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Genistein Directly Represses the Phosphorylation of STAT5 in Lactating Mammary Epithelial Cells

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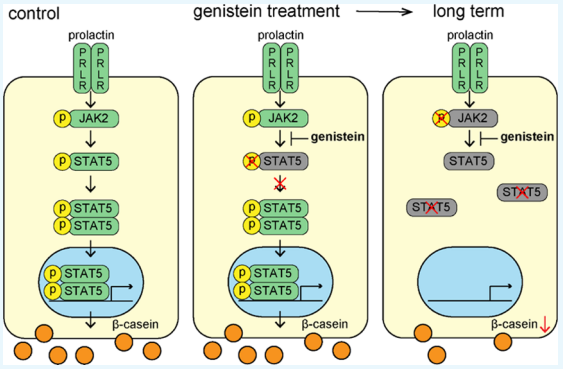
ABSTRACT: Genistein is a soy isoflavone and shows various physiological activities, such as affinities for estrogen receptors (ERs) and inhibitory effects on the epidermal growth factor receptor (EGFR) pathway. A previous study reported that genistein downregulates milk production ability in mammary epithelial cells (MECs) while decreasing the phosphorylation of STAT5. The ER and EGFR pathways indirectly regulate STAT5. In this study, the repressing mechanism of genistein against the phosphorylation of STAT5 was investigated using a culture model of mouse MECs with milk production ability. The results revealed that genistein did not influence the behavior of ERα and ERβ, whereas genistein immediately repressed the phosphorylation of ERK1/2. However, the decrease in phosphorylated STAT5 occurred independent of the phosphorylation of EGFR. Genistein repressed new phosphorylation of STAT5 by prolactin without influencing the phosphorylation of JAK2. In conclusion, this study indicates that genistein directly inhibits the phosphorylation of STAT5 in lactating MECs.



INTRODUCTION

Genistein is a soy isoflavone that participates in various physiological activities.[1,2] In previous studies, we reported that genistein inhibits production of β-casein, which is a representative milk component, in vitro and in vivo mammary epithelial cells (MECs).[3,4] The milk production ability of MECs during lactation is primarily regulated by the prolactin-JAK2-STAT5 pathway.[5−7] The tyrosine residue of STAT5 becomes phosphorylated by the phosphorylation of JAK2, and phosphorylated STAT5 translocases into nucleus when JAK2 is phosphorylated by the binding of prolactin to the prolactin receptor. Next, phosphorylated STAT5 in the nucleus regulates the transcription of target genes related to the milk production ability of MECs. In addition to the STAT5, the AKT-mTOR pathway by insulin stimulation and the glucocorticoid receptor (GR) also upregulate the milk production ability of MECs.[8−10] Therefore, various cellular pathways are involved in the milk production ability of MECs. However, how genistein inhibits the milk production ability of MECs remains unclear.

Genistein has affinities for estrogen receptors (ERs).[11] There are several types of ERs, such as ERα, ERβ, and G protein-coupled ER.[12] As a component of genomic action, translocation of ERs into the nucleus occurs when they bind to their ligands, such as estradiol.[13] ERs regulate the transcription of target genes. As part of non-genomic action, ERs rapidly regulate transcription through cellular pathways, such as AKT or ERK1/2.[14,15] Genistein also affects cell viability and proliferation via the alteration of AKT or ERK1/2 through ERs. For example, genistein inhibited cell proliferation through the ER pathway in a neonatal pituitary gland explant culture.[16] Genistein also repressed cell viability through a decrease in phosphorylation of AKT in lung and bile duct cancer cells.[17,18] Genistein inhibited cell proliferation by decreasing the phosphorylation of AKT and increasing the phosphorylation of ERK1/2, with a decrease in the mRNA expression of ERα in endometrial cancer cells.[19] In addition, genistein, like estradiol, repressed cell proliferation while decreasing the phosphorylation of AKT and ERK1/2 in breast cancer cells.[20] Therefore, genistein can influence the behavior of ER and its downstream cellular pathways in MECs.

Genistein inhibited cell proliferation by exerting an inhibitory effect on tyrosine kinase activity through stimulation of the epidermal growth factor (EGF) in colon cancer cells.[21] The EGF receptor (EGFR) activates MEK1/2-ERK1/2, p38, and JNK through tyrosine kinase activity when their ligands, such as EGF, bind to EGFR. Genistein also exerted an inhibitory effect on tyrosine kinase activity via EGF in epidermoid carcinoma.[22] In addition, genistein repressed rectifier K^+ current to the human atria through an inhibitory

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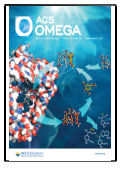

effect of tyrosine kinase activity in EGFR.[23,24] In MECs, genistein downregulated the expression of EGFR.[25,26] Therefore, genistein can influence the EGFR pathways in MECs.

In a previous study, we reported that genistein downregulated the milk production ability of MECs while decreasing the phosphorylation of STAT5.[3] STAT5 was phosphorylated by stimulation of EGF in trophoblastic cells,[27] and estradiol increased the amount of STAT5 in the mammary glands.[28] However, it was unclear whether genistein directly or indirectly represses the phosphorylation of STAT5 in MECs. In this study, we investigated the mechanism by which genistein affects STAT5, including the EGFR pathway and behavior of ERs.

RESULTS

Genistein-Downregulated β-casein Production and Phosphorylation of STAT5 in MECs. The effect of genistein in β-casein production and on JAK2-STAT5 pathway was determined based on the amount of secreted β-casein in the medium and the amounts of intracellular β-casein, pJAK2, JAK2, pSTAT5, and STAT5 in MECs treated with genistein at 5 μM or 25 μM for 24 h, which were assessed by western blot and densitometry analyses (Figure 1A). MECs treated with genistein at 25 μM decreased intracellularly and secreted β-casein to approximately one-third of the amount of β-casein in the control group. Genistein at 25 μM also decreased the amounts of pJAK2, pSTAT5, and STAT5 to 80, 40, and 80%, respectively, of those in the control group. In contrast, MECs treated with genistein at 5 μM increased the amount of STAT5 to 1.2-fold of that in the control group.

To investigate the influence of genistein on the cellular pathways that are related to the milk production ability of MECs, the cells were treated with genistein at 25 μM for 1, 3, or 6 h. Next, the amounts of pJAK2, JAK2, pSTAT5, STAT5, pGR, GR, pAKT, AKT, pmTOR, and mTOR were assessed by western blot and densitometry analyses (Figure 1B). After 6 h of genistein treatment, the amount of pSTAT5 decreased to less than 60% of that of the control group. In contrast, genistein did not influence the amounts of pJAK2, JAK2, STAT5, pGR, GR, pAKT, AKT, pmTOR, or mTOR within 6 h.

Therefore, these results indicate that genistein decreased the amount of phosphorylated STAT5 in lactating MECs within 6 h.

Genistein did not Influence the Behavior of ERα and ERβ in MECs. Genistein binds to ERs as a ligand.[11] To evaluate the effect of genistein on ERs, the amounts of pERα, ERα, and ERβ were investigated by western blot and densitometry analyses (Figure 2A). The results revealed that genistein did not influence the amounts of pERα, ERα, and ERβ for the first 6 h. In addition, the localization patterns of ERα and ERβ were investigated by immunostaining. In the control group, ERα localized in both the nucleus and the cytoplasm, whereas ERβ localized primarily in the nucleus (Figure 2B). The same localization patterns were observed in the genistein group. Genistein was observed to have no influence on the distribution of ERα and ERβ in the nucleus and the cytoplasm based on the results of western blot and densitometry analyses (Figure 2C).

Therefore, these results indicate that genistein did not influence the behavior of ERα and ERβ in lactating MECs within 6 h.

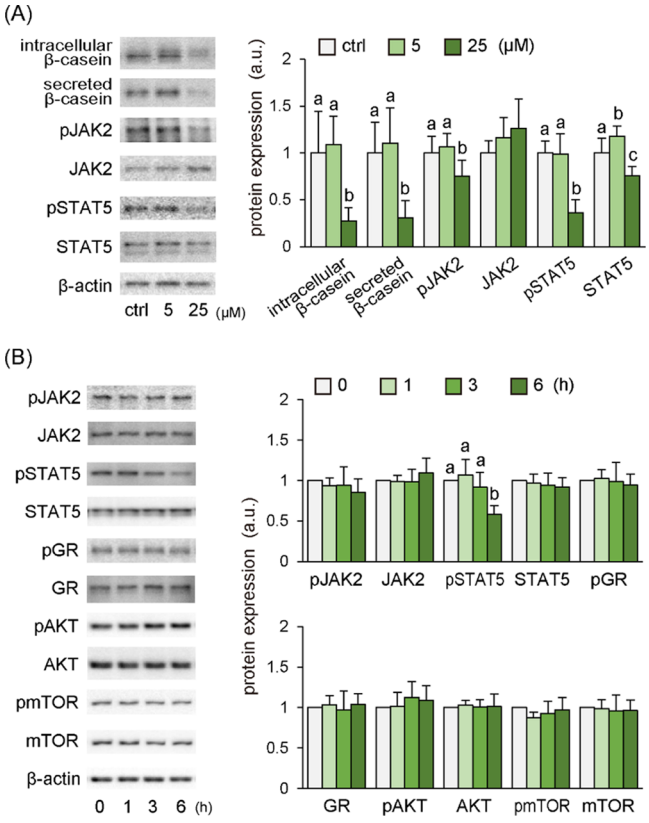

Figure 1. Genistein inhibits β-casein production and the JAK2-STAT5 pathway in MECs (A) MECs were treated with genistein at 5 μM or 25 μM for 24 h. Bands detected by western blot show β-casein in MECs (intracellular) and in the culture medium (secreted), and pSTAT5, STAT5, pJAK2, and JAK2 in the MECs. The graph shows the results of the densitometric analyses. (B) MECs were treated with genistein at 25 μM for 1−6 h. Bands detected by western blot reveal pSTAT5, STAT5, pJAK2, JAK2, pGR, GR, pAKT, AKT, pmTOR, and mTOR in the MECs. The graph shows the results of the densitometry analyses. β-actin was used as an internal control. Data are presented as the mean ± SD, and the expression levels are relative to the control (n = 8−12). Significance values were calculated using one-way analysis of variance followed by post-hoc Tukey's test. Different alphabetical letters (a−c) indicate significant differences (p < 0.05).

Genistein Influenced the EGFR-MEK1/2-ERK1/2 in MECs. Genistein inhibits the protein tyrosine kinase activity in the EGFR pathway.[22] The effect of genistein on the EGFR pathway was investigated by western blot and densitometry analyses (Figure 3). MECs treated with genistein for 6 h increased the amounts of pEGFR, pMEK1/2, and pERK1/2 to approximately 1.4-, 1.4-, and 1.2-fold compared with the amounts of those in the control group (Figure 3A). In contrast, genistein did not influence the amounts of EGFR, MEK1/2, ERK1/2, pp38, p38, pJNK, or JNK.

The 6 h genistein treatment increased the amount of pEGFR, pMEK1/2, and pERK1/2 in MECs; therefore, the influence of genistein treatment on the EGFR pathway was evaluated for a shorter time period (Figure 3B). The results showed that the amounts of pMEK1/2 and pERK1/2 in MECs treated with genistein for 5 min decreased to 70 and 40%, respectively, of those in the control group. In contrast, 60 min genistein treatment increased the amounts of pEGFR and pERK1/2 to more than 1.2-fold compared with those in the

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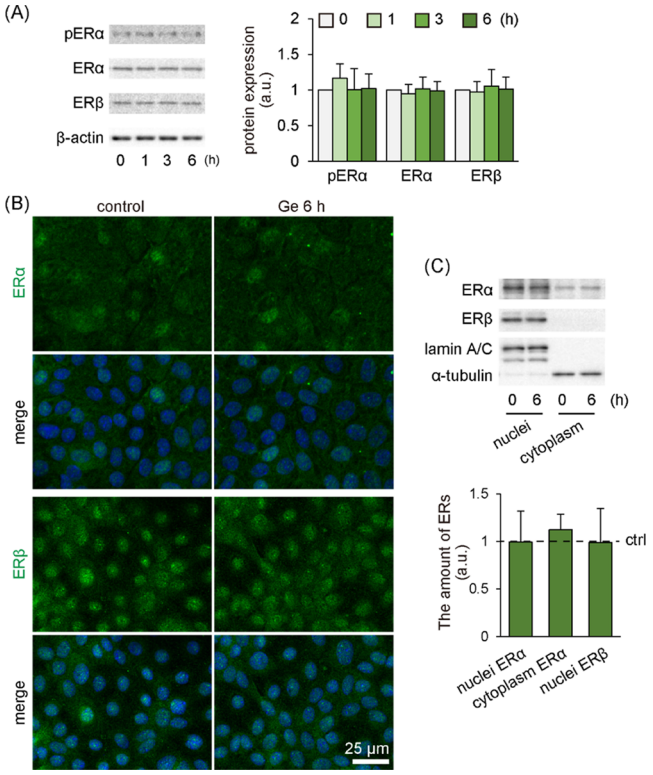

Figure 2. Influence of genistein on ERs in MECs. MECs were treated with genistein (Ge) at 25 μM for 1−6 h. (A) Bands detected by western blot reveal pERα, ERα, and ERβ in MECs. The graph shows the results of the densitometry analyses. β-actin was used as an internal control. (B) Localization of ERα and ERβ (green) with DAPI (blue; nuclei) in MECs. Scale bar shows 25 μm. (C) Bands detected by western blot reveal ERα and ERβ. The graph shows the results of the densitometry analyses. Lamin A/C and α-tubulin were used as internal controls. Data are presented as the mean ± SD, and the expression levels are relative to the control (n = 8−12). Significance values were calculated using one-way analysis of variance followed by post-hoc Tukey's test (A) or Student's t-test (C).

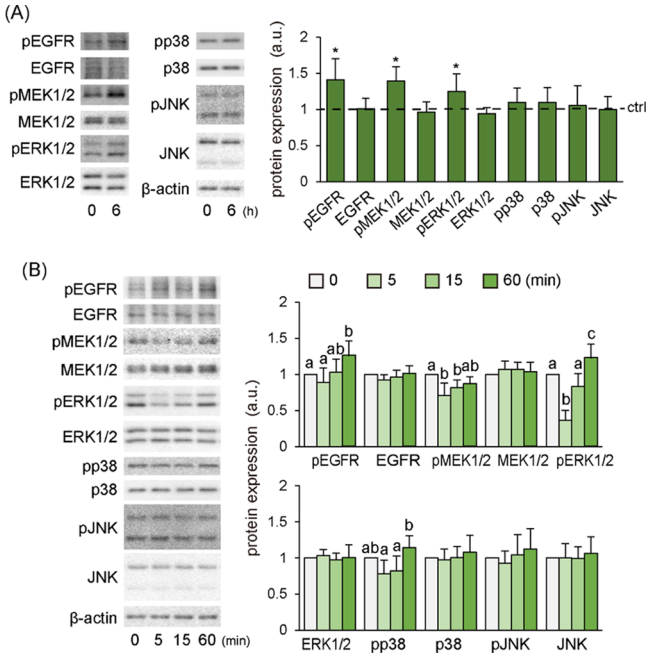

Figure 3. Influence of genistein on the EGFR pathway in MECs. (A) MECs were treated with genistein at 25 μM for 6 h. (B) MECs were treated with genistein at 25 μM for 5−60 min. Bands detected by western blot reveal pEGFR, EGFR, pMEK1/2, MEK1/2, pERK1/2, ERK1/2, pp38, p38, pJNK, and JNK. The graphs show the results of the densitometry analyses. β-actin was used as an internal control. Data are presented as the mean ± SD, and the expression levels are relative to the control (n = 8−12). Significance values were calculated using one-way analysis of variance followed by post-hoc Student's t-test (A) or Tukey's test (B). Asterisks and different alphabetical letters (a−c) indicate significant differences (p < 0.05).

control group. However, the amounts of p38, pJNK, and JNK were not affected by genistein within 60 min of treatment.

Therefore, these results indicate that genistein influenced the amount of phosphorylated ERK1/2, phosphorylated MEK1/2, and phosphorylated EGFR in lactating MECs within 60 min.

Decrease in the Phosphorylated ERK1/2 by Genistein did not Influence the Decrease in Phosphorylation of STAT5. Genistein drastically decreased pERK1/2 within 5 min and decreased pSTAT5 after 6 h. To investigate whether the effect of the EGFR-MEK1/2-ERK1/2 by genistein influenced the decrease in pSTAT5, the MECs with milk production ability were cultured in EGF-free differentiation medium for 2 days. Next, those MECs were cultured with an EGF-free differentiation medium containing 100 nM AG1478 (EGFR tyrosine kinase inhibitor) for 2 h prior to genistein treatment (Figure 4A). MECs cultured with the EGF-free differentiation medium containing 100 nM AG1478 were not detected in the pEGFR band, and they decreased the amounts of EGFR, pMEK1/2, MEK1/2, pERK1/2, and ERK1/2 to approximately 50, 60, 75, 45, and 75%, respectively, of those of the control group, which was cultured using the normal differentiation medium (Figure 4A′). In addition, MECs cultured in EGF-free medium decreased the amounts of pJAK2 and STAT5 to approximately 60 and 70%, respectively, of those in the control group. In the inhibition of the EGFR-MEK1/2-ERK1/2, MECs treated with genistein for 6 h decreased the amount of pSTAT5 to approximately 50% of that of the control group cultured with the EGF-free medium (Figure 4A″).

To confirm whether the inactivation of ERK1/2 by genistein for 5 min was related to the decrease in pSTAT5, MECs were cultured in a normal differentiation medium for 6 h after 5 min treatment with genistein (Figure 4B). MECs treated with genistein for 6 h decreased the amount of pSTAT5 to approximately 60% of that of the control group, whereas MECs treated with genistein for 5 min decreased the amount of pERK1/2 to approximately 20% of that of the control group. In contrast, MECs cultured in a normal differentiation medium for 6 h after treatment with genistein did not influence the amounts or phosphorylation of STAT5 and ERK1/2.

Therefore, these results indicate that genistein decreases the amount of phosphorylated STAT5 in lactating MECs independent of the decrease in phosphorylated ERK1/2.

Genistein Inhibited New Phosphorylation of STAT5 by Prolactin. To investigate whether genistein directly inhibited the JAK2-STAT5, MECs were pretreated with genistein for 6 h and then stimulated by prolactin to activate the JAK2-STAT5 pathway (Figure 5A). In the prolactin-free medium, the pSTAT5 band was not detected by western blot, regardless of genistein treatment, although the bands of pJAK2, JAK2, and STAT5 were detected. After 5 min stimulation by prolactin, the pSTAT5 band was detected by western blot, and the band was maintained for 60 min. The amounts of pJAK2, JAK2, pSTAT5, and STAT5 in the MECs that were stimulated

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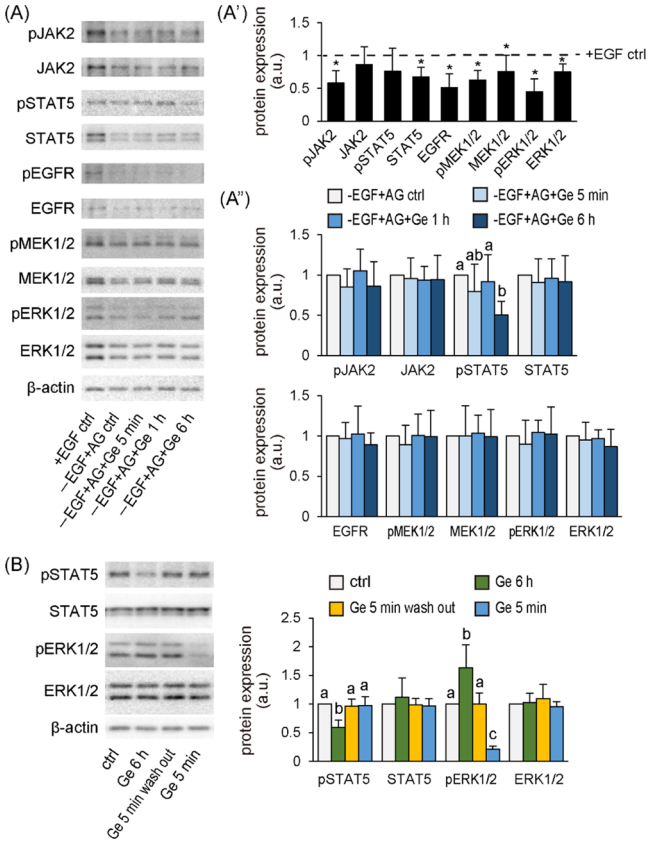

Figure 4. Phosphorylation of EGFR or the inactivation of ERK1/2 by genistein is not related to the decrease in phosphorylation of STAT5. (A) MECs with milk production ability were cultured in an EGF-free differentiation medium for 2 days. Next, these MECs were cultured with the EGF-free differentiation medium containing 100 nM AG1478 (AG; EGFR tyrosine kinase inhibitor) for 2 h before genistein (Ge) treatment. Bands detected by the western blot show pJAK2, JAK2, pSTAT5, STAT5, pEGFR, EGFR, pMEK1/2, MEK1/2, pERK1/2, and ERK1/2. (A′) Graph shows the results of the densitometry analyses comparing −EGF + AG ctrl with +EGF ctrl. (A″) Graphs show the results of the densitometry analyses comparing −EGF + AG ctrl with the Ge treatment. (B) MECs were treated with genistein for 5 min, or 6 h, or were cultured in a normal differentiation medium for 6 h after treatment with genistein for 5 min. Bands detected by western blot reveal pSTAT5, STAT5, pERK1/2, and ERK1/2. The graph shows the results of the densitometry analyses. β-actin was used as an internal control. Data are presented as the mean ± SD (n = 8−12). Significance values were calculated using one-way analysis of variance followed by post-hoc Student's t-test (A′) or Tukey's test (A″ and B). Asterisks and different alphabetical letters (a−c) indicate significant differences ($p < 0.05$).

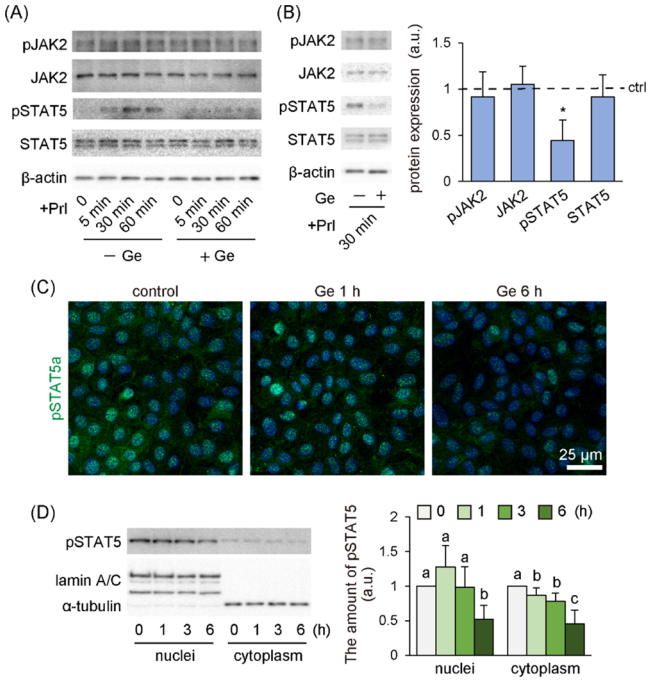

Figure 5. Genistein inhibits new phosphorylation of STAT5 by prolactin. (A,B) MECs were pretreated with genistein (Ge) for 6 h and then were stimulated by prolactin (Prl) for 5−60 min. Bands detected by western blot reveal pJAK2, JAK2, pSTAT5, and STAT5. The graph shows the results of the densitometry analyses. β-actin was used as an internal control. (C,D) MECs were treated with Ge for 1−6 h. (C) Localization of pSTAT5a (green) with DAPI (blue; nuclei) in MECs. The scale bar shows 25 μm. (D) Band detected by western blot reveals pSTAT5. The graph shows the results of the densitometry analyses. Lamin A/C and α-tubulin were used as internal controls. Data are presented as the mean ± SD (n = 8−12). Significance values were calculated using one-way analysis of variance followed by post-hoc Student's t-test (B) or Tukey's test (D). An asterisk and different alphabetical letters (a−c) indicate significant differences ($p < 0.05$).

by prolactin for 30 min were investigated by western blot and densitometry analyses (Figure 5B). MECs that were pretreated with genistein for 6 h exhibited less than half of the amount of pSTAT5 of MECs 30 min after prolactin treatment without genistein. In contrast, pretreatment with genistein did not influence the amount of pJAK2, JAK2, or STAT5 in MECs by prolactin stimulation.

The localization pattern of pSTAT5a in MECs was observed by immunostaining (Figure 5C). In the control group and the 1 h genistein treatment group, most nuclei of the MECs exhibited a positive reaction for pSTAT5a. In contrast, most nuclei of MECs treated with genistein for 6 h exhibited a negative reaction for pSTAT5a. The influence of genistein on the distribution of pSTAT5 in the nucleus and cytoplasm was investigated by western blot (Figure 5D). The amount of pSTAT5 in the nucleus of MECs treated genistein for 6 h decreased to approximately 50% of that of the control group. The amount of pSTAT5 in the cytoplasm gradually decreased due to the genistein treatment, with its amount after 6 h genistein treatment decreased to less than half of that of the control.

Therefore, these results indicate that genistein directly represses the phosphorylation of STAT5 in lactating MECs.

DISCUSSION

In this study, the mechanism of the role of genistein in decreasing the amount of phosphorylated STAT5 in MECs was investigated. The prolactin-JAK2-STAT5 pathway is the primary regulator of milk production in MECs during lactation, but also regulates the development of mammary alveolar structure during pregnancy.[29] In previous studies, we reported that genistein inhibits β-casein production in vitro and in vivo MECs.[3,4] In this study, lactating MECs treated with genistein for 24 h decreased intracellular and secreted β-casein while decreasing the phosphorylation of STAT5, as was observed in previous studies. The milk production ability of MECs is also upregulated by the AKT-mTOR pathway and GR.[5,6,8−10] In addition, genistein's influence on the develop-

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ment of mammary structure during pregnancy via the ER pathway has been demonstrated by some studies, and its influence on the EGFR pathway has been revealed by other studies.[20,21] The results of the current study revealed that genistein decreased the amount of phosphorylated STAT5 and influenced EGFR-MEK1/2-ERK1/2, although genistein did not influence the AKT, mTOR, and GR. In addition, genistein did not influence the behavior of ERs. Estrogen, which promotes the development of mammary structure during pregnancy binds to ERs and phosphorylates ERs to activate ERs.[13] The activation of ERs phosphorylates AKT or ERK1/2 to activate their pathways.[14,15] After parturition, estrogen in the blood diminishes and mammary glands initiate the milk secretion. However, the MECs express ERs during lactation and their ligands can influence the behavior of ERs.[30] In this study, genistein did not drastically influence the behavior of ERs in lactating MECs. Therefore, these results indicate that genistein decreases the amount of phosphorylated STAT5 without influencing the behavior of ERs.

Activation of the EGFR pathway influences the milk production ability of MECs.[8,31] The influence of genistein on the EGFR-MEK1/2-ERK1/2 correlated with the decrease in the amount of phosphorylated STAT5 was investigated. Genistein significantly decreased the phosphorylation of ERK1/2 in MECs after 5 min. This result indicates that genistein inhibits the EGFR-MEK1/2-ERK1/2 in lactating MECs, as was shown in previous studies.[22] However, genistein decreased the amount of phosphorylated STAT5 in the EGFR inactivated-MECs, which were cultured in EGF-free medium containing an EGFR inhibitor. Moreover, the temporary decrease in phosphorylated ERK1/2 by genistein did not influence the decrease in the amount of phosphorylated STAT5. Therefore, these results suggest that genistein decreases the amount of phosphorylated STAT5 independent of the phosphorylation of EGFR. In addition, both the EGFR inactivated-MECs and MECs treated with genistein for 24 h each decreased the amount of total STAT5. Therefore, inactivation of the EGFR-MEK1/2-ERK1/2 by genistein may be related to inactivation of the STAT5 pathway followed by a decrease in β-casein production in MECs.

Various protein kinases are involved in the milk production ability of MECs via prolactin stimulation.[32,33] To investigate whether genistein can directly inhibit the phosphorylation of STAT5, MECs were pretreated with genistein and then stimulated by prolactin to activate STAT5. The increase in the amount of phosphorylated STAT5 by prolactin stimulation was repressed by pretreatment of genistein. Moreover, the amount of pSTAT5 in cytoplasm decreased depending on the treatment time of genistein. Therefore, these results indicate that genistein inhibits new phosphorylation of STAT5 via prolactin in MECs. It has been reported that genistein inhibits the phosphorylation of STAT3, which is also downstream of JAK2.[34,35] Therefore, it is assumed that genistein directly inhibits the phosphorylation of STAT5 like STAT3. In this study, JAK2 in MECs was already phosphorylated before prolactin stimulation, which contradicts the findings of previous studies.[36,37] JAK2 is also phosphorylated by EGF.[38] In this study, MECs were cultured in a medium containing EGF and MECs with an inactivated EGFR that decreased the amount of phosphorylated JAK2. Consequently, JAK2 might have already been phosphorylated before prolactin stimulation. Therefore, these factors suggest that genistein inhibits new phosphorylation of STAT5 by prolactin stimulation without influencing the phosphorylation of JAK2.

Various hormones and its downstream cellular pathways are involved in the development of the mammary gland structure during pregnancy and milk production in MECs during lactation.[39,40] The cellular pathways in MECs are complicated as a cellular pathway is regulated by multiple factors. For example, the JAK2-STAT5 is influenced by not only prolactin but also EGF−EGFR or estrogen−ERs.[41] In addition, the effects of hormone on its downstream cellular pathway are altered by the presence of other hormones or treatment term.[31,42,43] In this study, the amount of pERK1/2 was increased by genistein after 60 min, although the amount of pERK1/2 was diminished by genistein after 5 min. Moreover, our previous study revealed that genistein has conflicting different effects on milk production ability in MECs with treatment concentration or term.[4] In this study, genistein at 5 μM increased the amount of STAT5 after 24 h treatment, although genistein at 25 μM decreased that. Therefore, a further study is necessary to reveal the interaction with cellular pathways in lactating MECs. In conclusion, this study provides evidence that genistein directly inhibits phosphorylation of STAT5 in lactating MECs. This study helps guide future development of therapeutic targets for excess milk production, such as hyperlactation.

MATERIALS AND METHODS

Animals. Virgin female ICR mice were purchased from Sankyo Labo Service Corporation (Shizuoka, Japan). The mice were housed under a 12 h light−dark interval at 24 °C. The mice (9−14 weeks) were decapitated, and their mammary glands were quickly dissected to isolate the MECs. All experiments were approved by the Animal Resource Committee of Hokkaido University (permission number: 18-0154) and were performed in according to the Hokkaido University guidelines for the Care and Use of Laboratory Animals.

Materials. The following primary antibodies were purchased for western blot. Rabbit polyclonal antibodies against AKT (Cell Signaling Technology, Danvers, MA, USA, 1:1200), phosphorylated AKT (pAKT; Ser473, Cell Signaling Technology, 1:1200), mTOR (Cell Signaling Technology, 1:1200), phosphorylated mTOR (pmTOR; Ser2448, Cell Signaling Technology, 1:1200), GR (Sigma-Aldrich, St. Louis, MO, USA, 1:1000), phosphorylated GR (pGR; Ser211, Cell Signaling Technology, 1:1000), JAK2 (Cell Signaling Technology, 1:750), phosphorylated JAK2 (pJAK2; Tyr1007/1008, Cell Signaling Technology, 1:750), STAT5 (Cell Signaling Technology, 1:1000), phosphorylated STAT5 (pSTAT5; Tyr694, Cell Signaling Technology, 1:1000), EGFR (Cell Signaling Technology, 1:1000), phosphorylated EGFR (pEGFR; Try1068, Cell Signaling Technology, 1:1000), p38 (Cell Signaling Technology, 1:1000), phosphorylated p38 (pp38; Thr180/Tyr182, Cell Signaling Technology, 1:1000), MEK1/2 (Cell Signaling Technology, 1:1000), phosphorylated MEK1/2 (pMEK1/2; Ser217/221, Cell Signaling Technology, 1:1000), ERK1/2 (Cell Signaling Technology, 1:1000), phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204, Cell Signaling Technology, 1:1000), JNK (Cell Signaling Technology, 1:1000), phosphorylated JNK (pJNK; Thr183/Tyr185, Cell Signaling Technology, 1:1000), ERα (Sigma-Aldrich, 1:500), phosphorylated ERα (pERα; Ser167, Sigma-Aldrich, 1:750), ERβ (Abcam, Cambridge, UK, 1:1000), mouse

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monoclonal antibodies against β-actin (Sigma-Aldrich, 1:20,000), lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000), α-tubulin (Sigma-Aldrich, 1:1000), and a goat polyclonal antibody against β-casein (Santa Cruz Biotechnology, 1:1000) were purchased and stored at −20 °C until use. The following primary antibodies were purchased for immunofluorescence staining. Rabbit polyclonal antibodies against pSTAT5a (Tyr694, Abcam, 1:300), ERα (1:200), and ERβ (1:400) and secondary horseradish peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse antibodies for western blot analyses (1:2500−5000) from Sigma-Aldrich. An Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Genistein and AG1478 (EGFR tyrosine kinase inhibitor) were purchased from ENZO Life Sciences, Inc. (Farmingdale, NY, USA).

Cell Culture. In this study, we prepared an MEC culture model following a previously established model, with some modifications.[3] Briefly, primary MECs were isolated using collagenase and trypsin. Cell selection was performed by centrifugation with fetal bovine serum. The isolated MECs were cultured on a 24-well cell culture plate (BD Biosciences, San Diego, CA, USA) in RPMI 1640 medium supplemented with 5% fetal bovine serum (Thermo Fisher Scientific), 5 μg/mL insulin (Wako, Osaka, Japan), and 10 ng/mL EGF (BD Biosciences) for six days. Next, the MECs were cultured in a differentiation medium containing 1% fetal bovine serum, 10 μg/mL insulin, 10 ng/mL EGF, 100 ng/mL prolactin (Protein Laboratories Rehovot Ltd., Rehovot, Israel), and 1 μM dexamethasone (Sigma-Aldrich) in RPMI 1640 medium for three days. Next, MECs were treated with 5 μM or 25 μM genistein in the differentiation medium.

Western Blot. Western blot was performed as previously reported, with some modifications.[3] Cultured MECs were lysed in a RIPA buffer (25 mM Tris-HCl [pH7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors). The lysates and conditioned medium were lysed in a Laemmli sodium dodecyl sulfate-solubilizing buffer and were then heated for 15 min at 70 °C. The samples (10−20 μg/lane) were separated on sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The immunosignals were detected using a Luminata-Forte Western HRP Substrate (Millipore, Billerica, MA, USA). Images of the bands were obtained using a ChemiDoc EQ densitometer (Bio-Rad). For quantification, the bands were analyzed using Quantity One software (Bio-Rad).

Immunofluorescence Staining. MECs were cultured on cover glasses and fixed in methanol for 10 min at −20 °C, followed by immersion in a mixture of 1% formaldehyde with phosphate-buffered saline (PBS) for 10 min at 4 °C. Next, the MECs were washed with PBS containing 0.05% Tween-20 (PBS-T). The MECs were immersed in PBS-T containing 5% bovine serum albumin (Sigma-Aldrich) for 1.5 h at room temperature. Next, the MECs were incubated overnight at 4 °C with primary antibodies diluted in PBS-T containing 2.5% bovine serum albumin. Next, the cells were washed in PBS-T and incubated with secondary antibodies diluted with PBS-T containing 2.5% bovine serum albumin for 1 h at room temperature. Images of the cells were obtained using a confocal laser-scanning microscope (TCS SP5) and processed with LAS AF software (Leica, Mannheim, Germany).

Fractionation of Cytoplasmic and Nuclear Proteins. MECs were lysed in a fractionation buffer (10 mM HEPES [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1% NP-40, and protease inhibitors). The lysate was centrifuged at 20,000g for 10 min at 4 °C. The supernatant was considered to be the cytoplasmic fraction. Next, the pellet was resuspended with a fractionation buffer and passed 30 times through a 20-gauge needle. The lysate was centrifuged at 500g for 10 min at 4 °C, and the pellet was considered to be the nuclear fraction.

Statistical Analysis. The data were expressed as the mean ± SD. Significance values were calculated using one-way analysis of variance followed by post-hoc Student's t-test (Figures 2C, 3A, 4A′, and 5B) or Tukey's test (other). Differences were considered to be significant at p-value < 0.05, which are indicated by asterisks or different alphabetical letters in the figures. All experiments were performed at least three times by using different MEC cultures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03107.

Representative membranes detected by western blot, bands showing intracellular or secreted β-casein at low magnification with other duplicate results (#1, #2, and #3), densitometry-analyzed bands using Quantity One software, relative value based on control normalized by β-actin, and other proteins detected and quantified as same (PDF)

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Notes

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■ ABBREVIATIONS

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptors; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEC, mammary epithelial cell; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription

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