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AMBRA1 is involved in T cell receptor-mediated metabolic reprogramming through an ATG7-independent pathway

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ABSTRACT

Metabolic reprogramming contributes to dynamic alteration of cell functions and characteristics. In T cells, TCR-mediated signaling evokes metabolic reprogramming and autophagy. AMBRA1 is known to serve in the facilitation of autophagy and quality control of mitochondria, but the role of AMBRA1 in T cell metabolic alteration is unknown. Here, we show that AMBRA1, but not ATG7, plays a role in TCR-mediated control of glycolytic factors and mitochondrial mass, while both AMBRA1 and ATG7 are required for autolysosome formation. Our results suggested that AMBRA1 is a core factor that controls both autophagy and metabolic regulation.

1. Introduction

Metabolic reprogramming contributes to dynamic alteration of cell functions and characteristics [1–4]. During differentiation and maturation, specific metabolic alterations must occur correctly to generate functional cells. Glycolytic inhibition is essential for neural progenitor cell (NPC) differentiation to neurons, in which mitochondrial metabolism is dominant [5]. In another case, resting macrophages mature into either pro-inflammatory, which mainly use glycolysis [6,7], or anti-inflammatory macrophages, which mainly use mitochondrial metabolism [8]. Interestingly, metabolic reprogramming also occurs during acquisition of pluripotency in induced pluripotent stem (iPS) cells; mitochondria in somatic fibroblasts have been shown to degrade via autophagy, allowing glycolysis to become dominant [9].

In T cells, several lines of evidence have shown that metabolic change is relevant to T cell functions [10–12]. Xu et al. demonstrated that autophagy is required for memory CD8 T cell formation through the production of lipids, substrates for fatty acid oxidation that activate mitochondrial metabolism [13]. Therefore, it is assumed that metabolic reprogramming and autophagy are closely linked and play pivotal roles in the integrated control of cell functions and characteristics. However, the molecular basis for the association of autophagy and metabolic control is not fully understood. AMBRA1 is an autophagic protein, and is reportedly involved in the initiation step with the PI3K complex including BECLIN1 and VPS34 [14–18]. Here, we show that AMBRA1 plays a role in TCR-mediated metabolic regulation; in the decrease in glycolytic factors and increase in mitochondrial mass in an ATG7-independent manner; and in autolysosome formation, along with ATG7. Our results suggested that AMBRA1 is a core factor that controls both autophagy and metabolic regulation.

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2. Materials and methods

2.1. Cell culture

OVA53 cells, $CD4^{+}CD8^{+}$ thymic lymphoma, were derived from RAG2-deficient T-cell receptor (TCR) transgenic mice backcrossed with p53 knockout mice [19]. Cells were cultured in RPMI1640 (#05918, Nissui) containing 10% fetal calf serum (FCS), 2 mM glutamate, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified 5% CO2 incubator. Cell number was counted using a CDA-1000 hematology analyzer (Sysmex). For autophagy induction, cells were cultured with rapamycin (100 nM) for 4 h, or in serum-free medium for 24 h. For delivering the TCR-mediated signal, cells were cultured with anti-mouse CD3ε(145-2C11) at 250 ng/mL on 24-well culture plates pre-coated with anti-hamster IgG (ab5738, Abcam) for 24 or 48 h.

2.2. Antibodies

The following antibodies were used for immunoblot analysis, flow cytometry analysis, and immunohistochemistry: rabbit anti-AMBRA1 (#24907, Cell Signaling Technology), rabbit anti-ATG7 (#8558T, CST), rabbit anti-LC3 (PM036, MBL), guinea pig anti-p62 (FNGP62-C, Progen), HRP-conjugated anti-Mouse IgG (NA9310V, GE Healthcare), HRP- or Alexa 594-conjugated anti-Rabbit IgG (NA9340V, GE Healthcare, or A-11072, Invitrogen), and HRP-conjugated anti-Guinea pig IgG (sc-2438, Santa Cruz).

2.3. Plasmids and transfection

pGCDNsamIREShuKO was kindly provided by Dr. M. Onodera [20]. The huKO fragment was replaced by an hNGFR fragment, into which human AMBRA1 cDNA (DNAFORM clone ID 5296472) was inserted. Retrovirus vector pBABE-puro mCherry EGFP carrying human LC3B gene was purchased from Addgene (plasmid #22418), and the LC3B fragment was amplified and inserted into a PiggyBac vector (pAZG IRES NeoR) [21]. Retrovirus was produced using plat-e virus-producing cell system [22]. After infection, infected cells were collected using FACSAria (BD). pAZG mCherry EGFP LC3 and pCX mPB were transfected using Neon (Invitrogen), and transfected cells were sorted using a FACSAria cell sorter (BD).

2.4. Generation of Ambra1-deficient and Atg7-deficient cells

The target sequence of sgRNA was designed at exon 2 of mouse *Ambra1* by optimized CRISPR DESIGN (http://crispr.mit.edu/). Target oligonucleotides were inserted into an sgRNA-expressing vector R450. Wild type Cas9 with the IRES-mCherry sequence was cloned into an R522 vector, which harbors the CMV promoter/enhancer, rabbit beta-globin intron, and polyA signal sequence. The mCherry coding sequence was derived from the pmCherry-N1 vector, purchased from Clontech (632523). Cas9 with sgRNA expressing vector for *Atg7* PX459 was kindly provided by Dr. T. Yahata (Tokai Univ.Sch.Med.). Cas9-and sgRNA-expressing vectors were transfected by electroporation using Neon (Invitrogen). After limiting dilution, clones were analyzed by immunoblot.

2.5. Immunoblot analysis

Total cell lysates were prepared using an RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) with a protease inhibitor cocktail (11873580001, Roche) and 0.5 μM PMSF (P7627, Sigma). Proteins were separated on SDS polyacrylamide gel and then transferred to a PVDF membrane. For blocking, 3% BSA/TBST or 5% Skim Milk/TBST was used. The membranes were incubated overnight at 4 °C with the primary antibodies diluted in blocking solution. After washing with TBST, the membrane was incubated with the secondary antibodies for 1 h. After washing, target proteins were detected using Immobilon Western Chemilum HRP substrate (WBKLS0100, Millipore). The band intensities were measured using ImageJ software (NIH).

2.6. Immunofluorescence staining

Cells were fixed and permeabilized by Fixation and Permeabilization Solution (554722, BD) according to the manufacturer's instruction. After staining, cells were plated on slide-glasses by cytospin. The cells were then embedded in DABCO containing DAPI, then observed and photographed with confocal microscopy (LSM700, Carl Zeiss).

2.7. FACS analysis

For the staining of mitochondria, cells were incubated with MitoTracker-Green (20 nM M7514, Invitrogen) and MitoTracker-Deep red (20 nM M22426, Invitrogen) at 37 °C for 30 min. Cell cycle analysis was performed using an APC BrdU Flow kit (552598, BD). Fluorescent intensities were measured using FACSVerse flow cytometer (BD) and analyzed using the FlowJo software (FLOWJO LLC). Before and after autophagy induction, fluorescence levels of mCherry EGFP LC3B-transfected cells were measured using an LSRFortessa cell analyzer (BD).

2.8. Electron microscopy

Cells were fixed in 2.5% glutaraldehyde/0.05 M phosphate buffer for 10 min at room temperature and directly centrifuged on glass slides using a cytospin centrifuge. Then the cells were post-fixed in 1% osmium tetroxide/0.05 M phosphate buffer for 1 h, and stained with ulanyl acetate. After dehydration with a graded ethanol series, specimens were embedded in Quetol 812. Ultrathin sections were stained with ulanyl acetate and lead citrate and observed using a transmission electron microscope (JEM 1400; JEOL).

2.9. qPCR analysis

Total RNA was extracted using TRIzol Reagent (15596026, Ambion). cDNA was synthesized with ReverTra Ace qPCR RT Master Mix (FSQ-201, Toyobo). SYBR Green-based qPCR was performed with a Fast7500 PCR system using the THUNDERBIRD qPCR Mix (QPS-101, Toyobo). Values were normalized to *Ubc (Ubiquitin C)*. Sequences of specific primers are listed in Table 1.

Table 1
Primer sequences for qPCR analysis.

qPCR primers		
Primers	Strand	Sequence 5′→3′
Ubc	Forward	agcccagtgttaccaccaag
	Reverse	acccaagaacaagcacaagg
Glut1	Forward	agccctgctacagtgtat
	Reverse	aggtctcgggtcacatc
Ldha	Forward	aaaggttacacatcctgggc
	Reverse	gatacatgggacactgaggaag
Pkm2	Forward	tgtctggagaaacagccaag
	Reverse	tcctcgaatagctgcaagtg
Hk2	Forward	caactccggatgggacag
	Reverse	cacacggaagttggttcctc
c-myc	Forward	cctagtgctgcatgaggaga
	Reverse	tccacagacaccacatcattt

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