PPARγ-AGPAT6 Signaling Mediates Acetate-induced mTORC1 Activation and Milk fat Synthesis in Mammary Epithelial Cells of Dairy Cows

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Supplementary File

Supplementary materials and methods

Mammary epithelial cells isolation

Mammary epithelial cells were isolated from mammary tissues of 3 lactating cows at 90 DIM as previously described (Lv et al., 2021; Yang et al., 2017). Briefly, the tissue samples were washed three times with D-Hank's solution and minced with surgical scissors. Then the minced samples were digested with collagenase III (C8490, Solarbio, Beijing, China) for 2 h at 37°C. The digest was filtered through a nylon mesh and the filtrate centrifuged at 100 × *g* for 10 min. The cell pellet was resuspended and cultured in DMEM/F12 (Life technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (BI Biological Industries, Kibbutz Beit-Haemek, Israel), penicillin (100U/mL),

and streptomycin (100μg/mL) at 37℃ with 5% CO_2. The primary cell cultures were detached with trypsin-EDTA (0.25%, 25200056, Life Technologies) at ~80% confluency and transferred to new culture flasks, which were used to remove fibroblasts. Pure mammary epithelial cells were obtained after 3 passages.

Immunofluorescence staining of cytokeratin 18

The expression of cytokeratin 18 was examined by seeding 2×10^5 cells in a 35-mm glass bottom cell culture dish. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and blocked with 10% goat serum in PBS for 1 h at 37°C. Then cells were incubated overnight at 4 ℃ with cytokeratin 18 antibody (1:50 dilution; A1022, ABclonal, Wuhan, China). Following three washes with PBS, cells were treated with fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (1:200 dilution; AS011, ABclonal) for 1h at 37°C. To stain the nuclei, cells were treated with DAPI for 10 min at room temperature, followed by three washes with PBS, and mounted with DABCO. Fluorescence was assessed by confocal microscopy (TCS SP8, Leica Microsystems, Wetzlar, GmbH, Germany).

BODIPY staining of intracellular lipid droplets

The mammary epithelial cells were seeded in 35-mm glass bottom cell culture dishes (2×10^5 cells each). For milk lipid droplets staining, cells were rinsed twice in PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and then stained with BODIPY 493/503 (stock concentration 1 mg/mL, working solution 1:1000 dilution, D3922, Invitrogen, Carlsbad, CA) for 20 min at 37°C. Following three washes with PBS, nuclei were labeled by DAPI for 10 min at room temperature. The cells were

mounted with DABCO, and the lipid droplets were imaged by confocal microscopy (TCS SP8, Leica Microsystems, Wetzlar, GmbH, Germany).

Acetate and rapamycin treatment of mammary epithelial cells

To detect the effect of acetate on mTORC1 signaling activation, mammary epithelial cells were seeded in 6-well plates (2×10^5 cells/well). When cells grew to ~90% confluence, the medium was changed to serum-free medium with 1% FA-free bovine serum albumin (Sigma-Aldrich) for 12 h. Then cells were stimulated 48 h with 12 mM sodium acetate. To detect the effect of rapamycin on acetate-induced mTORC1 signaling activation, after being serum starved overnight, mammary epithelial cells were stimulated 48 h with 100 nM rapamycin in the presence of 12 mM sodium acetate.

Transfection

Mammary epithelial cells were cultured in 6-well plates at 2×10^5 cells/well. After being serum starved overnight, transient transfection was performed using Lipofectamine 2000 (Life technologies) according to the manufacturer's instruction. For RAPTOR knockdown, cells were transfected with 1.58 ng of RAPTOR siRNA (sense = GCAGCAAUGACUACUACAUTTAUGUAGUAGUCAUUGCUGCTT). Cells transfected with scrambled siRNA (sense =UUCUCCGAACGUGUCACGUTT) were used as negative control. All siRNAs were purchased from Gene Pharma (Shanghai, China). After transfection, 12 mM sodium acetate was added to the medium for another 48h.

Plasmid constructions

To construct AGPAT6 luciferase reporter gene construct, genomic DNA was extracted

from bovine mammary epithelial cells by a mammalian genomic DNA extraction kit (SparkJade Inc., Jinan, China). Bovine AGPAT6 promoter fragments with -800, -400, and -110 bp were obtained by PCR, and the promoter fragment with -40 bp was obtained by chemical synthesis. Then these fragments were subcloned into the luciferase reporter vector pGL3 (Promega Corporation, Madison, WI) at the Xho I/Hind III sites separately. To generate the mutant sequence, the pGL3-AGPAT6 (-110) was used as a template. The construct with mutation in the RXRα consensus binding site (located between -104 bp and -96 bp) was produced by displacing the sequence GTTGAACTC with TGATTGACC by PCR. The primers used were displayed in Supplementary Table S1. The integrity of plasmid DNA was verified by sequencing.

Luciferase reporter assays

Mammary epithelial cells were plated in 24-well plates at 1×10^4 cells/well and transiently transfected with 48 ng of each luciferase reporter gene vector or co-transfected with pGL3-AGPAT6(-110) and PPARγ-specific shRNA (1.25 ng) as described above. After transfection, the medium was changed to a medium containing 12 mM sodium acetate. After 48 h, cells were collected to measure the luciferase activity using the Luciferase Assay Dual System (Promega). Each transfection was done in triplicate and three independent experiments were performed for each construct.

ChIP Assay

Mammary epithelial cells (1×10^7 cells) were treated with 12 mM sodium acetate for 48h. Then cells were cross-linked with 1% formaldehyde (v/v) for 15 min at room temperature, and quenched with 125 mM glycine (Beyotime Biotechnology, Shanghai,

China) for 5 min at room temperature. Cells were harvested and sonicated with 0.1% SDS containing protease inhibitor (Solarbio) to shear DNA to an average length ranging from 200 to 500 bp. Then the fragmented DNA was incubated with 1 μg of PPARγ antibody (2435, Cell Signaling Technology, Beverly, MA) or IgG (as a negative control) overnight at 4 °C, and immunoprecipitated with 40 μL of protein A+G agarose (Beyotime Biotechnology). Final DNA extractions were used for quantitative-PCR (qPCR) analysis to determine whether acetate had an effect on the interaction between PPARγ and AGPAT6. qPCR was performed using the following primers: forward = 5'-CTGGCAGCATGTTGAACTCTTC-3' ; reverse = 5'-CTCCTGCCCAAGAGCATTTAG-3'.

Western blot analysis

Mammary epithelial cells were treated with RIPA buffer containing 1% protease inhibitor cocktail (Solarbio) on ice. The protein concentrations were determined using a BCA protein assay kit (TaKaRa). Then ~30 μg of protein was subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. The membranes were incubated for 2 h with 5% skim milk in TBST and then overnight at 4°C with antibodies specific to RAPTOR (1:1000 dilution, 2280, Cell Signaling Technology), mTOR (1:1000 dilution, 2983, Cell Signaling Technology), P70S6K (1:1000 dilution, 2708, Cell Signaling Technology), 4E-BP1 (1:1000 dilution, 9644, Cell Signaling Technology), p-mTOR (1:1000 dilution, 2971, Cell Signaling Technology), p-P70S6K (1:1000 dilution, 9234, Cell Signaling Technology), p-4E-BP1(1:1000 dilution, 2855, Cell Signaling Technology), AGPAT6 (1:1500 dilution,

16762-1-AP, Proteintech, Wuhan, China), PPARγ (1:1000 dilution, A0270, ABclonal), and β-ACTIN (1:1000 dilution, AC038, ABclonal). After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:3000 dilution, ABclonal) at 37℃ for 1h. The blots were probed by enhanced chemiluminescence detection system (Tanon, Shanghai, China). The bands were quantified using Image-Pro Plus 6.0 (Media Cybernetics Inc., Warrendale, PA), and each protein expression was normalized to β-ACTIN expression.

Measurement of cellular TAG content

Mammary epithelial cells were collected and the intracellular TAG were detected by a TAG assay kit (Applygen Technologies Inc., Beijing, China). The protein concentration of corresponding cell sample was determined by a BCA method (TaKaRa Inc., Dalian, China). The TAG level was normalized to the protein concentration of each sample.

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA). Comparisons between two groups were carried out using 2-tailed unpaired *t*-test. Comparisons more than 3 groups were assessed using one-way ANOVA with post-Bonferroni's multiple comparisons. Differences were considered significant at $P < 0.05$.

References

Lv, H., Meng, Q., Wang, N., Duan, X., Hou, X., & Lin, Y. (2021). Cell death-inducing DNA fragmentation factor-alpha-like effector C (CIDEC) regulates acetate- and beta-hydroxybutyrate-induced milk fat synthesis by increasing FASN expression in mammary epithelial cells of dairy cows. *J Dairy Sci*, *104*(5), 6212-6221. https://doi.org/10.3168/jds.2020-18975

Yang, Y., Lin, Y., Duan, X., Lv, H., Xing, W., Li, Q., Gao, X., & Hou, X. (2017). The effects of cell death-

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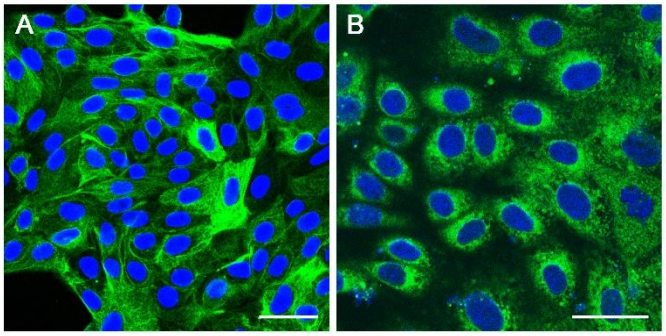
Supplementary Table S1 Primers used for AGPAT6 promoter PCR[1]

Gene	Primer sequence (5′ to 3′)
AGPAT6-800-XhoI	F: CCGCTCGAGGGTGGACAAACCACTACATGTAATG
	R: CCCAAGCTTGGCACTGCTTCCAGAAGTTTC
AGPAT6-400-XhoI	F: CCGCTCGAGTGCCTGGGATCACACTCCC
	R: CCCAAGCTTGGCACTGCTTCCAGAAGTTTC
AGPAT6-110-XhoI	F: CCGCTCGAGAGCATGTTGAACTCTTCTTG
	R: CCCAAGCTTGGCACTGCTTCCAGAAGTTTC
AGPAT6-110mut-XhoI	F: CCGCTCGAGAACCTTCTTGGTACTTCTGGCAAT
	R: CCCAAGCTTGGCACTGCTTCCAGAAGTTTC

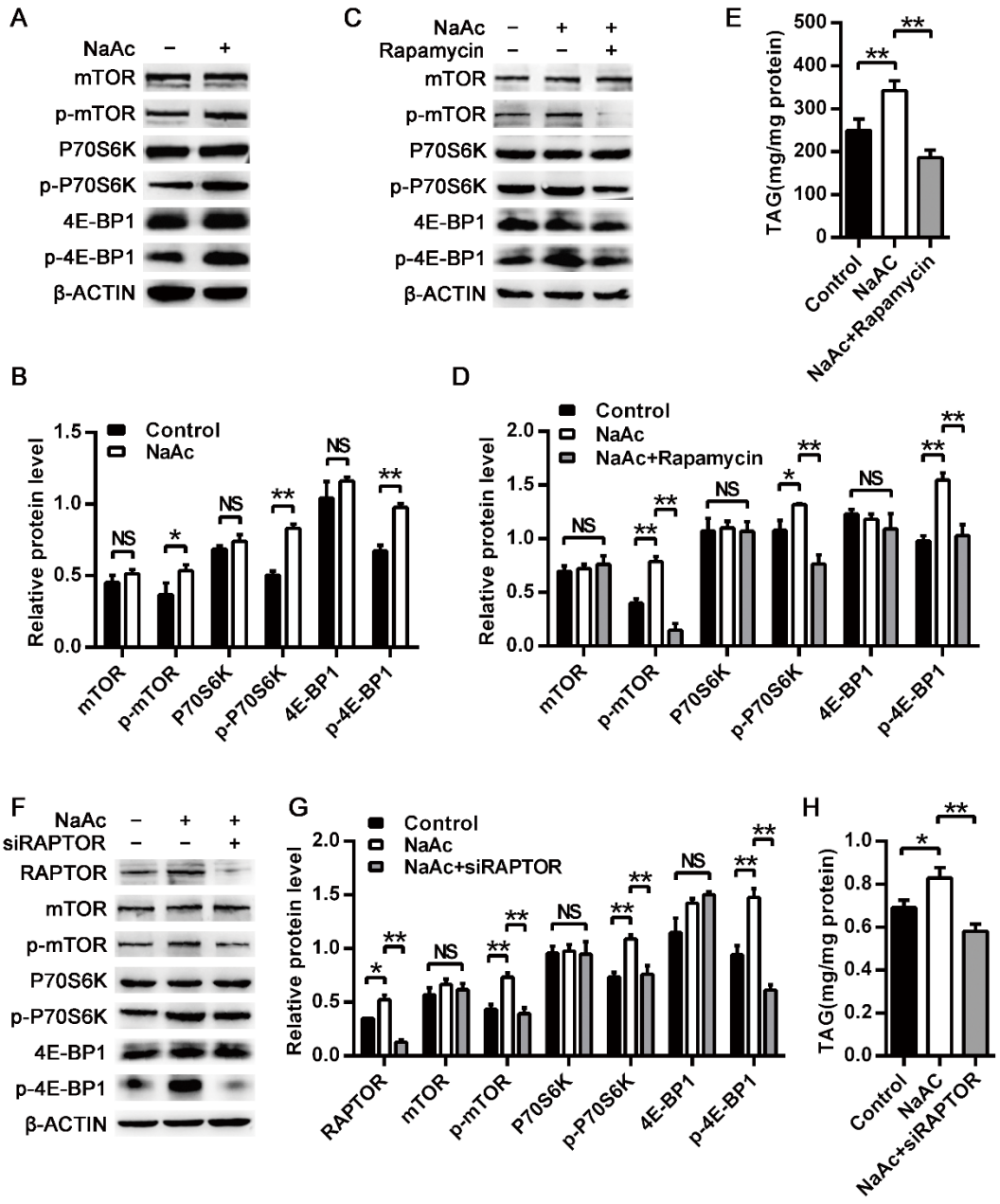
[1]F = forward; R = reverse; mut=mutation

Supplementary Fig. S1.



Supplementary Fig.S1. The detection of cytokeratin 18 and intracellular lipid droplets in dairy cow mammary epithelial cells. (A) Immunofluorescence staining showing the expression of cytokeratin 18 in dairy cow mammary epithelial cells. Cytokeratin 18 was labeled by FITC (green), and nuclei were labeled by DAPI (blue). Scale bar = 30 μm. (B) BODIPY staining showing the lipid droplets in dairy cow mammary epithelial cells. Lipid droplets were labeled by BODIPY 493/503 (green), and nuclei were labeled by DAPI (blue). Scale bar = 30 μm.

Supplementary Fig. S2.



Supplementary Fig.S2. Effects of mTORC1 signaling on acetate-induced milk fat synthesis in mammary epithelial cells of dairy cows. (A) Effect of acetate on mTORC1 signaling molecules expression. (B) Quantification of mTORC1 signaling molecules expression from western blots in panel A. (C) Effect of rapamycin on acetate-induced mTORC1 signaling molecules expression. (D) Quantification of mTORC1 signaling molecules expression from western blots in panel C. (E) Effect of rapamycin on acetate-

induced TAG synthesis. (F) Effect of *RAPTOR* siRNA on acetate-induced mTORC1 signaling molecules expression. (G) Quantification of mTORC1 signaling molecules expression from western blots in panel F. (H) Effect of *RAPTOR* siRNA on acetate-induced TAG synthesis. Results are representative of 3 independent experiments, each with n = 3. *$P < 0.05$; **$P < 0.01$.