Am J Transl Res 2020;12(10):6665-6681
www.ajtr.org /ISSN:1943-8141/AJTR0111021

Original Article

L-type amino acid transporter 1 promotes proliferation and invasion of human chorionic trophoblast and choriocarcinoma cells through mTORC1

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Received March 13, 2020; Accepted June 23, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: L-type amino acid transporter 1 (LAT1) is a neutral amino acid transporter expressed in trophoblast giant cells onembryonic day 8 in mice. LAT1 is responsible for metabolism in blastocysts and cancer cells. Despite research concerning the aberrant high expression and indispensable function of LAT1 in various cancers, little is known about the role of LAT1 in regulating the behaviors of human trophoblast cells under different physiological and pathological conditions. The HTR8-SVneo human trophoblast cell line and JEG-3 and JAR choriocarcinoma cell lines are used as models for trophoblast cell biological research. The proliferation and apoptosis of these cells were assayed using the CCK-8 assay and flow cytometry, respectively. Transwell-chambers were used to observed migration and invasion of the cells. Immunofluorescent staining, western blot, and RT-PCR assays were used to determine the possible mechanism of LAT1 on human trophoblast cell behaviors with small interfering RNA or signal agonists and antagonist treatments. LAT1 was expressed in the trophoblast and choriocarcinoma cells. LAT1 was involved in regulating behaviors of these cells, such as cell proliferation, apoptosis, migration, and invasion. Detailed results suggested that LAT1 modulated trophoblast cell functions by mediation of mTORC1 signaling pathways. Our results implicate LAT1 as a very important regulator in human trophoblast cell behaviors at the maternal-fetal interface.

Keywords: L-type amino acid transporter 1 (LAT1), trophoblast cell, mTORC1

Introduction

The placenta is an important organ in material exchange, immune protection, and providing stable environment between a mother and fetus. Three primary types of trophoblasts have been identified: cytotrophoblasts (CTBs), syncytiotrophoblasts (STBs) and extrovillous trophoblasts (EVTs). They are involved in the processes of pregnancy establishment and placentation [1]. During early placentation, CTBs are involved in invasion and proliferation and STBs display endocrine activity by secreting human chorionic gonadotropin (hCG), progesterone, and estrogen [2, 3]. Abnormal development and dysfunction of the placental trophoblast cells are associated with severe pregnancy and fertility pathology, such as spontaneous miscarriages, preeclampsia (PE), intrauterine growth restriction, and choriocarcinoma. Gestational choriocarcinoma is a gestational trophoblastic neoplasia that grows rapidly and invades different organs, including the lung, brain, liver, kidney, intestine, pelvis, and vagina.

L-type amino acid transporter 1 (LAT1) is responsible for the transport of many essential neutral amino acids to support their continuous metabolism in somatic cells, blastocysts, and even cancer cells [4-7]. Accumulating evidence indicates that LAT1 is indispensable for both normal and malignant cells, and aberrant high

expression of LAT1 has been observed in variouscancer, including colon cancer, prostate cancer, pulmonary cancer, and esophageal cancer [8-12]. Cancer cells predominantly take up a large amount of amino acids to maintain their survival and sustain their biological behaviors. LAT1 might promote cancer cell proliferation and invasion by stimulating the uptake of amino acid which is inportant for protein synthesis. Functional transport of LAT1 depends on the assembly of a heterodimeric complex with another glycoprotein (heavy chain CD98hc), which recruits the light chain of Slc7A5 in the plasma membrane through covalent association [7, 13]. As a result, the inhibition of LAT1 activity might be a potential therapeutic strategy for cancer treatment.

Mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that is involved in cancer cell survival and invasion [14]. Abnormal activation of mTOR can disrupt G1-S phase transition in a wide variety of tumor cells. Many studies have demonstrated that mTOR signaling regulates the activities of matrix metalloproteinase (MMP)2/9 to control the invasion of human bladder and breast cancer cells [15, 16]. mTOR forms two distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is very sensitive to rapamycin and can be activated by many upstream factors, including insulin, growth factors, and amino acids [17]. In many kinds of cancer cells, LAT1 transports many neutral amino acids including high-affinity leucine, which activates mTORC1 to facilitate cell proliferation [18-21].

Amino acid are essential during maternal-fetal interface development in both humans and rodents. Recent studies have implicated LAT1 as being crucial in trophoblast proliferation and invasion. Chrostowski et al. reported that the transport of leucine and arginine by LAT1 promotes mouse trophoblast outgrowth, invasion, as well as implantation by stimulating mTOR activity at the blastocyst stage [22, 23]. In mouse trophoblasts, LAT1 is crucial in mediating in cell cycle arrest, apoptosis, and viability of placental cells [24].

We previously reported that LAT1 might promote the outgrowth of ectoplacental cones (EPCs) during the early placenta establishment and participate in the maternal-fetal interface by modulating the controlled invasion of trophoblasts in mice [26-28]. The transport of nutrients transport is critical in the development of trophoblasts, even in gestational choriocarcinoma cell destiny. However, few studies have investigated the role of LAT1 in regulating human trophoblast cell behaviors under different physiological and pathological conditions. In this report, we demonstrate that LAT1 regulates the proliferation, migration, and invasion of human extravillous trophoblasts and choriocarcinoma cells proliferation, migration by modulation the mTORC1 signaling pathway.

Materials and methods

Cell culture and chemicals

The HTR8-SVneo human extravillous trophoblast cell line and the JEG-3 and JAR choriocarcinoma cell lines were kindly provided by Professor Hongmei Wang (Institute of Zoology, Chinese Academy of Sciences). The three cells types were observed from the placenta, which is a key organ responsible for the exchange of nutrients at the maternal-fetal interface. HTR8-SVneo cells have been widely used as a physiological model for the first trimester invasion and migration of EVTs, while JEG-3 and JAR cell lines have been used to examine many pathological aspects of placental physiology and biology. HTR8-SVneo and JAR cells were cultured in RPMI 1640 medium (Gibco, Waltham. MA, USA). JEG-3 cells were cultured in DMEM-F12 (1:1) medium (Gibco). The media were supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin G, and 100 mg/ml streptomycin and maintained at 37°C in an atmosphere of 5% CO_2. The reference concentrations of mTOR agonist rapamycin (V900930; Sigma-Aldrich, St. Louis, MO, USA) and antagonist MHY1485 (5005540001; Sigma-Aldrich) were related to general descriptive categories.

Cell treatment

Following the addition of inhibitor (2-amino-2-norbornanecarboxylic acid, BCH; Sigma-Aldrich), cells were plated (4×10^5 cells/50 cm^2) in the dishes. The samples were randomly assigned to Group 1 (blank control without any treatment), Group 2 (0.1 μM BCH), Group 3 (1 μM BCH), and Group 4 (4 μM BCH). Each group was cultured for 24 or 48 h.

To assess short hairpin (sh) RNA interference and over-expression of LAT1, prevalidated PCONGV102000025 shRNA sequences targeting *LAT1* were obtained from Genechem (Shanghai, China). The sequences are provided in our previous study [27]. H4509 *LAT1* cDNA was purchased from Fulen Gene (Guangzhou, China). The cDNA was used as we have previously detailed. Cells cultured to 40%-50% confluence were transfected with shRNAs and pEGFP-N1-*LAT1* plasmid in serum-free medium according to the Lipofectamine® 2000 Transfection Reagent protocol (11668019; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 6 μL Lipofectamine® 2000 was mixed with 3 μg shRNA or pEGFP-N1-*LAT1* plasmid to form complexes. After 4 h, the medium was replaced by complete medium. The control was validated sequence shRNA or empty vector.

Semi-quantitative RT-PCR

Total RNA was extracted from cells using TRIzol lysis buffer (Invitrogen) and purified according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed in 20 μL of reaction mixture containing 4 μL $MgCl_2$, 25 mM; 2 μL Reverse Transcription 10 × buffer; 2 μL dNTP mixture, 10 mM; 0.5 μL Recombinant RNasin® Ribonuclease Inhibitor, 15 U AMV Reverse Transcriptase (High Concentration), and 0.5 μg random primers (A3500; Promega, Madison, WI, USA). PCR was performed in a total volume of 25 μL containing 12.5 μL GoTaq® Green Master Mix (M7122; Promega), 0.5 μM primers, and 1 μL cDNA for over 20 cycles using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, or for25 cycles for *LAT1*. The primers used in this study were Lat1 (NM_003486.5) (Forward: 5'-GGAGGCTGCTGTGAAAACTC-3', Reverse: 5'-AGGAGAAAGGAAGGCTCCTG-3') and GAPDH (Forward: 5'-GTCAAGGCTGAGAACGGGAA-3', Reverse: 5'-AAATGAGCCCCAGCCTTCTC-3'). Amplifications were analyzed by electrophoresis on agarose gels containing ethidium bromide. The quantity of the PCR products was determined by Quantity One software (Tanon Science and Technology Co., Shanghai, China) and wasnormalized to GAPDH.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS for 15 min each time, permeabilized with 0.1% Triton X-100 (T8532; Sigma-Aldrich), and exposed to 5% bovine serum albumin for 1 h at room temperature. Cells were incubated with rabbit anti-human LAT1 (1:600, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C and then incubated for 1 h at room temperature with anti-rabbit labeled FluoProbe 594 (1:200 dilution; Abbkine, Wuhan, China). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) for 3 min. Images were captured usingconfocal microscopy (Leica, Wetzlar, Germany).

Western blotting

Total cell protein was isolated from the four groups and loaded on 12.5% Tris-glycine SDS-PAGE gels (20 μg protein per well). The resolved proteins were transferred to a nitrocellulose membrane (Hybond™-C; Amersham Bio-Sciences, Piscataway, NJ, USA). After blocking with 5% non-fat milk at room temperature for 2 h, membraneswere incubated primary antibodies: anti-LAT1 (0.2 μg/mL, sc-134994; Santa Cruz Biotechnology), anti-mTOR (0.5 μg/mL, ab2732, Abcam), anti-phospho-mTOR (0.5 μg/mL, ab84400, Abcam), anti-4E-BP-1 (1 μg/mL, 2855S; Cell Signaling Technology), and anti-phospho-p4E-BP-1 (1 μg/mL, 9644S; Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H + L) were used at 1:2000 dilution (A0208; Beyotime, Nantong, China) for 60 min at room temperature. Signals were developed by chemiluminescence (BeyoECL Plus, P0018; Beyotime).

Proliferation assay

Proliferation was measured using the CCK-8 assay kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to protocol. Briefly, 5×10^3 cells were seeded onto a 96-well plate for each condition. Following treatment for 24 and 48 h, 10-μL CCK-8 reagent was added to each well and incubated for 2-4 hours. The absorbance of each sample was measured at 450 nm using an EL × 800 enzyme-labeled instrument featuring high-speed 8-channel filter-based absorbance.

Flow cytometryanalysis of cell cycle distribution and apoptosis

Cells were collected by trypsinization. The cells (at least 1×10^5) were washed twice in PBS at 24 or 48 h fixed in ice-cold 70% ethanol for a minimum overnight incubation for cell cycle

analysis. Otheraliquots were re-suspended in cold PBS for cell cycle measurement. Cell apoptosis was determined by Annexin V Apoptosis Detection Kit (KeyGEN BioTEC, Nanjing, China). The cell cycle distribution and apoptosis were analyzed using a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell migration and invasion assay

Cell migration assay was carried out by using the QCMTM 24-Well Colorimetric Cell Migration or Invasion Assay Kit (Millipore, Billerica, MA, USA), while the upper side of the filters was coated with 100 μL Matrigel (1 mg/ml) for invasion analysis. Briefly, 1×10^5 cells in 300-μL serum-free medium were added to the upper chamber, and 10% FBS-containing medium was used as chemo-attractant in the lower chamber. After incubating for 24 h, the cells on the upper side of the insert were scraped off using a cotton swab and then fixed in methanol for 10 min. Cells that invaded or migrated to the other side of the insert were stained after 24 h and checked by light microscopy (Olympus, Tokyo, Japan).

Statistical analyses

Quantitative data are presented as mean ± standard deviation (SD). Statistical significance ($P < 0.05$) was determined by the Student's t-test or analysis of variance (ANOVA) using the Statistical Package for the Social Sciences for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

The tempospatial LAT1 expression in human trophoblast-derived cell lines

We analyzed the expression of LAT1 in trophoblast-derived cell lines. LAT1 mRNA could be detected in HTR8-SVneo human extravillous trophoblast cells, and JEG-3 and JAR choriocarcinoma cells (**Figure 1A**). Western blot data further confirmed the presence of LAT1 proteins in all three cell lines (**Figure 1B**). Immunohistochemistry data revealed that LAT1 were strongly located in the cytoplasm (**Figure 1C**). Thus, these three cell lines could be used as in vitro model systems to study the function of LAT1 in trophoblasts.

Manipulating LAT1 expression affects cell proliferation and apoptosis

To investigate the cellular function of LAT1 in all three cell lines, BCH, a competitive inhibitor of LAT1, was added to the culture medium before evaluating cell proliferation, cycle distribution, and apoptosis, and LAT1 expression. LAT1 protein was remarkably decreased following the 24-h BCH treatment (**Figure 1F**). This result suggested that BCH could directly regulate LAT1 expression. LAT2 is another important L-type amino acid transporter. BCH treatment could also inhibit the function of LAT2. To assess the LAT1 function distinct from that of LAT2, shRNA targeting LAT1 or LAT1 was used. Three specially over-expressing plasmids harboring shRNA-targeting *LAT1* were transfected into the three cells, followed by western blot evaluations. The results clearly showed that one of the shRNAs (SiLAT1-3) could significantly reduce the expression of LAT1 (**Figure 1D**), with elevated LAT1 protein detected in all three cell lines after pEGFP-N1-LAT1 transfection (**Figure 1E**).

Cell proliferation was assessed using the CCK-8 reagent assay. Flow cytometry was used to analyze the cycle distribution and apoptosis. As shown in **Figure 2A** and **2B**, 1 μM and 4 μM BCH treatment for 24 h or 48 h could inhibit cell proliferation and exhibited dose-dependent effects in JAR cells. Similarly, the proliferation of HTR8-SVneo and JEG-3 cells were also suppressed by 4-μM BCH treatment for 24 h or 48 h.

Flow cytometry showed that 4 μM BCH treatment for 24 h (**Figure 2C**) or 48 h (**Figure 2D**) aided HTR8-SVneo and JEG-3 cellsin overriding G2/M phase. A significant exposure-response relationship was evident (**Figure 2C** and **2D**). JAR cell cycle distribution was also disrupted by 4-μM BCH treatment for 24 h or 48 h. More cells were arrested at the G0/G1 phase with only a few cells remaining in the S phase, as shown in **Figure 2C** and **2D**. Cells cycle analysis revealed that HTR8-SVneo and JAR cells were arrested at the G0/G1 stageafter LAT1 silencing, with arrest at the G2/M stage after LAT1 over-expression (**Figure 2E**). In JEG-3 cells, cell cycle distribution was arrested at the G0/G1 in cells transfected with SiLAT1-3, while their time at the G0/G1 phase was shortened with arrest

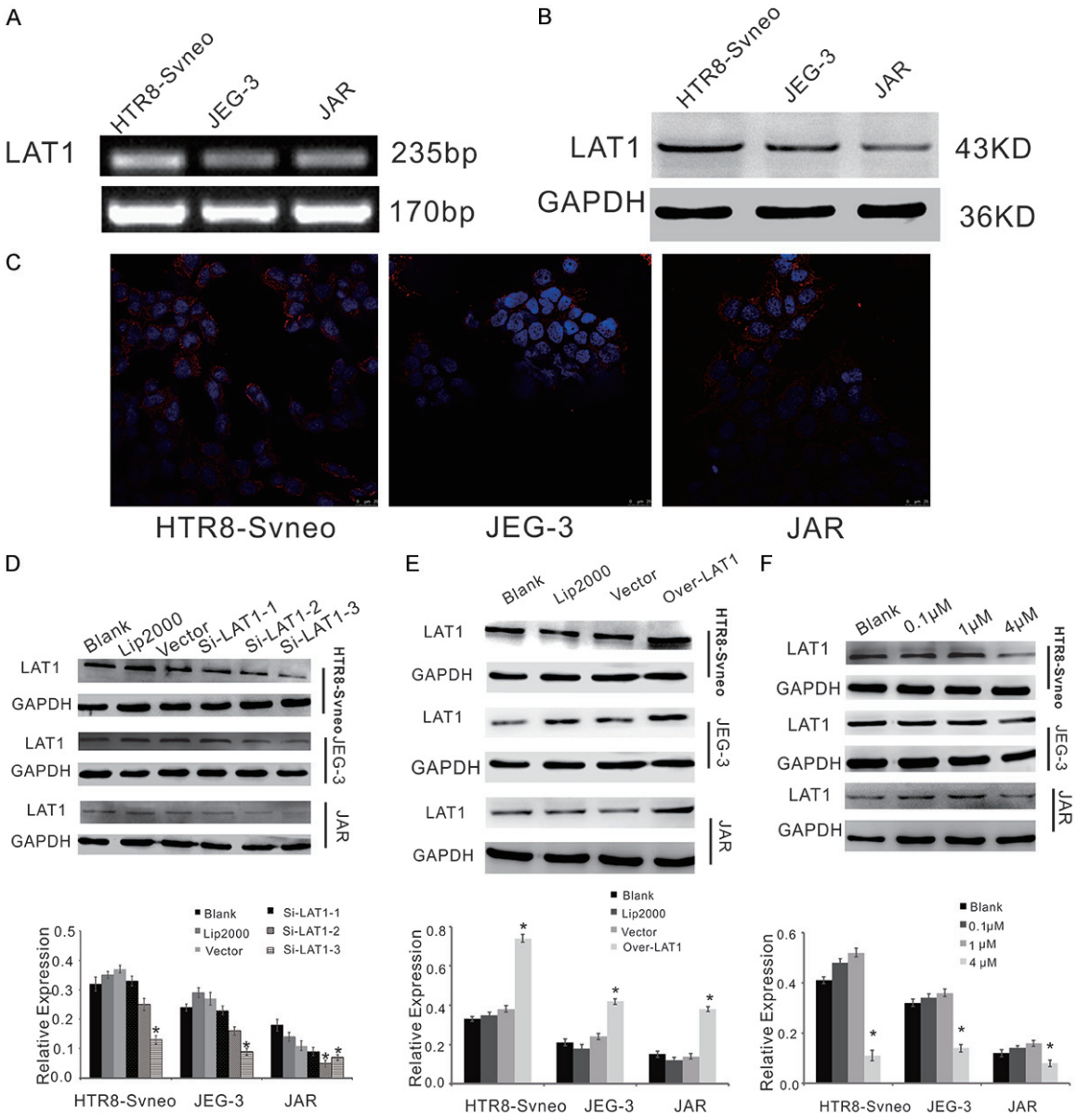
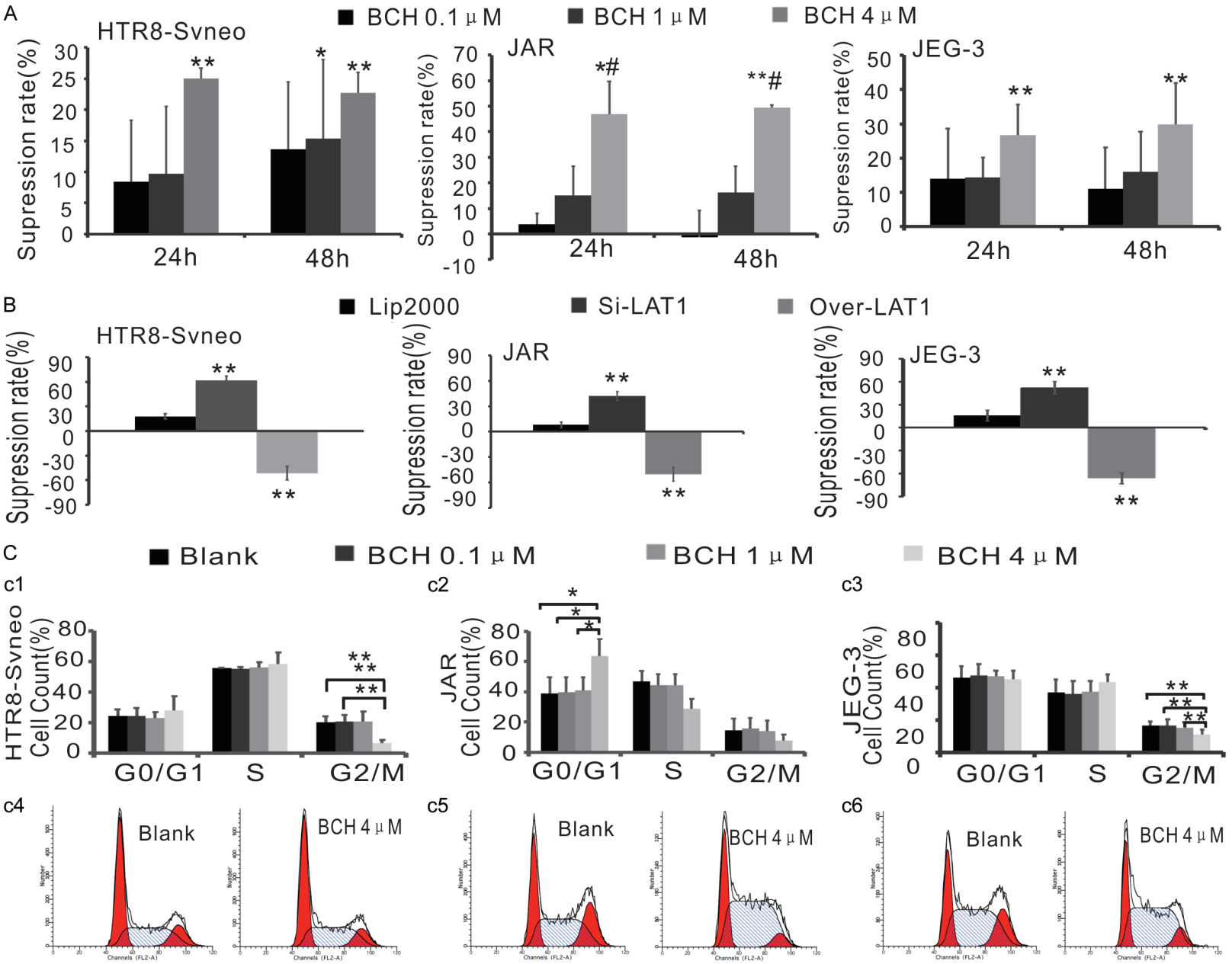


Figure 1. Expression of LAT1 in human placental cell lines. Expression of LAT1 in three trophoblast cell lines analyzed by semi-quantitative RT-PCR (A) and western blotting (B), respectively. GAPDH was used as an internal control for RT-PCR or loading control for western blotting. Immunofluorescence of LAT1 in three trophoblast cell lines (C). LAT1 signals were visualized as red and strongly located in the cytoplasm in HTR8-SVneo cells, JEG-3 cells, and JAR cells, respectively. The nuclei were highlighted by DAPI staining (blue). (D) Three sequences of shRNA targeting LAT1 were separately transfected in the three cell lines and the efficiency of silencing was evaluated by western blotting. The three sequences (SiLAT1-3) could effectively silence LAT1 expression and were used as interfering plasmid in follow-up experiments. *P < 0.05, as compared with blank. (E) The protein levels of LAT1 were enhanced in the three cell lines transfected with pEGFP-N1-LAT1 plasmid compared to those transfected with empty vector. *P < 0.05, as compared with vector. (F) The expression of LAT1 was decreased by 4 μM BCH (competitive inhibitor of LAT1), indicatingthat BCH could directly regulate LAT1 protein expression in the three cell lines. *P < 0.05, as compared with blank. Bar = 25 μM. Representative western blot images are shown with original western experiments.

at the G2/M phase by transfecting with pEGFP-N1-LAT1 at 48 h (**Figure 2E**). After the 24 h BCH treatment, a sharp increase in cell apoptosis was observed in JAR and JEG-3 cells, but not in HTR8-SVneo cells (**Figure 3A**). After 48 h BCH treatment, the ratios of apoptotic cells of all three cell lines were notably increased and all exhibited exposure-response relationship, as shown in **Figure 3A**. Similar to the results of BCH experiments, silencing LAT1 could pro-


A
BCH 0.1 μ M
BCH 1 μ M
BCH 4 μ M
HTR8-Svneo
JAR
JEG-3
Supression rate(%)
24h
48h
B
Lip2000
Si-LAT1
Over-LAT1
HTR8-Svneo
JAR
JEG-3
Supression rate(%)
C
Blank
BCH 0.1 μ M
BCH 1 μ M
BCH 4 μ M
c1
c2
c3
HTR8-Svneo Cell Count(%)
JAR Cell Count(%)
JEG-3 Cell Count(%)
G0/G1
S
G2/M
c4
c5
c6
Blank
BCH 4 μ M
Number
Channels (FL2-A)

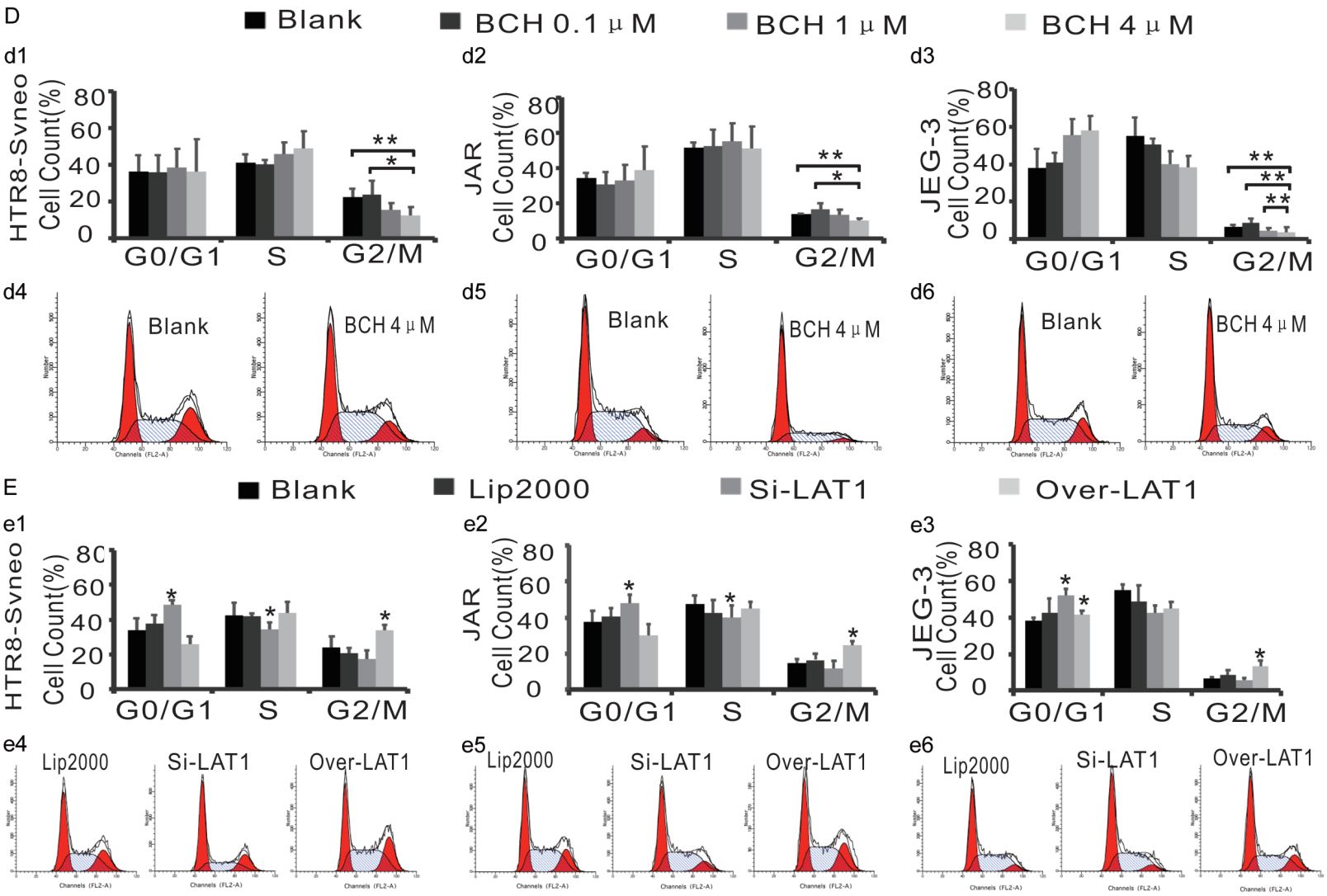


Figure 2. Effects of LAT1 on the proliferation and cell cycle distribution of the three trophoblast cell lines. (A) Down-regulation of LAT1 expression with 4 μM BCH suppressed cell proliferation in all three cell lines and exhibited a dose-effect relationship in JAR cells at 24 h or 48 h of treatment. (B) Down-regulation of LAT1 expression upon transfection with SiLAT1-3 plasmid decreased, while up-regulation of LAT1 with transfection of pEGFP-N1-LAT1 plasmid increased the proliferation in the three cell lines. Control group was transfected with invalid interference fragment. (C and D) Down-regulation of LAT1 disturbed the cell cycle distributions of all three cell lines after 24 h (C) and 48 h (D) of treatment. The statistical bar graphs showing down-regulation of LAT1 expression with 4 μM BCH. Obvious effects on cell cycle distribution are evident at 24 h and 48 h. Representative images of cell cycle distribution assayed by flow cytometry are shown at 24 h and 48 h. In HTR8-SVneo and JEG-3 cells, down-regulation of LAT1 with 4-μM BCH treatment significantly shortened the G2/M phase and exhibited a dose-effect relationship at 24 h or 48 h. In JAR cells, down-regulation of LAT1 with 4 μM BCH treatment arrested cells at the G0/G1 phase and shortened the S phase at 24 h or 48 h. (E) Up- and

down-expression of LAT1 regulated the cell cycle distributions of the three cell lines at 48 h. Statistical bar graphs showing the increased or decreased expression of LAT1 upon transfection with plasmids and the obvious effects on cell cycle distribution. Representative images of cell cycle distribution assayed by flow cytometry. In HTR8-SVneo and JAR cells, the cell cycle distributions were shortened at the S phase and arrested at the G0/G1 phase in cells transfected with SiLAT1-3, while cells transfected with pEGFP-N1-LAT1 were arrested at the G2/M phase at 48 h. In JEG-3 cells, cell cycle distribution was arrested at the G0/G1 phase in cells transfected with SiLAT1-3, while were shortened at the G0/G1 phase and arrested at the G2/M phase in cells transfected with pEGFP-N1-LAT1 at 48 h. Blank group was not treated. *$P < 0.05$, as compared with blank, **$P < 0.01$, as compared with blank, #$P < 0.05$, as compared with 0.1 μM BCH.

mote cell apoptosis in all three cell lines, but ectopically over-expressed LAT1 inhibited cell apoptosis (**Figure 3B**). These results indicated that LAT1 plays a key role in regulating trophoblast and choriocarcinoma cell behavior, and might be the main target of BCH treatment.

Presence of LAT1 promotes cell migration and invasion

To explore the functions of LAT1 on cell migration and invasion, Transwell matrigel invasion chambers and migration chambers were used for experiments. In the cell migration assay, the number of cells that migrated to the lower side of the membrane was decreased in all three cell lines after treatment with 4 μM BCH (competitive inhibitor of LAT1) or SiLAT1-3 transfection. Cells over-expressing pEGFP-N1-LAT1 plasmid presented eminent migration and invasion potential compared to cells expressing control vector (**Figure 4**). These results suggested the involvement of LAT1 in regulating the motility of human extravillous trophoblasts and choriocarcinoma cells.

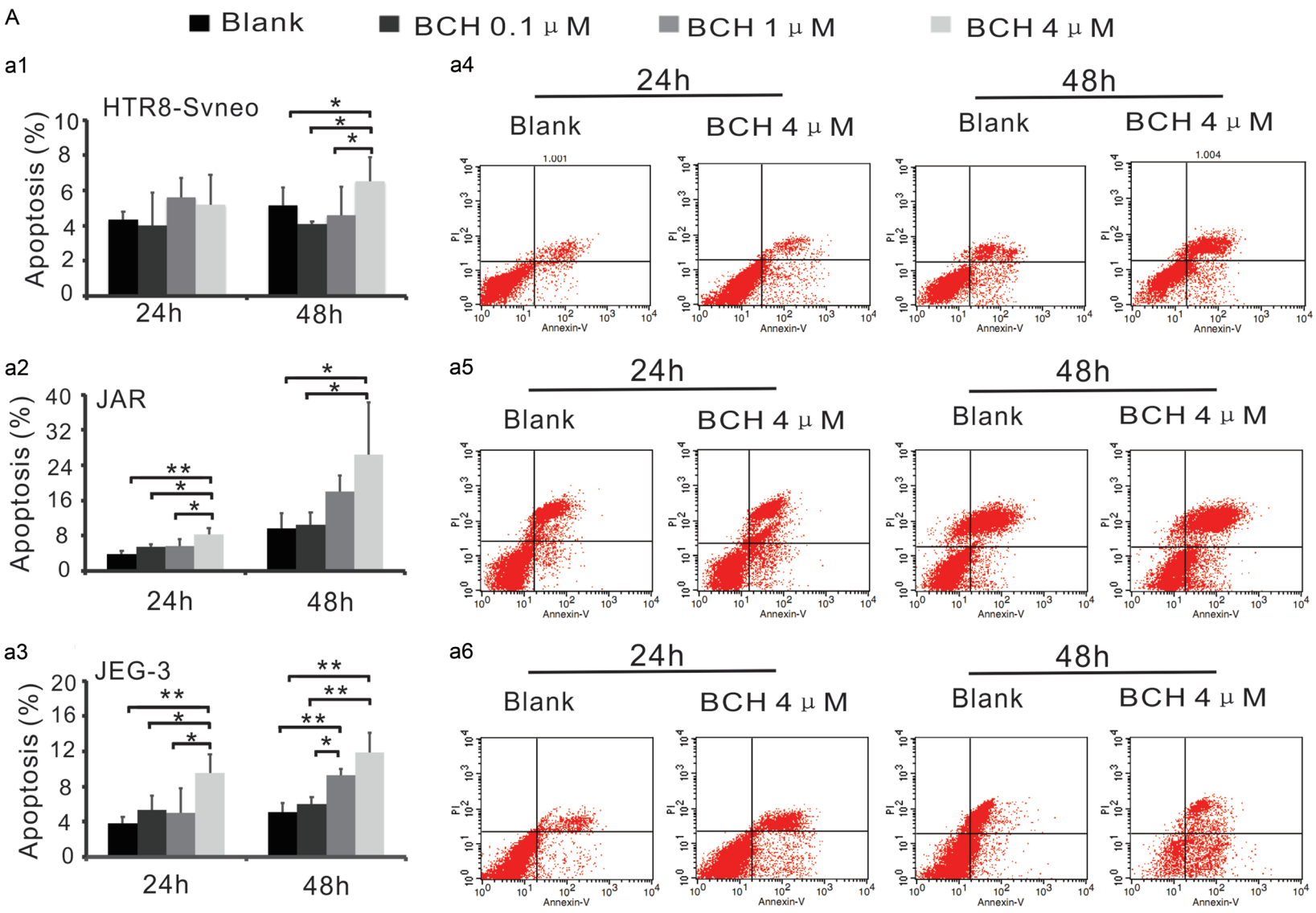
LAT1 positively regulates mTORC1 signaling pathway through p-4E-BP1

mTOR is an important regulator of cell proliferation and survival. The mTOR signaling pathway also plays a significant role in modulating cell invasion. Amino acids including leucine or amino acid prodrugs were transported into cell by LAT1, which then triggered mTORC1 activation. The results suggested that manipulation of LAT1 could be a new therapeutic strategy for tumor treatment. To assess this, it was necessary to investigate the interaction between LAT1 and mTOR signaling on cell growth or survival. Rapamycin and MHY1485 are an antagonist and agonist, respectively, of mTORC1 activity. We designed several experiments to clarify if these compounds could regulate LAT1 expression or rescue the phenotypes caused by different LAT1 expression level. As shown in **Figure 5**, the expression of LAT1 was significantly increased after treatment with 100 nM MHY1485, while being remarkably decreased after treatment with 100 nM rapamycin for 30 min or 24 h. Aberrant LAT1 expression in all three cell lines disrupted mTORC1 activity. To this end, the effects of up-regulation of LAT1 on mTORC1 activity were blocked by treatment with 100 nM rapamycin in all three cell lines. Depletion of LAT1 in the three cell lines transfected with SiLAT1-3 or treated with 4 μM BCH suppressed mTORC1 activity. The activity was rescued by 100 nM MHY1485, while the total protein levels of these molecules were unaffected following treatment. Down-regulation of LAT1 expression with SiLAT1-3 or treatment with 4 μM BCH suppressed mTOR, p-mTOR, and p-4E-BP1 expression. However, these expressions were blocked by treatment with 100 nM MHY1485. Up-regulation of LAT1 expression by transfection with the over-expressing pEGFP-N1-LAT1 plasmid promoted mTOR, p-mTOR, and p-4E-BP1 expression. These expressions were blocked by treatment with 100 nM rapamycin. Representative western blot images are shown with original western experiments.

Discussion

LAT1 gene expression and protein production were observed in HTR8-SVneo human trophoblasts and JEG-3 and JAR choriocarcinoma cells. Further investigation revealed the involvement of LAT1 in stimulating cell proliferation, inhibiting cell apoptosis, and promoting cell migration and invasion in both human trophoblasts and choriocarcinoma cells. Interestingly, mTORC1 signaling was a critical regulator of all these cell behaviors.

We previously demonstrated that LAT1 acts as a positive regulator of decidualization and the outgrowth of ectoplacental cones (EPCs) in a mouse model [25-27]. These phenomena suggested that the role of LAT1 could be dissected


A
Blank
BCH 0.1 μM
BCH 1 μM
BCH 4 μM
a1
HTR8-Svneo
Apoptosis (%)
24h
48h
a2
JAR
a3
JEG-3
a4
a5
a6
Annexin-V
PI
1.001
1.004

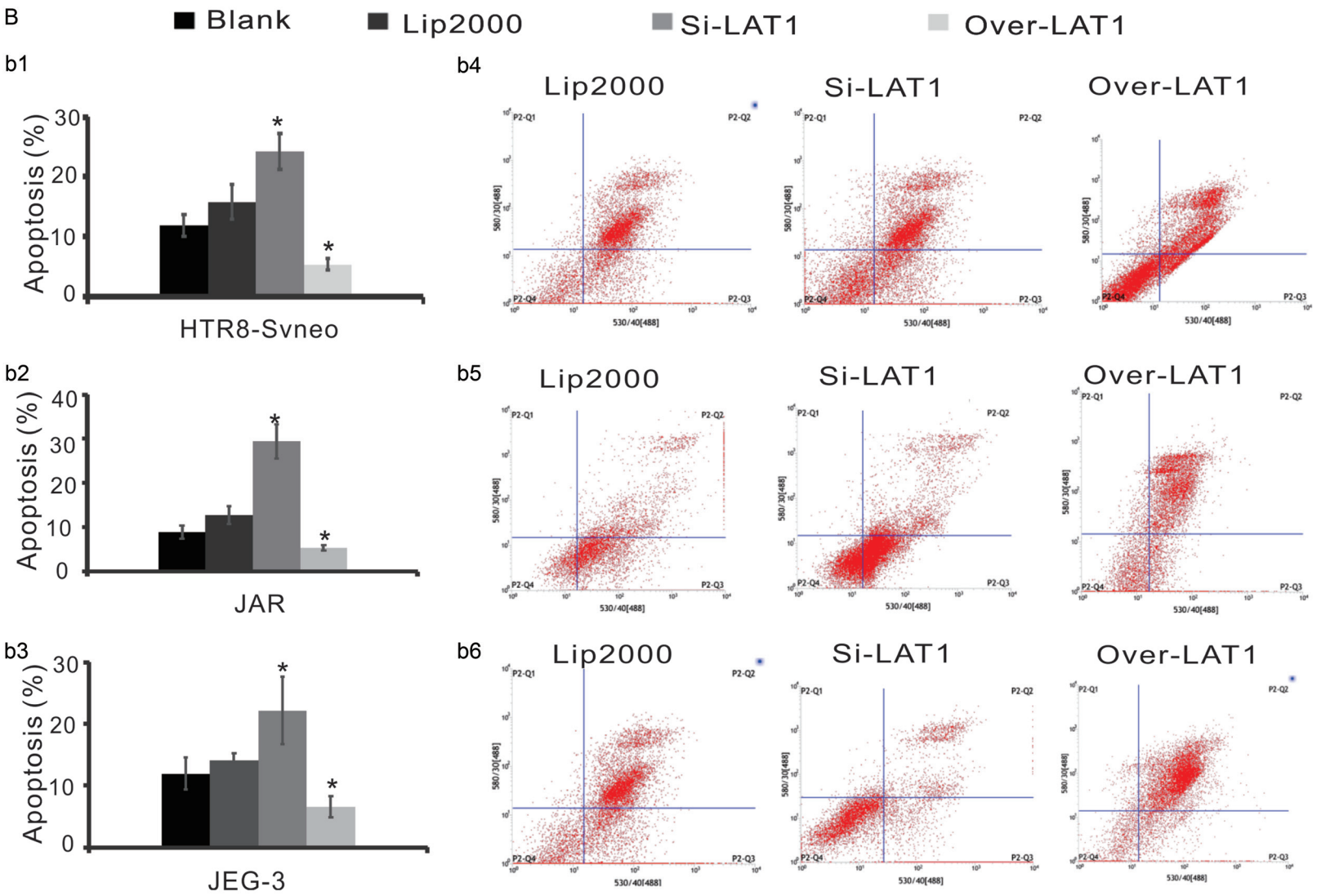
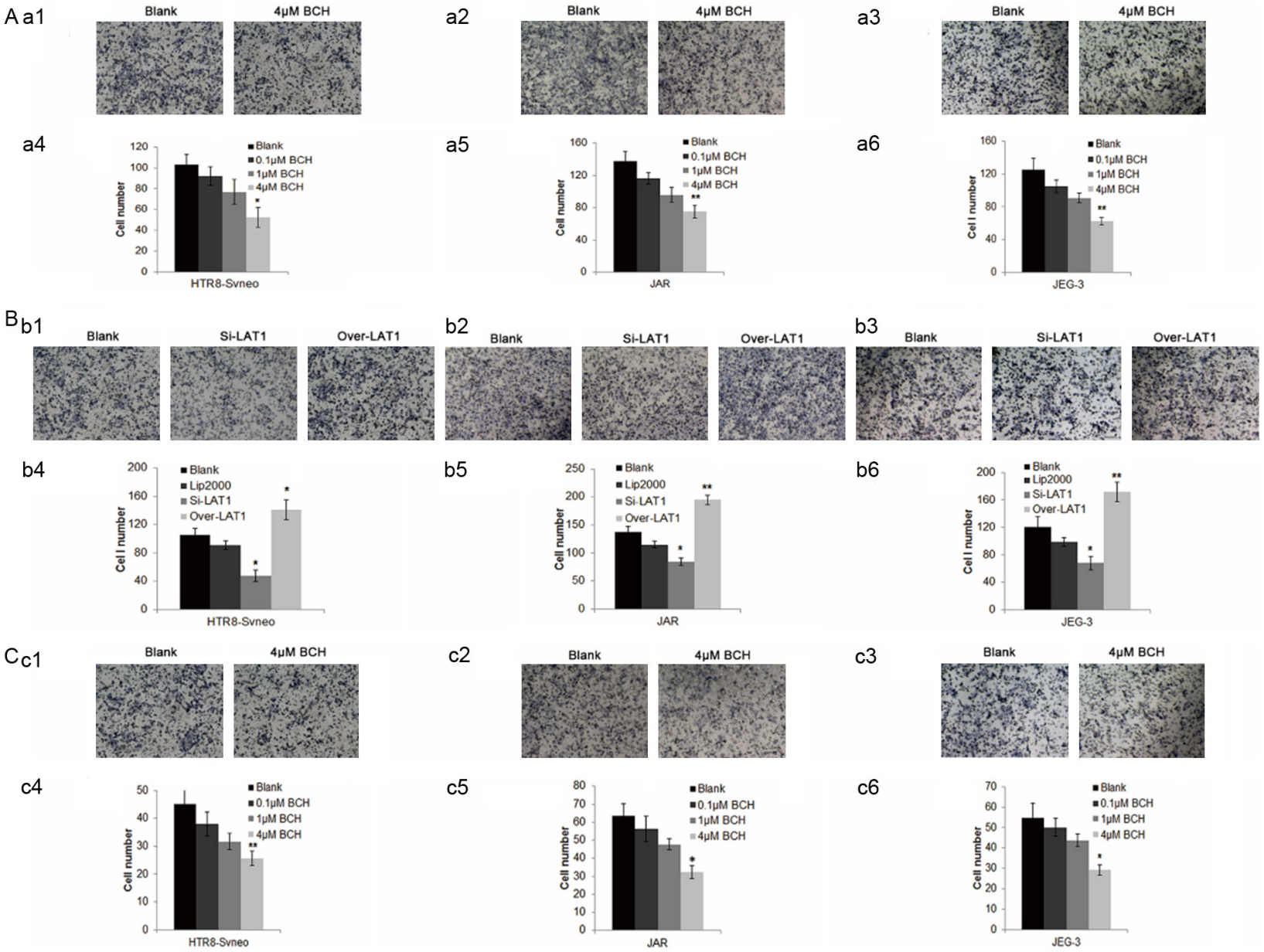


Figure 3. Effects of LAT1 on the apoptosis of the three trophoblast cell lines. (A) Down-regulation of LAT1 upon treatment with 4 μM BCH promoted apoptosis in the three cell lines at 24 h and 48 h of treatment, with a time-effect relationship. (a1-a3) The statistical bar graphs showing the rate of cell apoptosis. Representative images of cell apoptosis assayed by flow cytometry at 24 h and 48 h (a4-a6). (B) Up- and down-expression LAT1 upon transfection with plasmid regulated apoptosis in the three cell lines. (b1-b3) Statistical bar graphs showing that increased expression of LAT1 by transfection with pEGFP-N1-LAT1 plasmid inhibited apoptosis, while the decreased expression of LAT1 by transfection with SiLAT1-3 plasmid promoted apoptosis in the three cell lines. (b4-b6) Representative images of cell apoptosis assayed by flow cytometry. *$P < 0.05$, as compared with blank, **$P < 0.01$, as compared with blank.


A a1
Blank
4μM BCH
a2
Blank
4μM BCH
a3
Blank
4μM BCH
a4
Cell number
Blank
0.1μM BCH
1μM BCH
4μM BCH
HTR8-Svneo
a5
Cell number
JAR
a6
Cell number
JEG-3
B b1
Blank
Si-LAT1
Over-LAT1
b2
Blank
Si-LAT1
Over-LAT1
b3
Blank
Si-LAT1
Over-LAT1
b4
Cell number
Blank
Lip2000
Si-LAT1
Over-LAT1
HTR8-Svneo
b5
Cell number
JAR
b6
Cell number
JEG-3
C c1
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4μM BCH
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c4
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1μM BCH
4μM BCH
HTR8-Svneo
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JAR
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Cell number
JEG-3

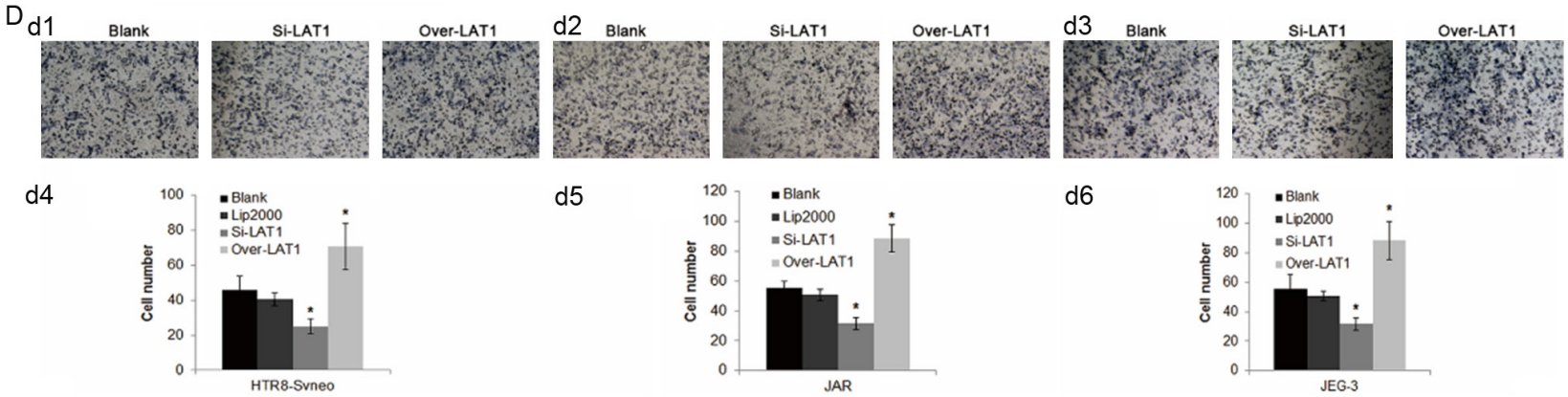
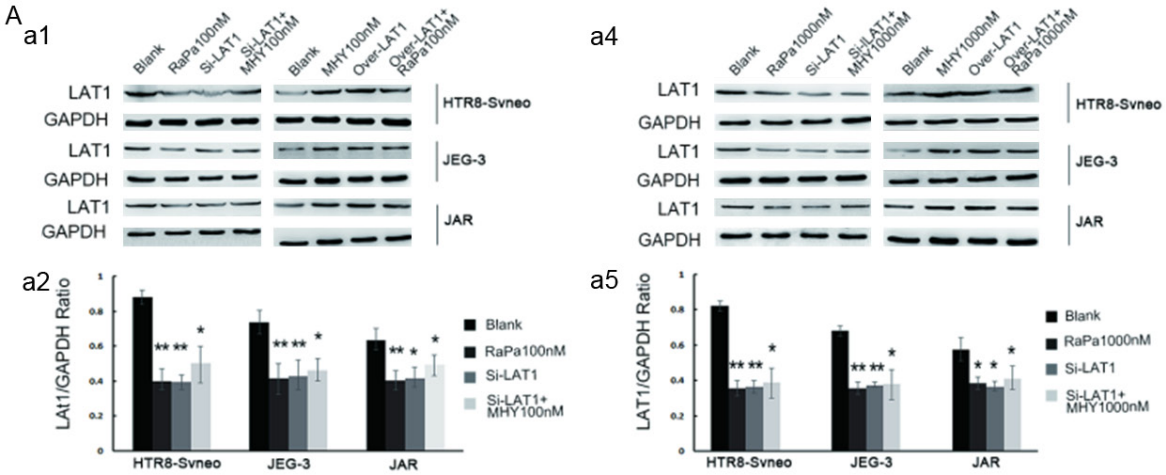


Figure 4. Effects of LAT1 on the migration and invasion of the three trophoblast cell lines. (A and B) cell migration of three trophoblast cell lines was promoted by up-regulation of LAT1 by transfection of pEGFP-N1-LAT1 plasmid, while migration was blocked by down-regulation of LAT1 by the transfection of SiLAT1-3 or treatment with 4 μM BCH. Representative hematoxylin stained images of invading cells in the Transwell cell migration assay with BCH treatment (a1-a3) or plasmid transfection (b1-b3). Statistical bar graphs showing the cell number in the lower side of the membrane with BCH treatment (a4-a6) or plasmid transfection (b4-b6). (C and D) Effects of LAT1 on the invasion of the three trophoblast cell lines. (a-c) Representative hematoxylin stained images of invading cells in the Transwell assay with BCH treatment (c1-c3) or plasmid transfection (d1-d3). Statistical bar graphs showing the increased cell numbers onthe lower side of the membrane with BCH treatment (c4-c6) or plasmid transfection (d4-d6). Treatment with 4 μM BCH or transfection with SiLAT1-3 in the three trophoblast cell lines decreased the number of cells that invaded to the lower side of the membrane. Over-expression of LAT1 by transfection with pEGFP-N1-LAT1 increased the invasion ability in the three trophoblast cell lines. *P < 0.05, as compared with blank, **P < 0.01, as compared with blank.



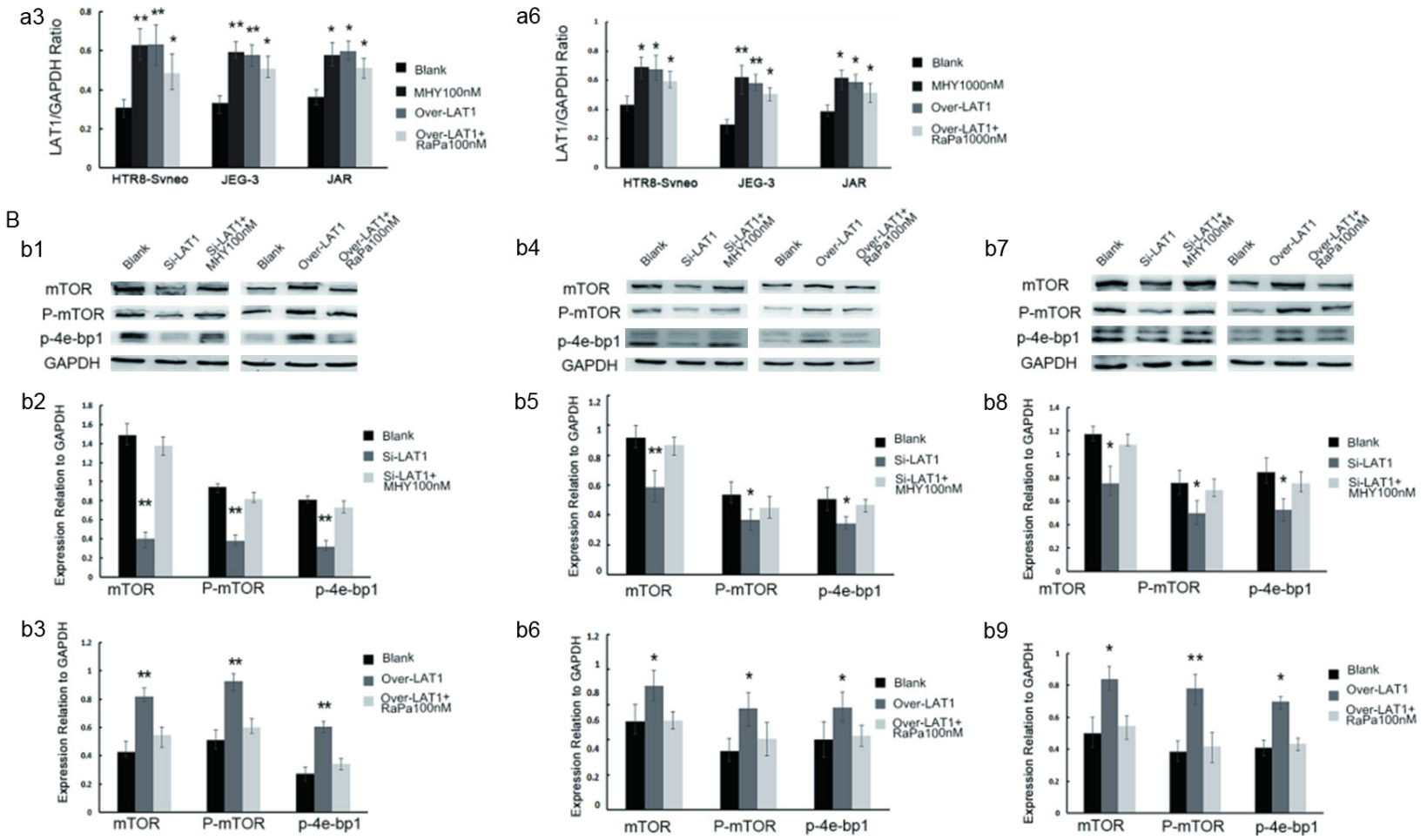


Figure 5. LAT1 interacts with mTORC1 cancer-related signaling pathways. Three cell lines were transfected with SiLAT1-3 or pEGFP-N1-LAT1 plasmids separately and then treated with rapamycin (mTORC1 signaling antagonist) or MHY1485 (mTORC1 signaling agonist) to determine if LAT1 interacts with the mTORC1 signaling pathways. A: Rapamycin decreased LAT1 expression and reversed over-expression of LAT1 by pEGFP-N1-LAT1 plasmids transfected in the three cell lines, while MHY1485 activated LAT1 expression and neutralized silencing of LAT1 expression by SiLAT1-3 plasmids transfected. B: LAT1 interacts with mTORC1 signaling pathways in the three cell lines. Down-regulation of LAT1 expression suppressed mTOR, p-mTOR, and p-4E-BP1 expression. The suppressed expression was blocked by treatment with 100 nM MHY1485. Up-regulation of LAT1 with transfection of pEGFP-N1-LAT1 plasmid promoted mTOR, p-mTOR and p-4E-BP1 expression. The promotion mTORC1 downstream of over-expression LAT1 was reversed by treatment with 100 nM rapamycin. *P < 0.05, as compared with blank. Representative western blot images are shown with original western experiments.

from a "maternal-fetal" perspective. The dynamic balance of trophoblast cell proliferation and invasion plays a key role in the establishment of the placenta and functional maturation. As main neutral amino acids transporter, trophoblast dysfunction has been related to many obstetrical issues. The role of LAT1 in human trophoblasts and its potential mechanism remains unclear. The proliferative and invasive properties of trophoblasts and tumor cells have several similarities involving growth factors, adhesion molecules, cytokines, and chemokines [3]. Our previous study showed that LAT1 and leucine promote the early establishment of the placenta and participate in the maternal-fetal interface in mice, indicating the active participation of LAT1 in supporting fetal development. In this study, the expression of LAT1 mRNA and protein of inHTR8-SVneo human trophoblasts indicated a possible correlation of LAT1 in maternal-fetal interface, such as proliferation and moderate invasion in normal pregnancy.

The aberrantly high expression of LAT1 is one of the most significant markers in various tumors, including mammary gland, thyroid, glioblastoma multiforme, and hemangiopericytomas [3, 28-31]. Suppression of LAT1 activity using selective compounds, such as BCH or (S)-2-amino-3-(4-((5-amino-2-phenylbenzo[d]oxazol-7-yl) methoxy)-3, 5-dichlorophenyl) propanoic acid (JPH203), might be considered novel molecular therapeutic strategies for tumor treatment [32]. Human placenta choriocarcinoma is rarely observed, but is a highly malignant neoplasm characterized by fast growth and broad transformation. In clinical conditions, choriocarcinoma is often found synchronously with metastasis. Unfortunately, we did not obtain any evidence that the expression of LAT1 in choriocarcinoma cells were abnormally higher than the expression in HTR8-SVneo trophoblasts. Due to limited gestational choriocarcinoma clinical specimens, we also lacked histological verification. Based on our current restricted knowledge, we might speculate that this phenomenon may be caused by differences in transport substrates. This needs to be assessed in further experiments. In this study, we failed to collect fresh choriocarcinoma tissues due to the limited research progress. However, we do report the oncogenic effects of LAT1 on choriocarcinoma JEG-3 and JAR cells. Both cell lines have been considered feasible for the study of the physiological and pathological effects on placental functions and human trophoblasts. The collective results we obtained are partially consistent with the observations of LAT1 expression in tumor cells with proliferative and invasive properties [28-31, 33].

HTR8-SVneo human extravillous trophoblasts are an established model to study first trimester EVT invasion and migration. In this study, BCH, a competitive inhibitor of the leucine transporter, was used to analyze the function of LAT1 proteins in HTR8-SVneo cells. BCH suppressed cell proliferation and promoted apoptosis, while inhibiting migration and invasion. BCH also inhibited LAT2. The inhibition of LAT2 also contributes to the cellular phenotype. We selectively knocked down LAT1 in HTR8-/SVneo cells, which resulted in significantly suppressed cell proliferation, cell migration, and cell invasion as well as massive cell apoptosis. The majority of cells were arrested in G0/G1 stage with a shortened S phase, which might be associated with disrupted mTORC1 signaling. We then over-expressed LAT1 in HTR8-SVneo cells. The cells remained in G2/M phase with increased cell proliferation, migration, and invasion, but suppressed apoptosis. Trophoblast invasiveness is regulated by cross-talk between trophoblasts and decidual cells in paracrine and autocrine manners [3]. The results provide direct evidence that LAT1 plays a major role in regulating human extravillous trophoblast cell behavior.

The mTOR signaling pathway regulates the function of the placental L-amino acid transporter in primary villous. Defective placental mTOR signaling might directly contribute to abnormal fetal growth in intrauterine growth retardation (IUGR) [34, 35]. In human trophoblasts, the mTOR signaling pathway was suggested a key role in the regulation of proliferation and differentiation in invasive trophoblasts [36]. Rapamycin regulates cell growth through mTORC1, but not mTORC2. The best-characterized downstream targets of mTORC1 are the ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) [14, 36]. However, we failed to detect the presence of S6K1 and its phosphorylated protein in the three cell lines. We observed the change of LAT1 expression after

treatment with rapamycin or its antagonist MHY1485 for 30 min or 24 h. Down-regulation of LAT1 suppressed mTOR, p-mTOR, and p-4E-BP1 expression. These suppressions were relieved by treatment with 100 nM MHY1485. Up-regulation of LAT1 could promote mTOR, p-mTOR, and p-4E-BP1 expressions, which were blocked by 100 nM rapamycin. These findings suggest that LAT1 functions as a powerful switch to control human trophoblast cell growth and differentiation in response to mTORC1.

The relationship between structure and function provides mechanistic and therapeutic insights into amino acid transport in the wider amino acid polyamine cation superfamily [37-40]. The solutable carriers LAT1-4F2hc (Slc7a5-Slc3a2) is a heterodimeric complex that plays a major role in transporting large branched and aromatic neutral amino acids, including leucine, past placental barriers [41]. Recent research shows 4F2hc is not only required for the cellular localization and stability of LAT1, but is also essential for its transport activity [13]. Due to its low expression and distribution in non-pathological cells and up-regulation in tumor cells, inhibitors of LAT1 would be introduced to impair or prevent cancer cell growth due to blocked amino acid uptake or induced deprivation. Moreover, LAT1-targeting amino-acid positron emission tomography tracers have been used in the tumor imaging [42, 43]. In this study, we detected co-expression of 4F2hc (Slc3a2) protein in HTR8-SVneo, JAR and JEG-3 cells (Supplementary Figure 1). Further revelations of the structure/function correlations will contribute to the exploration of new target drugs for pregnancy-related diseases.

We demonstrated that impeded LAT1 transporter activity or knockdown LAT1 expression in JEG-3 and JAR cells could partially suppress cell growth by inhibiting mTORC1 signaling. LAT1 suppression significantly decreased cellular leucine uptake, which might also contribute to mTOR signaling inhibition [44]. The relationship between intracellular leucine levels and mTOR signaling will be an interesting topic to study in the near future. It was beyond the scope of this paper. Irrespective of their relationship, LAT1 could be a potential marker in early diagnosis or ongoing therapeutic candidate for choriocarcinoma.

Conclusions

This is one of the few studies to investigate the role of LAT1 in human extravillous trophoblast and choriocarcinoma cells. LAT1 was validated as a key pathophysiological regulator in human pregnancy to control trophoblast and choriocarcinoma cell growth, survival, migration, and invasion. Thus, LAT1 may be considered a potential candidate involved in mTORC1 signaling pathway for further research in human trophoblast differentiation regulation and even in choriocarcinoma treatment. Further studies are needed to determine whether LAT1 exists in blood or other body fluids, and whether it has the potential to be used as a biomarker or a therapeutic molecular target for PE and choriocarcinoma in pregnancy.

Acknowledgements

Thanks are due to Professor Hongmei Wang (Institute of Zoology, Chinese Academy of Sciences, China) who kindly provided cell lines (HTR8-SVneo, JEG-3, and JAR). This work was supported by grants from the Chongqing Municipal Commission on Science & Technology (No. cstc2017jcyjAX0434), National Natural Science Foundation of China (No. 31301221; No. 31601206; No. 31571510), and Chongqing Yuzhong District Commission on Science & Technology (No. 20160118).

Disclosure of conflict of interest

None.

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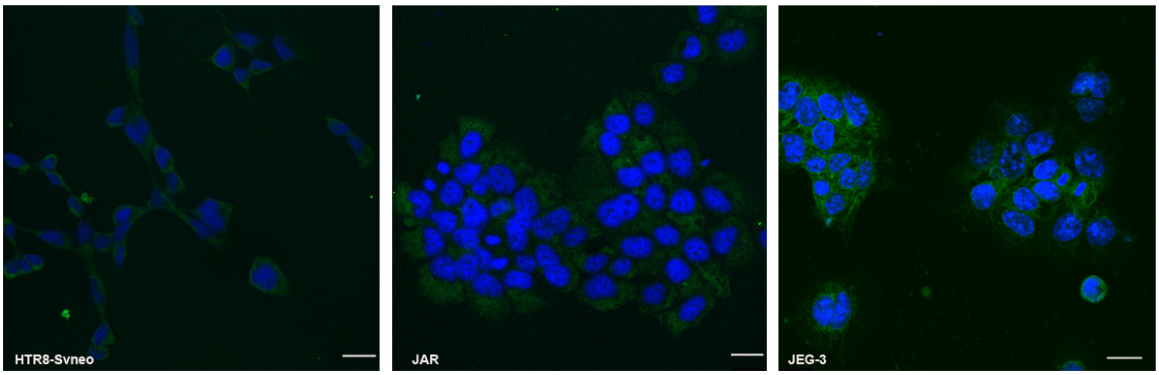

Supplementary Figure 1. Expression of Slc3a2 in human placental cell lines. Immunofluorescence of Slc3a2 in three trophoblast cell lines. Slc3a2 signals were visualized as red and strongly located in the cytoplasm in HTR8-SVneo cell, JEG-3 and JAR cell line, respectively. The nuclei were shown by DAPI staining (blue). Bar = 25 μM.