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ORIGINAL ARTICLE

An active glutamine/α-ketoglutarate/HIF-1α axis prevents pregnancy loss by triggering decidual IGF1^{+}GDF15^{+}NK cell differentiation

Shao-Liang Yang[1,2] · Hai-Xia Tan[3] · Zhen-Zhen Lai[1] · Hai-Yan Peng[4] · Hui-Li Yang[1] · Qiang Fu[5] · Hai-Yan Wang[2] · Da-Jin Li[1,6] · Ming-Qing Li[1,6,7]

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Abstract

Deficiency of decidual NK (dNK) cell number and function has been widely regarded as an important cause of spontaneous abortion. However, the metabolic mechanism underlying the crosstalk between dNK cells and embryonic trophoblasts during early pregnancy remains largely unknown. Here, we observed that enriched glutamine and activated glutaminolysis in dNK cells contribute to trophoblast invasion and embryo growth by insulin-like growth factor-1 (IGF-1) and growth differentiation factor-15 (GDF-15) secretion. Mechanistically, these processes are dependent on the downregulation of EGLN1-HIF-1α mediated by α-ketoglutarate (α-KG). Blocking glutaminolysis with the GLS inhibitor BPTES or the glutamate dehydrogenase inhibitor EGCG leads to early embryo implantation failure, spontaneous abortion and/or fetal growth restriction in pregnant mice with impaired trophoblast invasion. Additionally, α-KG supplementation significantly alleviated pregnancy loss mediated by defective glutaminolysis in vivo, suggesting that inactivated glutamine/α-ketoglutarate metabolism in dNK cells impaired trophoblast invasion and induced pregnancy loss.

Keywords Glutamine metabolism · α-Ketoglutarate · Decidual NK cells · Spontaneous abortion · IGF-1 · GDF-15

Abbreviations

α-KG	α-Ketoglutarate
ACTB	β-Actin
BPTES	Inhibitor of glutaminase
dNK	Decidual NK cells
DIC	Decidual immune cells
DMKG	Dimethyl alpha-ketoglutarate
DMOG	Dimethyloxallyl glycine, inhibitor of HIF prolyl-hydroxylase
DSC	Decidual stromal cells
eNK	Endometrial NK cells
EGCG	Epigallocatechin gallate sulfate, inhibitor of glutamate dehydrogenase
EGLN1	Egl nine homolog 1
EP300	E1A binding protein p300
ESC	Endometrial stromal cells

Shao-Liang Yang and Hai-Xia Tan contributed equally.

✉ Hai-Yan Wang
haiyanwang2002@163.com

✉ Da-Jin Li
djli@shmu.edu.cn

✉ Ming-Qing Li
mqli@fudan.edu.cn

[1] Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, China

[2] Department of Gynecology of Integrated Traditional Chinese and Western Medicine, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, China

[3] Department of Obstetrics and Gynecology, Zhangye People's Hospital of HeXi College, Zhangye, Gansu, China

[4] Department of Gynecology and Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China

[5] School of Pharmacy, Binzhou Medical University, Yantai, Shandong, China

[6] Key Laboratory of Reproduction Regulation, Shanghai Institute for Biomedical and Pharmaceutical Technologies, Fudan University, Shanghai, China

[7] Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, China

ESR1	Estrogen receptor 1
FGF-19	Fibroblast growth factor-19
GABA	γ-Aminobutyric acid
GDF15	Growth differentiation factor-15
GLS	Glutaminase
GLUD	Glutamate dehydrogenase
HIF-1α	Hypoxia inducible factor-1α
IGF1	Insulin-like growth factor-1
IUGR	Intrauterine growth restriction
OXPHOS	Oxidative phosphorylation
PBMC	Peripheral blood mononuclear cells
pNK	Peripheral NK cells
TCA	Tricarboxylic acid
TET	Ten-eleven translocation methylcytosine dioxygenase

Introduction

Spontaneous abortion occurs in 10% of clinically recognized pregnancies in humans [1]. A variety of studies have identified various causes (e.g., chromosomal abnormalities, endocrine abnormalities, uterine anomalies, infectious etiologies, thrombophilia and immune factors) of spontaneous abortion [2]; however, the cases of a large proportion of spontaneous abortions remain unexplained.

Decidualization denotes the transformation that the stromal compartment of the endometrium must undergo to accommodate a successful pregnancy [3]. Additionally, there is a large number of peripheral $CD56^{dim}CD16^{+}CXCR4^{+}$ NK cells with high cytotoxicity, which are recruited into the decidua and further differentiate into decidual $CD56^{bright}CD16^{-}$ NK cells during early pregnancy [4, 5]. More importantly, these decidual NK (dNK) cells with a high capacity to secrete various cytokines and chemokines constitute approximately 70% of the lymphocytes in the decidua and play important roles in maintaining immune tolerance at the maternal–fetal interface, promoting the decidualization, remodeling of decidual vessels, and the development of placenta and embryo growth, contributing to a successful pregnancy [6–10]. Deficiencies in NK-cell number and function are associated with reproductive diseases and pregnancy complications, including female infertility, spontaneous abortion, intrauterine growth restriction (IUGR) and preeclampsia [8–12]. Unlike dNK cells, there were very limited numbers of ILCs in decidua during early pregnancy, and no significant change of ILCs in decidua between normal and spontaneous abortions [9]. With the development of single cell sequencing technology at the maternal–fetal interface, the characteristics of dNK cells have been explored more deeply. dNK cells were divided into different subgroups based on gene expression, including resting NK ($MKI67^{-}$ $TOP2A^{-}$), proliferating NK ($MKI67^{+}$ $TOP2A^{+}$), peripheral NK ($CD16^{+}PLAC8^{+}$) and dNK1 ($CD39^{+}CYP26A1^{+}B4GALNT1^{+}$), dNK2 ($ANXA1^{+}ITGB2^{+}$) and dNK3 ($CD160^{+}KLRB1^{+}CD103^{+}CD127^{-}$) [13–16]. Recently, emerging evidences show that metabolic reprogramming plays a crucial role in the differentiation and functional fate of NK cells, especially in the tumor microenvironment [17–20]. A heightened capacity for glucose metabolism through glycolysis and oxidative phosphorylation (OXPHOS) supports NK cells with robust cytotoxic capacity. dNK1 cells, which contain more cytoplasmic granules and express higher levels of PRF1, GNLY, GZMA and GZMB, also show higher levels of enzymes involved in glycolysis [14]. Despite this, the role of metabolism in regulating the function and differentiation of decidual NK cells is still very limited. The specific mechanism by which metabolic factors exert their remains to be further explored.

As the most abundant and versatile amino acid in the body, the rate of glutamine consumption by immune cells is similar to or greater than that of glucose [21, 22]. Glutamine is an essential immunonutrient for lymphocyte proliferation and cytokine production, and the phagocytic and secretory activities of macrophages [23–25]. It has been reported that glutamine maintains the cell growth and cell response of mouse NK cells in cMyc-dependent and glutaminolysis-independent manners [26]. However, the role of glutamine in NK cell phenotype and function is rarely reported, especially in human tissues.

Therefore, the purpose of this study was to investigate the metabolic characteristics and regulatory mechanism of dNK cell differentiation and function in early pregnancy, and the glutamine metabolism disorders of dNK cells in the pathogenesis of unexplained recurrent spontaneous abortion, and to explore potential intervention strategies.

Materials and methods

Tissue collection

All of the tissues, including decidua, endometria and serum, were collected from patients in the Obstetrics and Gynecology Hospital of Fudan University from February 2018 to June 2021 and were approved by the Ethical Committee (No. 2018-25). Informed consent was obtained from the patients. Decidual tissues of normal early pregnant women ($n = 108$, age 20–43 years, gestational age of 6–10 weeks) were obtained from patients admitted to the hospital to terminate pregnancy, with no history of spontaneous abortion, stillbirth or other adverse pregnancies. The decidua of unexplained recurrent spontaneous abortion patients ($n = 10$, age 25–37 years, gestational age of 6–9 weeks) were collected from those who experienced

two or more consecutive spontaneous abortions, without distributing factors such as fetal chromosome abnormality, reproductive tract infection or cervical incompetence. Endometrium was collected from patients (age 34–51 years, $n = 15$) who were diagnosed with curettage or undergoing hysterectomy for benign reasons unrelated to endometrial dysfunction as healthy controls. All of the tissues were stored in ice-cold DMEM/F-12 (HyClone, USA) under sterile conditions and transported to the laboratory in 1 h to further isolate endometrial stromal cells (ESCs), decidual stromal cells (DSCs), and dNK cells as previously described [27]. Peripheral blood samples were obtained from healthy volunteers (age 26–39 years $n = 36$) and transported to the laboratory immediately under sterile conditions to further isolate peripheral NK (pNK) cells as previously described [27, 28]. Decidual tissue was digested, filtered and density gradient centrifuged to obtain decidual immune cell (DIC). Peripheral blood was density gradient centrifugated to obtain peripheral blood mononuclear cells (PBMCs). Then, pNK and dNK cells were isolated from PBMCs or DIC according to the protocol of the NK-cell isolation kit (MiltenyiBiotec, Bergisch Gladbach, Germany). In brief, immune cells were labeled with NK-cell biotin-antibody cocktail and NK-Cell microbead cocktail separately, incubated in the refrigerator (2–8 °C), and separated through the magnetic field of the MACS separator. Then NK cells were enriched in the unlabeled cells. The purity of isolated NK cells was detected by flow cytometry. NK cells were used in further studies only if the purity was above 90%.

Cell culture

pNK and dNK cells were cultured with or without ESCs, DSCs, or a human trophoblast cell line (HTR-8/SVneo, purchased from the Chinese Academy of Sciences Cell Bank, Shanghai, China) with DMEM/F12 (HyClone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum (Gibco Cell Culture, Carlsbad, CA, USA) and 1% antibiotic–antimycotic (Gibco Cell Culture, Carlsbad, CA, USA). NK92 cells (a cell line of NK cells derived from peripheral NK cells of a non-Hodgkin lymphoma patient) transfected with IL-2 were provided by Professor Qiang Fu at the Department of Immunology, Binzhou Medical College and were cultured with L500 Serum-Free Medium for Lymphocytes (Dakewe Biotech Co., Ltd., Shanghai, China) containing 20% fetal bovine serum and 1% antibiotic–antimycotic. Cells were passaged every two or three days depending on their densities. The temperature of the incubator was set as 37 °C, and the CO_2 concentration was 5%. The O_2 concentration under normoxic conditions was 20%, and under hypoxic conditions, it was 1%.

LC–MS

The pNK and dNK cells treated in different groups were collected. After washing with PBS, the cells were centrifuged at 1500 rpm at 4 °C for 10 min, and the deposit was fully extracted with 80% methanol precooled at − 80 °C overnight. The supernatant was collected and transferred to another EP tube and dried in a rotary volatilizer. The dried powder was collected for mass spectrometry detection (TSQ-Vantage, Thermo) by the Institute of Biomedical Sciences, Fudan University. Every sample was tested three times, and the results were analyzed in Analyst Software.

Glutamine detection

The glutamine concentration was detected with a glutamine/glutamate determination kit (#GLN1, Sigma-Aldrich Co. LLC, USA) according to a standard procedure. In brief, cell lysate or tissue grinding fluid was first deaminated, and then dehydrogenated, and the absorbance of the reaction products was measured. The glutamine concentration was calculated according to the curve of glutamine standards.

Flow cytometry

In animal experiments, mouse uteruses were mechanically cut, digested with collagenase, and filtrated by sieve to prepare monoplast suspensions. Cells in different groups were collected by centrifugation, washed with PBS and then incubated with different panels of antibodies. The intracellular molecules were stained after fixation and permeation by FOXP3 Fix/Perm Buffer (BioLegend).

Antibodies used in the research are as follows: APC-conjugated anti-human CD56 (#318310), BV421-conjugated anti-human CD45 (#304032), APCCY7-conjugated anti-human CD3 (#344818), FITC-conjugated anti-human CD16 (#360716), PE-conjugated anti-human NKP30 (#325208), PECY7-conjugated anti-human NKG2D (#320806), BV510-conjugated anti-human IFN-γ (#502532), BV421-conjugated anti-human perforin (#308122) and PE-conjugated anti-human Ki-67(#350504, all from Biolegend); rabbit anti-glutaminase antibody (#ab93434) and goat secondary antibody to rabbit IgG Alexa Fluor 647(#ab150079, both from abcam); carboxyfluorescein diacetate succinimidyl ester (CFSE, eBioscience, San Diego, CA, USA), and Annexin V/7-AAD apoptosis kit (BD Biosciences, San Jose, CA, USA); and APCCY7-conjugated anti-mouse CD3 (#100222), Percp-conjugated anti-mouse CD45 (#103132), PE-conjugated anti-mouse NK1.1 (#108707, from Biolegend), Alexa Fluor 488-conjugated anti-mouse IGF-1 (#NBP2-34679AF488, Novus Biologicals, CO, USA) and APC-conjugated anti-mouse GDF-15 (#32572–05161, Assaypro LLC, MO, USA).

Data were collected in a FACS Calibur flow cytometer (Beckman Coulter CyAn ADP or Beckman Coulter Cytoflex, North Carolina, USA) and analyzed with FlowJo 7.6 (BD Biosciences, San Jose, CA, USA). Each experiment was performed three times independently. Statistical analysis was performed by using isotype matched controls as references. Typically, less than 1% positive cells were permitted beyond the statistical marker in the appropriate controls.

Immunofluorescence

The paraffin sections were dehydrated in graded ethanol, retrieved by citric acid buffer or Tris–EDTA buffer, and disposed with 3% hydrogen peroxide. Then, the section was incubated with 5% BSA at room temperature for 1 h, and incubated with anti-NCAM1 (CD56, 1: 200; abcam ab9018), anti-GLS (1:100; abcam ab93434), anti-cytokeratin 7 (1:4000; abcam ab181598), anti-F4/80 (1:500; abcam ab60343), anti-CD86 (1:300; abcam ab 119,857) or rabbit IgG isotypes at 4 °C overnight. After washing with PBS three times, the sections were incubated with fluorescence conjugated secondary antibody at room temperature for 1 h, and stained with DAPI for 10 min. The sections were observed, and pictures were taken under a fluorescence microscope (Leica, Munich, Germany).

RT-PCR

The total RNA of NK cells was extracted by RNAiso Plus reagent (TaKaRa Biotechnology) according to the manufacturer's instructions. Five hundred nanograms of RNA from every sample was reverse transcribed into cDNA using a reverse transcription kit (TaKaRa Biotechnology). Then, the transcription levels of genes were verified by RT−PCR (ABI QuantStudio 6 Flex, USA). The fold change in gene expression was calculated using the change in cycle threshold value method (ΔΔCt). All values obtained were normalized to the values obtained for β-actin (ACTB). The primer sequences were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and are listed in Supplementary Table 2.

Western blot

Cells were washed with cold PBS, lysed with lysis buffer (Beyotime Biotechnology, Shanghai, China), diluted with loading buffer (Beyotime Biotechnology) and heated to 95 °C for 10 min. Protein concentrations were detected by a BCA protein assay kit (Beyotime Biotechnology). For western blotting, equal amounts of protein were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels (Epizyme Scientific, Shanghai, China), transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% non-fat milk for 2 h at room temperature, and incubated with corresponding primary SLC1A5 (1:1000, Cell Signaling Technology, D7C12), GLS (1:1000, abcam, ab93434), Actin (1:3000, abcam, ab8226), IGF-1 (1:500, BioVision, 5119–100), GDF-15 (1:500, GeneTex, 10A3) and HIF-1α (1:1000, abcam, ab179483) antibody overnight at 4 °C. The membrane was washed three times and incubated with HRP-linked Anti-rabbit IgG (1:3000, Cell Signaling Technology, 7074) for 1 h at room temperature. After washing for three times, the protein bands were wetted with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Darmstadt, Germany) and detected by Luminescent Image Analyzer LAS 4000 (FUJIFILM, Japan).

Matrigel transwell assay

Matrigel (BD Bioscience) was diluted with DMEM/F12 at a ratio of 1:8, and 35 μL of diluted Matrigel was added to the transwell upper chamber (8 μm, Corning) and placed in a 24-well plate for 1 h at 37 °C. Two hundred microliters of HTR-8 suspension (2×10^4 [4] cells/well) without FBS was added to the upper chamber, and 600 μL medium containing 10% FBS or dNK cells (1×10^5 5 cells/well) in different groups or not was added to the lower chamber. The cells were treated as described in the article. After 48 h, the upper chamber medium and non-penetrating cells were gently wiped off. Cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 20 min. Random photographs were taken under an inverted microscope (Leica, Munich, Germany), and 5 visual fields were counted in each chamber. The number of invaded cells was counted using the Qupath (Centre for Cancer Research & Cell Biology at Queen's University Belfast).

ELISA

The concentrations of IGF-1, GDF-15 and FGF-19 were detected by ELISA (R&D Systems, No. DG100, DGD150 and DF1900). Supernatants of dNK cells in different groups were collected and detected according to the manufacturer's instructions.

Cytokine array

dNK cells treated with BPTES (10 μM, MedChemExpress) or 1‰ DMSO were centrifuged and the supernatant was collected. A cytokine array was performed by H-Wayen Biotechnology, Shanghai, China using the Human Cytokine Array AAH-BLG-507(RayBiotech, Norcross, GA, USA) as previously described [29].

Bioinformatics analysis

The intracellular sensing protein of α-KG was predicted based on the molecular structure (http://www.swisstargetprediction.ch). The protein interaction network was constructed using an online database (https://version11.string-db.org/). Transcription factor and target gene prediction was based on the databases TransFac (http://gene-regulation.com/pub/databases.html), TRED (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and TRRUST (https://www.grnpedia.org/trrust/).

The mRNA of pNK and dNK cells ($n=3$) was collected, and transcriptome sequencing was performed by Shanghai Ritz Biotechnology Co., Ltd., as previously described [29].

Animal model and treatment

All of the mice used in this research were of the C57BL/6J strain (from Shanghai Jieijie Experimental Animal Co., Ltd.) and were raised in an SPF experimental animal facility. The Animal Care and Use Committee of Fudan University approved all animal protocols. Eight-week-old female mice and 8-week-old male mice were caged at a ratio of 2:1, and whether the female mice were pregnant was confirmed on the morning of the morning based on vaginal plugs (marked as 0.5 days of gestation). Pregnant mice were randomly divided into different groups, intraperitoneally injected with BPTES (#HY-12683, 12.5 mg/kg, MedChemExpress), EGCG (HY-13653, 10 mg/kg, MedChemExpress), DMOG (HY-15893, 40 mg/kg, MedChemExpress) or PBS at 0.5 days, 4.5 days, 7.5 days and 10.5 days of gestation, and fed a control diet or 5% calcium 2-oxoglutarate (FC40259, Sigma) supplementary diet (MolDiets, Shanghai, China). At 7.5 days of gestation, mice were sacrificed, and the numbers for embryo implantation, embryo absorption and IGF-1 and GDF-15 expression in uterine NK cells were recorded. Similarly, at 13.5 days of gestation, mice were sacrificed, and the number of embryos implanted, embryo absorption, placenta weight and crown-rump length were observed. In addition, the invasion depth of trophoblasts in the uterus was measured by immunofluorescence.

NK depletion and adoptive transfer

The depletion of NK cells in pregnant mice was performed by tail vein injection of anti-NK1.1 or isotype antibody (#108759 and #401508, from Biolegend, 50 μg in 200 μl PBS per mouse) at day 0.5 and day 4.5 of gestation, and adoptive transfer was conducted at Day 5.5 with spleen NK cells (10^6 [6] cells per mouse in 100 μl PBS) isolated from non-pregnant female mice that were consecutively intraperitoneally injected with Ctrl, BPTES or EGCG for 7 days. At 13.5 days of gestation, mice were sacrificed, and the number of embryos implanted, embryo absorption, placental weight and crown-rump length were observed.

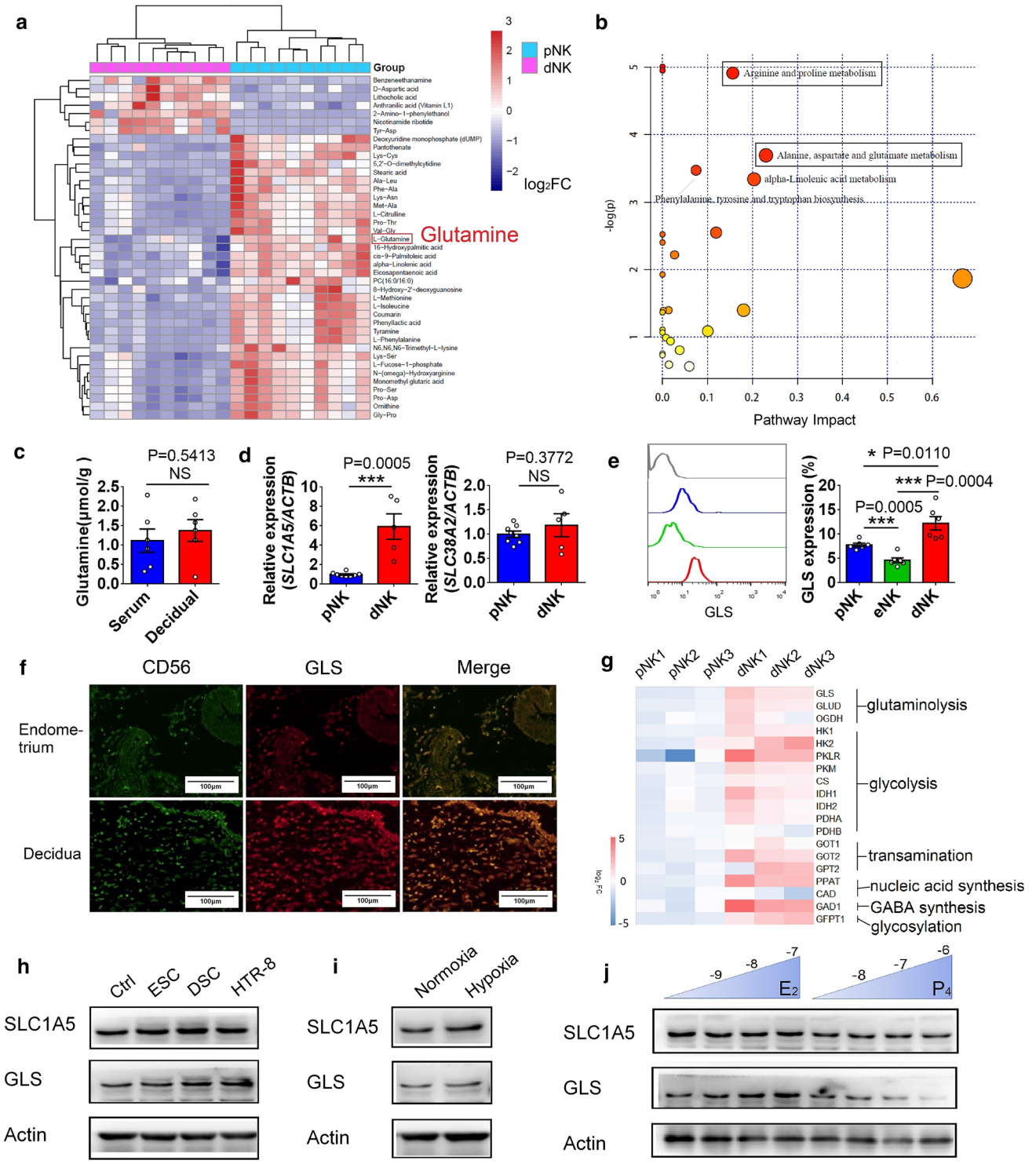
Statistical analysis

All of the data are shown as the mean ± SEM or median and min to mix. Comparisons between two groups were analyzed by Student's t test or the Mann−Whitney U test. Comparisons among three or more groups were analyzed by one-way ANOVA. When the data were non-normally distributed, the Mann−Whitney rank sum test, the Wilcoxon paired test or the Kruskal–Wallis test were generally used. Comparisons of rates or proportions among different groups were analyzed by the chi-square test. All of the analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) for Windows. Differences were considered to be significant at $P<0.05$.

Results

Local unique microenvironment induces activated glutamine metabolism in dNK cells at the maternal–fetal interface

To evaluate the metabolite profiles and differences between peripheral blood NK (pNK) and dNK cells, NK cells from 10 normal early pregnant women were recruited. As shown, metabonomic results showed that there were more than 40 differential metabolites between pNK and dNK cells from normal early pregnant women (Fig. 1a), especially activated arginine/proline metabolism and alanine-aspartate/glutamate metabolism (Fig. 1b). In our data of single-cell RNA sequence (data not shown), we observed dNK cells rarely express ARG1 and P5CS (key factors for arginine/proline metabolism), but a high level of glutaminase (GLS). In both metabolic pathways, glutamine was the key metabolite, which was markedly increased in dNK cells (Fig. 1a, b). To explore the reasons for glutamine enrichment in dNK cells, we further compared the surrounding glutamine concentration, glutamine transport and endogenous synthesis of pNK and dNK cells. As shown, there was no significant difference in the normalized glutamine concentration between serum and decidual tissues from normal early pregnant women (Fig. 1c). As endogenous glutamine synthesis in NK cells was quite low (Supplementary Fig. 1a, b), the main glutamine transporters (SLC1A4, SLC1A5, SLC38A1, SLC38A2, SLC38A5, SLC38A7) of NK cells were detected [26]. We found that SLC1A5, but not SLC38A2, was increased in dNK cells (Fig. 1d), which may contribute to glutamine enrichment. More importantly, we observed that GLS expression was significantly increased in dNK cells compared with pNK



and endometrial NK (eNK) cells (Fig. 1e, f). Additionally, the results of RT−PCR showed that enzymes involved in glutamine metabolism, including glutaminolysis, glycolysis, transamination, and γ-aminobutyric acid (GABA) synthesis, were highly expressed in dNK cells (Fig. 1g), suggesting that dNK cells display an activated and unobstructed glutamate metabolism.

To explore the potential mechanism for the activation of glutamate metabolism in dNK cells during early pregnancy, a coculture system of NK92 cells and decidual stromal cells (DSCs) or human trophoblast HTR-8/SVneo cells was constructed in vitro. As shown, DSCs and embryo-derived trophoblast cells upregulated GLS expression in NK92 cells. Interestingly, estrogen upregulated GLS expression in NK92

◂**Fig. 1** **A** unique local microenvironment induces activated glutamine metabolism in dNK cells at the maternal–fetal interface. **a**, **b** The metabolites in dNK and pNK cells from women with normal pregnancy ($n=10$) were detected by LC−MS, and the KEGG metabolic pathway of differential metabolites was analyzed. **c** Glutamine in serum and decidua from women ($n=6$) with normal pregnancy was detected by the glutamine determination kit. **d** The expression of the glutamine transporters SLC1A5 and SLC38A2 in dNK ($n=5$) and pNK ($n=8$) cells from women with normal pregnancy was measured by RT−PCR. **e**, **f** GLS expression in pNK and dNK cells from women ($n=6$) with normal pregnancy, and endometrial NK (eNK) cells from control non-pregnant women ($n=6$) was tested by flow cytometry and immunofluorescence (green: CD56; red: GLS). **g** The key enzymes related to glutamine metabolism in dNK and pNK cells from women ($n=3$) with normal pregnancy were detected by RT−PCR, and the relative expression is shown in the heatmap. **h–j** The effect of the coculture system, hypoxia and sex hormones (estrogen and progesterone) on SLC1A5 and GLS expression in NK92 cells was measured by western blot. ESC: coculture of ESC and NK92 cells; E2: estrogen; P4: progesterone. Data are presented as the mean ± SEM and were analyzed by *t* test, Mann−Whitney *U* test or one-way ANOVA. $^{*}P<0.05$, $^{***}P<0.001$, *NS* no significance

cells in a dose-dependent manner, whereas progestogen downregulated GLS expression. To our surprise, SLC1A5 expression seemed to be not affected significantly by coculture, hypoxia or hormones, suggesting that the increased SLC1A5 expression in dNK cells may be induced by other factors (Fig. 1h–j). These findings demonstrate that glutamine metabolism is activated in dNK cells and is induced by the local environment at the maternal–fetal interface.

IGF-1^{+}GDF-1^{+}dNK cells induced by activating glutamine metabolism enhance trophoblast invasion

To investigate the role of glutamine, dNK cells were treated with or without the GLS inhibitor BPTES or cultured with glutamine-free medium. As shown, BPTES obviously decreased the expression of Ki67 and the cell size of dNK cells (Fig. 2a, b). In contrast, glutamine supplementation increased Ki67 expression and cell size (Fig. 2c, d). Additionally, both GLS inhibition and glutamine withdrawal significantly promoted the apoptosis of NK92 cells (Fig. 2e). Interestingly, dNK cells notably promoted the invasion of HTR-8/SVneo cells in vitro, which was significantly impaired when dNK cells were pretreated with BPTES (Fig. 2f). In vivo, trophoblast invasion was impaired in the BPTES-treated mice at D13.5 of pregnancy (Fig. 2g), suggesting that activated glutamine metabolism contributes to dNK cell survival and further enhances trophoblast invasion.

Further analysis of the cytokine array showed that there were 89 differential cytokines in Ctrl and BPTES-treated dNK cells. Among these cytokines, 21 were growth factors (Fig. 3a). Subsequently, 5 abundant cytokines with significant differences (IGF-1, GDF-15, VEGF-C, FGF-23, FGF-19) were selected, and the ELISA results showed that the secretion levels of insulin-like growth factor-1 (IGF-1), growth differentiation factor-15 (GDF-15) and fibroblast growth factor-19 (FGF-19) in dNK cells were decreased after BPTES treatment (Fig. 3b). In vivo, intraperitoneal injection of BPTES also decreased the expression of IGF-1 and GDF-15 in mouse uterine NK cells (Fig. 3c, d). Recombinant human IGF-1 protein and GDF-15 protein were found to promote the proliferation of HTR-8 cells within the range of certain concentrations (Supplementary Fig. 2a–c). Notably, treatment with IGF-1 or GDF-15 significantly enhanced the invasiveness of HTR-8/SVneo cells (Fig. 3e, f). The neutralization of IGF-1 and GDF-15 significantly restricted the stimulatory effect of dNK cells on trophoblast invasion (Fig. 3g, h). To confirm the effect of GDF-15, and avoid the contamination with TGF-β [30], we have added the experiment, and found that the pro-invasion effect of GDF-15 could be abrogated by anti-GDF-15 neutralizing antibody. Therefore, the effect of recombinant protein in our study was caused by GDF-15 (Supplementary Fig. 2d). These data suggest that dNK cells with activated glutamine metabolism promote the invasion of trophoblasts by secreting IGF-1 and GDF-15.

Glutamine/α-ketoglutarate metabolism promotes IGF-1^{+}GDF-15^{+} dNK differentiation

To investigate whether glutamine can be utilized in the tricarboxylic acid cycle, we measured the oxygen consumption and acid production rate of NK cells after supplementation with different substrates. As shown, glutamine supplementation increased the oxygen consumption of NK92 cells immediately, and BPTES markedly inhibited this effect (Fig. 4a). Additionally, the availability of glucose influenced the utilization of glutamine. In the presence of glucose, NK cells were less dependent on glutamine (Fig. 4a). Glucose dramatically increased the glycolysis of NK cells, while glutamine was mainly involved in aerobic respiration (Fig. 4a). To further explore the mechanism of glutamine metabolism on the differentiation of IGF-1^{+}GDF-15^{+}dNK cells, the downstream metabolites of dNK cells treated with or without BPTES were analyzed. As shown, BPTES caused the accumulation of glutamine, a reduction in glutamate and α-ketoglutarate and a compensatory increase in acetyl coenzyme A and citrate (Fig. 4b). Further analysis showed that α-KG increased the secretion of IGF-1 and GDF-15 in a time- and/or dosage-dependent manner (Supplementary Fig. 3a–d), and supplementation with dimethyl alpha-ketoglutarate (DMKG) reversed the inhibitory effect of BPTES (Fig. 4c). Similarly, epigallocatechin gallate sulfate (EGCG, an inhibitor of glutamate dehydrogenase that converts glutamate into α-KG) significantly decreased IGF-1 and GDF-15 production, and DMKG also partly abolished this effect (Fig. 4d). Uterine NK cells from pregnant mice

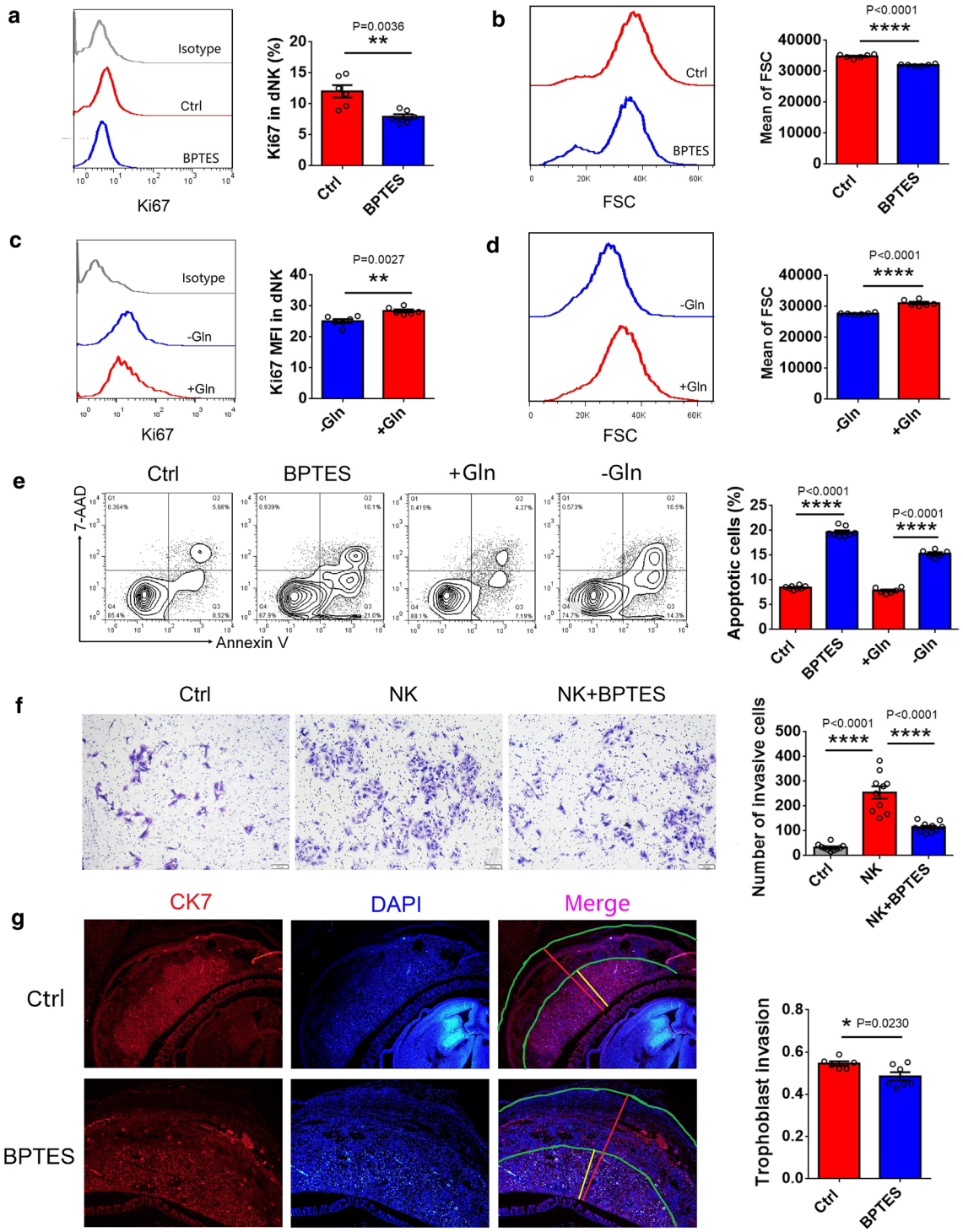


Fig. 2 Glutamine metabolism promotes proliferation, inhibits apoptosis of NK cells, and contributes to the invasion of trophoblast cells. **a**–**d** Ki67 and cell size were detected by flow cytometry in dNK cells ($n=6$) treated with control medium, BPTES (10 μM, 48 h), glutamine-free medium or glutamine (2 mM, 48 h). **e** Apoptosis was detected by flow cytometry in NK92 cells treated with control medium, BPTES, glutamine-free medium or glutamine. **f** The invasion of HTR-8 cells was detected by Matrigel invasion assay after coculture with or without control dNK cells or BPTES-pretreated dNK cells ($n=6$) for 48 h. **g** The trophoblast infiltration depth in the placenta of pregnant mice treated with or without BPTES (12.5 mg/kg) was measured by immunofluorescence on D13.5 (on the 13.5th day of pregnancy, $n=6$). The ratio of trophoblast infiltration (yellow line) to the depth of the entire placenta was analyzed. Data are presented as the mean ± SEM and were analyzed by *t* test, Mann−Whitney *U* test or one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$

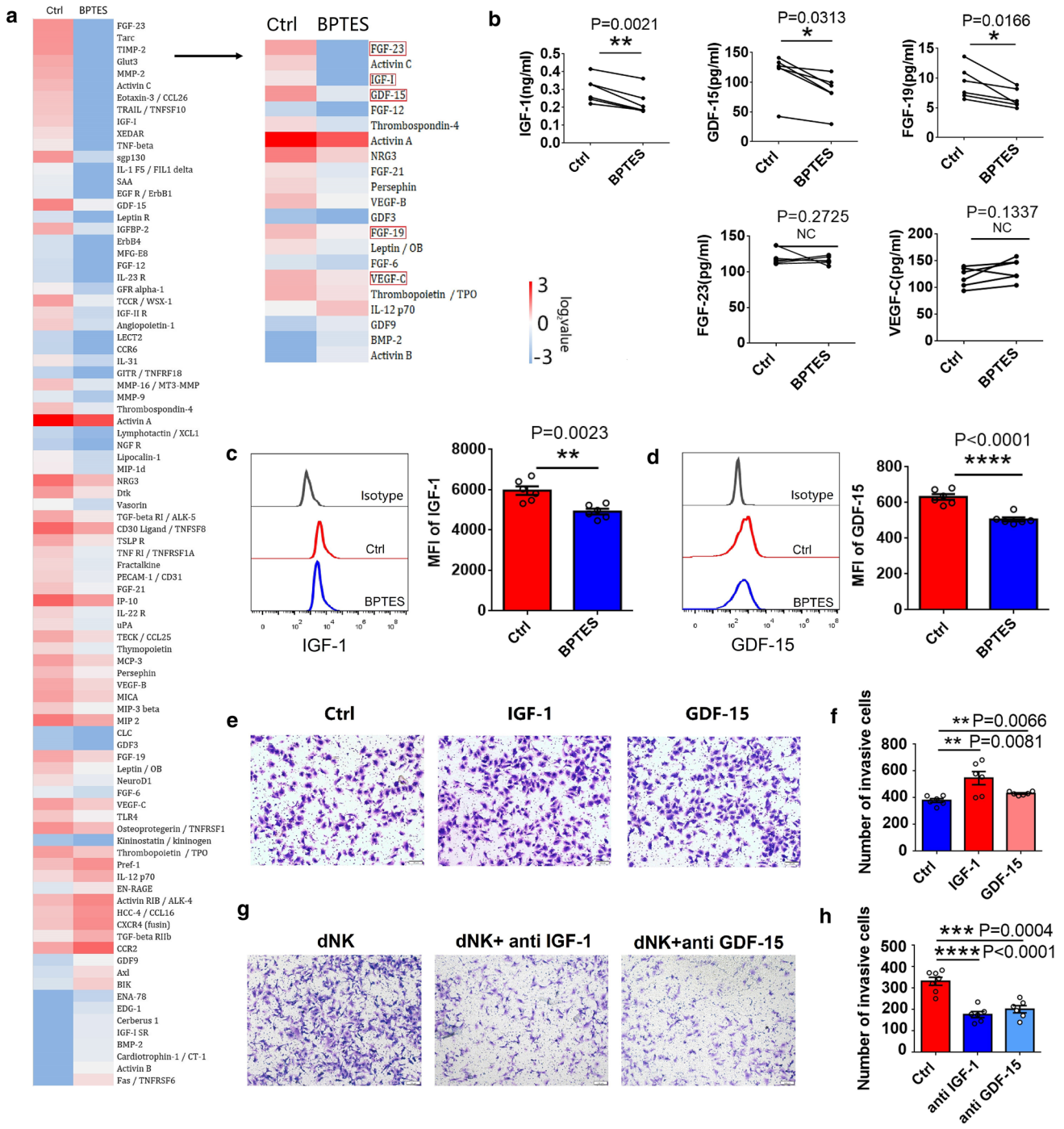


Fig. 3 Glutamine metabolism in dNK cells promotes trophoblast invasion through IGF-1 and GDF-15. **a**, **b** A cytokine array was performed in dNK cells ($n=6$) treated with BPTES (10 μM, 48 h) or not. The secretion levels of IGF-1, GDF-15, FGF19, FGF23 and VEGF-C were validated by ELISA. **c**, **d** After intraperitoneal injection with or without BPTES, IGF-1 and GDF-15 expression in uterine NK cells of pregnant mice ($n=6$) on D7.5 was analyzed by flow cytometry. **e**, **f** The invasion of HTR-8 cells treated with or without IGF-1 (20 ng/ml, 48 h) or GDF-15 (50 ng/ml, 48 h) was observed by Matrigel invasion assay. **g**, **h** HTR-8 cell invasion in the presence of dNK cells treated with anti-IGF-1 (2 μg/ml, 48 h), anti-GDF-15 (5 μg/ml, 48 h) neutralizing antibody or isotype antibody ($n=6$) was observed by Matrigel invasion assay. Data are presented as the mean ± SEM and were analyzed by *t* test, Mann−Whitney *U* test or one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

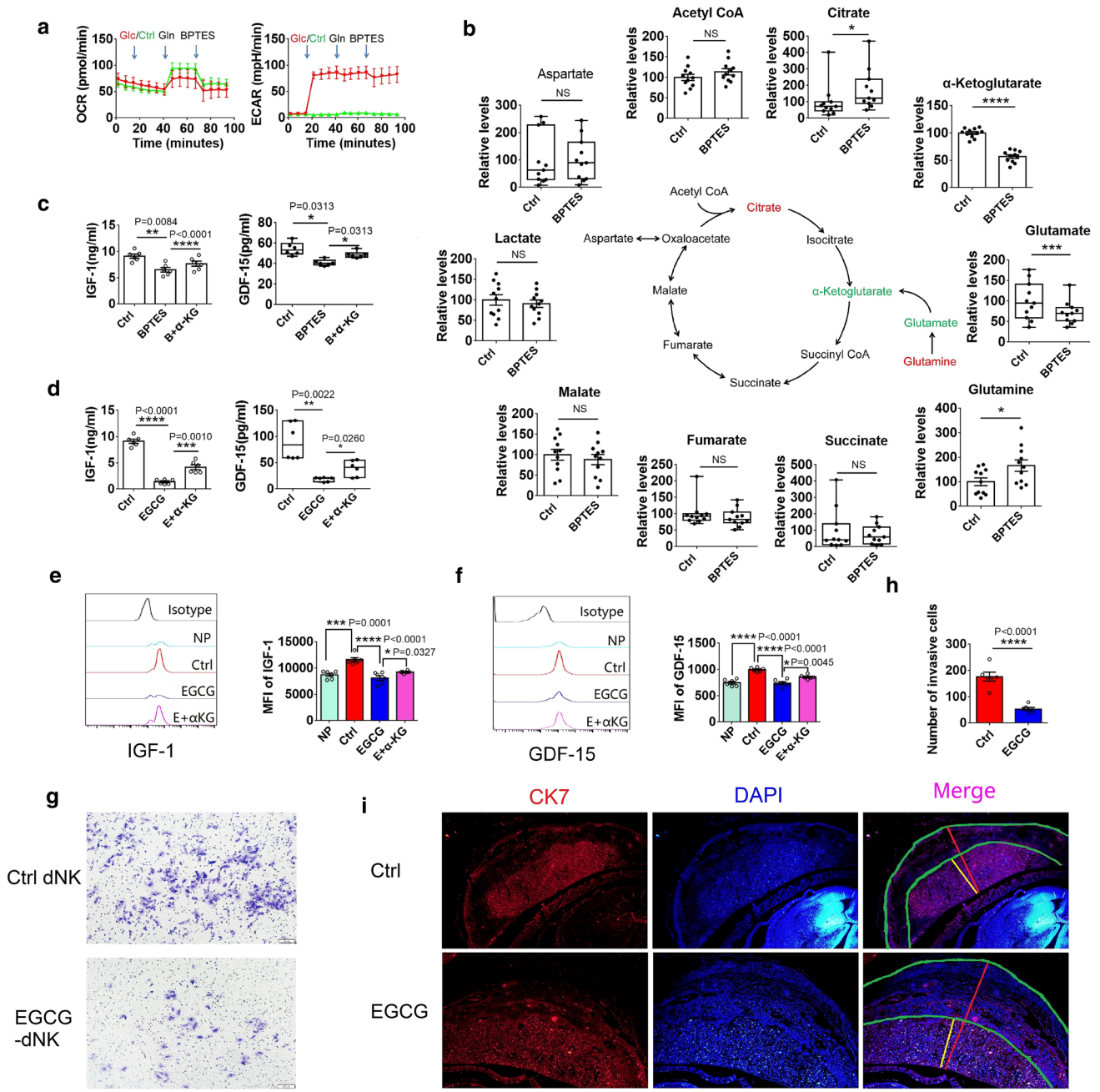


Fig. 4 Glutamine/α-ketoglutarate metabolism promotes IGF-1^{+}GDF-15^{+} dNK differentiation. **a** The oxygen consumption and extracellular acidification rate of NK92 cells after providing the substance shown in the picture were measured by the Seahorse XFe96 Analyzer. **b** The metabolites of dNK cells ($n=11$) treated with or without BPTES were extracted and detected by LC−MS. **c**, **d** The concentrations of IGF-1 and GDF-15 in the supernatant of dNK cells ($n=6$) treated with BPTES or EGCG (50 μM, 48 h) with or without dimethyl-α-ketoglutarate (2 mM, 48 h) were tested by ELISA. **e**, **f** IGF-1 and GDF-15 expression in uterine NK cells of pregnant mice ($n=6$) on D7.5 intraperitoneally injected with PBS or EGCG (30 mg/kg) with or without an α-KG diet (2%, w/w) was analyzed by flow cytometry. **g**, **h** HTR-8 cell invasion was observed in the coculture system with control or EGCG-pretreated dNK cells ($n=6$). **i** The trophoblast infiltration depth in the placentas of pregnant mice treated with or without EGCG was measured by immunofluorescence on D13.5. The ratio of trophoblast infiltration (yellow line) to the depth of the entire placenta was analyzed. Data are presented as the mean ± SEM or median and min to mix and were analyzed by *t* test, Mann−Whitney *U* test, one-way ANOVA or Wilcoxon test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, *NS* no significance

produced more IGF-1 and GDF-15 than those from non-pregnant mice (Fig. 4e, f). Intraperitoneal injection of EGCG impaired IGF-1 and GDF-15 expression in uterine NK cells, and α-KG supplementation in the diet partly rescued it in pregnant mice (Fig. 4e, f). Treatment with EGCG weakened the invasion of HTR-8/SVneo cells stimulated by dNK cells in vitro (Fig. 4g, h) and decreased the infiltration depth of trophoblasts in pregnant mice in vivo (Fig. 4i). These results indicate that α-KG derived from glutamine promotes IGF-1 and GDF-15 production in dNK cells and further enhances trophoblast invasion.

α-KG-triggered IGF-1 and GDF-15 production is dependent on HIF-1α degradation in dNK cells

To further explore the mechanism of α-KG on IGF-1 and GDF-15 expression, we predicted the intracellular sensory proteins of α-KG by Swiss Target Prediction (http://swisstargetprediction.ch/) (Supplementary Table 1), analyzed the interaction network of these sensing proteins with IGF-1 and GDF-15, and found that α-KG might regulate the expression of IGF-1 and GDF-15 through Egl Nine Homolog 1 (EGLN1)-hypoxia inducible factor-1α (HIF-1α) (Fig. 5a). Based on three databases, we predicted the potential transcription factors IGF-1 and GDF-15, and observed that most of these transcription factors were significantly upregulated in dNK cells (Fig. 5b). Further analysis showed that five of the transcription factors (TWIST1, TWIST2, EP300, ESR1, TP53) of IGF-1 and GDF-15 could be regulated by HIF-1α (Fig. 5c). It has been reported that α-KG promotes the hydroxylation and degradation of HIF-1α [31]. As an inhibitor of HIF prolyl hydroxylase, dimethyloxallyl glycine (DMOG) significantly increased the accumulation of HIF-1α and decreased the expression and secretion of IGF-1 and GDF-15 in dNK cells (Fig. 5d, e). In vivo, intraperitoneal injection of DMOG inhibited the expression of IGF-1 in uterine NK cells, while the expression of GDF-15 was not significantly changed (Fig. 5f, g). Additionally, DMOG suppressed the stimulatory effects of α-KG on IGF-1 and GDF-15 expression in dNK cells (Fig. 5h), suggesting that the positive regulation of IGF-1 and GDF-15 production by α-KG is dependent on HIF-1α degradation.

Defective glutamine/α-KG metabolism in dNK cells leads to pregnancy loss

To evaluate the possible differences in glutamine metabolism in dNK cells between normal pregnancy and unexplained recurrent spontaneous abortion, we detected the glutamine concentration and key enzymes in dNK cells from women with normal pregnancy and unexplained recurrent spontaneous abortion. As shown, the level of glutamine and the expression of SLC1A5 and glutamate dehydrogenase (GLUD) were decreased in dNK cells from women with unexplained recurrent spontaneous abortion (Fig. 6a, b), along with a decrease in IGF-1 expression (Fig. 6c). In the pregnant mouse model, intraperitoneal injection of BPTES did not influence the number of embryos implanted, while the rate of embryo absorption was significantly increased in the BPTES-treated group on the 13.5th day of pregnancy (D13.5) (Fig. 6d–f). Additionally, BPTES significantly decreased the crown-rump length and placenta weight (Fig. 6e, g). Of note, EGCG injection led to a decrease in mouse embryo implantation number on D7.5, comparable embryo absorption on D13.5 (Fig. 6h, i), and significant decreases in crown-rump length and placental weight on D13.5 (Fig. 6j, k). These results suggest that α-KG deficiency due to defective glutamine metabolism impairs placental development and embryo growth and contributes to pregnancy loss.

To further validate the glutamine metabolism of uterine NK cells in successful pregnancy, we depleted NK cells by tail vein injection of anti-NK1.1 antibody at D0.5 and D4.5 (Fig. 7a) and observed that depletion of NK cells significantly increased the embryo absorption rate and decreased the placental weight (Fig. 7b–e). Adoptive transfer of NK cells from BPTES- or EGCG-treated mice led to more embryo absorption and/or decreased placental weight compared with transfer of NK cells from control mice, especially NK cells from EGCG-treated mice (Fig. 7f–i). These results demonstrate that defective glutamine/α-KG metabolism in uterine NK cells increases the risk of pregnancy loss.

α-KG supplementation rescues pregnancy loss caused by the defective glutaminolysis in dNK cells

To explore the possible value of α-KG in the treatment of spontaneous abortion caused by uterine NK cells with defective glutaminolysis during pregnancy, BPETS- or EGCG-injected mice were fed an α-KG supplementary diet. As shown, the α-KG supplementary diet decreased the risk of embryo absorption in BPTES-treated pregnant mice on D13.5 (Fig. 8a, b). More importantly, α-KG resulted in an increase in the embryo implantation number in the EGCG-treated mice on D7.5, as well as the placental weight on D13.5 (Fig. 8c, d). In addition, the infiltration depth of trophoblasts in the placenta was reversed in the α-KG supplementary diet group (Fig. 8e–h). To explore the immunoregulatory effect of dNK cells, we detected the infiltration of macrophages in the uterus by immunofluorescence. As shown, the proportion of macrophages was decreased in the BPTES and EGCG-treated groups, and α-KG partly restored the number of macrophages, while the expression of CD86 seemed to be not significantly changed (Supplementary Fig. 4). These results suggest that the deficiency of α-KG from defective glutamine/glutamate metabolism severely disrupts embryo trophoblast invasion

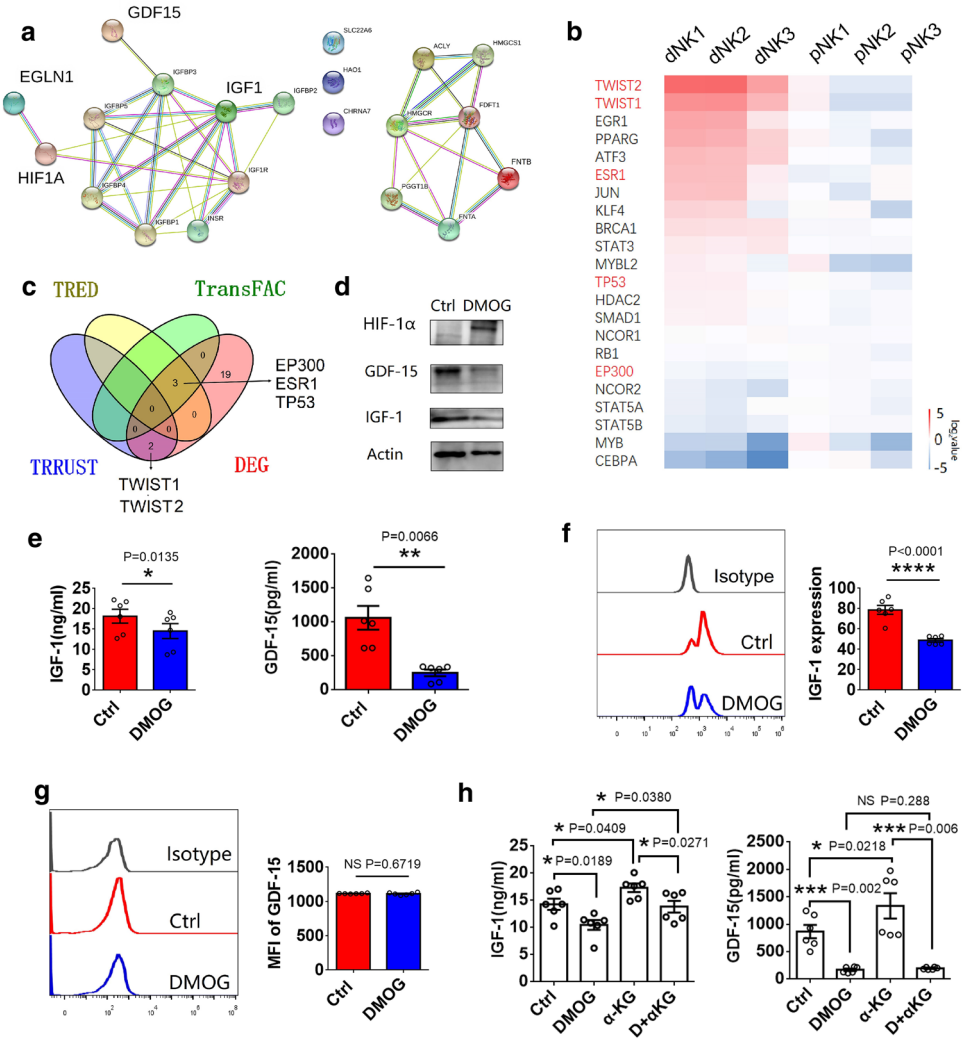


Fig. 5 α-KG-triggered IGF-1 and GDF-15 production is dependent on HIF-1α degradation in dNK cells. **a** The protein interaction network between intracellular sensory proteins of α-KG and IGF-1 and GDF-15 was analyzed by Swiss Target Prediction (http://swisstargetprediction.ch/). **b** The expression of transcription factors regulating IGF-1 or GDF-15 was analyzed based on the transcriptome sequencing of dNK and pNK cells ($n=3$). **c** The upstream transcription factors IGF-1 and GDF-15, which can be regulated by HIF-1α, were analyzed according to online databases. **d** The expression of HIF-1α, GDF-15, and IGF-1 was measured by western blot in dNK cells ($n=6$) treated with or without DMOG (1 mM, 48 h). **e** The concentrations of IGF-1 and GDF-15 in the supernatant of dNK cells ($n=6$) treated with or without DMOG were tested by ELISA. **f**, **g** After intraperitoneal injection with PBS or DMOG (40 mg/kg, $n=6$), IGF-1 and GDF-15 expression in uterine NK cells of pregnant mice on Day 7.5 was analyzed by flow cytometry ($n=6$). **h** The concentrations of IGF-1 and GDF-15 in the supernatant of dNK cells treated with DMOG, α-KG, or DMOG + α-KG or not were tested by ELISA ($n=6$). Data are presented as the mean ± SEM analyzed by *t* test, Mann−Whitney *U* test, or one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, *NS* no significance

and contributes to impaired embryo implantation and placental development, and the occurrence of pregnancy loss. α-KG supplementation rescues the trophoblast invasion and pregnancy loss caused by inactivated glutaminolysis.

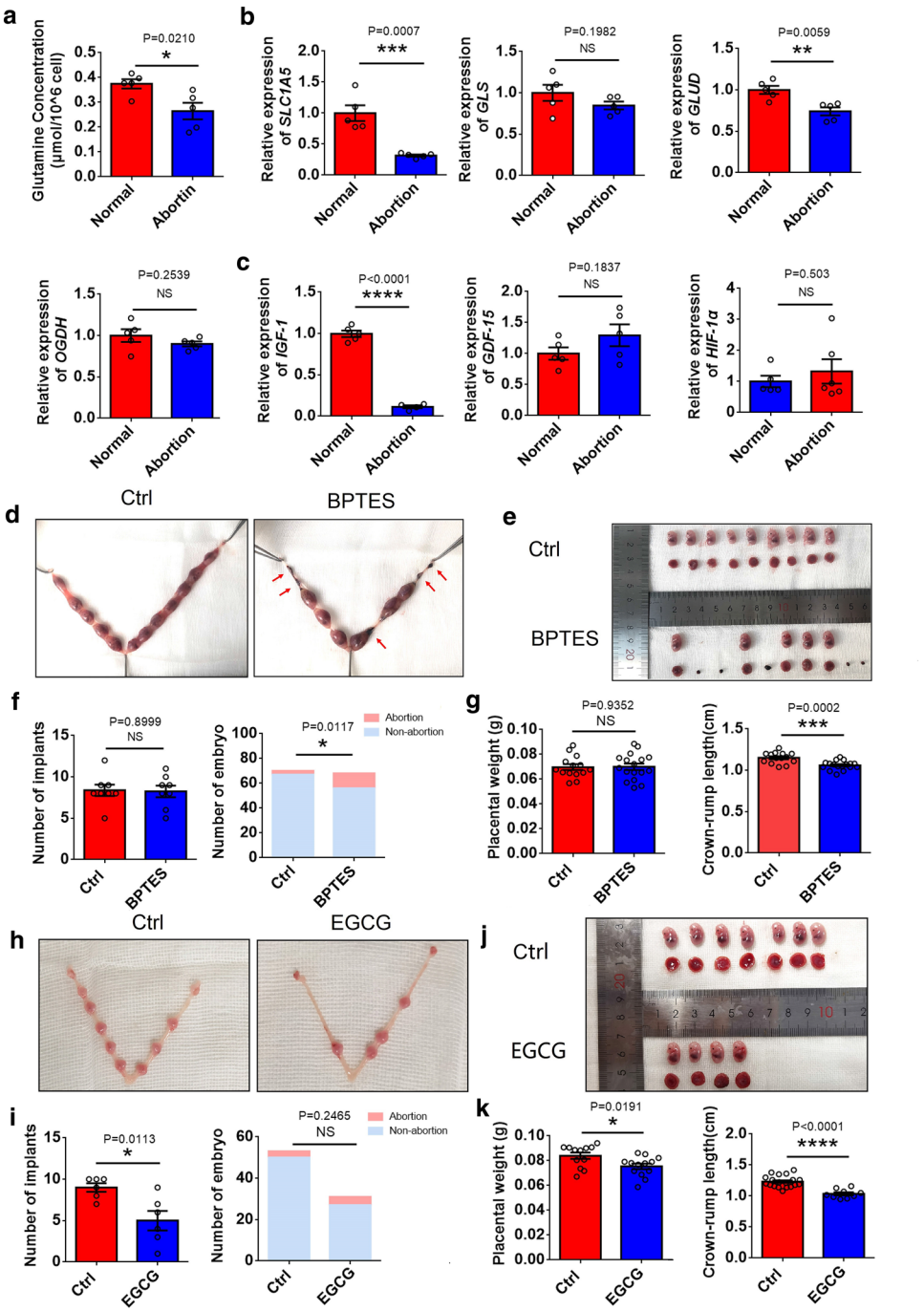


Fig. 6 Defective glutamine/α-KG metabolism leads to pregnancy loss. **a**–**c** The glutamine concentration, relative expression of glutamine metabolism enzymes, and expression of IGF-1, GDF-15 and HIF-1a were detected in dNK cells from women ($n=5$) with normal pregnancy and recurrent spontaneous abortion. **d**–**g** After intraperitoneal injection with or without BPTES, the number of embryos implanted, embryo absorption rate, placenta weight and crown-rump length were observed in mice ($n=8$) on D13.5. **h**–**k** After intraperitoneal injection with or without EGCG, the number of embryos implanted was counted on D7.5, and the embryo absorption rate, placenta weight and crown-rump length were observed in mice ($n=6$) on D13.5. Data are presented as the mean ± SEM and were analyzed by *t* test, Mann−Whitney *U* test or Chi-square test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, *NS* no significance

Discussion

The importance of glutamine in the development and differentiation of cells has been gradually emphasized, especially cells with rapid growth and huge demand for energy and nutrients, such as tumor cells and activated immune cells. In tumor cells, glutamine was even more preferentially absorbed and utilized than glucose [22]. In T-cell development, glutamine metabolism, and its effects on chromatin, were proven to promote Th17 but constrain Th1 and CTL effector cell differentiation [32]. Despite the fact that the application of single cell sequencing technology at the maternal–fetal interface has greatly accelerated our understanding of dNK cells in recent years, the role of glutamine metabolism in NK-cell function is still very limited [13–15]. Loftus et al. reported that fueling the tricarboxylic acid (TCA) cycle to generate ATP may not be the most important role of glutamine metabolism, rather the large number of metabolites such as succinic acid, fumaric acid and malic acid derived from glutamine through the TCA cycle may

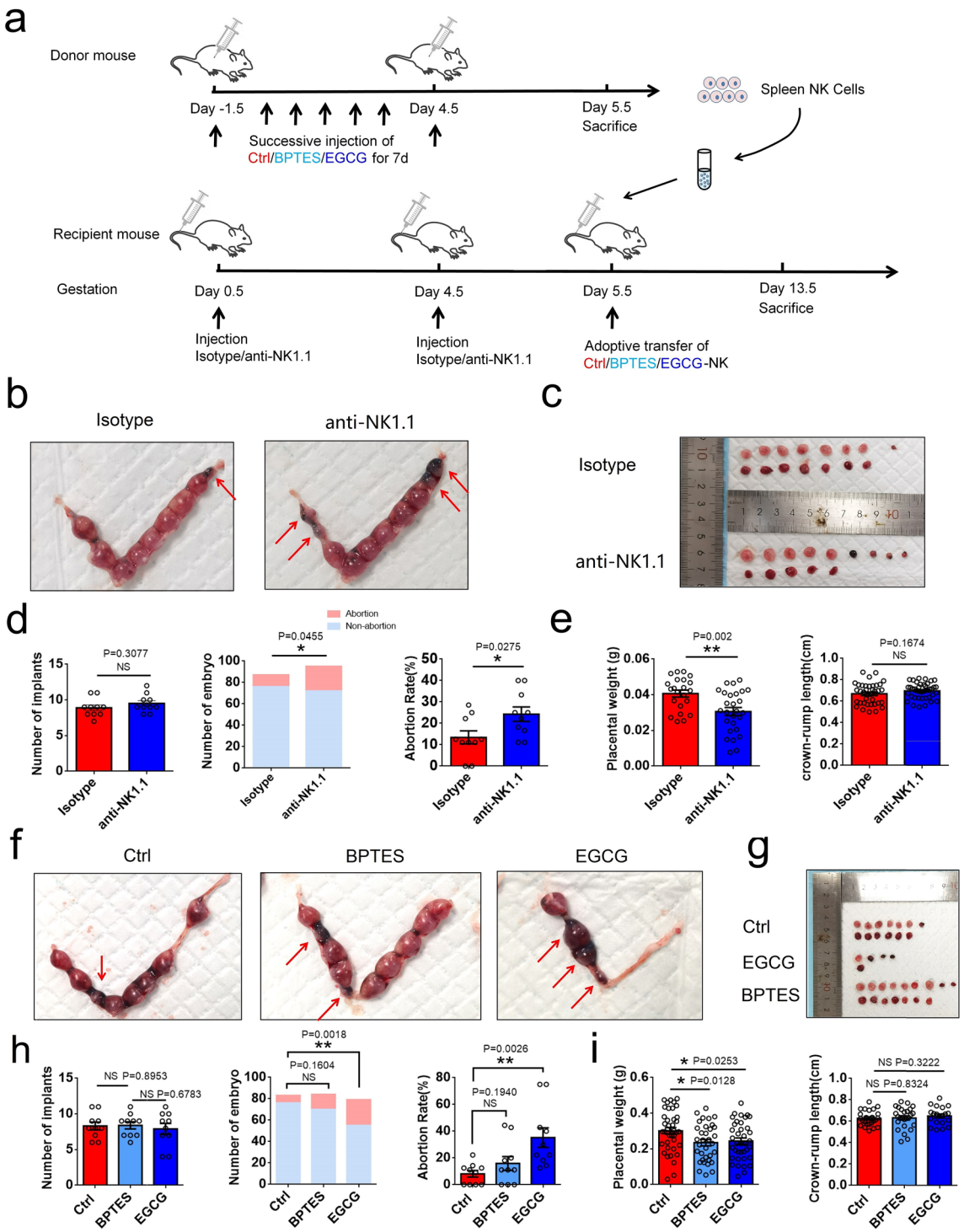


Fig. 7 Impairment of glutamine/α-KG metabolism in uterine NK cells leads to pregnancy loss. **a** The procedure of NK depletion and adoptive transfer in mice. The donor mice accepted intraperitoneal injection for seven consecutive days with Ctrl, BPTES or EGCG ($n=5$), and spleen NK cells were isolated at day 5.5 of gestation (D5.5). Then, these NK cells were transferred to recipient pregnant mice ($n=10$) whose NK cells were depleted by tail vein injection of anti-NK1.1 or isotype antibody at D 0.5 and D4.5. **b–e** The embryo implantation number, embryo absorption rate, placenta weight and crown-rump length were observed in mice ($n=10$) with or without NK depletion at D13.5. **f–i** The embryo implantation number, embryo absorption rate, placenta weight and crown-rump length were observed in mice ($n=10$) with NK depletion on D13.5. Data are presented as the mean ± SEM and were analyzed by *t* test, Mann−Whitney *U* test, Chi-square test or one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, *NS* no significance

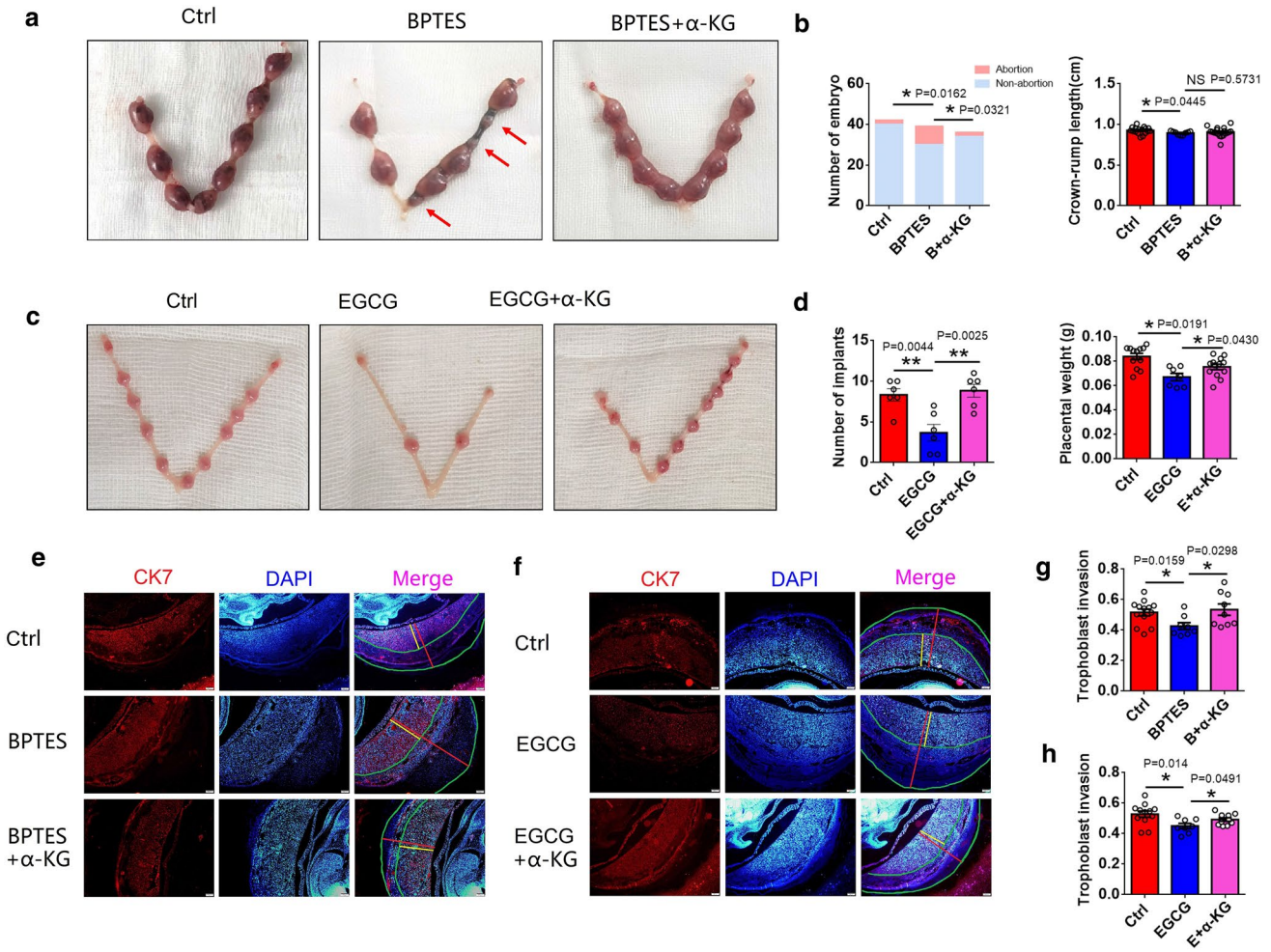


Fig. 8 α-KG supplementation rescues the pregnancy loss caused by BPTES and EGCG. (**a**, **b** After intraperitoneal injection with PBS or BPTES, supplemented with α-KG diet or not (2%, w/w), the embryo absorption rate and crown-rump length were observed in mice ($n=6$) on D13.5. **c**, **d** After intraperitoneal injection with PBS or EGCG, supplied with α-KG diet or not (2%, w/w), the number of embryos and placental weight were observed in mice ($n=6$) on D7.5 and D13.5. **e**–**h** The trophoblast infiltration depth in the placenta of pregnant mice treated with PBS, BPTES or EGCG, supplied with α-KG diet or not (2%, w/w), was measured by immunofluorescence on D13.5. The ratio of trophoblast infiltration (yellow line) to the depth of the entire placenta was analyzed. Data are presented as the mean ± SEM analyzed by one-way ANOVA or the Chi-square test. $^*P<0.05$, *NS* no significance

be more pivotal in regulating the phenotype and function of NK cells [26]. In the presence of glucose, notably, we observed that NK cells were less dependent on glutamine. More importantly, a low oxygen concentration (hypoxia) is part of normal embryonic implantation and development. BPTES led to significant decreases in aerobic respiration in NK92 cells, suggesting that glutamine metabolism should be an important resource of aerobic respiration.

dNK cells showed an activated glutamine metabolism in our study, represented as increased expression of glutamine transporter SLC1A5 and upregulated key enzymes of glutaminolysis. GLS expression was induced by estrogen and the co-culture system, while SLC1A5 was not significantly changed in our study. Common regulatory factors in NK cells include mTOR, c-MYC, IL-2, IL-15 and the process of ubiquitination-mediated protein degradation [33]. HIF-2α was also shown to induce the expression of SLC1A5 under hypoxic conditions, while this variant of SLC1A5 exists only in mitochondria [34]. Additionally, the relationship between glutamine and glucose metabolism has become increasingly evident in recent years. Lactate was shown to stimulate SLC1A5 expression in cervical cancer cells [35], and inhibition of glucose metabolism was reported to reduce the glycosylation of SLC1A5, suggesting the existence of coordinated regulation of glucose and glutamine metabolism [36].

Our study showed that activated glutamine metabolism enhanced the invasiveness of trophoblasts in vitro and in vivo, depending on the secretion of IGF-1 and GDF-1 in dNK cells. In contrast, blocking glutaminolysis with EGCG

or BPTES led to early implantation failure or spontaneous abortion with impaired trophoblast infiltration in the mouse uterus. In addition, we noted that the effect of BPTES and EGCG differed in embryo implantation and fetal abortion. EGCG showed a decrease in implantation number and placental weight, but no change in embryo abortion. While BPTES increased the risk of embryo abortion and placental growth inhibition, it showed no change in implantation number. We attributed these differences to the timing and extent of the glutamine-α-KG metabolism block and trophoblast function. Severe impairment of trophoblast function leads to implantation failure, while moderate and persistent impairment of trophoblast function may result in growth restriction.

α-KG is the key point that links glucose metabolism and amino acid metabolism. Metabolites are recognized as important epigenetic modulators. Some metabolites including α-KG can serve as substrates for enzymes that modify chromatin and nucleic acids, and others such as 2-hydroxyglutarate can modify enzyme activity often by competitively inhibiting substrate utilization [37, 38]. αKG-dependent dioxygenases consume the metabolite α-KG as an obligate cosubstrate and are emerging as key mediators of metabolic control of cell fate [38]. Dioxygenases can catalyze the demethylation or hydroxylation reactions of the target gene or enzyme. DNA methylation and transcription mediated by ten-eleven translocation(TET) methylcytosine dioxygenase is an important way for α-KG to participate in epigenetic regulation of genes [39]. In this study, however, BPTES and EGCG had no influence on IGF-1 and GDF-15 methylation, and the methylation levels of IGF-1 and GDF-15 were not obviously different in dNK cells between normal pregnant women and RSA patients (data not shown). Based on the bioinformatics analysis, we predicted that HIF-1α was controlled by EGLN1 (an α-KG-dependent dioxygenase) [40], possibly regulating the expression of IGF-1 through ESR1, and GDF-15 through TWIST. Further studies verified that DMOG significantly increased the accumulation of HIF-1α and decreased the expression and secretion of IGF-1 and / or GDF-15 in dNK cells in vitro and in vivo. However, the mechanism remains to be further studied.

Decidual NK cells were divided into different subgroups in the latest single cell sequencing studies. dNK4 ($CD16^{+}PLAC8^{+}$) cells are regarded as cytotoxic decidual NK cells [13]. Granzyme and perforin are the main functional molecules for NK cells to play immune defense and cell killing and play a role in resisting bacterial and viral infection during pregnancy. The latest research showed that decidual NK cells could transport granzymin to trophoblasts through nanotubes to resist bacterial infection [41]. Here we observed that blocking glutamine metabolism with BPTES slightly downregulated the expression of cytotoxicity-related molecules (NKP30, NKP46, perforin, CD107a and Granzyme B), and decreased cell viability in vitro (Supplementary Fig. 5a, b). Regulatory dNK cells have a powerful capacity of cytokine secretion. Through IFN-γ, decidual NK cells are involved in the regulation of the invasion of extravillous trophoblasts [42], the remodeling of placental vessels, the inhibition of Th17-cell differentiation [43, 44], and the maintenance of monocyte and neutrophil immune tolerance [44]. Growth factors are another kind of functional molecule in dNK cells, including VEGF and GM-CSF. With the deepening of research, more growth factors secreted by dNK cells have been discovered, among which pleiotrophin and osteolysin were found to play important roles in promoting fetal growth and bone and joint development [10]. Interestingly, we found that activated glutamine metabolism and α-KG induced the secretion of IGF-1 and GDF-1 in dNK cells and further enhanced the invasiveness of trophoblasts.

IGF-1 is an anabolic hormone that is mainly synthesized by the liver under the stimulation of growth hormone during the non-gestational stage [45]. In acute effects, IGF-1 can promote the synthesis of proteins and carbohydrates, promote the uptake of amino acids in skeletal muscle, and promote the absorption and utilization of glucose in tissues. It also promotes the growth and development of various tissues, such as the heart, bone, muscle and embryo [45–48]. Additionally, IGF-1 can regulate the immune response through autocrine and paracrine pathways. For example, IGF-1 exerts anti-inflammatory, antioxidant and antiatherogenic effects by suppressing macrophage accumulation [49]. Recently, we reported that WISP2/IGF1 produced by DSCs impaired the cytotoxicity of dNK cells [50]. In pregnancy, IGF-1 was significantly positively correlated with birthweight in both healthy controls and patients with type 1 diabetes [51]. IGF-1 can activate mTOR signaling in the placenta, promoting protein synthesis, mitochondrial function and nutrient transport, which may increase fetal nutrient supply and contribute to fetal growth [52]. Knockdown IGF-1 was found to suppress trophoblast cell migration and invasion [53, 54]. As a member of the TGF-β superfamily, GDF-15 is highly expressed in the placenta under physiological conditions and weakly expressed in most other tissues [55]. Similarly, we observed that GDF15 was mainly expressed by epithelial cells at the maternal–fetal interface, especially extravillus trophoblast (EVT) in our data of single-cell RNA sequence (data not shown). These findings suggest that GDF15 secreted by trophoblasts plays a more important role in the regulation of the biological behavior of trophoblasts. GDF-15 was also associated with the development of NK-cell dysfunction during systemic inflammation [56]. GDF-15 was thought to induce immune tolerance at maternal interface and tumor microenvironment [55, 57], and promotes prostate cancer cell migration and invasion [57, 58]. Here, we identified a subset of dNK

cells, IGF-1$^+$GDF-15$^+$ dNK cells, that contribute to trophoblast invasion, embryo implantation and growth under the positive regulation of activated glutamine metabolism and α-KG.

Collectively, as described in Supplementary Fig. 6, the high levels of glutamine transporters and metabolic enzymes involved in glutaminolysis result in activated glutamine metabolism in dNK cells under the influence of local environmental factors. Activated glutamine metabolism promotes proliferation and maturation, inhibits apoptosis, and promotes aerobic respiration in NK cells. More importantly, α-KG derived from glutamine promotes the differentiation of IGF-1$^+$GDF-15$^+$dNK cells through EGLN1/HIF-1α, enhances the invasion of trophoblasts, and further supports embryo growth. However, impaired glutaminolysis leads to a lack of glutamine-derived α-KG, contributing to embryo implantation failure, spontaneous abortion or restricted fetal growth with impaired trophoblast invasion and placental development to different degrees. Patients with unexplained recurrent spontaneous abortion had a decrease in glutamine and defective glutamine metabolism. Interestingly, the α-KG supplementary diet rescued the trophoblast invasion and mouse pregnancy loss caused by inactivated glutaminolysis. Therefore, the potential therapeutic value of α-KG in unexplained biochemical pregnancy and spontaneous abortion should be emphasized due to the activation of IGF-1$^+$GDF-15$^+$dNK cell-mediated trophoblast invasion during early pregnancy. Despite all this, there were some limitations in this study. The detailed mechanism of IGF-1 and GDF-15 in glutamine-α-KG-induced trophoblast invasion and embryo development in vivo still needs to be studied.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04639-x.

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Author contributions SLY and HXT conducted all experiments and prepared the figures and the manuscript. ZZL, HYP and HLY assisted with cell sorting, in vivo experiments, prepared the figures and the manuscript. QF edited the manuscript. MQL, DJL and HYW initiated and supervised the project and edited the manuscript. All the authors were involved in writing the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki and all experiments were approved by The Animal Care and Use Committee of Fudan University.

Consent for publication All authors consent to the publication of this study.

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