

# Arginine Supplementation Recovered the IFN- $\gamma$ -Mediated Decrease in Milk Protein and Fat Synthesis by Inhibiting the GCN2/eIF2 $\alpha$ Pathway, Which Induces Autophagy in Primary Bovine Mammary Epithelial Cells

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During the lactation cycle of the bovine mammary gland, autophagy is induced in bovine mammary epithelial cells (BMECs) as a cellular homeostasis and survival mechanism. Interferon gamma (IFN- $\gamma$ ) is an important antiproliferative and apoptogenic factor that has been shown to induce autophagy in multiple cell lines *in vitro*. However, it remains unclear whether IFN- $\gamma$  can induce autophagy and whether autophagy affects milk synthesis in BMECs. To understand whether IFN- $\gamma$  affects milk synthesis, we isolated and purified primary BMECs and investigated the effect of IFN- $\gamma$  on milk synthesis in primary BMECs *in vitro*. The results showed that IFN- $\gamma$  significantly inhibits milk synthesis and that autophagy was clearly induced in primary BMECs *in vitro* within 24 h. Interestingly, autophagy was observed following IFN- $\gamma$  treatment, and the inhibition of autophagy can improve milk protein and milk fat synthesis. Conversely, upregulation of autophagy decreased milk synthesis. Furthermore, mechanistic analysis confirmed that IFN- $\gamma$  mediated autophagy by depleting arginine and inhibiting the general control nonderepressible-2 kinase (GCN2)/eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) signaling pathway in BMECs. Then, it was found that arginine supplementation could attenuate IFN- $\gamma$ -induced autophagy and recover milk synthesis to some extent. These findings may not only provide a novel measure for preventing the IFN- $\gamma$ -induced decrease in milk quality but also a useful therapeutic approach for IFN- $\gamma$ -associated breast diseases in other animals and humans.

## INTRODUCTION

Bovine mammary epithelial cells (BMECs), which have a lactation function, can be obtained by different isolation and cultivation methods and can be a cellular model for studies of mammary gland development and the mechanisms that regulate lactation (Bian et al., 2015; Hu et al., 2009; Li et al., 2009). Currently, milk protein and milk fat percentages, two important dairy quality indicators, are lower in China than in developed countries. Many dietary, hormonal, environmental and management factors affect dairy cow mammary gland functions at the molecular and cellular levels, and these factors can significantly affect milk production and quality. A better understanding of the regulation of dairy cow milk synthesis and quality will facilitate strategies for optimizing milk quality and drive the profitability of the dairy industry (Bian et al., 2015). Increasing evidence suggests that diet can increase the release of proinflammatory cytokines in the mammary gland, and the concentrations of cytokines in mammary venous plasma are negatively correlated with milk production efficiencies (Wu, 2013; Zhou et al., 2014). IFN- $\gamma$  is primarily a proinflammatory cytokine that is produced by T cells and natural killer cells, but its levels are also influenced by diet (Borden et al., 2007). IFN- $\gamma$  modulates several cellular activities, including the cell cycle and cell growth, by regulating transcription (Boehm et al., 1997; Maher et al., 2007). Recently, a genome-wide ribosome profiling approach showed that the translational regulation of IFN- $\gamma$  selectively affected pathways important for cytokine expression, protein synthesis and cell metabolism (Marino and Lopez-Otin, 2004). Emerging evidence suggests that IFN- $\gamma$  signaling and autophagy interact (Harris, 2011).

Autophagy is a major protective mechanism that has important functions in resistance to starvation, growth control, maintenance of cellular functions, and removal of anomalous cellular components; it also helps defend organisms against degenerative, infectious, inflammatory, and neoplastic diseases (Kroemer et al., 2010; Rubinsztein et al., 2011). Normal cells can employ autophagy as the means to survive nutrition and growth factor deprivation. Autophagy also generally functions to maintain cellular homeostasis through nutrition recycling and

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protein quality control (Marino and Lopez-Otin, 2004). Numerous lines of evidence showed that basal autophagy plays a critical role in the maintenance of cellular homeostasis and genomic integrity (Hannigan and Gorski, 2009). Increasing evidence suggests that dysfunction in the autophagy pathway is implicated in a growing number of diseases (Kimmelman, 2011; Ravikumar et al., 2010).

In the mammary gland, autophagy was observed during the lactation cycle, and its activity is the highest in the dry period (Motyl et al., 2007; Sobolewska et al., 2009). The autophagic effect of TGF- $\beta$ 1 has been shown directly *in vitro* in bovine BME-UV1 MECs (Gajewska et al., 2005). In addition, IGF-I and EGF are involved in suppressing autophagy via the mTOR kinase pathway, whereas 17 $\beta$ -estradiol and progesterone seem to function as inducers in BME-UV1 cells (Sobolewska et al., 2009). Moreover, the induction of autophagy contributes to the survival of epithelial cells during anoikis and to lumen formation (Sobolewska et al., 2011). However, the direct effects of IFN- $\gamma$  in regulating autophagy and the role of autophagy have not yet been investigated in BMECs.

Therefore, we hypothesized that IFN- $\gamma$  might regulate milk synthesis by activating autophagy. In this study, primary BMECs were cultured *in vitro* to explore the effects of IFN- $\gamma$  on milk synthesis and its underlying mechanisms. We demonstrated that IFN- $\gamma$  inhibited milk synthesis in BMECs *in vitro* in an autophagy-dependent manner. Furthermore, arginine supplementation rendered the cells resistant to autophagy, which was triggered by arginine depletion and activation of GCN2/eIF2 $\alpha$  signaling pathway, and recovered milk synthesis to some extent. Our findings may form the basis of a proper nutritional program for maintaining the function of the mammary gland and provide a reference for human breast health.

## MATERIALS AND METHODS

### Reagents

Bovine IFN- $\gamma$  was purchased from the Kingfisher Group (Kingfisher Biotech, Inc., USA). Bafilomycin A1 and E64d were purchased from Abcam (UK). 3-Methyladenine (3-MA) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Germany). Chloroquine and rapamycin were purchased from Cell Signaling Technology (USA). The amino acids were purchased from Nanjing Keygen Biotech. Co., Ltd.

### Cell culture

Primary BMECs were isolated and purified as previously described (Hu et al., 2009). The BMECs were maintained in DMEM/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Arginine was added at no more than 4 times (4  $\times$ ) the normal plasma concentrations. The normal concentration (1-fold) of each amino acid was ( $\mu$ M): L-Asp, 12.1; L-Ser, 65.4; L-Gln, 152.5; L-Gly, 204.3; L-Ala, 179.6; L-Val, 272.0; L-Met, 19.0; L-Ile, 128.1; L-Leu, 171.9; L-Tyr, 105.0; L-Phe, 46.7; L-Lys, 91.2; L-His, 51.2; L-Pro, 80.2; L-Thr, 105.0; and L-Arg, 83.9 (Angcjas et al., 2014; Mackle et al., 2000).

### Quantification of the target genes

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. The relative transcript levels for the target genes were determined using the 2<sup>- $\Delta\Delta$ CT</sup> method (Fredericksen et al., 2015).  $\beta$ -actin is part of the actin cytoskeleton, and its expression level in most cells is rela-

tively stable; thus, it is frequently used as housekeeping gene for quantitative analyses (Bougarn et al., 2011). Therefore,  $\beta$ -actin was used as a control gene for normalization in the study. The primers of the target genes were: beta-casein (CSN2): sense 5'-TGGGCAAAGGGGTGGATT-3'; antisense 5'-ACCTGGTGAGATTGTGGAAAGC-3'; Acetyl CoA Carboxylase alpha (ACACA): sense 5'-TCCTGCTGCTATTGCTACTCCA-3'; antisense 5'-CAGTCCCCGCACTCACATAA-3'; microtubule-associated proteins 1 light chain 3B (MAP1LC3-II): sense 5'-AGCAGCATCCTACCAAAATCCC-3'; antisense 5'-GAATCCATCTTCATCCTTCTCGC-3'; and  $\beta$ -actin: sense 5'-GCCCTGAGGC-TCTCTTCCA-3'; antisense 5'-GCGGATGTCGACGTCACA-3.

### Western blot analysis

Proteins from the BMECs were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. The primary antibodies used in this study were: anti-MAP1LC3 (Cell Signaling Technology, USA; Cat 2775), anti-ATG5 (Novus Biologicals, USA; Cat NB110-53818), anti-SQSTM1/p62 (Santa Cruz Biotechnology Inc., Germany; Cat sc-25575), anti-GAPDH (Cell Signaling Technology; Cat 2118), anti-total mTOR (Cell Signaling Technology, Cat 2983), anti-phospho-mTOR (Cell Signaling Technology, Cat 5536), anti-phospho-4E-BP1 (Cell Signaling Technology, Cat 2855), anti-total EIF2S1 (Abcam, Cambridge Science Park, UK; Cat ab70542), anti-phospho-EIF2S1 (Abcam, Cat ab32157), anti-ATF4 (Abcam, Cat ab1371), anti-total GCN2 (Abcam, Cat ab134053), and anti-phospho-GCN2 (Abcam, Cat ab75836). The luminescent fluid was prepared according to the manufacturer's protocol, and protein expression was detected using the alpha chemiluminescent gel imaging system FluorChem E.

### Detection of CSN2

The M-PER Mammalian Protein Extraction Reagent (Thermo 78503, USA) was added to monolayers of cells after the cells were washed twice in ice-cold PBS on ice for 10 min. The lysate was centrifuged at 10,000  $\times$  *g* at 4°C for 20 min to remove the insoluble material, and supernatants were subsequently employed for CSN2 detection using the ELISA Kit (Jingma Tech Inc., China), according to the manufacturer's instructions.

### Detection of triglyceride secretion

Cell-free supernatants were measured in the triglyceride assay using the Triglyceride (TG) GPO-POD assay Kit (Applygen Tech Inc., China), according to the manufacturer's instructions.

### Preparation of BMEC cytoplasmic proteins and mass spectrometry

The cytoplasmic proteins were extracted from 1  $\times$  10<sup>7</sup> BMECs using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFinnigan, USA) according to the manufacturer's instructions. The protein concentration was determined using a BCA assay kit (ThermoFinnigan, USA), and 50  $\mu$ g of protein were applied to a 12% bis-Tris gel. After visualization via Coomassie brilliant blue staining, entire gel lanes were subjected to in-gel tryptic digestion. The extracted peptides from each gel piece were analyzed using an Ettan™ MDLC system (GE Healthcare). The acquired MS/MS spectra were automatically searched against the protein database for Bovidae proteins in NCBI using BioworksBrowser rev. 3.1 (Thermo Electron, USA). The results were extracted from the SEQUEST outfiles with BuildSummary.

### Transmission electron microscopy

The BMECs were seeded in 24-well plates at a density of 1  $\times$  10<sup>5</sup>

cells/ml. After being treated with IFN- $\gamma$  (10 ng/ml) at the indicated time points, the bilayer membrane vesicle structures of the autophagosomes were observed with a JEM-1200 EX TEM (JEOL), as previously described (Pei et al., 2014).

### Observation of autophagosome

To detect the autophagosomes, the BMECs were seeded in 24-well plates and cultured to 70% confluency in DMEM/F12. After 24 h, the vAd-GFP-RFP-LC3 adenovirus constructs (Han Heng Biotechnology, China) were added to the cells. The cells were treated with IFN- $\gamma$  at 24 h post-transfection, and then fixed with 4% paraformaldehyde at 24-72 h post-treatment. The cell nucleus was counterstained with DAPI. A laser scanning confocal microscope Olympus FV300 (Olympus, Japan) was used to observe and image the cells.

### Extraction of the free amino acids from the BMECs

After the BMECs were washed twice in ice-cold PBS,  $1 \times 10^7$  BMECs were digested with trypsin and harvested via centrifugation at 1,000 rpm for 10 min. The cell pellets were thoroughly degreased with lipase and organic solvent. Subsequently, the cells were washed in ice-cold PBS and disrupted with ultrasound. The proteins present in the supernatant were precipitated using alcohol and filtered through a 0.22- $\mu$ m pore size membrane, and the concentrations of 16 different free amino acids in the BMEC supernatants were determined using an automatic amino acid analyzer (L-8900, HITACHI, Japan).

### Statistical analysis

All experiments were performed at least three times, and the samples were analyzed in triplicate. All of the data are expressed as the mean  $\pm$  SD. The statistical analysis was performed using one-way ANOVA and SPSS (Statistical Package for the Social Sciences) software (version 16.0). In every case,  $P < 0.05$  was considered significant;  $P < 0.01$  was considered very significant.

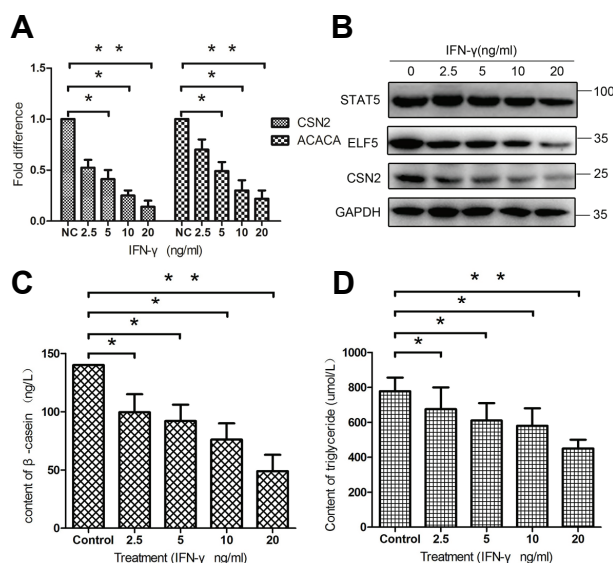
## RESULTS

### IFN- $\gamma$ downregulates milk synthesis in primary BMECs *in vitro*

Milk stably expresses high levels of beta-casein (CSN2). Acetyl CoA Carboxylase alpha (ACACA) is a key enzyme for the first step in the fatty acid biosynthesis pathway. In this study, CSN2 and ACACA were elected as detection indices for the lactation performance of cultured BMECs. To explore the effect of IFN- $\gamma$  on milk synthesis, we first measured the changes in the CSN2 and ACACA mRNA levels using qPCR. As shown in Fig. 1A, the CSN2 and ACACA mRNA levels were decreased in a dose-dependent manner in the IFN- $\gamma$ -treated BMECs. The Western blot analysis showed that IFN- $\gamma$  inhibited the expression of genes that regulate milk protein synthesis and decreased the CSN2 protein levels in the BMECs (Fig. 1B). Similarly, the ELISA also confirmed that the levels of CSN2 were reduced in the cell culture supernatants from the IFN- $\gamma$ -treated groups (Fig. 1C), and the triglyceride content was also decreased in a dose-dependent manner (Fig. 1D). Taken together, these results strongly suggested that IFN- $\gamma$  significantly inhibited milk synthesis in BMECs.

### IFN- $\gamma$ activates autophagy in primary BMECs *in vitro*

To determine whether IFN- $\gamma$  induces autophagy *in vitro*, we used a Western blot analysis to detect the type of MAP1LC3 protein that was present in the cells. The BMECs were treated

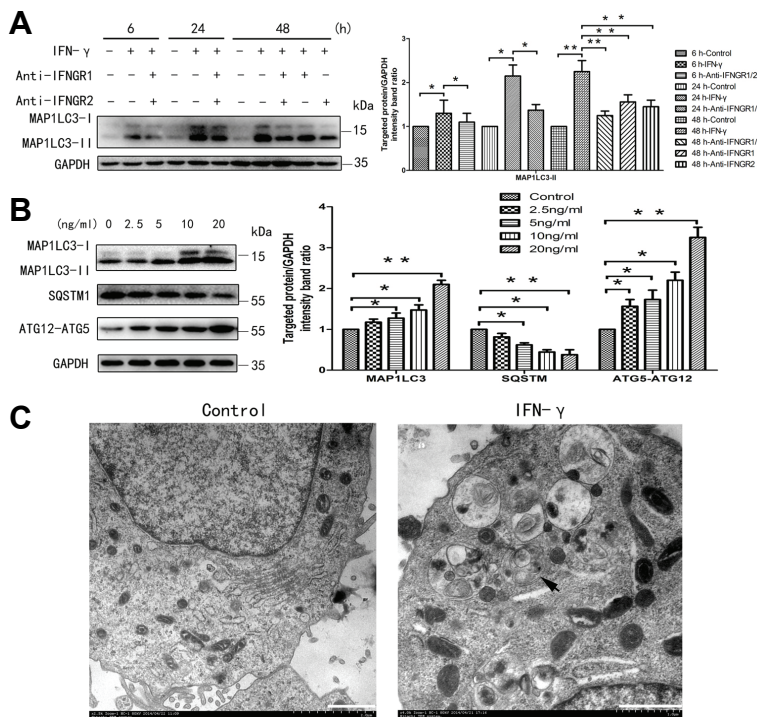


**Fig. 1.** IFN- $\gamma$  downregulates milk synthesis in primary BMECs *in vitro*. (A) IFN- $\gamma$  induces the expression of the CSN2 and ACACA mRNAs. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$ . (B) The dose-dependent induction of STAT5, ELF5 and CSN2 expression the IFN- $\gamma$ -treated BMECs was investigated using Western blot analysis; GAPDH was used as a loading control. (C) The CSN2 content was detected by ELISA. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$ . (D) The triglyceride content in the cell-free supernatant was determined using the TG GPO-POD assay. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$ .

with 10 ng/ml of IFN- $\gamma$  for 6, 24 and 48 h, and the results showed that MAP1LC3-II expression was significantly increased in a time-dependent manner (Fig. 2A). Moreover, when the cells were treated with 2.5 ng/ml, 5 ng/ml, 10 ng/ml or 20 ng/ml of IFN- $\gamma$  for 24 h, the expression of MAP1LC3-II significantly increased in a concentration-dependent manner (Fig. 2B). Furthermore, ATG12-ATG5 complex formation was increased, and SQSTM1 expression exhibited a decreasing trend (Fig. 2B). To confirm that the observed effects were a result of the IFN- $\gamma$  treatment, the BMECs were treated with IFN- $\gamma$  in the presence or absence of an anti-IFNAR1 or anti-IFNAR2 monoclonal antibody alone or in combination with an anti-IFNAR1/2 monoclonal antibody. As shown in Fig. 2A, after the BMECs were treated with the indicated anti-IFNGR antibodies, the IFN- $\gamma$ -induced conversion of soluble MAP1LC3-I into MAP1LC3-II was significantly disrupted. TEM images showed that the control BMECs had few or no autophagosomes (Fig. 2C). In contrast, the TEM images displayed many autophagosomes at various developmental stages in the IFN- $\gamma$ -treated cells (Fig. 2C). These data indicate that IFN- $\gamma$  specifically functions upstream of autophagy in BMECs *in vitro*.

The Western blot analysis showed that the protease inhibitor E64d increased the levels of MAP1LC3-II and SQSTM1 in the BMECs compared with the control group (Fig. 3A). To determine





ng/ml IFN- $\gamma$ . Representative images of the typical untreated control cells and cell populations at 48 h after treatment were shown. The arrows indicate the double-membrane structures. Scale bar, 10  $\mu$ m.

**Fig. 2.** IFN- $\gamma$  activates autophagy in primary BMECs *in vitro*. (A) The BMECs were treated with IFN- $\gamma$ , anti-IFNGR1, and anti-IFNGR2 alone or in combination for 6, 24 and 48 h. The MAP1LC3, ATG12-ATG5 and GAPDH expression levels were analyzed by Western blot analysis with specific antibodies. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05, \*\* $P$  < 0.01. (B) The dose-dependent induction of MAP1LC3, SQSTM1, ATG12-ATG5 and GAPDH expression in the BMECs treated along with concentrations (0 ng/ml, 2.5 ng/ml, 5 ng/ml, 10 ng/ml and 20 ng/ml) was investigated by Western blot analysis with specific antibodies. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05, \*\* $P$  < 0.01. (C) Transmission electron micrographs of IFN- $\gamma$ -treated BMECs presenting morphological evidence of autophagy. All treated cells were incubated with 10

whether IFN- $\gamma$  induced autophagosome formation, the BMECs were infected with a tandem GFP-RFP-LC3-expressing adenovirus construct. Confocal laser scanning microscopy was used to detect the intracellular expression and distribution of GFP-RFP-LC3. Double-positive autophagosomes were first identified at 24 h post-transfection (Fig. 3B). The proportion of autophagosome-containing cells and the number of double-positive autophagosomes per cell dramatically increased in the IFN- $\gamma$ -treated BMECs (Fig. 3B). Based on normal autophagosome maturation, the number of autolysosome-containing cells and the number of autolysosomes per cell increased over time, as expected (Fig. 3B). Collectively, the data show that IFN- $\gamma$  triggers complete autophagic flux in BMECs.

#### IFN- $\gamma$ downregulates CSN2 synthesis and the triglyceride content via an autophagy-dependent mechanism

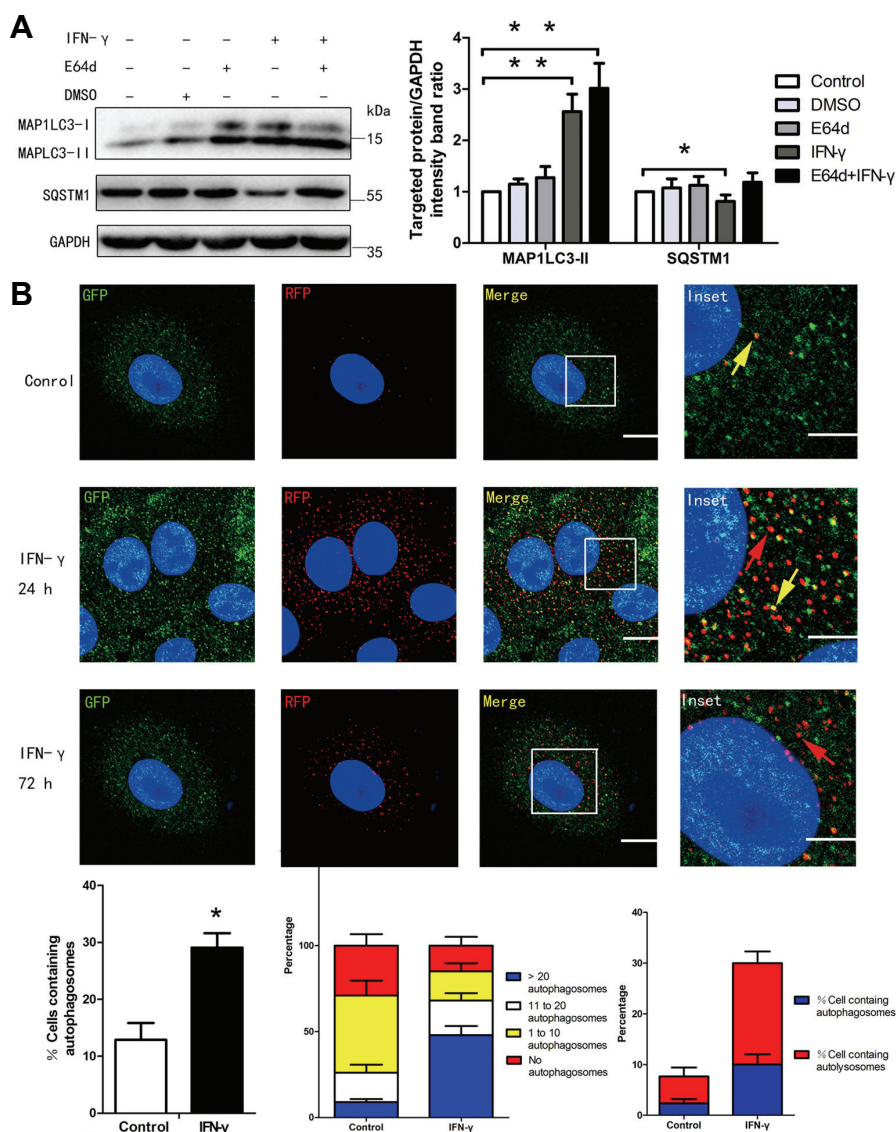
We next tested whether IFN- $\gamma$ -induced autophagy could interfere with milk synthesis in BMECs; we first measured the changes in the mRNA levels of CSN2 and ACACA using qPCR. As shown in Fig. 4A, compared with the control, the CSN2 and ACACA mRNA levels were decreased in the IFN- $\gamma$ -treated groups. After treatment with bafilomycin A1 or chloroquine, inhibitors of autophagy, the levels of CSN2 and ACACA could be recovered to some extent. Consistent with the changes in the mRNA levels, decreases in the CSN2 protein levels were also observed in the IFN- $\gamma$ -treated BMECs using Western blot analysis (Fig. 4B). However, the CSN2 and triglyceride levels in the bafilomycin A1- or chloroquine-treated cells were increased compared with the IFN- $\gamma$ -treated group (Fig. 4B). Furthermore, the expression of STAT5 and ELF5 exhibited the same decrease as CSN2. Similarly, the ELISA also confirmed that the CSN2 levels were reduced in the cell culture supernatants from

the IFN- $\gamma$ -treated groups (Fig. 4C), and the CSN2 levels and triglyceride content were recovered to some extent when autophagy was inhibited with bafilomycin A1 or chloroquine (Figs. 4C and 4D). Taken together, these results strongly suggested that IFN- $\gamma$  significantly inhibited milk synthesis via an autophagy-dependent mechanism in BMECs.

#### Arginine depletion and activation of the GCN2/eIF2 $\alpha$ pathway mediated IFN- $\gamma$ -induced autophagy

We attempted to explore the mechanisms by which IFN- $\gamma$  induced autophagy in BMECs. One of the master regulators of protein synthesis is mTORC1, which is a good candidate “gate keeper” of the autophagy pathway because it functions as a sensor for amino acids and ATP and can integrate hormonal stimuli via the class I PI-3K/PKB pathway. To examine the effects of IFN- $\gamma$  on mTORC1 activity, we analyzed the phosphorylation status of mTOR (p-mTOR) and 4E-BP1 in response to IFN- $\gamma$ . We found that the mTOR inhibitor rapamycin inhibited mTOR phosphorylation but enhanced 4E-BP1 phosphorylation. Compared with rapamycin treatment, we did not observe any modifications in the phosphorylation status of 4E-BP1 following IFN- $\gamma$  exposure (Fig. 5A), suggesting that IFN- $\gamma$  does not interfere with mTOR signaling in BMECs.

To further explore the mechanisms by which IFN- $\gamma$  may induce autophagy in BMECs, we used a shotgun LC-MS/MS proteomics technique to identify a differentially expressed protein, eukaryotic initiation factor 2- $\alpha$  (eIF2 $\alpha$ ) (Fig. 5B), which also regulates autophagy. The mechanisms by which eIF2 $\alpha$  activates autophagy involve the induction of the expression of the transcription factors CHOP and ATF4, which subsequently promote the expression of the autophagy genes ATG5 and MAP1LC3-II. Here, IFN- $\gamma$  promoted eIF2 $\alpha$  phosphorylation and



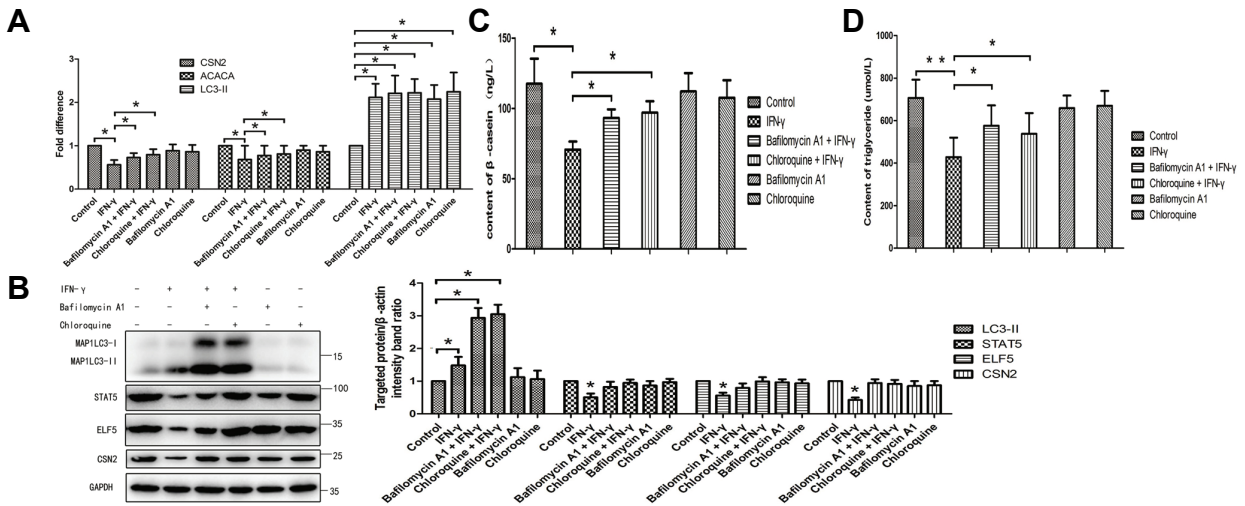
**Fig. 3.** IFN- $\gamma$  enhances autophagic flux in BMECs. (A) The cells were treated with IFN- $\gamma$  in the presence or absence of E64d (10  $\mu$ g/ml). After 24 h, cell lysates were prepared and analyzed via immunoblotting using anti-MAP1LC3, anti-SQSTM1 and anti-GAPDH antibodies. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05, \*\* $P$  < 0.01. (B) The BMECs were transiently transfected with the tandem GFP-RFP-LC3 adenovirus construct, and fluorescent vesicles were visualized 24 or 72 h later. Representative images are shown. Scale bar: 5  $\mu$ m. The error bars indicate the mean  $\pm$  SD of three independent experiments. One-way ANOVA; \* $P$  < 0.05; \*\* $P$  < 0.01.

expression of the ATF4 protein in the Western blot analysis (Figs. 5C and 5D). We also found that IFN- $\gamma$  promoted GCN2 phosphorylation (Figs. 5C and 5D), which is a kinase for eIF2 $\alpha$  and a sensor of amino acid starvation. Therefore, we next determined whether IFN- $\gamma$  induced an amino acid starvation response in BMECs. The automatic amino acid analysis showed that the IFN- $\gamma$  treatment resulted in rapid decreases in the intracellular free arginine concentrations (Fig. 5E). These results demonstrate that the IFN- $\gamma$  treatment led to arginine depletion and activation of the GCN2/eIF2 $\alpha$  pathway, which mediated IFN- $\gamma$ -induced autophagy in BMECs.

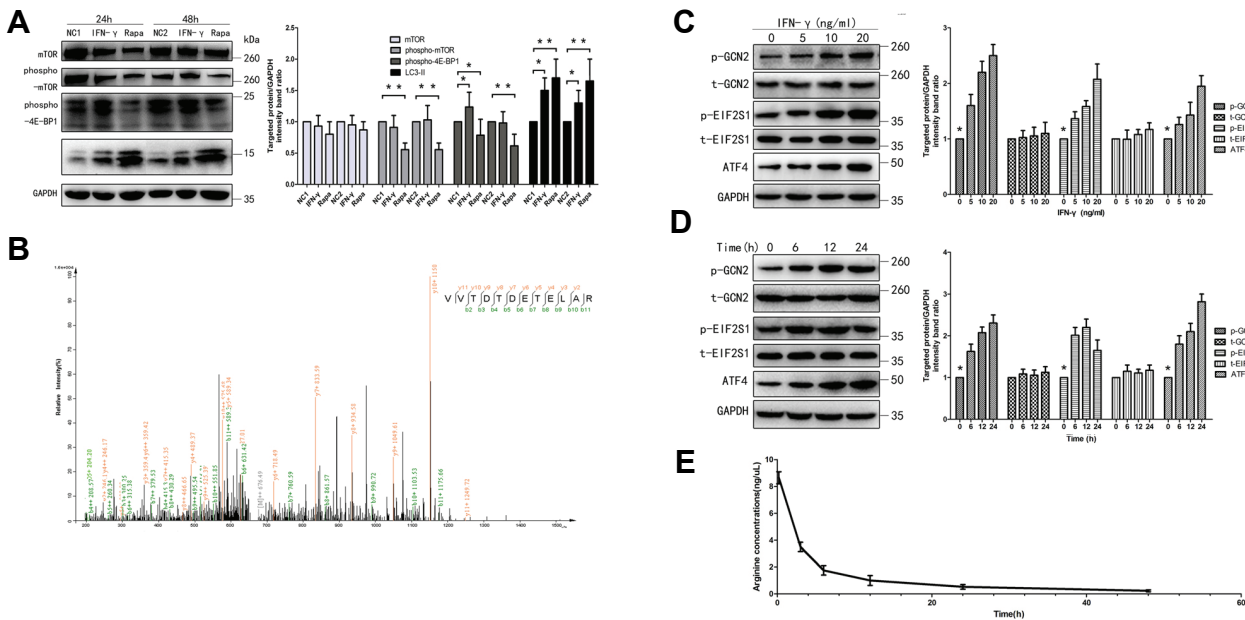
#### Arginine supplementation assists in recovering milk synthesis that had been reduced by IFN- $\gamma$ -induced autophagy

Because IFN- $\gamma$  treatment resulted in arginine depletion and induced autophagy in primary BMECs, we added extra arginine to the cultured BMECs to investigate whether arginine supplementation affected autophagy and milk synthesis. We found that the phosphorylation of GCN2 and eIF2 $\alpha$  were reversed by

arginine supplementation (Fig. 6A). In addition, arginine supplementation reduced LC3II accumulation during IFN- $\gamma$  exposure (Fig. 6A), which suggests that the accumulation of autophagosomes in the IFN- $\gamma$ -treated BMECs depends on the availability of arginine. Furthermore, we elucidated the effect of arginine supplementation on the expression of the CSN2, ELF5, and STAT5 genes in IFN- $\gamma$ -treated BMECs. As shown in Fig. 6A, IFN- $\gamma$  downregulated STAT5, ELF5 and CSN2 expression. However, arginine supplementation attenuated an IFN- $\gamma$ -induced decrease in STAT5, ELF5 and CSN2 expression. Furthermore, the ELISA also confirmed that the CSN2 levels were reduced in the BMECs from the IFN- $\gamma$ -treated groups, and the CSN2 levels were recovered to some extent following arginine supplementation (Fig. 6C). The triglyceride content was significantly decreased after the IFN- $\gamma$  treatment, and arginine supplementation increased the triglyceride content to some extent (Fig. 6D). These beneficial effects were specific, as similar effects were not observed with glycine supplementation; the effects of 15 other amino acids on autophagy are shown in Fig.

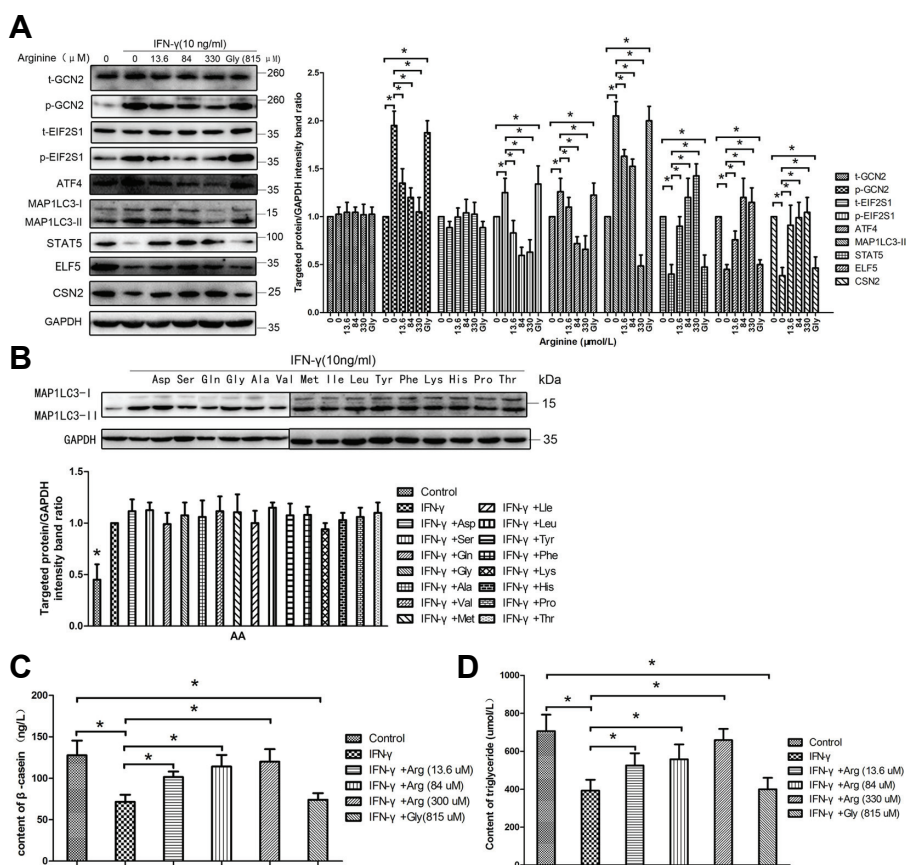


**Fig. 4.** IFN- $\gamma$  downregulates CSN2 synthesis and decreases the triglyceride content via an autophagy-dependent mechanism. (A) IFN- $\gamma$  induces the expression of the CSN2 and ACACA mRNAs. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05, \*\* $P$  < 0.01. (B) The expression of MAP1LC3, STAT5, ELF5 and CSN2 in the IFN- $\gamma$ -treated BMECs was investigated using Western blot analysis. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05. (C) The CSN2 content was detected by ELISA. (D) The triglyceride content in the cell-free supernatant was determined using the TG GPO-POD assay. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05.



**Fig. 5.** Activation of the GCN2/eIF2 $\alpha$  pathway mediated IFN- $\gamma$ -induced autophagy. (A) IFN- $\gamma$  does not interfere with mTOR signaling. The BMECs were incubated with 10 ng/ml of IFN- $\gamma$  or 10 nM rapamycin or were left untreated for 24 or 48 h. Whole-cell lysates were prepared and analyzed via Western blot analysis using anti-mTOR, anti-phospho-mTOR, anti-phospho-4E-BP1, anti-MAP1LC3 and anti-GAPDH antibodies. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05, \*\* $P$  < 0.01. (B) MS/MS spectra used for the identification of eIF2 $\alpha$ . The sequence VVTDTDETELAR was used to identify eIF2 $\alpha$ . The dose- (C) and time-dependent (D) induction of t-GCN2, p-GCN2, p-eIF2S1, t-eIF2S1, ATF4 and GAPDH expression in the IFN- $\gamma$ -treated BMECs was investigated using blot analysis. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P$  < 0.05. (E) The time course of free arginine metabolism in the IFN- $\gamma$ -treated BMECs was investigated using an automatic amino acid analyzer.





**Fig. 6.** Arginine supplementation assists in recovering milk synthesis that had been reduced by IFN- $\gamma$ -induced autophagy. (A) Arginine supplementation partially inhibits the effects of IFN- $\gamma$  on BMECs. The BMECs were incubated with 10 ng/ml IFN- $\gamma$  alone or with the indicated concentration of arginine or glycine (as control) or were left untreated. The expression levels of the t-GCN2, p-GCN2, t-EIF2S1, p-EIF2S1, ATF4, MAP1LC3, STAT5, ELF5, CSN2 and GAPDH proteins were evaluated by immunoblotting. The relative levels of the target proteins were estimated using densitometry, and the ratios were calculated relative to the GAPDH control. (B) Effects of 15 different amino acid supplements on LC3II accumulation. The BMECs were incubated with 10 ng/ml IFN- $\gamma$  alone or with a 4-fold increase in the endogenous level of Asp, Thr, Ser, Gln, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, Lys, His and Pro for 24 h. At the end of the treatment, the expression levels of MAP1LC3-II and  $\beta$ -actin (loading control) were analyzed by immunoblotting with specific antibodies. The relative levels of the target proteins were estimated using densitometry, and the

ratios were calculated relative to the GAPDH control. (C) The CSN2 content was detected by ELISA. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P < 0.05$ . (D) The triglyceride content in the cell-free supernatant was determined using the TG GPO-POD assay. The data represent the mean  $\pm$  SD of 3 independent experiments. Each bar represents the mean of three independent experiments. One-way ANOVA; \* $P < 0.05$ .

6B. These results suggested that arginine supplementation may partially assist in recovering milk synthesis that had been reduced by IFN- $\gamma$ -induced autophagy.

## DISCUSSION

In the current study, our findings are the first to demonstrate that arginine depletion and the GCN2/eIF2 $\alpha$  pathway mediate IFN- $\gamma$ -induced autophagy, which, in turn, modulates milk synthesis in BMECs. Moreover, arginine supplementation inhibits autophagy and contributes to the partial restoration of milk synthesis *in vitro*, suggesting that immunometabolism, autophagy and milk synthesis are correlated. These findings provide new insights into potential autophagy-based therapeutic approaches for improving milk synthesis and even nutritional, metabolic, and other diseases in animals and humans.

Type I and II interferon have been reported to promote autophagy in various cells by depleting tryptophan and activating the ATF6-C/EBP- $\beta$ -signaling pathway (Fougeray et al., 2012; Schmeisser et al., 2013). IGF-I and EGF were shown to inhibit autophagy via the mTOR kinase pathway, while the steroid hormones 17 $\beta$ -estradiol and progesterone stimulated autophagy in BME-UV1 cells (Sobolewska et al., 2009). In the present study, we did not observe reduced phosphorylation of 4E-BP1

or mTOR in BMECs following IFN- $\gamma$  treatment, suggesting that IFN- $\gamma$ -induced autophagy via a non-mTOR kinase pathway. Furthermore, we demonstrated that IFN- $\gamma$  activated the GCN2/eIF2 $\alpha$  pathway and induced autophagy, indicating a novel function of IFN- $\gamma$  in the regulation of autophagy.

The IFN- $\gamma$  level may be increased by the diet, resulting in long-term pressure of high IFN- $\gamma$  levels in some individuals. During lactation, mammary epithelial cells have a strong metabolism, the lobules of the mammary glands expand and the tissue density is increased; thus, mammary glands have an increased risk of pathogenesis (Ahmadinejad et al., 2013). In the present study, the free amino acid analysis showed that IFN- $\gamma$  treatment resulted in a rapid decrease in the intracellular free arginine concentration and also induced autophagy in primary BMECs. More importantly, the activation of autophagy downregulated CSN2 synthesis and decreased the triglyceride content. In bovines, previous reports confirmed that casein gene transcription is mediated by STAT5, and its activity increases during lactation, mostly due to phosphorylation (Bionaz and Loor, 2011; Yang et al., 2000). ELF5 regulates milk protein expression (Bionaz and Loor, 2011). ELF5-deficient mammary glands fail to undergo alveolar morphogenesis and accumulate luminal progenitor cells. Conversely, forced expression of ELF5 in mice causes the formation of alveolar structures and induces

milk production (Lee et al., 2011). The decrease in STAT5 and ELF5 expression via an autophagy-dependent mechanism in this study might suggest that protein synthesis was inhibited. Our findings provide new insights into the role and regulation of autophagy in mammary gland physiology.

Amino acids are the most well-known nutrient regulators of autophagy, but the amino acids that exert direct effects vary depending on the cell type and physiological conditions (Angcajas et al., 2014). The present study showed that arginine supplementation attenuated the IFN- $\gamma$ -induced increase in GCN2 and EIF2S1 phosphorylation and ATF4 expression and inhibited the MAP1LC3 modification, implying that autophagy was suppressed. At the same time, STAT5 and ELF5 expression was upregulated in the arginine-treated group compared with the IFN- $\gamma$ -treated group, and the CSN2 and triglyceride content was recovered to some extent. These results indicated that arginine supplementation appeared to render the cells resistant to IFN- $\gamma$ -induced autophagy and could recover milk synthesis.

In summary, the present study demonstrated an avenue for cross-talk in the signal transduction between the arginine depletion-mediated, IFN- $\gamma$ -induced autophagy and milk synthesis regulatory pathways. A better understanding of the physiological benefits of the signaling mechanisms underlying the strong link between autophagy and metabolic regulation would provide new insights into potential autophagy-based therapeutic approaches for improving milk synthesis and even nutritional, metabolic, and other diseases in animals and humans.

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