TCA-related metabolites as well as various nucleotide/nucleoside-related metabolites (Figure 4F).

We then tested the effects of LXR623 in an Ishikawa cell-based xenograft model. Oral gavage of LXR623 dramatically prevented

the enlargement of the tumour xenografts (Figure 4G and H; Figure S9A). Histological analysis revealed a decrease in Ki67 signals in LXR623-treated xenografts (Figure S9B and C). Targeted metabolomics analysis revealed dramatic changes in cellular metabolism (Figure S9D and Table S14). The top-50 differentially abundant metabolites are presented in a heatmap (Figure S9E). In particular, we observed increases in the abundances of various methyl-branched fatty acids and decreases in the abundances of various nucleotide/nucleoside-related metabolites with LXR623 treatment (Figure 4I). These results suggest that targeting *de novo* lipogenesis is a reasonable strategy for treating EHA/EA.

DISCUSSION

Understanding the biological features of proliferative endometrium in different malignant statuses is key to elucidating the pathogenesis of EA/EHA and developing specific therapies for endometrial neoplasms. In the present study, we identified the abnormally elevated levels of glutamine and glutamate in EA/EHA as an important factor for the development of endometrial neoplasms. Glutamine promoted the growth of the endometrial neoplasms by enhancing c-MYC translation and glutaminolysis simultaneously. Reshaping the metabolism of glutamine towards *de novo* lipogenesis may prevent the development of endometrial neoplasms.

Amino acids are recognized as important types of metabolites involved in the development of tumours. For example, the metabolism of tryptophan has marked effects on T-cell-based immunological regulation of cancer cells (Platten et al., 2019). Aspartate has been recognized as an endogenous inhibitor of cancer growth (Sullivan et al., 2018). The abundance of serine can also significantly impact the outcomes of patients with malignant tumours (Kang, 2020; Montrose et al., 2021; Yang and Vousden, 2016). The relationship between glutamine and cancer, especially the addiction of cancer cells to glutamine, has been reported in lung, liver, or ovarian cancer studies (Edwards et al., 2021; Jin et al., 2020; Wu et al., 2021). The correlation between glutamine/glutamate abundance and endometrial malignancy in our study further highlights the importance of amino acid metabolism, especially for the development of neoplasms in the uterus.

Metabolic reprogramming has been demonstrated to be a promising strategy for the treatment of cancer. Specifically, limiting the intake of dietary amino acids or proteins has been proven to be an effective way to treat various types of cancer (Kang, 2020; Wanders et al., 2020; Yin et al., 2018). However, the lack of amino acid supplementation may also lead to undesired side effects on non-malignant tissues. In the present study, we found that inhibiting the glutamine transporter, whose ectopic expression was identified in endometrial neoplasms, can prevent the proliferation of Ishikawa cells. With the help of tumour-targeted drug delivery technologies, it is possible to develop specific therapies that prevent the uptake of glutamine by endometrial neoplasms.

In addition to regulating the intracellular abundance of amino acids, our study demonstrated that reshaping the metabolism of glutamine to *de novo* lipogenesis can also regulate the development of neoplasms. As obesity is a risk factor for endometrial neoplasms, many previous studies have suggested that the suppression of lipogenesis can be a strategy to prevent the development of cancer (Lu et al., 2022a; Song et al., 2020). However, the findings of our study indicated that the precise enhancement of lipogenesis may actually provide benefits for patients with endometrial neoplasms. The effects of LXR623 on preventing the growth of EHA/EA were not due to lipid toxicity (Gaschler and Stockwell, 2017; Schulze et al., 2016), as the direct supplementation of fatty acids had no specific effects on cancer cell viability. The relationship we revealed between amino acid and lipid metabolism will further inspire the research community to develop novel metabolic therapies for endometrial neoplasms.

In summary, our study revealed the addiction of endometrial neoplasms to glutamine via its roles in c-MYC translation and energy metabolism. The development of metabolic reprogramming-based therapies for endometrial neoplasms is feasible.

MATERIALS AND METHODS

Reagents

The reagents involved in this study are described in Table S15.

Clinical sample collection and organoid establishment

The four-stage cohort with 27 samples was collected from the Obstetrics and Gynecology Hospital of Fudan University. The organoids were established in a published study and the experiments were performed according to the literature (Ren et al., 2022). All patients recruited in the study provided written informed consent. NE, EH and EHA/EA samples were obtained from women who came to the clinic with abnormal bleeding and received a uterine dilation and curettage (D&C) operation. EE samples were collected from infertile patients after estrogen action who routinely undergo hysteroscopy and require an endometrial biopsy.

Cell culture and cell viability assay

The Ishikawa cells were maintained in DMEM medium (Gibco) with 10% FBS (Gibco) and 1% penicillin/streptomycin mixture (Gibco). Ishikawa cells were seeded at a density of 1.5×10^4/mL in 96-well plates. Twenty-four hours later, cell culture media were replaced by serum free DMEM and fasting for 24 h before drug treatments. Then cells were treated with various chemicals for 0, 24, 48, 72 or 96 h. Cell proliferation rates were subsequently assessed using the cell counting kit-8 (CCK-8) (Yeasen, China) according to the manufacturer's instructions.

Western blotting

Ishikawa cells were lysed in 1% NP40 buffer with protease and phosphatase inhibitors. Total protein was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Proteins were then separated by 10% SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Germany). The PVDF membranes with proteins were incubated with specific primary antibodies overnight at 4°C and then incubated with HRP-conjugated secondary antibodies for 60 min. ECL Reagent (Thermo Fisher Scientific) and a Clinx ChemiScope 6000 (Clinx Science Instruments Co., Ltd, China) were used for visualizing protein bands.

RNA-seq assay

Total RNA was extracted from endometrial samples using TRIzol reagent (Thermo Fisher Scientific). Extracted RNA was then reversely transcribed using the PrimeScript™ RT reagent Kit (TaKaRa, USA). RNA-seq libraries for expression analysis were constructed using KAPA RNA HyperPrep Kit KR1350 v1.16 according to the vendor's protocol and paired-end 2× 150 bp reads were sequenced using the Illumina HiSeq platform. The raw data were aligned and quantified by HISAT2 (Kim et al., 2019). The raw data will be deposited in the Gene Expression Omnibus once the manuscript is accepted. Differentially expressed genes were determined by DESeq2 (Love et al., 2014) and P value<0.05 and Log_2 fold change >2 were recognized as the cutoff.

Lipid and metabolite extraction

The extraction method was modified from a published article (Huang et al., 2020) and briefly described here. Endometrial samples were homogenized in 700 μL High Performance Liquid Chromatography (HPLC) grade water/methanol (2/5) mixture by tissue grinder for 2 min at 4°C, and transferred to a new glass centrifuge tube. Then 500 μL HPLC grade methanol, and 5 mL HPLC methyl tert-butyl ether (MTBE) were vortexed for 1 min. The mixture was then incubated on a rotator for 1 h at room temperature, then 1.25 mL HPLC grade water was mixed and vortexed for 1 min. The mixture was separated into two phases after centrifuging at 1,000×g for 10 min at 4°C; non-polar lipids and aqueous metabolites were contained in the upper phase and the lower phase, respectively. A SpeedVac was used to dry the non-polar lipids and aqueous metabolites at room temperature. The dried lipids or metabolites were stored at −80°C until analysis by Liquid Chromatograph Mass Spectrometer (LC-MS).

Untargeted lipidomics

A mixture of 2-propanol/acetonitrile/water (30/65/5; 200 μL) was used to reconstitute the nonpolar lipids. A 5 μL reconstituted sample was used for analysis by Liquid Chromatograph Mass Spectrometer (LC-MS). The untargeted lipidomics method was modified from a published method (Breitkopf et al., 2017) by using a C30 column (Acclaim C30, 3 μm, 2.1 mm×150 mm). The mass-spec data were acquired using an Orbitrap Exploris 480 (Thermo Fisher Scientific) utilizing the polarity switching approach with the data-dependent acquisition (DDA) mode. All lipidomics RAW files were processed on *LipidSearch* 4.0 (Thermo Fisher Scientific) for lipid identification.

Targeted metabolomics

A total of 100 μL acetonitrile/water (50/50) was used to reconstitute the aqueous metabolites. A 5 μL reconstituted sample was used for analysis by LC-MS. The targeted metabolomics method was modified from a published protocol (Yuan et al., 2012) by using an amide Hydrophilic interaction liquid chromatography (HILIC) column (XBridge Amide 3.5 μm, 4.6 mm×100 mm). The mass data were acquired by a QTRAP 5500+ (AB Sciex, USA) using the polarity switching approach. The LC-MS/MS peak integration was determined on MultiQuant (AB Sciex) and data were transferred to the metabolomics spreadsheet.

Metabolomic and lipidomic data analyses

The MetaboAnalyst 5.0 and LINT-web websites were utilized to analyze metabolomic and lipidomics results. In brief, the data was normalized to the median value, log-transformed and auto-scaled. Then the relative abundance was calculated based on the processed data. The details of how these tools work were described in the literature (Li et al., 2021; Pang et al., 2020).

^{13}C-labeled glucose/lactate/glutamine treatment

The Ishikawa cells were seeded in 10 mm plates at a density of 2×10^5/mL. Fasting with high DMEM medium without serum, glutamine, pyruvate and lactate for 12 h, the cell culture media were replaced by complete DMEM contained ^{13}C-labeled glucose (10 mmol L^{-1})/lactate (20 mmol L^{-1})/glutamine (4 mmol L^{-1}), and extracted cell metabolites at different time points. Cell metabolites were analyzed by targeted Metabolomics described above.

RT-qPCR

Total RNA was extracted from Ishikawa cells using TRIzol (Thermo Fisher Scientific). Extracted RNA (500 ng) was converted into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa). Quantitative RT-PCR (qRT-PCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems, USA) in an Applied Biosystems QuantStudio 5. The internal reference gene *ACTB* served as the internal reference gene. The primer sequences are provided in Table S15.

Metabolic rate measurement in seahorse experiment

In vitro cell metabolic alterations, such as OCR and ECAR, were monitored with the Seahorse XFp Flux Analyzer (Seahorse Bioscience, USA), according to the manufacturer's instructions.

CRISPR-mediated gene knockouts

Cas9-expressing Ishikawa cells were generated by infecting cells with lenti-cas9-Blast (Addgene). The infected cells were selected with blasticidin (10 μg mL^{-1}) for at least 5 d. We then infected the Cas9-expressing Ishikawa cells with LentiGuide-Puro carrying sgRNAs targeting negative control regions or the coding region of *SLC1A5*. The infected cells were selected with puromycin (2 μg mL^{-1}) for at least 2 d before experiments. The sgRNA sequences are provided in Table S15.

Animal experiments

Six-week-old BALB/c nude mice were obtained from GemPharmatech. Mice were housed and adapted to the environment for one week before the experiment. A total of 1×10^7 of Ishikawa cells were suspended in 100 μL PBS and subcutaneously injected into nude mice. Mice were divided into 2 groups when the xenograft grew to a diameter of about 5 mm. Mice in the experimental group were given 50 mg kg^{-1} LXR623 daily by gavage treatment, and the control group was given placebo treatment daily. Body weight and transplantation tumor size were measured every 2–3 d. The experiment was terminated when the maximum diameter of the tumor reached 1.5 cm. The

tumor was peeled intact for analysis.

Histology

The procedure of immunohistochemistry (IHC) was described briefly as follows: the xenograft was fixed in 10% formalin for paraffin-embedding, sectioning and staining. Picrosirius red staining was used to identify the collagen histochemistry with a picro-sirius red reagent (Servicebio, China). The Ki67 staining used an anti-Ki67 mouse monoclonalantibody (mAb) (Servicebio) and the secondary antibodies are HRP-goat antirabbit IgG (Servicebio). The sections were reacted with DAB kit (DAKO, Denmark). Histochemical staining of the xenograft was visualized with a microscope (Chongqing COIC Industrial Co., Ltd) using a 20× objective lens.

Statistics

GSEA was performed according to its guidelines (Mootha et al., 2003; Subramanian et al., 2005). The chi-square test was used for the c-MYC positive rate analysis in Figure S3A. Student's *t*-test with two-way ANOVA correction was used in this study unless specified.

Data availability statement

The raw data will be provided by Jin Li (li_jin_lifescience@fudan.edu.cn) upon reasonable request.

Compliance and ethics

The authors declare that they have no conflict of interest. This study was approved by the Institutional Review Board of Obstetrics and Gynecology Hospital of Fudan University (2021-132). The EHA/EA cohort with 52 samples was collected during the surgeries in Qilu Hospital of Shandong University and approved by the Institutional Review Board (KYLL-202209-049-1). All animal experiments were performed according to procedures approved by the Obstetrics and Gynecology hospital of Fudan University ethical committee.

Acknowledgement

This work was supported by the National Key Research and Development Program of China (2022YFC3600901, 2020YFA0803800, 2020YFA0803601, 2018YFA0801300 and 2019YFA0801900), the National Natural Science Foundation of China (82171633, 32071138 and 92057115), Natural Science Foundation of Shanghai (20ZR1408800), and Shanghai Sailing Program (20YF1402600). We thank Single Cell Quantitative Metabolomics and Lipidomics Core Facility of IMIB at Fudan University for LC-MS/MS analysis.

Supporting information

The supporting information is available online at https://doi.org/10.1007/s11427-024-2761-y. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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