

miR-574-5p regulates milk synthesis and cell survival activities via DAG-DGKs-PA pathway in GMECs

Yuhan Liu

College of Animal Science and Technology, Northwest A&F University <https://orcid.org/0000-0001-5619-9299>

Meng Zhang

Northwest Agriculture and Forestry University

Dan Guo

Northwest Agriculture and Forestry University

Jiangang Wang

Northwest Agriculture and Forestry University

Binyun Cao (✉ caobinyun@126.com)

<https://orcid.org/0000-0003-2814-9256>

Xiaopeng An

Northwest Agriculture and Forestry University

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Abstract

miRNA-regulated gene expression is momentous for mammary development and lactation performance. Previous work has revealed that miR-574-5p is capable of functioning on goat mammary epithelial cells, but the potential molecular mechanism remains to be further understood. According to transcriptome sequencing results, we focused on DGKI and DGKH, which both were down-regulated differentially expressed unigenes by miR-574-5p. miR-574-5p targeted seed sequences of the DGKI 3'UTR and interdicted mRNA and protein levels of DGKI and DGKH, correspondingly. It's known that diacylglycerol kinases phosphorylate diacylglycerol to produce phosphatidic acid then regulate a range of biological processes. Detection kits showed that DGKI and DGKH induced diglycerides conversion to phosphatidic acid, and DGKI facilitated triglycerides and β -casein production while DGKH only acted as a promoter of triglycerides. DGKI inhibited cell apoptosis and induced cell proliferation, and co-expression of miR-574-5p with DGKI proved an adiabatic impact. However, DGKH had an anti-proliferative and pro-apoptotic effects on GMECs. Additionally, DGKI motivated both AKT-mTOR and Raf-1-ERK pathways while DGKH showed negative impacts on AKT-mTOR, Raf-1-ERK and IKK-NF κ B pathways in GMECs. It suggested that miR-574-5p not only inactivated PA-mTOR pathway and contributed to regressive secretion of triglycerides and β -casein via depletion of DGKI, but also arrested cell proliferation and enhanced cell apoptosis by inhibiting DGKI-PA-ERK pathway. All these established a farther regulatory mechanism of miR-574-5p in milk synthesis and mammary development.

Introduction

Diacylglycerol kinases (DGKs) are a multiple flock of lipid – metabolizing enzymes that phosphorylate diacylglycerol (DAG) to produce phosphatidic acid (PA) [1]. DAG and PA are pivotal forerunners of triacylglycerol (TAG), phospholipids and valid catalyst of plentiful signaling proteins, many of which, when motivated can regulate a wide range of biological processes [2, 3]. DAG is a pivotal second messenger counted in multifarious cellular responses, such as cell proliferation, apoptosis and secretion [4]. DAG triggers activation of conventional and novel protein kinase C isoforms (PKCs) and Ras guanyl nucleotide – releasing proteins (RasGRPs) [5]. PA, a lipid second messenger and adjuster of the mitogenic action of hormones and growth factors, takes part in diverse cell processes and then functions a cluster of signaling proteins, such as protein kinases and phosphatases [6]. PA can regulate phosphatidylinositol – 4-phosphate 5 – kinase, Ras – GAP, C – Raf and atypical PKC [3]. Otherwise, PA mediates the protein deemed as mammalian object of rapamycin (mTOR) emphatically, a primary controller of various intracellular signaling events [7]. Hence, DGKs exert effects not only by depressing DAG – modulated signals but also by the production of PA, which accordingly well positioned to function in signal transduction and complex lipid biosynthesis.

Ten DGK isozymes (α , β , γ , δ , η , κ , ϵ , ζ , ι , and θ) have been recognized and categorized into five subfamilies in view of their structural characteristics, furnishing abilities to mediate precise DAG and PA pools and participate in various signaling axes [8]. DGKI, also named DGKI ι , belongs to type IV DGKs, the other closely correlative isoform is DGK ζ [9]. Early report reveals that DGKI combines to RasGRP3 and

blocks Rap1 motivation by metabolizing DAG. This depletion thus promotes Ras signaling as that Rap1 antagonize the obligation of Ras [10] DGK ζ also suppresses RasGRPs 1, 3 and 4 and prominently mediates Ras signaling [11]. In Jurkat T cells, DGK ζ restrains activated Ras, ERK and AP – 1 and acceleration of CD69, a motivation marker [12]. Previous experiments in which DGK ζ is overexpressed in HEK293 cells exhibit that DGK ζ elevates PA production and activates S6K, a downstream target of mTOR, in a sence reliance on mTOR's PA binding domain [13]. Type II DGKs consist of DGK δ , DGK η (also named DGKH), and DGK κ . Deficiency of DGKH in HeLa cells brings about apparently negative cell proliferation via restraining C – Raf motivation and ERK1/2 and MEK1/2 phosphorylation, which implicates DGKH as a regulator of Ras – Raf – MEK – ERK pathway [14]. In Hek cells, DGKH improves G – Protein coupled receptor signaling via blockade of PKC activation [15]. In consequence, we undertook to inquire the effects of DGKI and DGKH in goat mammary epithelial cells (GMECs) in this study.

miRNAs, a flock of single – stranded small noncoding endogenous RNAs with about 22 nucleotides (nts) in length, are ascertained as momentous post – transcriptional regulators [16]. Currently, miRNAs interdict gene expression via interacting with the 3'–UTR (untranslated region) of target messenger RNAs, triggering translation deficiency and/or mRNA blockage [17]. A single miRNA is forecasted to mediate plentiful genes, thus regulating a myriad of biological processes including cell development, proliferation, apoptosis and milk performance [18 – 20]. miR – 574 – 5p was verified that it prominent impacted on lactation performance and mammary development drew on sequence consequences compared mammary gland transcriptome data during common milk with colostrum stages [21]. Studies about miR – 574 – 5p have specialized in its anti – oncogenic and anti – metastatic capabilities [22 – 24]. Although we also have clarified a circRNA-miR-574-5p-mRNA network that affects GMECs, the function of miR-574-5p required to be profoundly revealed.

In our prevenient work, small RNA libraries were framed via transfection of miR-574-5p mimics or negative control into GMECs utilizing RNA sequencing. Herein, among down-regulated differentially expressed unigenes (DEGs), we selected DGKI and DGKH for further studies, owing to its potential biological functions. The hodiernal study furnished new insights into the molecular mechanisms involved in miR-574-5p in GMECs.

Materials And Methods

PcDNA3.1-DGKI/DGKH plasmids construction

The CDS regions of DGKI ([XM_018046893.1](#)) and DGKH ([XM_018056794.1](#)) were amplified via PCR from [extractive](#) GMECs genomic cDNA. The PCR fragments were digested and cloned into pMD™19-T vector (TaKaRa, Beijing, China). Afterwards, the eukaryotic expression pcDNA3.1(+) vector (Thermo Fisher, Shanghai, China) was used to construct DGKI and DGKH overexpression plasmids. The entire DGKI and DGKH CDS sequences were then introduced into the numerous cloning spots of the pcDNA3.1 vector, and the constructs were ascertained using DNA sequencing.

Forward primer of DGKI: EcoR I 5'-CGGAATTCGCCGCGGATGGATGCTG-3', and reverse primer: Xho I 5'-CCCTCGAGCAGGGGAAAAGTGAATGCATAGGGG-3'. Forward primer of DGKH: Kpn I 5'-CATGGTACCGACAGGATGGCCGGGGC-3', and reverse primer: Not I 5'-ATGCGGCCGCGTATGATTACACCTCAGGC-3'.

Animals, cell culture and transfection

Xinong Saanen dairy goats in the experimental farm of Northwest A&F University of China were used. After collected mammary glands and maintained in PBS replenished with 100 µg/mL penicillin and 100 µg/mL streptomycin, then samples were disposed in the laboratory within 1 h. The GMECs were cultivated in DMEM/F12 medium (Gibco, CA, USA) containing 5 mg/mL insulin, 10% foetal bovine serum, 100 U/mL streptomycin/ml penicillin, 10 ng/mL epidermal growth factor 1 (EGF-1, Gibco, CA, USA), and 0.3 mmol/L hydrocortisone at 37 °C in a humidified atmosphere with 5% CO₂. GMECs were filtrated and cultured in accordance with former studies [25]. GMECs were seeded and cultivated in 6-well plates at 2-4 × 10⁵ cells/cm² till cell density arrived at 80-90%. In terms of the manufacturer's specifications, NC, miR-574-5p mimics, inhNC, miR-574-5p inhibitors, siRNA-DGKI/DGKH (GenePharma, Shanghai, China), pcDNA3.1 and pcDNA3.1-DGKI/DGKH vectors were transfected into GMECs by Lipofectamine 2000 (Invitrogen, CA, USA). In brief, 4 µL miR-574-5p mimics/inhibitors or siRNA-DGKI/DGKH with 4 µL Lipofectamine 2000, 4 µg pcDNA3.1 or pcDNA3.1-DGKI/DGKH vectors with 6 µL Lipofectamine 2000, 2 µL miR-574-5p mimics and 2 µg pcDNA3.1-DGKI/DGKH vectors with 6 µL Lipofectamine 2000 were attenuated by 200 µL Gibco Opti-MEM I medium and the compound was cultured for 20 min. Approximately 4 h post-transfection, the medium was altered to DMEM/F12 complemented with 10% FBS. Then cells were transfected in 24 h or 48 h and selected for farther detection. miR-574-5p mimics/inhibitors, inhNC, NC and siRNA of DGKI and DGKH sequences were showed in Table S1.

RNA extraction and quantitative real-time PCR (RT-qPCR)

After cells treatment, the total RNA was separated with TRIzol reagent (Invitrogen, CA, USA). Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) assessed the concentration and fineness of RNA. Reverse transcription was performed using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA, Beijing, China) and the methods were referred to previous report. Quantification of mRNA criterion in diverse genes was measured through SYBR Premix Ex Taq II (TAKARA, Beijing, China) and examination was fulfilled using the CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). Table S2 listed the recognized primers for real-time PCR. The RT-PCR factors were: 95 °C for 10 min and then 40 cycles at 94 °C for 15 s, 60 °C for 30 s, followed by 72 °C for 30 s. β-actin mRNA and U6 small nuclear RNA were used as internal controls for DGKI or DGKH and miR-574-5p mRNA, separately. All of experiments were performed in triplicate. Relative expression was metered via the $2^{-\Delta\Delta C_t}$ approach.

Luciferase reporter assay

About 284 bp sequences of DGKI 3'UTR containing the calculated target sites of miR-574-5p were amplified via PCR. Afterwards, the PCR compound was inlet into psiCHECK-2 vectors (Addgene, CA, USA)

with particular Xho I and Not I restriction enzymes for dual-luciferase assay. Forward primer of DGKI wild type: Xho I 5'-CCCTCGAGGCCCCCTTCTTTCAAGTTC-3', reverse primer: Not I 5'-AAGCGGCCGCAAATGGAGAGAAAGTAGGAG-3', forward primer of DGKI mutant type: Xho I 5'-CCCTCGAGCAGAACTCAATTAGAGCCCCCTT-3', reverse primer: Not I 5'-AAGCGGCCGCAATGCACACCCCAAACAAA-3'. To measure the connections between miR-574-5p and its target DGKI, GMECs were seeded in a 48-well plate co-transfected with 0.33 mg luciferase reporter gene constructs and 10 pmol miR-574-5p mimics (and NC) or inhibitors (and inhNC) with Lipofectamine 2000 for 24h. Then GMECs were dissolved and 10 μ L cell lysate was placed into a 96-well enzyme label plate, and the renilla and firefly luciferase activities were estimated through thermo scientific varioskans flash (Thermo scientific, USA) using the Dual-Glo luciferase assay system (Promega, USA).

Flow cytometry assay

Flow cytometry method (FCM) was used to examine the apoptosis of GMECs. The apoptotic effects were detected utilizing Annexin V-FITC PI staining apoptosis assay kit (SeaBiotech, Shanghai, China). Cells were seeded in six-well plates, after 24 h post-transfection, quantities of apoptotic cells were tested by flow cytometry in 30 min behind staining with annexin V-FITC and propidium iodide (PI), in virtue of the manufacturer's agreement.

CCK8 assay

The viability of GMECs was investigated by CCK8 approach. GMECs were seeded in 96-well plates at a concentration of 1×10^4 cells per well in a 150 μ L volume with six repetitions. Subsequently, GMECs were treated diversely in 24, 48 and 72h. 15 μ L CCK8 reagents (ZETATM life, USA) were accelerated in each well and cultured in dark at 37°C for 2 h. Then mixtures were oscillated for 20 min and Epoch microplate reader (Biotek, Winooski, USA) examined the absorbance at 450 nm.

EdU staining

After 24 h post-transfection in 96-well plates, GMECs proliferation analysis was implemented with EdU dyeing. In brief, cells were stained with EdU (Ribobio, Guangzhou, China) at an ultimate 50 μ M content for 2 h and further with DAPI for 15 min in 37°C. Images were resolved using fluorescence microscopy.

Determination of diglycerides, PA, triglycerides and β -casein

Cell-free supernatants and cell lysates were assembled when GMECs pro-treated with 24 h and then used to evaluate diglycerides production by diglyceride Enzyme linked immunosorbent assay (ELISA) kit (Fankew, China), PA production by total phosphatidic acid assay kit (Cayman Chemical, Shanghai, China), triglycerides secretion using triglycerides quantitative assay kit (Applygen, Beijing, China) and β -casein concentrations using goat β -casein ELISA kit (Tongwei, Shanghai, China). Based on detective specifications, the absorbance at 450 nm, 530 nm, 550 nm and 450 nm in utilization of 50 μ L supernatants, 40 μ L supernatants, 10 μ L cell lysate and 50 μ L supernatants was appraised via Epoch

microplate reader (Biotek, Winooski, USA), respectively. Calculated the corresponding concentrations of samples using the equation obtained from the linear regression of the standard curve.

Western blot

Cell lysates were assembled and extracted on ice utilizing RIPA lysis buffer (Biotek, Beijing, China) to which protease inhibitors had been appended 0.1 mg/mL phenylmethanesulfonyl fluoride (PMSF, Solarbio, Beijing, China) subsequently GMECS treated in 48 h. Protein concentrations in the samples were verified using BCA protein assay kit (Vazyme Biotech, Nanjing, China). About 40 µg total protein was subjected to a 12% SDS-PAGE. The proteins were separated on a 12% SDS-PAGE by electrophoresis and then diverted into a polyvinylidene difluoride (PVDF, Merck Millipore, MA, USA) membrane. After the incubation in 10% skim milk powder in Tris-buffered saline containing 0.1% Tween 20 (pH 7.6) for 1 h at room temperature, membranes were incubated with referential primary antibodies at 4 °C overnight. Then adequate horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit or goat were hatched for 1 h at 4 °C. Table S3 exhibited the **homologous** primary antibodies. β-actin was used as an endogenous reference gene. All proteins were examined using facilitated chemiluminescence (Advansta, USA). Quantification was performed by the Quantity One program (Bio-Rad, California, USA).

Statistical analysis

Analysis of statistics was fulfilled and calculated using SPSS 19.0 (Beijing, China) software. All data were exposed as the mean ± SE (standard error) of three independent experiments. Comparisons between treatments were measured by t tests for two groups or one-way and two-way analysis of variance (ANOVA) for more than two groups. Statistical significance was viewed at * p < 0.05 and ** p < 0.01.

Results

miR-574-5p impaired DGKI directly and DGKH indirectly in GMECs

To authenticate the novel underlying molecular mechanism of miR-574-5p affecting GMECs, we detected miR-574-5p putative targets via Target Scan (<http://www.targetscan.org/>). Among them, DGKI drew our attention due to following reasons: (1) TargetScan forecasted that there was one miR-574-5p target site at nucleotides 11361 to 11369 of DGKI-3'UTR; (2) DGKI was a differentially expressed unigene in terms of transcriptome sequencing and (3) DGKI was regarded as a momentous regulator for diversiform metabolic processes, which upon activation converted DAG into PA [9, 10] To further validate that DGKI is transcriptionally mediated by miR-574-5p, two-tier luciferase reporters linked the psiCHECK-2 (psC) vector with the wild-type (wt)-DGKI-3'UTR or mutant (mut)-DGKI-3'UTR were used (Figure 1A). Indeed, miR-574-5p mimics or inhibitors attenuated or facilitated relative luciferase activities significantly, nevertheless reporters mutated in DGKI-3'UTR failed to react to miR-574-5p expression (Figure 1B). Then RT-qPCR and western blot were used to screen the mRNA and protein expressions of DGKI. Compliance with above-mentioned observation, DGKI mRNA level was apparently depressed by re-expression of miR-574-5p, whereas it improved when miR-574-5p interference in antisense molecules (Figure 1C). Cells post-

transfected with miR-574-5p mimics or inhibitors emerged a remarkable decrease or increase of relative protein expression of DGKI in comparison with contrasts (Figure 1D). All the evidence indicated that miR-574-5p served as a demotivated mediator of DGKI via bonding to the 3'-UTR directly in GMECs.

As RNA-seq results indicated that the expression of DGKH was reduced in miR-574-5p-induced cells, we wanted to affirm their connections. RT-qPCR and western blot results showed that miR-574-5p inhibited the mRNA and protein expressions of DGKH, coinciding with those of transcriptome sequencing (Figure 1C, D). It suggested that miR-574-5p took negative regulatory impacts on DGKH expression.

miR-574-5p regulated milk synthesis via DGKI and DGKH in GMECs

We next screened for DGKI and DGKH that were involved in regulating milk synthesis. Since DGKs could convert DAG to PA, we identified the secretion of diglycerides and phosphatidic acid in the cell-free supernatants using detection kits. As expected, overexpression of DGKI and DGKH both inhibited diglycerides production and promoted PA production (Figure 2A, B), while depressed DGKI and DGKH exhibited reverse secretion (Figure 2C), notably. We also found DGKI and DGKH attracted the assuasive effects of miR-574-5p on the content of diglycerides and PA (Figure 2A, B). Moreover, DGKI and DGKH showed induced production of triglycerides whether GMECs treated with overexpression plasmids or siRNAs (Figure 2D, E, F). The negative effects of miR-574-5p on triglycerides content were counteracted by DGKI or DGKH (Figure 2D, E). ELISA results revealed that DGKI acted as a promoter of secretion of β -casein and alleviated that negative impact of miR-574-5p in GMECs (Figure 2D, F). However, there was no change in β -casein secretion after GMECs treated with various expressions of DGKH (data not shown). All clues made certain that miR-574-5p regulated the production of diglycerides, PA and triglycerides via DGKI and DGKH and blocked β -casein secretion via DGKI in GMECs.

miR-574-5p depressed the proliferation and improved the apoptosis of GMECs via DGKI

In our previous work, we made certain that miR-574-5p depressed the proliferation and improved the apoptosis of GMECs. Thus we next sought to determine whether miR-574-5p were inclusive of modulating survival capabilities of GMECs via its negativity to DGKI or DGKH. siRNA-DGKI/DGKH and vectors carrying DGKI/DGKH coding sequences (CDS), were introduced into GMECs for the blockage and [motivation](#) of DGKI or DGKH. CCK8 assay after cells cultivated for 24, 48 and 72h exhibited that inducement of DGKI promoted the cell viability (Figure 3A), while interference of DGKI using siRNA restrained the cell viability compared to contrasts (Figure 3C). Figure 3B, C showed that DGKH appeared a negative role on GMECs viability. Subsequently, the EdU staining assay was conducted to investigate the proliferation of GMECs. We observed a prominent increase in cellular proliferation following DGKI overexpression (Figure 3D, G), which was conspicuously impaired by si-DGKI in comparison with negative control (Figure 3F, G). Expectably, DGKI moderated miR-574-5p-weakened effects on GMECs proliferation via decreasing cell viability and EdU positive cells (Figure 3A, D, G), clarifying that miR-574-5p suppressed cell proliferation through DGKI in GMECs. However, DGKH functioned as an oppressive regulator on the proliferation of GMECs when we treated cells with distinct expressions of DGKH (Figure 3E, F, G), which meant miR-574-5p didn't regulate cellular proliferation by DGKH.

On behalf of affirming the impact of DGKI and DGKH on GMECs apoptosis, the extents of apoptosis were measured utilizing Annexin V-FITC/PI staining and relative expression of decisive apoptotic genes. Flow cytometry showed that 24 h following transfection of GMECs, the apoptotic rates were higher in si-DGKI group and lower after DGKI overexpressed, significantly (Figure 4A, C, S1A, C). Since incremental documents revealed that Bcl2 and Bax were diverse valid apoptotic genes [26, 27], we implemented western blot analysis and DGKI inhibited pro-apoptotic Bax protein expression and improved anti-apoptotic Bcl-2 protein expression, separately (Figure 4D, E). DGKI topically rescued the stimulative impact of miR-574-5p on GMECs apoptosis (Figure 4A, E, S1A). All these indicated that miR-574-5p answered for facilitating cell apoptosis via its mediation of DGKI. Compared with respective control groups, interference of DGKH diminished the cells apoptotic rates (Figure 4C, S1C), and on the contrast, inducing DGKH elevated count of apoptotic cells (Figure 4B, S1B). Loss of DGKH enhanced the protein level of Bcl-2 cooperated with down-regulated Bax expression (Figure 4D), while **motivated** DGKH exhibited the **opposite** results (Figure 4F). All the data affirmed that DGKH promoted the apoptosis of GMECs.

DGKI and DGKH effected AKT-mTOR signaling pathways in GMECs

Searching for the mechanism by which DGKI and DGKH affected milk synthesis, we turned to AKT-mTOR signalling, a potent classical pathway that modulated mammary development [28, 29]. DGKI overexpression clearly increased the activation of PI3K, AKT, mTOR, S6K1, RPS6, EIF4B and 4EBP1, meanwhile, neutralized the passive influence of miR-574-5p (Figure 5A). Oppositely, cells treated with miR-574-5p mimics and si-DGKI reduced these phosphorylation levels (Figure 5C), which meant miR-574-5p suppressed the expression of milk protein synthesis-relevant proteins via down-regulating DGKI in GMECs. Afterwards, we inquired whether DGKH could affect AKT-mTOR pathway. Deficiency of DGKH triggered activation of AKT, mTOR, S6K1, RPS6, EIF4B and 4EBP1, which are components of downstream targets of mTOR (Figure 5C). Interestingly, PI3K was inactivated in si-DGKH treated cells (Figure 5C). The diagrams exhibited opposite corresponding protein levels after cells transfected with pcDNA3.1-DGKH vectors (Figure 5B). We hence suggested that DGKH could attenuate AKT-mTOR signalling in GMECs.

DGKI and DGKH effected ERK and NFKB signaling pathways in GMECs

It's well known that NFKBs, MAPKs, incorporating ERK, p38 MAPK and JNK, are potent signaling molecules partook in cellular processes and modulate cell proliferation and cell apoptosis [30-33]. To elucidate the mechanism by which DGKs effected **cellular** activities, the crucial members activation in Raf-ERK signaling pathway in DGKs-induced or -silenced GMECs was subsequently measured. The key members' activation was evaluated by their phosphorylation via western blot. In cells overexpressing DGKI, phosphorylation of Raf-1, MAP2K1&2, ERK, RSK1 and Bad were greater than that seen in control cells (Figure 6A). DGKI also partially abrogated the negative effects of miR-574-5p on aforesaid key proteins motivation (Figure 6A). Consistent with our prior observation that DGKI promoted ERK signaling, we found that DGKI depletion significantly restrained the increase in p-Raf-1, p-MAP2K1&2, p-ERK, p-RSK1 and p-Bad (Figure 6C). To further pursue the impacts of DGKI on GMECs, we assessed the activation in

IKK-NFκB signaling. Astonishingly, Figure 6D showed that induced DGKI inhibited the expression of p-IKKα/β, p-IKBα/β and NFκB1. Cells treated with si-DGKI and miR-574-5p uncovered reverse results (Figure 6F), indicating that DGKI and miR-574-5p both arrested IKK-NFκB signaling pathway. Thus we confirmed that miR-574-5p was capable of regulating cell survival capabilities via the DGKI effects on Raf-ERK signaling, but not IKK-NFκB signaling in GMECs. With regard to DGKH functions, we compared ERK signaling in GMECs transfected with si-DGKH or pcDNA3.1-DGKH plasmids with controls. Blockage of DGKH caused up-regulation of phosphorylation of Raf-1, MAP2K1&2, ERK, RSK1 and Bad (Figure 6C), whereas contrary results were exhibited after DGKH enhanced (Figure 6B), supporting a critical role of DGKH[1] in the blockage of the Raf-ERK signaling cascade. We also measured that the expression of p-IKKα/β, p-IKBα/β and NFκB1 was reduced or improved when DGKH overexpressed or knocked-down (Figure 6E, F). All clues affirmed that DGKH acted on a catalyzer in cell apoptosis and promoted cell proliferation via impairing ERK pathway and NFκB pathway.

Crosstalk between mTOR and ERK pathways

Enhanced documents reveal about novel signaling connections between mTOR and ERK pathways [34, 35]. To suppress the ERK pathway, we used either pharmacological UO126, an effective inhibitor of MAP2K1&2 or siRNAs of MAP2K1 and MAP2K2. As expected, the phosphorylation of ERK was diminished under these conditions (Figure 7A, B). UO126 with different concentrations in 24 h triggered inactivation of mTOR, S6K1 and 4EBP1 in GMECs, intriguingly, we observed a notable increase in phosphorylation of AKT (Figure 7A). Cells transfected with siRNA-MAP2K1&2 also showed the same results compared with NC (Figure 7B). Afterwards, we examined the effects of the mTOR inhibitor, rapamycin, and the mTOR catalyzer, MHY1485 on GMECs in 1, 5 and 10 μM. The results exhibited that the expression of p-S6K1 and p-4EBP1 was down-regulated via rapamycin accompanied by induced phosphorylation of AKT, MAP2K1&2 and ERK (Figure 7C). Significantly, MHY1485 treatment gave rise to positive phosphorylation of S6K1 and 4EBP1, while AKT, MAP2K1&2 and ERK was inactivated (Figure 7D). These data supported a role for ERK motivation of mTOR-S6K1/4EBP1 pathway and ERK signaling might lie upstream mTOR signaling. Since S6K1 is one of the downstream targets of mTOR, we introduced siRNA targeted against S6K1 and found higher levels of p-AKT and p-ERK in GMECs (Figure 7F, G), which meant S6K1 arrested both ERK and AKT pathways. Then we suspected whether AKT motivation via ERK inhibition was reliant on mTOR-S6K1. Western blot showed that UO126 didn't further improved p-AKT from enhanced phosphorylation of AKT levels by rapamycin-blocked mTOR or knockdown of S6K1 (Figure 7E, F). It suggested that mTOR-S6K1 signaling was essential for ERK inhibition-induced AKT motivation in GMECs. At the same time, rapamycin-induced ERK motivation was dependent on S6K1 (Figure 7G), providing evidence that S6K1 was a central medium of ERK inhibition-enhanced AKT activation and mTOR inhibition-enhanced ERK activation.

Discussion

For decades, studies have been devoted to promoting the composition of goat milk to meet the cumulatively elaborate nutritional demands. The mammary gland acquires the function of milk synthesis

during lactation. Our previous work from comparative appraisal of sequence data with foregone mammary gland transcriptome data during common milk and colostrum stages shows that miR-574-5p predominantly influences mammary gland development and lactation performance [21]. According to high-throughput sequencing results, we have identified DEGs mediated by miR-574-5p directly or indirectly in GMECs. We have made certain that miR-574-5p not only demotivates the MAPK pathways, which leads to enhanced cell apoptosis and suppressed cell proliferation, but also arrests AKT-mTOR pathway and β -casein and triglycerides expression by binding to MAP3K9 directly. To explore novel feasible molecular principle of miR-574-5p in GMECs, we prioritize and focus on DGKI and DGKH, which both are down-regulated DEGs for further research.

Our work sniffed out that miR-574-5p objected seed sequences of one of the DEGs, DGKI and further blocked its mRNA and protein levels. miR-574-5p also restrained the mRNA and protein expression of DGKH indirectly. Since DGKs could catalyze the phosphorylation of DAG to produce PA, they have been confirmed as rising crucial regulators of a variety of cell signaling systems [4, 14]. Hence, we probed into whether miR-574-5p functioned in GMECs via its deficiency of DGKI and DGKH. In our work, upon transfection of GMECs with si-DGKI/H and pcDNA3.1-DGKI/H vectors, we found that DGKI and DGKH both inhibited diglycerides secretion or promoted phosphatidic acid and triglycerides concentrations. The respective effects of miR-574-5p on the content of diglycerides, PA and triglycerides were counteracted by DGKI or DGKH. DGKI also acted as a promoter of β -casein production and alleviated that depressive impact of miR-574-5p on GMECs. Hence we speculated that miR-574-5p regulated the secretion of diglycerides, PA and triglycerides via DGKI and DGKH and blocked the secretion of β -casein via DGKI in GMECs. In mammalian cells, DAG is a canonical allosteric motivator of RasGRP1 and then triggers the activation of AKT pathways [12]. It's known that PA, which is competitive with mTOR inhibitor rapamycin, could interact with mTOR and then motivate mTOR signaling [6]. Researches over the foretime have emphasized the roles for PI3K/AKT signaling in mediating milk components integration such as triglycerides and caseins [29]. As a primary regulatory element in cell signal transduction, AKT can give rise to the mTOR phosphorylation and then active downstream S6K1 and 4EBP1 [36]. Motivation of S6K1 brings about the abundance of protein integrated signaling and 4EBP1 induces the initiation of translation by bonding of EIF4G to EIF4E [37, 38]. At the same time, EIF4B and RPS6 are widespread downstream targets of S6K1, reliable for translation initiation [39, 40]. Based on these studies, we estimated varieties in the protein expression levels of PI3K, AKT, mTOR, S6K1, RPS6, EIF4B and 4EBP1. We found that phosphorylation expression of above - mentioned proteins were enhanced by DGKI. Interestingly, DGKH blocked the expression of p - AKT, p - mTOR, p - S6K1, p - RPS6, p - EIF4B, p-4EBP1, and activated PI3K. These observations supported our hypothesis that miR-574-5p inhibited milk synthesis by depressing DGKI motivation of PA-mTOR-S6K1/4EBP1 pathway in GMECs, but not via DGKH. However, DGKH showed negative impacts on AKT-mTOR pathway, which suggested that the induced-production of triglycerides owing to other mechanisms.

Proliferation and apoptosis of GMECs are closely related to the development of mammary gland [41]. Accordingly we specialized in the mechanism of miR - 574 - 5p in cellular survival capabilities of GMECs. Results demonstrated that DGKI elevated cell proliferation and restrained cell apoptosis, and co -

expression of miR - 574 - 5p with DGKI indicated an adiaborous impact. Whereas, DGKH had an anti - proliferative and pro - apoptotic effects on GMECs. All these revealed that miR - 574 - 5p regulated cellular states via DGKI, but not DGKH in GMECs. Hence we wonder why DGKI and DGKH have that reverse functions. Recent work has clarified that RasGRP1 and PKCs can be activated by DAG. RasGRP1 in turn contributes to Raf - 1 motivation, additionally, PKCs lead to the activation of IKK and NFKB [11, 14, 42]. PA could also interact with the serine - threonine kinase Raf - 1 directly [3]. Raf - 1 is known to active ERK via phosphorylating and stimulating the MEKs (MAP2K1 and MAP2K2) in vivo and then motivates the ERK signaling [43]. As a pivotal ERK downstream kinase, RSK is able to phosphorylate Bad, which attenuates its pro - apoptotic capabilities via promoting its decomposition of Bcl - xL [44]. So then Raf - 1, MAP2K1&2, ERK, Rsk1 and Bad protein levels were measured and our results showed that DGKI motivated their expressions, indicating that miR - 574 - 5p facilitating cell apoptisis via DGKI through PA - ERK - Bad pathway in GMECs. Inversely, DGKH gave decline to the phosphorylation of Raf - 1, MAP2K1&2, ERK, Rsk1 and Bad in GMECs, which suggested a mechanism of cell apoptisis owing to inhibiting DAG-ERK-Bad pathway. Our data uncovered the view that DGKI inhibited pro-apoptotic Bax and promoted anti-apoptotic Bcl-2 expressions, while DGKH exhibited the inverse results. Since we have identified Bcl-2 was motivated via ERK, accompanying with down-regulaion of Bax, here we showed an ERK-Bcl-2/Bax inhibited-apoptisis mechanism. IKB kinases (IKK α and IKK β) could catalyze the phosphorylation of IKBs and then trigger rapid ubiquitination and proteolysis of IKB [45]. After the degradation of IKBs, NFKB following translocates into the nucleus to motivate target genes [30]. NFKB is an omnipresent transcription element that induces genes related to anti - apoptotic responses. NFKB acts as an anti - apoptotic factor utilizing inducement of Bcl - 2 and Bcl - xL expression [46]. Western blot showed that DGKI and DGKH both blocked the activation of IKK α / β , IKB α / β and NFKB1, meanwhile, DGKH elevated Bax expression and diminished Bcl - 2 expression. That provided evidence that DGKH elevated cell apoptisis and arrested cell proliferation via functions on DAG - NFKB - Bcl - 2/Bax pathway destructively. However, although DGKI also inactivated IKK signaling, it showed anti - apoptotic impacts on GMECs. Thus we speculated that DGKI performed its main functions by induced PA, not of DAG blockage. On account of antecedent studies that ERK signaling could mediate mTOR signaling, and S6K1 is a vital element of the S6K/mTOR negative - feedback loop [47], we then looked into the feasibility that AKT motivation by ERK deficiency was regulated via S6K1 in GMECs. We used pharmacological or siRNA methods and found that ERK could promote mTOR signaling activation. ERK inhibition-induced AKT motivation and mTOR inhibition-enhanced ERK motivation was dependent on S6K1, implicating connections between mTOR and ERK pathways. Thus we speculated that DGKH depressed ERK signaling activation and accordingly triggered the blockage of AKT-mTOR pathway via inhibiting DAG expression.

Conclusions

In summary, we elucidated the function and the regulatory mechanism of miR-574-5p in GMECs. miR-574-5p blocked DGKI directly and DGKH indirectly, which were both down-regulated DEGs. miR-574-5p not only repressed DAG-PA-mTOR pathway and the expression of triglycerides and β -casein, but also inactivated DAG-PA-ERK pathway, which brought about depressive cell proliferation and stimulative cell

apoptosis via depletion of DGKI (Fig. 8). DGKH also inhibited DAG-mTOR pathway, and exhibited anti-proliferative and pro-apoptotic capacities via diminishing DAG-ERK and DAG-IKK-NFKB pathways in GMECs (Fig. 8). Simultaneously, the irritative roles of DGKH on triglycerides remained to be further understood. Farther comprehending of molecular techniques in terms of these characteristics will undertake effective cognition in majorization of milk qualities in dairy goats.

Abbreviations

miRNA: microRNA; DGKs: diacylglycerol kinases; DAG: diacylglycerol; PA: phosphatidic acid; TAG: triacylglycerol; PKCs: protein kinase C isoforms; RasGRPs: Ras guanyl nucleotide-releasing proteins; GMECs: goat mammary epithelial cells; nts: nucleotides; UTR: untranslated region; DEGs: differentially expressed unigenes; NC: negative control; inhNC: inhibitor negative control; FCM: flow cytometry method; PI: propidium iodide; ELISA: enzyme linked immunosorbent assay; PMSF: phenylmethanesulfonyl fluoride; PVDF: polyvinylidene difluoride

Declarations

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Author Contributions

YL designed the research. YL and MZ cultured cells, constructed plasmids and performed luciferase assays, ELISA, bioinformatics analyses, and co-wrote the manuscript. YL, DG and JW performed CCK8 assay, EdU staining, flow cytometry assay, and accompanying analysis, analyzed data. BC and XA were major contributors to data analysis and co-wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement

The Review Committee for the Use of Animal Subjects of Northwest A&F University, Yang Ling, China validated the animal use and care protocol. All surgeries were performed with a trial to minimize martyrdom to animals.

Author details

¹College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, *P.R.* China.

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Figures

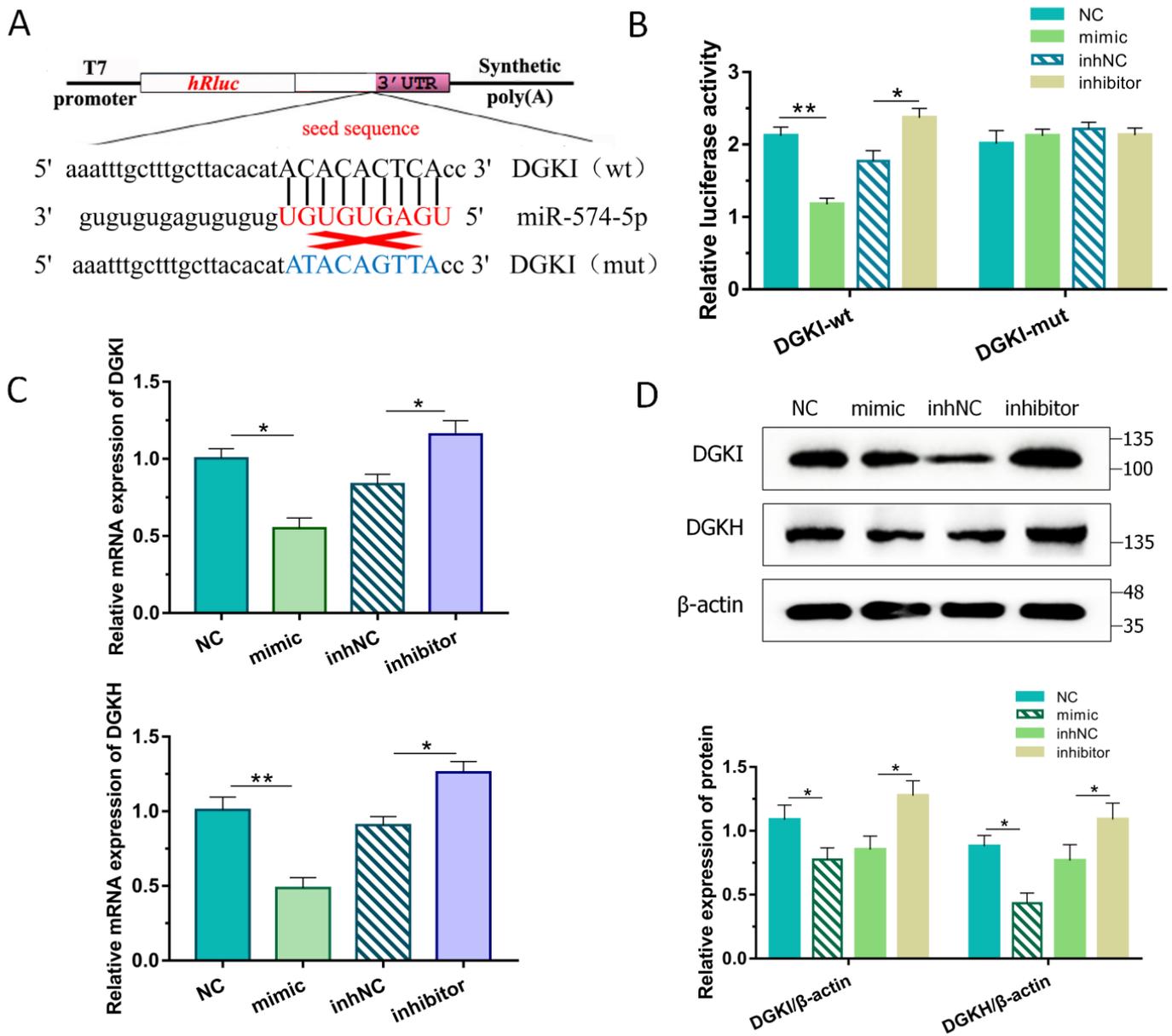


Figure 1

Figure 1

miR-574-5p impaired DGKI directly and DGKH indirectly in GMECs. (A) Binding sites for miR-574-5p in the DGKI 3'-UTR and the structure of the luciferase expression vector (Luc) melted with the DGKI 3'-UTR. wt delegates the wt DGKI 3'-UTR (11361 to 11369) Luc reporter vector; mut presents the mutated Luc reporter vector at the miR-574-5p target sites in DGKI 3'-UTR. (B) GMECs were transfected luciferase vectors with miR-574-5p mimics/NC or inhibitors/inhNC in 24 h and then the relative luciferase activities were examined. The relative mRNA (C) and protein (D) expression of DGKI and DGKH in GMECs transfected with miR-574-5p mimics or inhibitors for 24 h or 48 h was quantified by RT-qPCR and western blot, respectively. β -actin was regarded as internal control. * $p < 0.05$; ** $p < 0.01$.

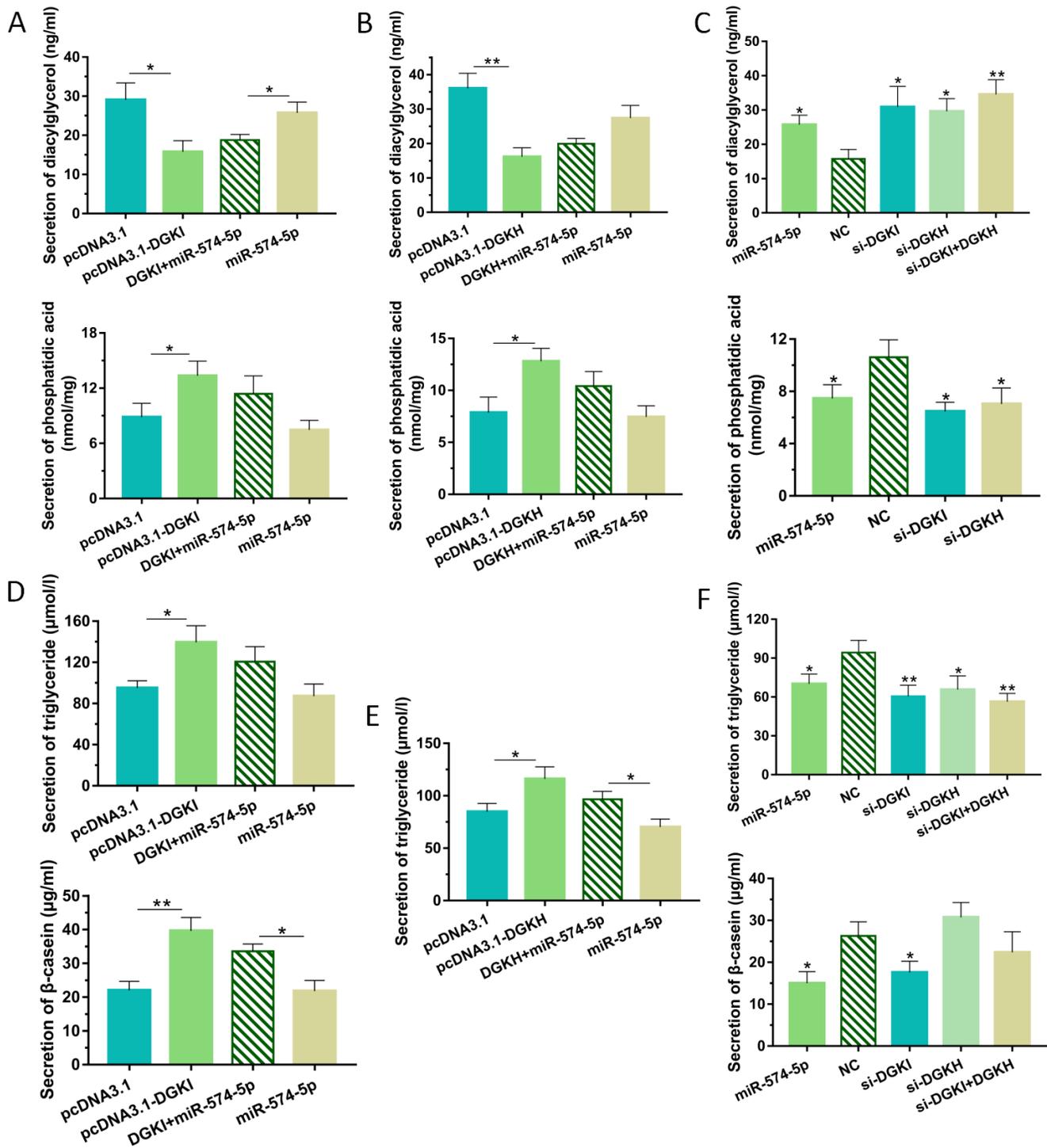


Figure 2

Figure 4

miR-574-5p regulated the milk synthesis via DGKI and DGKH in GMECs. GMECs were post-treated with miR-574-5p mimics, si-DGKI, si-DGKH, pc-DGKI, pc-DGKH expression vectors or co-transfected DGKI or DGKH with miR-574-5p mimics for 24 h. Secretion of diglyceride and phosphatidic acid in the cell-free supernatants in pcDNA3.1-DGKI (A) or pcDNA3.1-DGKH (B) plasmids or siRNAs of DGKI or/and DGKH (C) treated GMECs was estimated via ELISA kit. Secretion of triglyceride or β -casein in the cell lysate or cell-

free supernatants in pcDNA3.1-DGKI (D) or pcDNA3.1-DGKH (E) plasmids or siRNAs of DGKI or/and DGKH (F) treated GMECs was estimated by detection kits. * $p < 0.05$; ** $p < 0.01$.

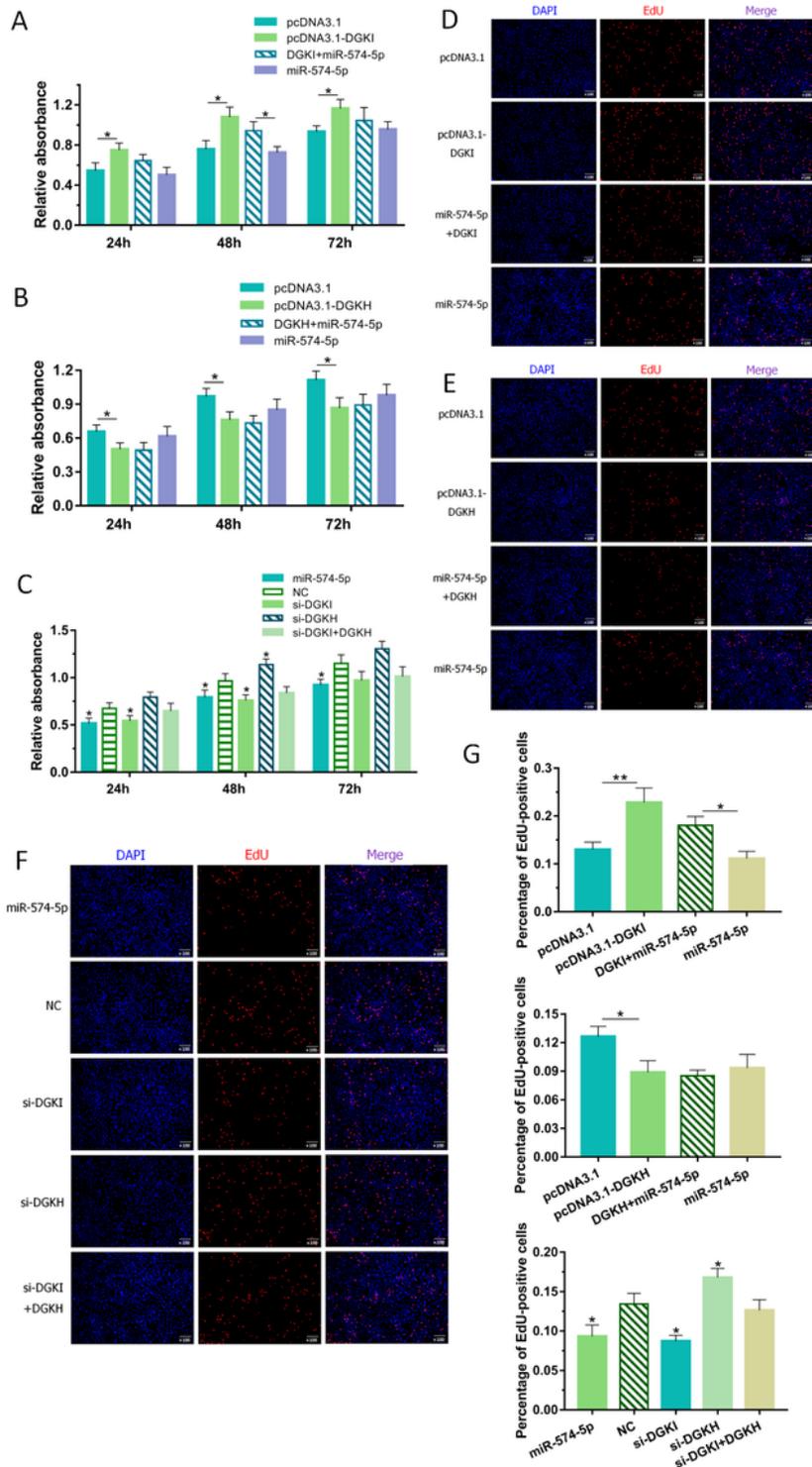


Figure 3

Figure 7

miR-574-5p depressed the proliferation of GMECs via DGKI. (A, B, C) Cell viability was measured via the CCK8 assay for 24 h, 48 h, and 72 h. GMECs were transfected with si-DGKI, si-DGKH, miR-574-5p mimics, pcDNA3.1-DGKI or pcDNA3.1-DGKH plasmids or co-transfected DGKI or DGKH with miR-574-5p. (D, E, F)

EdU staining assay was performed to detect the proliferation of GMECs. After 24 h transfection, GMECs in the S-phase were stained with EdU in red, while cell nuclei were dyed with DAPI in blue. (G) The histogram of percentage of EdU-positive cells. * $p < 0.05$; ** $p < 0.01$.

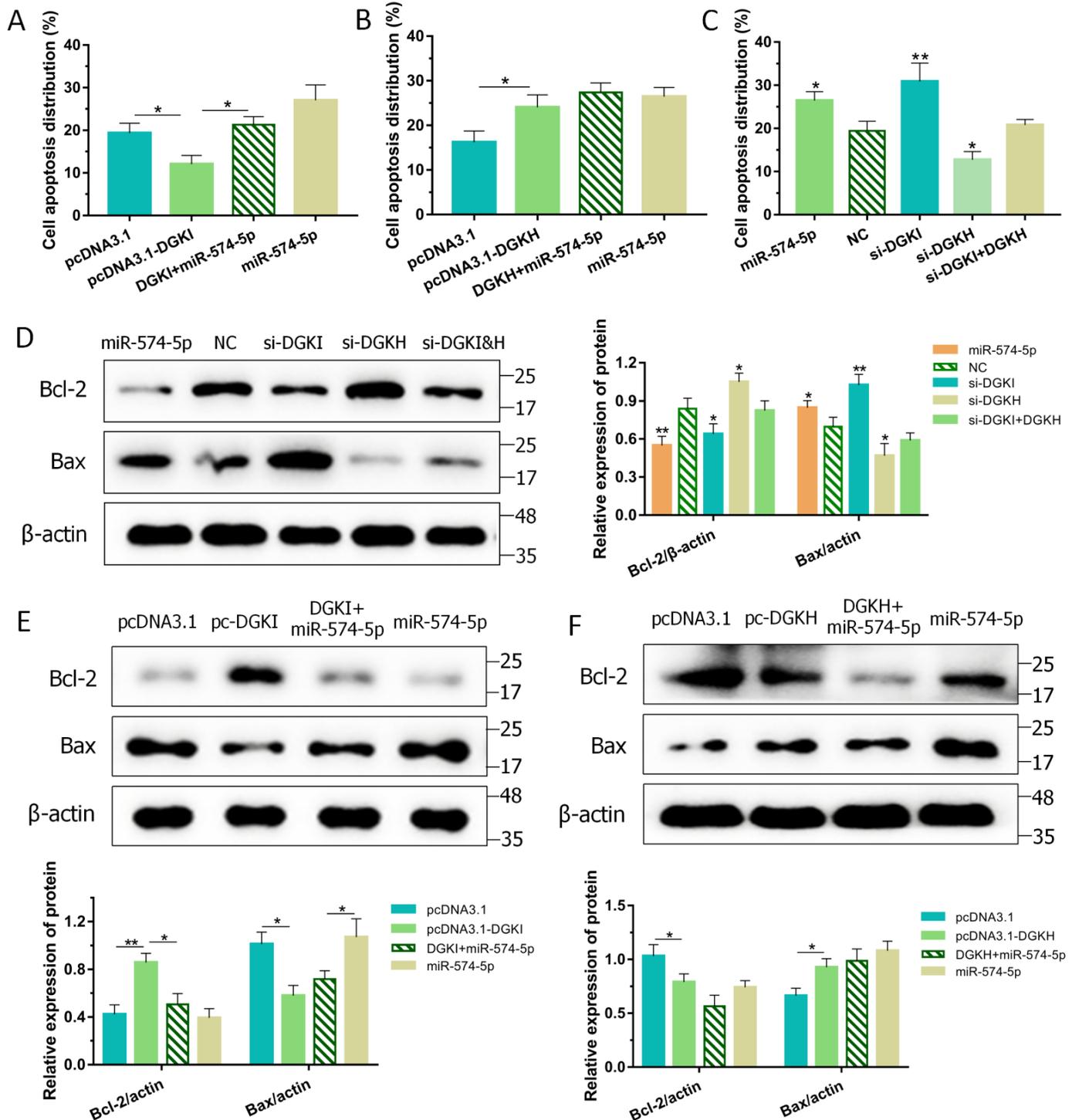


Figure 4

Figure 10

miR-574-5p improved the apoptosis of GMECs via DGKI. (A, B, C) The apoptosis of GMECs treated with si-DGKI, si-DGKH, miR-574-5p mimics, pcDNA3.1-DGKI or pcDNA3.1-DGKH plasmids or co-transfected DGKI

or DGKH with miR-574-5p for 24 h. The histogram exhibited the apoptotic cell percentage measured using FCM, and error bars denote mean \pm SD. After 48 h transfection, (D, E, F) western blot analysis was used to measure Bcl-2 and Bax protein expression in GMECs. β -actin was detected as a loading control. * $p < 0.05$; ** $p < 0.01$.

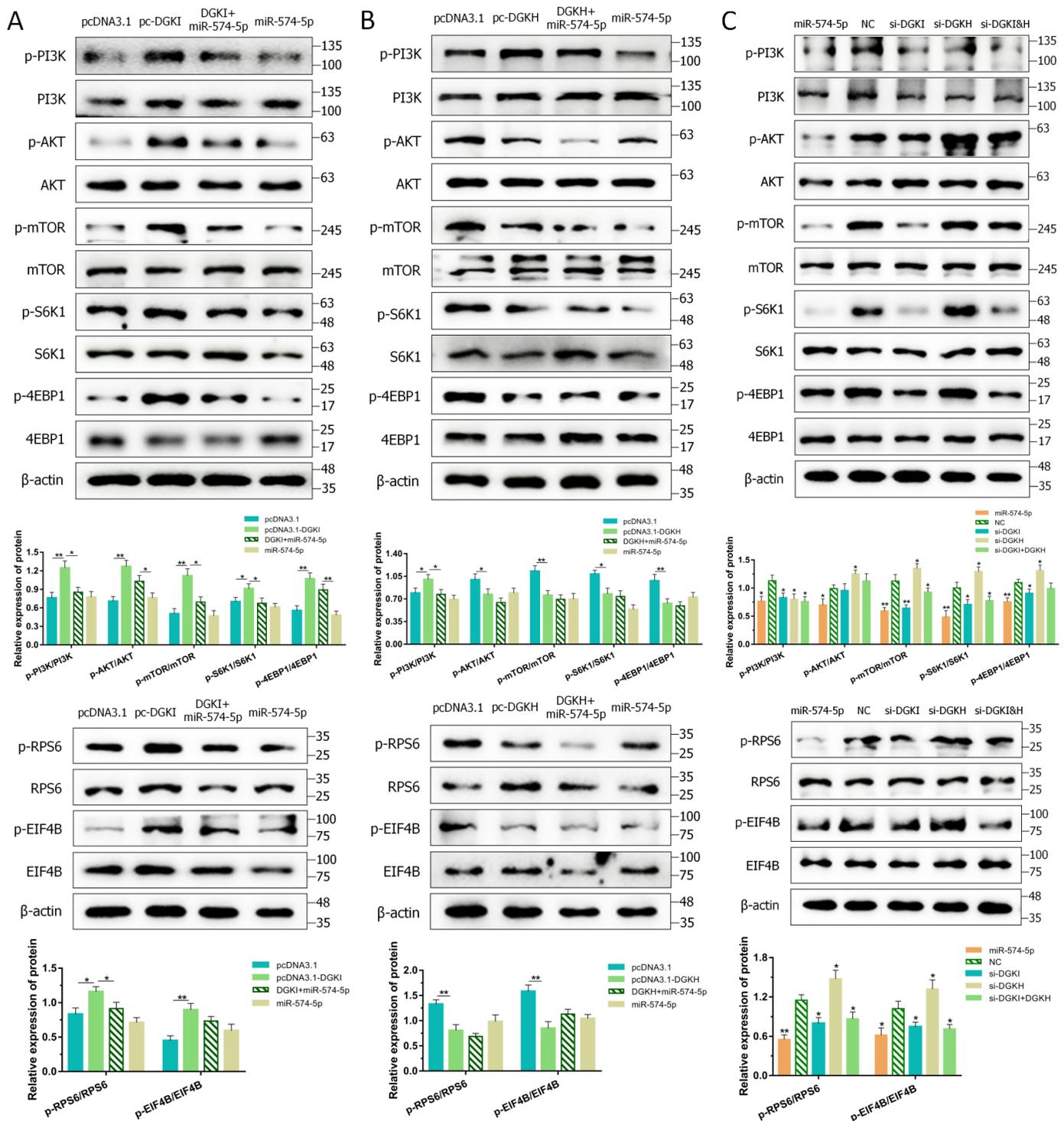


Figure 5

Figure 13

DGKI and DGKH effected AKT-mTOR signaling pathways in GMECs. GMECs were post-transfected with miR-574-5p mimics, (A) pcDNA3.1-DGKI plasmids or (B) pcDNA3.1-DGKH plasmids or (C) siRNAs of DGKI or/and DGKH for 48 h. Cytosolic proteins and related phosphorylation levels were analyzed by western blot for PI3K, AKT, mTOR, S6K1, RPS6, EIF4B and 4EBP1. β -actin was detected as a loading control. * $p < 0.05$; ** $p < 0.01$.

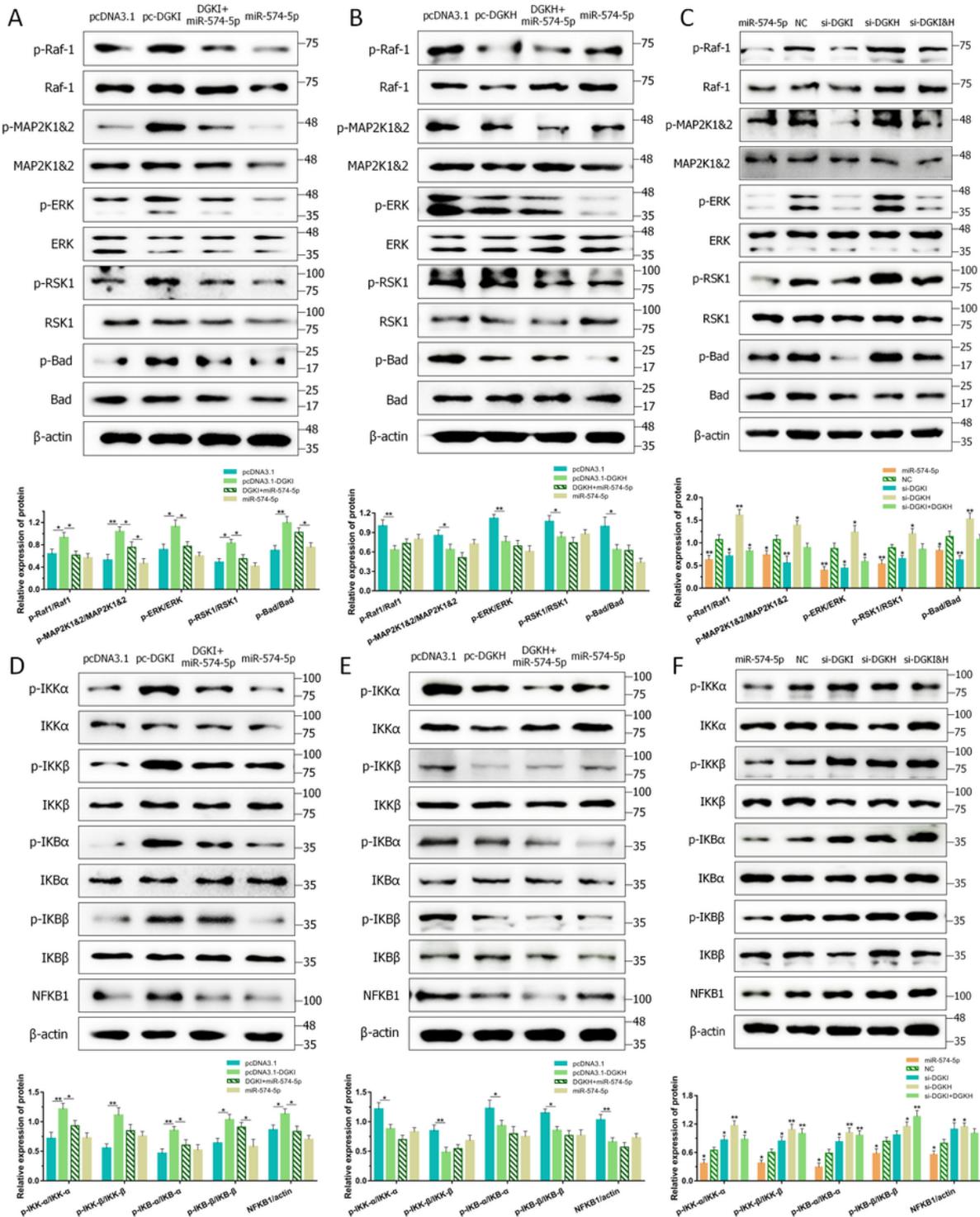


Figure 6

Figure 16

DGKI and DGKH effected ERK and NFKB signaling pathways in GMECs. GMECs were transfected with si-DGKI, si-DGKH, miR-574-5p mimics, pcDNA3.1-DGKI or pcDNA3.1-DGKH plasmids or co-transfected DGKI or DGKH with miR-574-5p for 48 h. (A, B, C) Cytosolic proteins were analyzed by western blot for Raf-1, MAP2K1&2, ERK, RSK1, Bad and related phosphorylation levels. Western blot detection of IKK α / β , p-IKK α / β , IKB α / β , p-IKB α / β and NFKB1 proteinlevels. β -actin was detected as a loading control. * $p < 0.05$; ** $p < 0.01$.

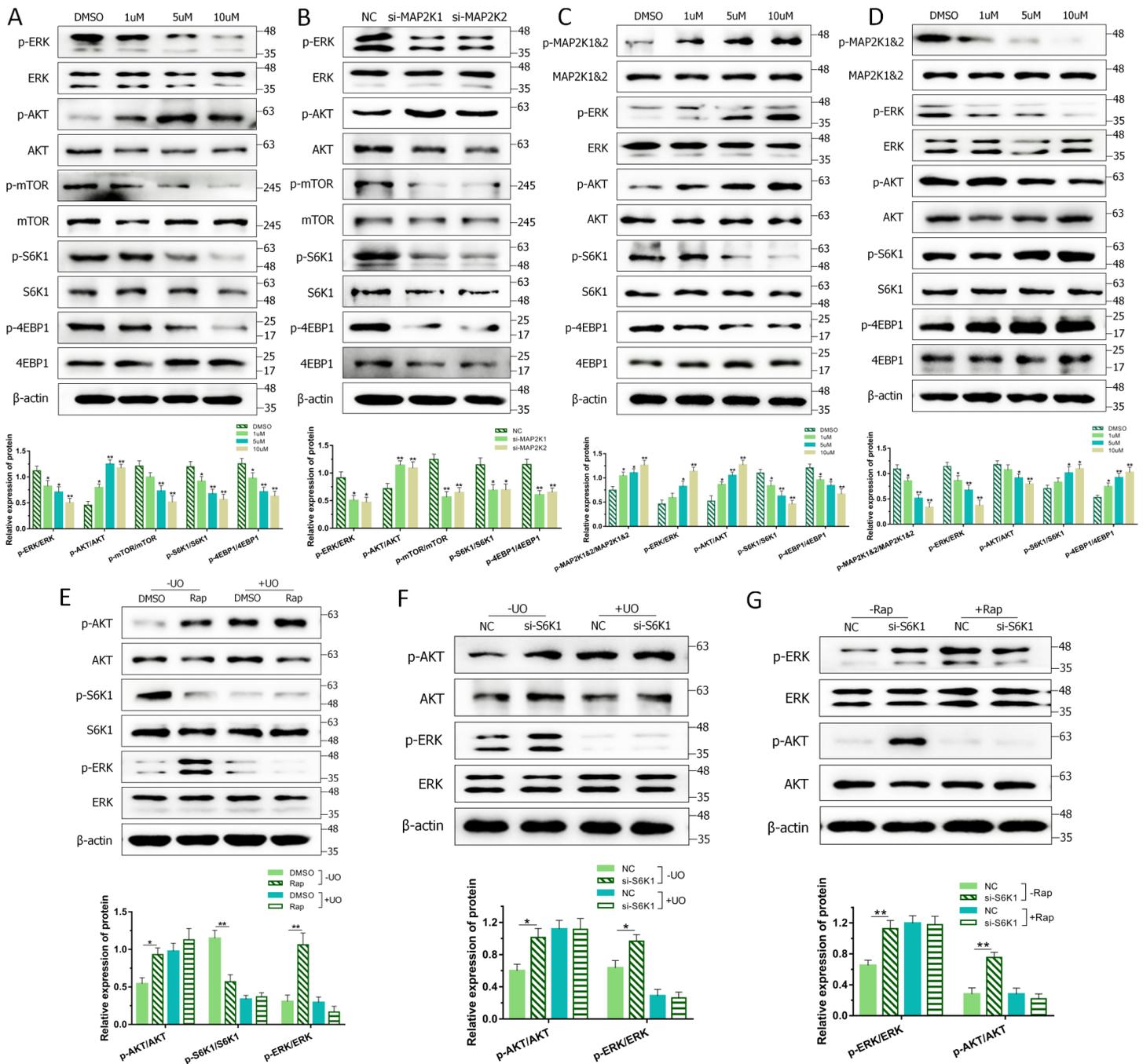


Figure 7

Figure 19

Crosstalk between mTOR and ERK pathways. GMECs were treated with (A) 1, 5 and 10 μ M UO126 (UO) in 24 h or (B) siRNAs of MAP2K1 or MAP2K2 in 48 h or (C) 1, 5 and 10 μ M rapamycin (Rap) in 24 h or (D) 1,

5 and 10 μM MHY1485 (MHY) in 24 h, then cell lysates were subjected to western blot analysis with the indicated antibodies. (E) GMECs were treated with 10 μM rapamycin for 24 h, then treated with 10 μM UO126 for 24 h. Western blot analysis was used to measure AKT, p-AKT, S6K1, p-S6K1, ERK and p-ERK protein expression. GMECs were transfected with si-S6K1. After 48 h transfection, cells were treated with (F) 10 μM UO126 or (G) 10 μM rapamycin for 24 h, then indicated protein levels were detected via western blot. β -actin was detected as a loading control. * $p < 0.05$; ** $p < 0.01$.

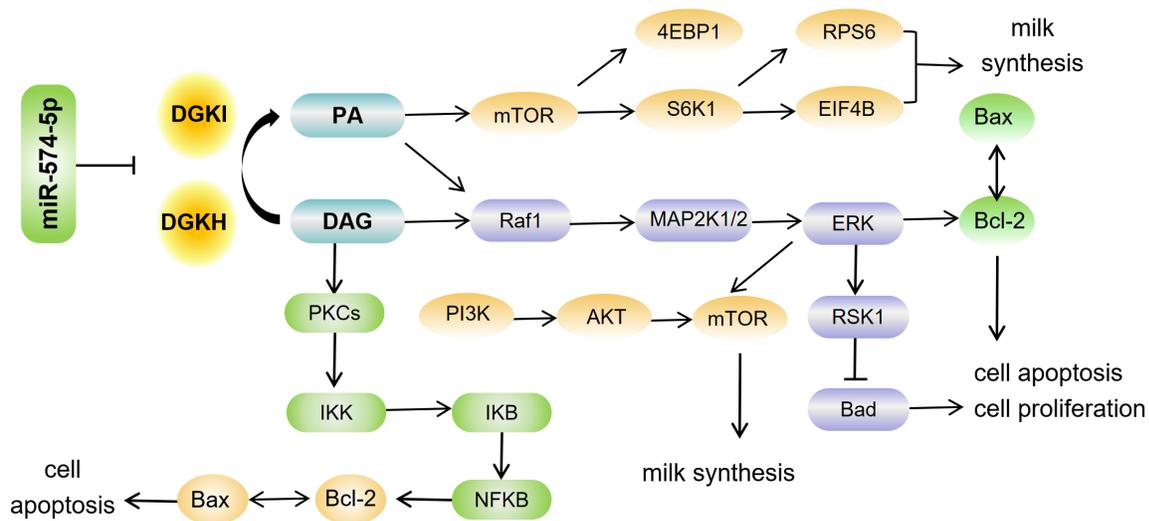


Figure 8

Figure 22

Proposed model linking the miR-574-5p locus to the biology of GMECs. miR-574-5p blocked DGKI directly and DGKH indirectly. DGKI and DGKH induced diglycerides conversion to phosphatidic acid and then mediate various signal pathways. DGKI not only activates DAG-PA-mTOR pathway resulting in triglycerides and β -casein production, but also induces DAG-PA-ERK pathway, which brought about stimulative cell proliferation and depressive cell apoptosis. DAG-IKK-NFKB pathway is arrested by DGKI, surprisingly. DGKH inhibits DAG-mTOR pathway, and exhibits anti-proliferative and pro-apoptotic capacities via depletion of DAG-ERK and DAG-IKK-NFKB pathways in GMECs.

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