

Kisspeptin-10 Promoting the Synthesis of Milk Fat by Inhibiting the AMPK/SIRT6 Signaling Pathway via the GPR54 in Bovine Mammary Epithelial Cells

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Research

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Abstract

Background Kp-10 is a peptide hormone mainly involved in the initiation tissue development in puberty. Recent studies have shown that Kp-10 is involved in fat synthesis. However, the role of Kp-10 in milk fat synthesis in lactating dairy cows has not been reported. Therefore, this study investigated the correlation between GPR54 and milk fat synthesis in dairy cows and to study the underlying mechanism in BMECs.

Results The results showed that the expression of GPR54, SREBP1 and FASN in mammary glands of high-milk fat dairy cows were significantly higher than those in mammary glands of low-milk fat dairy cows. Meanwhile, 10nM Kp-10 can significantly inhibit AMPK/SIRT6 signaling pathway and promote milk fat synthesis in BMECs through its receptor GPR54. Overexpression of SIRT6 significantly reduced the acetylation level of SREBP1 and milk fat synthesis in BMECs.

Conclusions These results suggested that Kp-10 inhibits the AMPK / SIRT6 signaling pathway by mediating GPR54, thereby increasing SREBP1 acetylation levels and increasing milk fat synthesis in BMECs.

Background

Milk fat is an important indicator for evaluating milk quality and an important nutrient for ensuring the growth of offspring[1]. Milk fat is composed of about 98% triglycerides and a small amount of other lipids[2]. This process of milk fat synthesis mainly involves the de novo synthesis of fatty acids and the transfer of long fatty acids[3]. This process is regulated by a variety of transcription factors[4]. It is known that SREBP1 can regulate milk fat synthesis[5-7]. Generally, immature SREBP1 is localized on the endoplasmic reticulum membrane, and then transformed into mature SREBP1 after the Golgi lysates are lysed, and transferred into the nucleus to induce de novo synthesis of fatty acids transcription by binding to the target gene promoter[5-8].

Milk fat synthesis is mainly affected by fatty acids and amino acids in the blood[3, 8, 9]. On the one hand, amino acids and fatty acids in the blood can directly enter mammary epithelial cells to participate in fatty acid synthesis[2, 3, 8, 9]. On the other hand, they bind specific membrane receptor proteins to inhibit the AMPK signaling pathway to promote downstream signal transduction pathways[8, 9]. However, hormone as an important regulatory factor in milk production is seldom studied in the synthesis of milk fat. It has been reported that hormones play an important role in lipid metabolism[10]. For example, knockout of ER α increases adipose tissue in mice[11]. IGF-I can significantly inhibit 3T3-L1 adipocyte adipogenesis through IGFBP-2[12]. Excessive glucocorticoids can induce increased visceral fat and diabetes[13]. In addition, some studies have reported the effects of some hormones on milk fat synthesis. Oxytocin can significantly increase the content of monoglyceride in milk fat[14]. Melatonin inhibits milk fat synthesis via MT1 in BMECs[15].

Kisspeptin-10 (Kp-10) is a peptide hormone encoded by kiss 1 gene, which is synthesized by hypothalamic neurons and play a variety of biological functions in the body through its receptor

GPR54[16]. Meanwhile, GPR54 regulates the release of GnRH and inhibits the synthesis of growth hormone[17, 18]. Knockout of GPR54 inhibits mammary gland and ovarian development in puberty mice[19]. In addition, GPR54 also has a regulatory effect on lipid metabolism, and GPR54 expression has been detected in humans and mice adipose tissue[20-22]. Knockout of GPR54 inhibits adipogenesis and inhibits adipocyte development and reduces weight in mouse[20, 23]. Meanwhile, a high-fat diet will reduce GPR54 gene expression, and under restricted diets will increase GPR54 mRNA expression[23]. Moreover, GPR54 is also expressed in BMECs, and Kp-10 promotes BMECs proliferation and milk protein synthesis through GPR54[24, 25].

In short, Kp-10 plays an important role in tissue formation and lipid metabolism. The effect of Kp-10 on milk fat synthesis in dairy cows has not been reported. Therefore, the purpose of this study is to initially demonstrate the effect of Kp-10 on milk fat synthesis, and to elucidate the intrinsic regulatory mechanism of Kp-10 in promoting milk fat synthesis through primary bovine mammary epithelial cells.

Materials And Methods

Animals and Tissue Collection

Twenty dairy cows were selected from a 5,000-cow dairy farm located in Suihua City, Heilongjiang Province, China based on the milk fat content in the milk. Selected lactating Holstein cows with similar numbers of lactations and body condition scores. The twenty dairy cows were divided into two groups: high milk fat (milk fat >3.5%) and low milk fat (milk fat ≤3.5%)[26](Table 1). Milk yield of lactating cows with high-fat milk was 34.55 ± 3.44 kg/d. Milk yield of lactating cows with low-fat milk was 34.33 ± 2.53 kg/d. Body condition scores are 2.80 ± 0.16.

All cows are guaranteed to be free from other diseases and are kept in separate pens, where they can drink freely. TMR full mixed diets were fed daily at 5:30 and 18:00 (Table S1). Milk samples were collected daily and added with preservative (1 mg/mL of potassium dichromate) for the detection of milk fat, milk protein and lactose. The 20 dairy cows were slaughtered at 60 DIM. After bloodletting and slaughtering, the mammary gland parenchyma was collected aseptically for cell culture, and a portion of the mammary gland parenchyma was frozen at -80°C and 4% formaldehyde for subsequent experiments.

Milk component	Dairy cows with low-fat milk (n=10)	Dairy cows with high-fat milk (n=10)
Milk fat (‰)	2.583±0.1991	4.490±0.2274
Milk protein (‰)	3.213±0.0873	3.123±0.1690
Lactose(‰)	4.827±0.1486	4.847±0.01667

Table 1
Milk components of lactating Hostern dairy cows

Cell Culture and Treatment

Isolation and culture of bovine mammary gland epithelial cells as previously described[27]. Briefly, lactating cows are slaughtered, mammary glands are collected and bovine mammary epithelial cells are cultured using adherent methods. Bovine mammary epithelial cells were purified using a [cell passage](#) method, and the resulting cells were identified for purity using K18. Mammary epithelial cells were cultured using DMEM medium containing 10% calf serum in a humidified atmosphere of 95% air and 5% CO₂.

For experimental assays, 25,000 cells were added to a 60×15 mm dish (Life Science, Oneonta, NY, USA), and different concentration of Kp-10 was added to the medium. To suppress GPR54 receptor, peptide-234 was added to the medium. In order to activate AMPK signaling pathway and overexpression SIRT6, AICAR and Over-expression plasmid of Sirt 6 were added to culture medium.

Triglyceride Content Assay

Milk fat content was measured using a triglyceride content kit according to the method previously described[28]. Briefly, BMECs were incubated with Kp-10 for 24 h. The medium was discarded and the cells were lysed with lysate, centrifuged at 2000 rpm for 5 min, and protein concentration was measured using BCA. A working solution was then prepared to measure and calculate the triglyceride content in 550nm.

Lipid Droplet Observation

BODIPY 493/503 (Invitrogen) was used to stain Lipid Droplets in BMECs[5]. Briefly, the cells were plated in 6-well plates with coverslips. After the cells were adhered, the cells were treated with different concentrations of Kp-10 and peptide-234 and incubated for 24 hours. Subsequently, the cells were washed 3 times with PBS, and the cells were incubated with Bodipy 493/503 for 15 min. Then the nuclei were stained with DAPI and mounted, and then observed with a fluorescence microscope (TCS SP5; Leica, Mannheim, Germany).

Western Blotting

Western blotting was performed as described[27]. Briefly, the cells were washed 3 times with PBS, then the cells were lysed with NP40 (Beyotime, Shanghai, China) (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; sodium orthovanadate; sodium fluoride; ethylenediaminetetraacetic acid, leupeptin; 1 mM PMSF). Protein concentrations were determined using an Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). The proteins were separated using 4% and 12% SDS-PAGE (20 µg/lane) and transferred to the PVDF membrane (Millipore, Darmstadt,

Germany). Subsequently, the PVDF membrane was blocked with 5% milk with TBST for 2 hours and incubated with primary antibody at 4 °C overnight. The primary antibodies were as follows: FASN (3180T), p-AMPK(2535s), AMPK (5832s) and Acetylated-lysine (9441s) were purchased from Cell Signaling Technology Inc. (USA). GPR54 (15505-1-AP), β -actin (66009-1-1g), SREBP1 (14088-1-AP) and SIRT6 (13572-1-AP) were purchased from Proteintech Co., Inc. (China). The PVDF membrane was washed 5 times with PBST for 10 min each, and incubated with HRP-labeled secondary antibody (Boster, BA1055/BA1051, USA) configured with 5% milk with TBST for 1 h. Protein bands were visualized using a Beyo Enhanced Chemiluminescence Reagent Kit (Beyotime).

Immunoprecipitation assay

Immunoprecipitation was performed as described[27]. Briefly, cell supernatant was collected in a 1.5 mL centrifugal tube using lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP 40 and 5% glycerol), and acetylated-Lysine (CST, USA) anti-body was added to each sample to form immune complexes. Then, 25 μ L Pierce protein A/G was added to each sample tube, and the magnetic beads were collected by a 12-tube magnetic separation rack (CST). Finally, 100 μ L eluent was added to elute the proteins on the magnetic beads, and the samples were prepared for Western blot experiments. The Co-IP test was performed using the Pierce™ Classic Magnetic IP/Co-IP Kit.

Real-time (RT) PCR

The BMECs were stimulated with Kp-10 for 24 hours, and the expression of FASN and SREBP1 mRNA was detected. RT-PCR was performed as described[27]. Briefly, total RNA in BMECs was extracted using TRIzol reagent according to the instructions (Life Technologies, California, USA). 2 μ g of total RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit (TaKaRa, Kusatsu, Japan). RT-PCR was performed on a CFX96 system using SYBR® Green Premix Ex Taq™ II (TaKaRa, Kusatsu, Japan) and 1 μ L of cDNA. Each PCR was run in triplicate at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and a melt curve was generated from 65 °C to 95 °C (0.5°C per 5 s). β -actin was used as a reference gene, and the amount of mRNA was calculated with the $2^{-\Delta\Delta CT}$ method. All RT-PCR analyses were repeated 3 times. The primer sequences were as follows as Table 2.

Gene	Primers	Length (bp)
FASN	(F) 5'-TCCTCCACCGCACACTCCATC-3'	141
	(R) 5'-CTCCTCCAGGTTCTCCGACTCAG-3'	
SREBP1	(F) 5'-CTCCGACACCACCAGCATCAAC-3'	122
	(R) 5'-GCAGCCCATTTCATCAGCCAGAC-3'	
β -actin	(F) 5'-CGTCCGTGACATCAAGGAGAAGC-3'	143
	(R) 5'-GGAACCGCTCATTGCCGATGG-3'	

Table 2

Primer sequences of FASN, SREBP1 and β -actin

Cell transfection

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The cells were plated in 60×15 mm dish at 1×10^6 cells/dish. For Sirt6 overexpression, 80% confluent cells were transfected with either 2.5 μ g of pcDNA3.1-Sirt6 or pcDNA3.1(+) as a negative control. All experiments were performed in triplicate. After 24 h of transfection, cells were harvested for lipid droplet immunostaining and TAG content assays. The following PCR primer pair was used:

Forward 5'-CGGGGTACCGCCACCATGTCGGTGAATTACGCG-3'

Reverse 5'-CCGGAATTCGCTAGGCACCACCTCGGTTTTTC-3'.

Statistical Analysis

Each experiment consisted of three sets of replicates, and the data were shown using mean \pm standard error. Data were analyzed for significance using Unpaired Student's t-tests (two groups) and ANOVA (general linear model) (more than two groups). Different symbols indicate differences between groups ($p < 0.05$).

Results

GPR54 expression in the mammary glands of dairy cows

To investigate the potential correlation between GPR54 and milk fat synthesis, we performed immunohistochemical and Western Blot detects on high-fat and low-fat mammary glands, respectively.

The results showed that the expression levels of SREBP1 and FASN were significantly higher in high-fat mammary gland than in low-fat mammary gland (Figure 1B, D, E). The above results show that the milk fat synthesis ability of high-fat mammary glands is higher than that of low-fat mammary glands. In addition, the expression of GPR54 in high milk fat mammary gland was significantly higher than that in low milk fat mammary gland (Figure 1A, B, C). The above results showed that the expression of GPR54 in mammary gland was significantly related to the synthesis of milk fat.

Kp-10 promotes milk fat synthesis in BMECs

To study the effect of Kp-10 on milk fat synthesis, BMECs was cultured with DMEM and supplemented with different concentrations of Kp-10 (0, 1, 10 and 100nM) for 1 day and detected with milk fat and BODIPY staining. The results showed that 10 nM Kp -10 had the most obvious effect on milk fat synthesis in BMECs (Fig A, G and H). Therefore, 10 nM Kp-10 was selected and used in subsequent studies. Moreover, 10 nM Kp-10 significantly increased SREBP1 and FASN gene and protein expression (Fig 1B-F). The above results indicate that Kp-10 may promote milk fat synthesis in BMECs by increasing the synthesis of proteins related to milk fat synthesis.

Kp-10 promotes milk fat synthesis by activating GPR54 in BMECs

Previous studies have shown that Kp-10 as a GPR54 endogenous receptor can promote the proliferation of BMECs and increase β -casein synthesis[24, 25]. Therefore, we speculate that Kp-10 may promote milk fat synthesis in BMECs through GPR54. Subsequently, we incubated BMECs with 10 nM Kp-10 for 1 day and detected the protein expression of GPR54. The results showed that Kp-10 significantly increased the expression of GPR54 protein (Figure 3A, B, D).

In order to elucidate the role of GPR54 in Kp-10 promoting the synthesis of milk fat in BMECs , we subsequently used the GPR54 inhibitor (Peptide-234) to inhibit the GPR54 receptor. The results showed that 1 μ M Peptide-234 significantly inhibited milk fat synthesis induced by Kp-10 in BMECs (Figure 4A, F, G). In addition, 1 μ M of Peptide-234 significantly reduced the expression of SREBP1 and FASN promoted by Kp-10 (Figure 4C, D). The above results indicate that Kp-10 promotes the synthesis of milk fat and the expression of FASN and SREBP1 proteins through GPR54 in BMECs.

Kp-10 promotes milk fat synthesis by Inhibiting the AMPK Signaling Pathway in BMECs

Previous studies have shown that inhibition of AMPK signaling pathway can effectively promote the synthesis of lipid and milk fat[9]. Therefore, we used 10nM Kp-10 to incubate BMECs for 24h and detect the phosphorylation level of AMPK. The results showed that 10nM Kp-10 significantly inhibited the

expression of p-AMPK(Figure 3A, C). In addition, Peptide-234 reversed the inhibitory effect of Kp-10 on AMPK signaling pathway (Figure 4B, E).

Subsequently, we investigated the role of AMPK signaling pathway in Kp-10-induced milk fat synthesis in BMECs using AICAR, an activator of the AMPK signaling pathway. The results showed that effect of Kp-10 on the expression of p-AMPK/AMPK, SREBP1 and FASN disappeared after pretreatment of BMECs with AICAR (Figure 5B, C, D, E).

Kp-10 promotes milk fat synthesis by inhibiting SIRT6 in BMECs

Previous studies have shown that the AMPK signaling pathway regulates lipid synthesis by mediating SIRT6[29]. Therefore, we detected the expression of SIRT6 by adding Kp-10 and AMPK to BMECs. The results showed that Kp-10 significantly inhibited the expression of SIRT6 (Figure 5A, B, F). In addition, AICAR also reversed the inhibition of Kp-10 on SIRT6 (Figure 5B, F).

To study the role of SIRT6 in milk fat synthesis, we subsequently overexpressed SIRT6 to study its effect on milk fat synthesis. The results showed that overexpression of SIRT6 significantly increased the expression of SIRT6 in BMECs (Figure 6A, B). Meanwhile, overexpression of SIRT6 significantly inhibited milk fat synthesis in BMECs (Figure 6E). In addition, overexpression of SIRT6 also inhibited the expression of SREBP1 and FASN (Figure 6A, C, D). The above results indicate that the high expression of SIRT6 inhibits the synthesis of milk fat.

Previous studies have shown that acetylation of SREBP1 increases lipid synthesis[30, 31]. Therefore, in order to investigate whether Kp-10 can increase the acetylation of SREBP1 by inhibiting SIRT6 and promote milk fat synthesis. We then used IP experiments to study the interaction between SIRT6 and SREBP1. The results showed that 10nM Kp-10 significantly increased acetylation level of SREBP1(Figure 6F). In addition, high expression of SIRT6 significantly reduced acetylation levels of SREBP1 (Figure 6G). The above results indicate that Kp-10 promotes milk fat synthesis by increasing SREBP1 acetylation by reducing SIRT6-SREBP1 complex levels.

Discussion

In this study, the expression of GPR54 in mammary gland of high-fat dairy cows was significantly higher than that in mammary gland of low-fat dairy cows. In addition, Kp-10 inhibits the AMPK signaling pathway by regulating its receptor GPR54, thereby reducing the deacetylation of SREBP1 by SIRT6 and promoting milk fat synthesis in BMECs.

Studies have found that knocking out the GPR54 receptor can reduce weight and triglyceride content in mice[20]. This indicates that Kp-10 has a regulatory effect on lipid metabolism. In our previous study, we found that the expression of GPR54 was significantly higher in mammary gland of high-quality milk dairy

cows (1.07 ± 0.18 kg / day of milk family yield) than in the mammary gland of low-quality milk dairy cows (0.67 ± 0.22 kg / day of milk family yield)[24]. This suggests that the difference in milk fat expression may be related to the expression of GPR54. In order to further confirm our hypothesis, we detected the expression of SREBP1 and FASN in mammary gland tissue. The results showed that the expression of SREBP1 and FASN in mammary gland of high-milk fat dairy cows were higher than that in mammary gland of low-milk fat dairy cows. Meanwhile, GPR54 is more expressed in high-milk fat mammary gland than in low-milk fat mammary gland, which further proves our speculate. Then we used different concentrations of Kp-10 to stimulate BMECs to explore the role of Kp-10 in milk fat synthesis. The results showed that 10nM had the best effect on the synthesis of milk fat in BMECs, and this concentration was consistent with the previous research results of Kp-10 promoting 3T3-L1 fat deposition. However, in LO2 cells, the effect of 1 nM Kp-10 on lipid deposition was more obvious[20]. In addition, 10nM Kp-10 also significantly increased the expression of SREBP1 and FASN in BMECs. It is suggested that Kp-10 may promote milk fat synthesis in BMECs by increasing the expression of SREBP1 and FASN.

As an endogenous ligand of GPR54, Kp-10 is known to be highly expressed in adipose tissue, and is involved in lipid metabolism and adipocyte differentiation and development[20, 21, 23]. In this study, 10nM Kp-10 can significantly promote the expression of GPR54 in BMECs. While previous studies have shown that Kp-10 can promote the proliferation of BMECs and β -casein synthesis through GPR54[24, 25]. This also indicates that Kp-10 may promote milk fat synthesis in BMECs through GPR54. We subsequently used Peptide-234 to inhibit GPR54. The results showed that Peptide-234 completely eliminated Kp-10 promoting milk fat synthesis and expression of milk fat synthesis-related proteins in BMECs.

AMPK is an important regulator of energy homeostasis[32, 33]. Studies have shown that the activation of AMPK can effectively inhibit diet-induced obesity, and has been used as a target for drugs to treat obesity[33]. Vitamin D relieves fat deposition in C2C12 cells through AMPK/SIRT1[29]. Ginsenosides Rg1 reduces fat deposition in adipose tissue and 3T3-L1 cells by activating AMPK[34]. Aged Oolong Tea reduces fat deposits caused by a high-fat diet by activating AMPK[35]. In addition, the role of AMPK in the synthesis of milk fat in lactating cows has been introduced in some studies. Trans-10, cis-12 conjugated linoleic acid can change fat metabolism in goat mammary epithelial cells through AMPK signaling pathway[9]. SIRT6, as a downstream regulator of AMPK, can also effectively inhibit body fat deposition[36]. In this study, Kp-10 significantly inhibited the phosphorylation level of AMPK and the expression of SIRT6. In order to further explain the effect of AMPK/SIRT6 signaling on milk fat synthesis in BMECs. Subsequently, we used AMPK agonists to observe the synthesis of milk fat in BEMCs. The results showed that the effect of Kp-10 on the expression of SREBP1 and FASN disappeared after AMPK activation. This indicates that Kp-10 promotes milk fat synthesis in BMECs by inhibiting the AMPK/SIRT6 signaling pathway.

In order to further explore the effect of AMPK/SIRT6 signaling pathway on milk fat synthesis in BMECs. Subsequently, we observed the change of milk fat synthesis by overexpression of SIRT6 in BMECs. The

results showed that overexpression of SIRT6 significantly reduced the content of milk fat in BMECs. Previous studies have shown that SIRT6 inhibits SREBP1 expression through a variety of pathways[30]. In addition, SIRT1, a member of the same family of SIRT6, can inhibit fat synthesis by reducing the level of SREBP1 acetylation[31]. Therefore, we use IP experiments to prove the relationship between SIRT6 and SREBP1. The results showed that Kp-10 significantly increased the acetylation level of SREBP1. The over-expressed SIRT6 eliminated the effect of Kp-10 on increasing SREBP1 acetylation. The above results showed that Kp-10 promoted the synthesis of milk fat by reducing the acetylation of SREBP1 in BMECs.

In summary, these results indicate that Kp-10 promotes SREBP1 acetylation by inhibiting AMPK/SIRT6 signaling pathway through GPR54, thereby promoting milk fat synthesis in dairy cows.

Abbreviations

BMECs: Bovine Mammary Epithelial Cells; Kp-10: Kisspeptin-10; SREBP1: sterol regulatory element binding protein 1; FASN: Fatty acid synthase;

Declaration

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Date Availability Statement

The expects data that support the findings of this study are available from the corresponding author upon reasonable request. The mandates data that support the findings of this study are available from the corresponding author upon reasonable request. The data that support the findings of this study are available from the corresponding author upon reasonable request data and peer reviews data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Yu Cao, Juxiong Liu and Shoupeng Fu designed experiments. Yu Cao, Lijun Ma, Qing Zhang, Jiaxin Wang, Wen Li, Yusong Ge, Zhanqing Yang and Ji Cheng carried out experiments. Yu Cao and Shoupeng Fu analyzed sequencing data. Yu Cao, Shoupeng Fu and Juxiong Liu wrote the manuscript.

Ethics approval and consent to participate

All animal experiments and care procedures were carried out in accordance with the Jilin University Institutional Animal Care and Use Committee (approved on 27 February 2015, Protocol No. 2015047).

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

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Figures

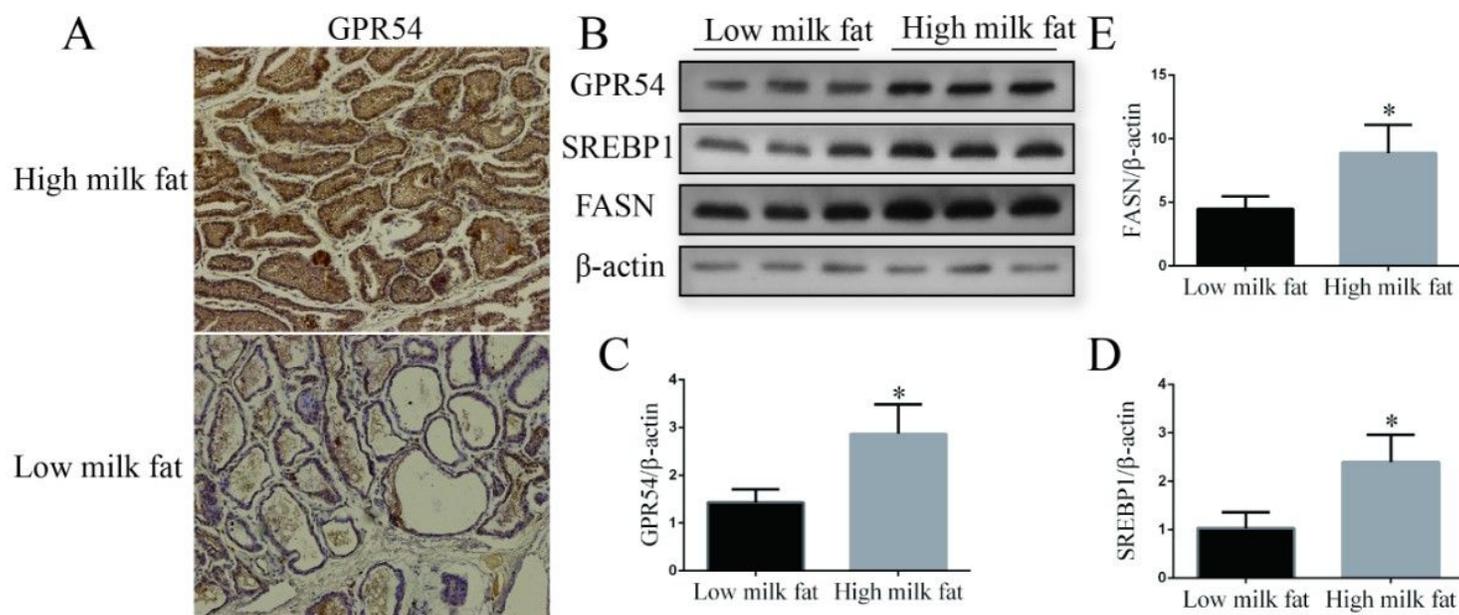


Figure 1

GPR54 Expression in Lactating Mammary Glands of Dairy Cows. (A) Immunohistochemical staining showing the expression of GPR54 in mammary tissues of dairy cows. (B) Western Blot analysis of GPR54, SREBP1, FASN and β -actin. (C, D, E) Relative protein abundance of GPR54, SREBP1 and FASN. The data were analyzed with paired t-tests and ANOVA, and expressed as the mean \pm SEM (n = 10 per group). *P < 0.05 versus the Low milk fat group.

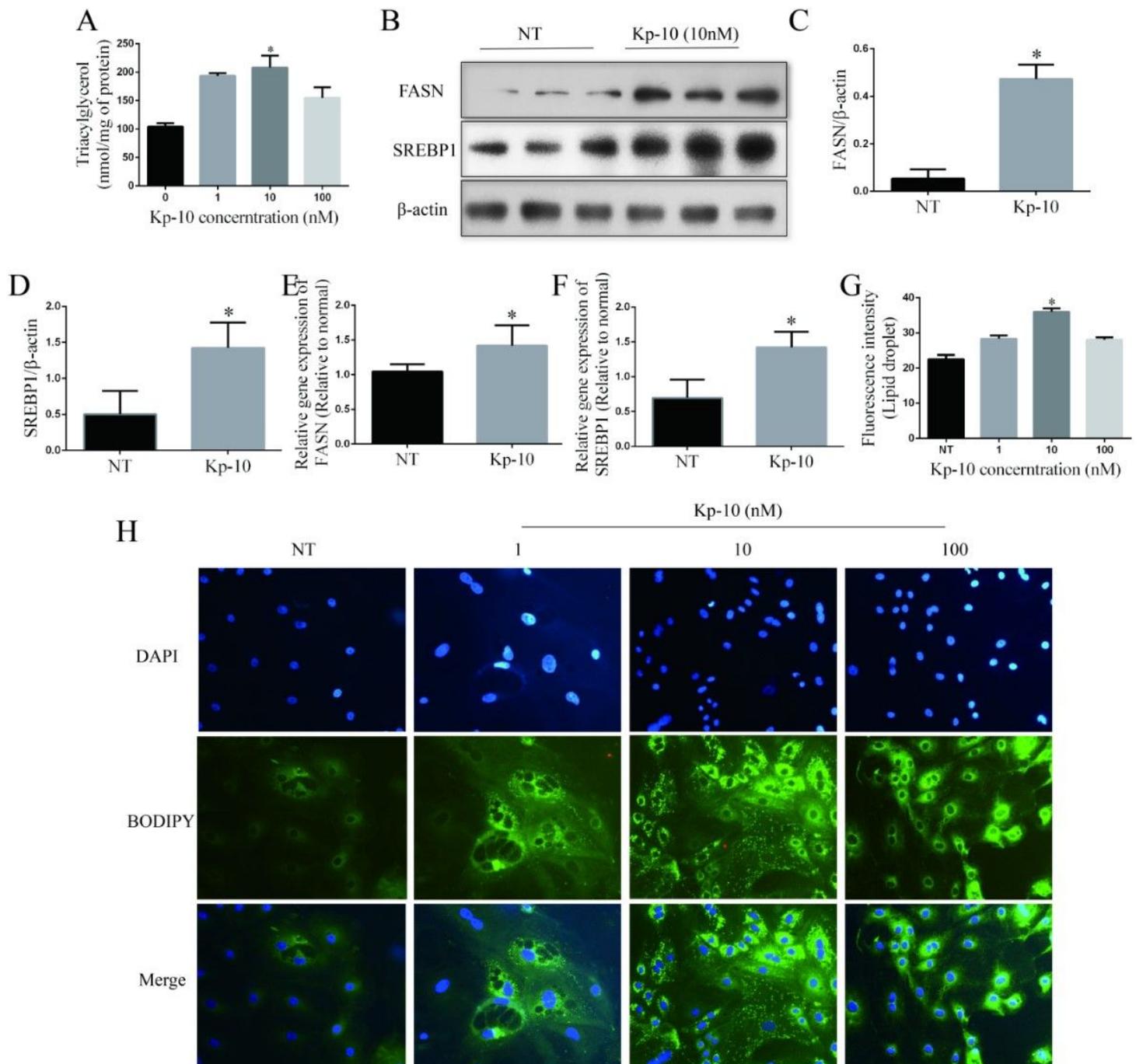


Figure 2

The effect of Kp-10 on milk fat synthesis in BMECs. (A) Cells were treated with 0, 1, 10 or 100 nM Kp-10 for 24 h. The content of TAG were determined by triglyceride content assay in BMECs. (B) Cells were treated with 10 nM Kp-10 for 24 h. Western Blot analysis of FASN and SREBP1. (C, D) Immunoblot bands of SREBP1 and FASN were digitized and are expressed as the ratios to β-actin. The data were analyzed with unpaired t-tests and ANOVA, and expressed as the mean ± SEM (n = 3 per group). *P < 0.05. (E, F) Cells were treated with 10 nM Kp-10 for 24 h. Relative mRNA abundance of FASN and SREBP1. (H) Cells were treated with 10 nM Kp-10 for 24 h. Milk fat were labeled by Bodipy 493/503. *P < 0.05 versus the no-treatment group.

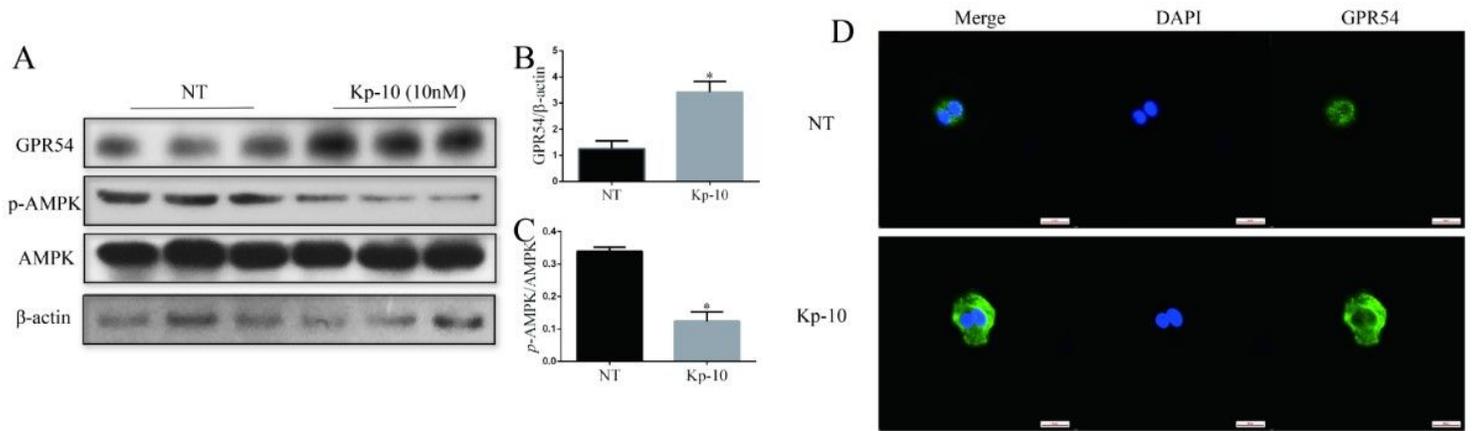


Figure 3

Expression and localization of GPR54 membrane receptor in BMECs. BMECs were treated with 10 M Kp-10 for 24 h. (A) Western Blot analysis of GPR54, p-AMPK and AMPK. (B, C) Immunoblot bands of GPR54 and p-AMPK/AMPK were digitized and are expressed as the ratios to β -actin and AMPK. (D) Immunofluorescence observation of the plasma membrane localization of GPR54 in BMECs. The data were analyzed with unpaired t-tests and ANOVA, and expressed as the mean \pm SEM (n = 3 per group). *P < 0.05 versus the no-treatment group.

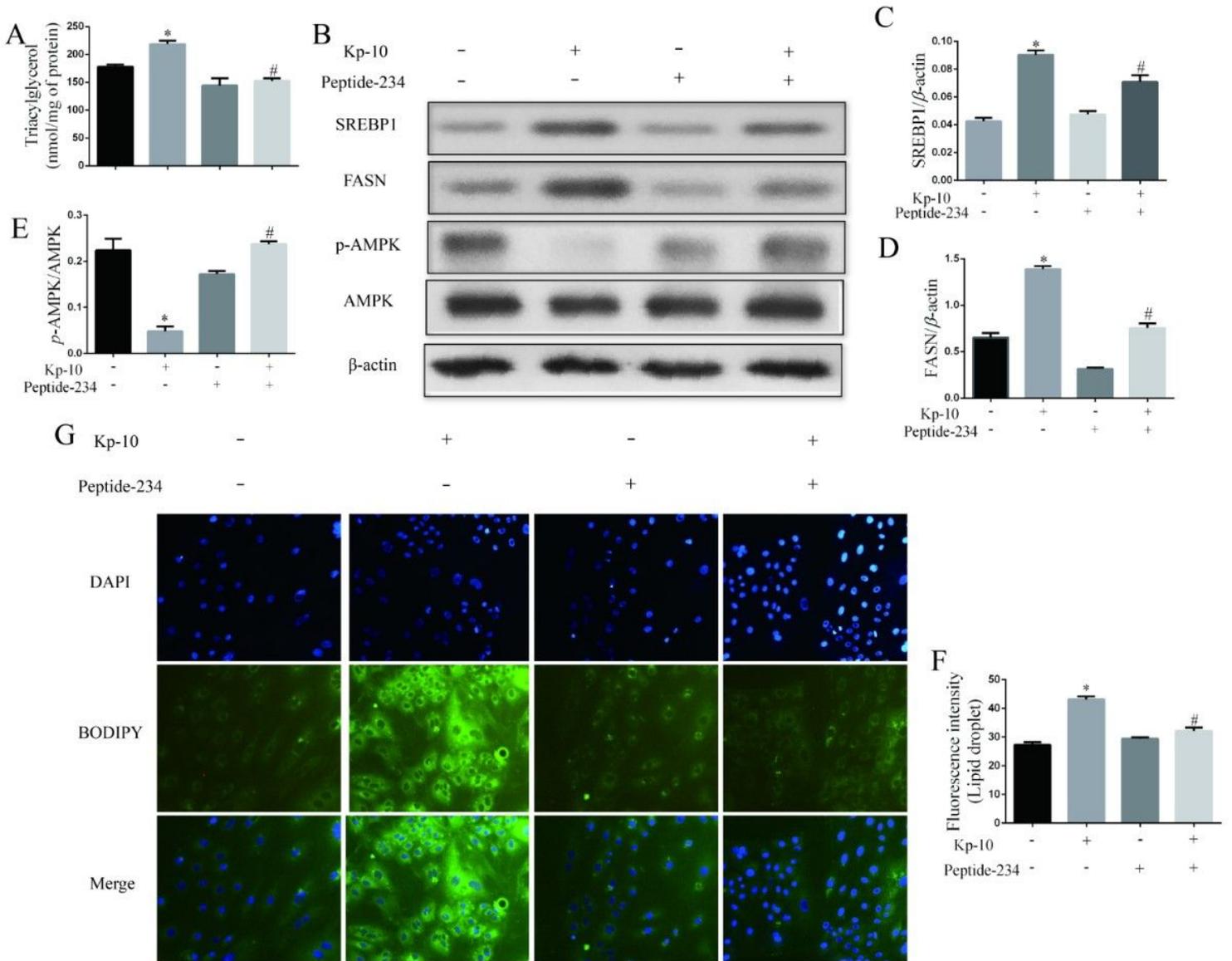


Figure 4

Inhibition of GPR54 blocked Kp-10 to promote milk fat synthesis in BMECs. The BMECs were pretreated for 1 h with Peptide-234 (1 μ M) and then incubated for 24h with Kp-10 (10 nM). (A) The content of TAG were determined by triglyceride content assay in BMECs. (B) Western Blot analysis of SREBP1, FASN, p-AMPK, AMPK and β -actin. (C, D, E) Immunoblot bands of SREBP1, FASN and p-AMPK/AMPK were digitized and are expressed as the ratios to β -actin. (F, G) Milk fat were labeled by Bodipy 493/503. The data were analyzed with ANOVA and expressed as the mean \pm SEM (n = 3 per group). *P < 0.05 versus the no-treatment group and #P < 0.05 versus the Kp-10 group.

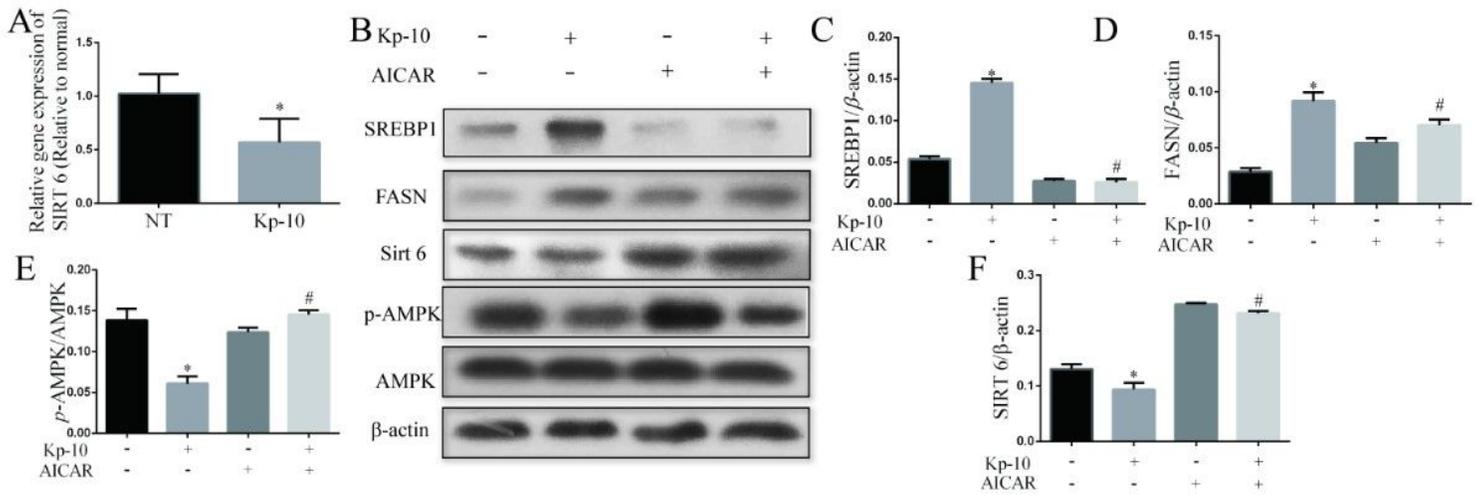


Figure 5

Activation of AMPK blocked Kp-10 to promote milk fat synthesis in BMECs. The BMECs were pretreated for 1 h with AICAR (1 μ M) and then incubated for 24h with Kp-10 (10 nM). (A) Relative mRNA abundance of FASN and SREBP1. (B) Western Blot analysis of SREBP1, FASN, Sirt 6, p-AMPK, AMPK and β -actin. (C, D, E) Immunoblot bands of SREBP1, FASN, Sirt 6 and p-AMPK/AMPK were digitized and are expressed as the ratios to β -actin and AMPK. The data were analyzed with unpaired t-tests and ANOVA, and expressed as the mean \pm SEM (n = 3 per group). *P < 0.05 versus the no-treatment group and #P < 0.05 versus the Kp-10 group.

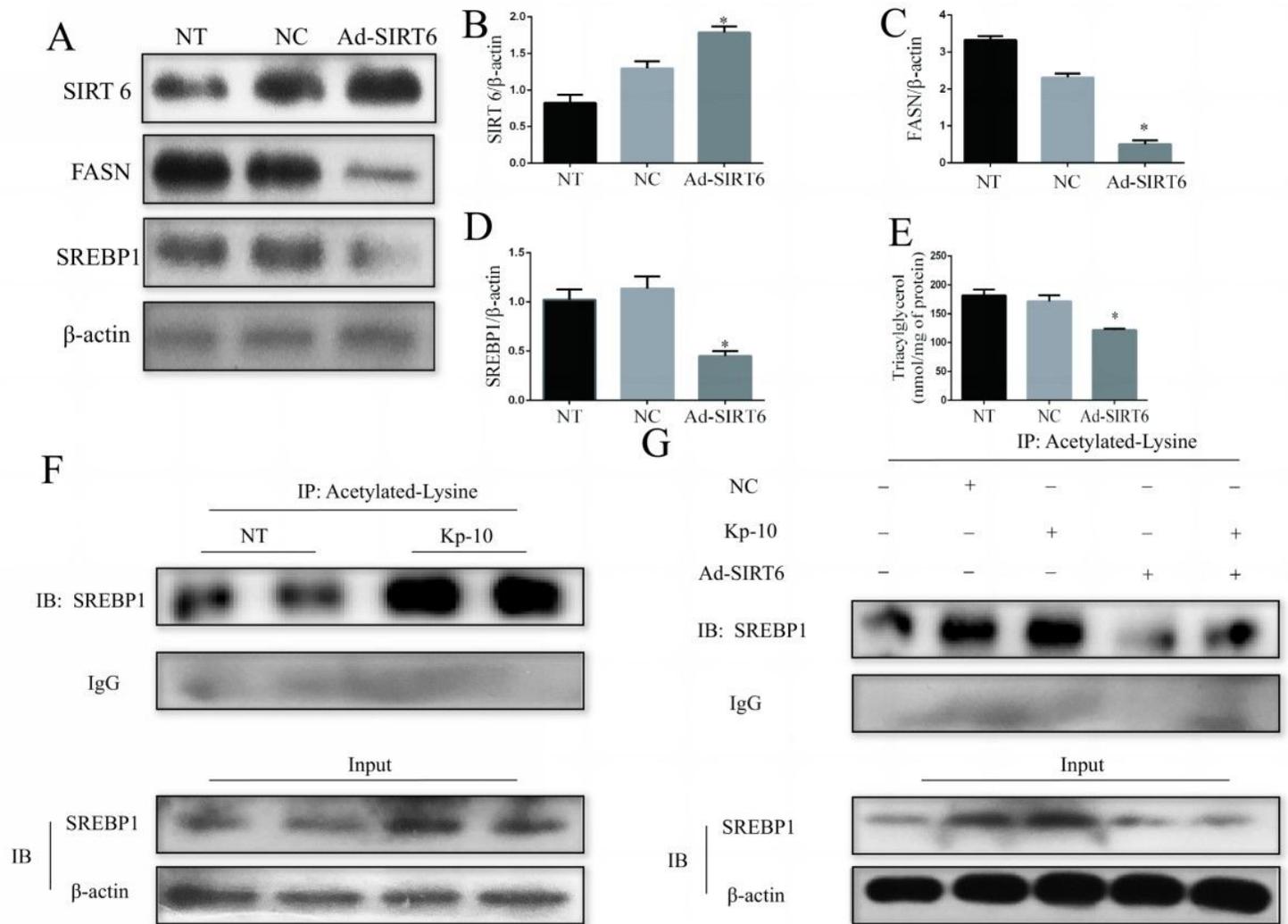


Figure 6

The effects of Sirt 6 overexpression on milk fat synthesis in BMECs. BMECs were incubated with Sirt 6 overexpression plasmid, NC and Kp-10 for 24 h. (A) Western Blot analysis of Sirt 6, FASN, SREBP1 and β-actin. (B, C, D) Immunoblot bands of Sirt 6, FASN and SREBP1 were digitized and are expressed as the ratios to β-actin. (E) The content of TAG were determined by triglyceride content assay in BMECs. (F, G) Immunoprecipitation (IP) with anti-Acetylated-Lysine or rabbit IgG (negative control)-conjugated magnetic G beads was carried out in the cell lysates from treated BMECs. Western blots were probed with anti-SREBP1. *P < 0.05 versus the NC group.

Supplementary Files

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- [tableS1.doc](#)