Isoflavones and their metabolites influence the milk component synthesis ability of mammary epithelial cells through prolactin/ STAT5 signaling

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Abbreviations

EGF, epidermal growth factor; ER, Estrogen receptor; FABP3, fatty acid binding protein 3; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; JAK2, Janus kinase 2; MEC, mammary epithelial cell; PBS-T, PBS containing 0.05% Tween-20; PRLR, prolactin receptor; RT, reverse transcription; SREBP1, sterol regulatory element binding protein 1; STAT5, signal transducer and activator of transcription 5; UGP2, UDP-glucose pyrophosphorylase 2; WAP, whey acidic protein

Keywords

Mammary epithelial cell, isoflavone, β-casein, prolactin receptor, STAT5

Abstract

Scope: Isoflavones are a class of polyphonic compounds present in legumes and are called phytoestrogens because of their estrogen-like activity. Estrogen influences the behavior of mammary epithelial cells (MECs) during pregnancy and lactation. In this study, we investigated the direct influences of isoflavones and their metabolites in milk production ability of MECs.

Methods and results: Mouse MECs were cultured with prolactin and dexamethasone (glucocorticoid analog) to induce milk production ability. Subsequently, lactating MECs were treated with each isoflavone. Coumestrol, biochanin A, genistein and formononetin decreased the intracellular and secreted β-casein. On the other hand, p-ethylphenol, daidzein and equol did not significantly influence β-casein production at any concentration. Coumestrol, biochanin A and genistein down-regulated the mRNA expression of whey acidic protein (WAP), lactoferrin and α-lactalbumin. In contrast, p-ethylphenol, daidzein and equol up-regulated β-casein and/or WAP with α-lactalbumin. Furthermore, coumestrol and genistein down-regulated the expression of prolactin receptor and signal transducer and activator of transcription 5 (STAT5) accompanied by a decrease in STAT5 phosphorylation.

Conclusions: Isoflavones and their metabolites influence the milk production ability of MECs through different interactions with prolactin/STAT5 signaling. Simultaneous intake of multiple isoflavones by consumption of legumes may induce promotive or adverse effects on lactating MECs.

1 Introduction

Isoflavones are a class of polyphonic compounds present in legumes, which show a diversity of isoflavone composition between species. For example, soybean contains abundant genistein and daidzein, whereas chickpea contains biochanin A. Alfalfa mainly contains coumestrol [1-5]. Isoflavones are absorbed into the body as aglycon-type isoflavones and metabolites after metabolic conversion by enteric bacteria [6]. There are two primary isoflavone metabolic pathways. One is associated with formononetin, daidzein, equol and the other with biochanin A, genistein, and p-ethylphenol [7, 8]. These isoflavones and metabolites each have various physiological activities such as anti-oxidative action or anti-inflammatory effects [9, 10]. Further, isoflavones are also called phytoestrogens because they are structurally similar to estradiol, and they can bind to estrogen receptors and affect estrogen signaling.

Several hormones and growth factors regulate the structural development of mammary epithelium during the process of gestation, and estradiol is known as a key player in the development of the mammary epithelium [11, 12]. In addition, Estradiol regulates MECs by bind to estrogen receptors (ERs) and MECs maintains the expression of ERs during lactation [13, 14]. Estradiol functionally inhibits milk secretion by mammary epithelial cells (MECs) by interfering with prolactin signaling during late pregnancy [15]. Some isoflavones are also known to regulate the structural development of mammary glands during pregnancy [16-18]. Isoflavones bind to ERs and also have anti-inflammatory activities [10, 19]. Inflammation induced by infection and obesity downregulates milk production ability of MECs in lactating mice [20, 21]. However, it remains unclear whether isoflavones and their metabolites are functionally involved in milk production ability in MECs during lactation.

During lactation, MECs produce various milk components such as proteins, lactose and triglyceride. Milk contains several milk-specific proteins, including caseins, whey acidic protein (WAP) and lactoferrin. Caseins are composed of several subtypes containing α-, β- and κ-casein. MECs secrete caseins as micelles into the mammary alveolar lumen through exocytosis [22]. Lactose is a milk-specific disaccharide consisting of glucose and UDP-galactose. Glucose transporters, GLUT-1 or GLUT-12, absorb glucose in MEC and glucose is converted into UDP-galactose by several enzymes, containing UDP-glucose pyrophosphorylase 2 (UGP-2) and phosphoglucomutase 2 [23-26]. In the presence of galactosyltransferase and α-lactalbumin, MECs synthesize lactose from glucose and UDP-galactose. Triglyceride is composed of glycerol and fatty acids and is either made by de novo synthesis in MECs or absorbed from the blood through fatty acid transporters such as SLC27A3 [27]. Triglyceride synthesis in MECs is facilitated by sterol regulatory element binding protein 1 (SREBP1) and fatty acid binding protein 3 (FABP3) [28, 29].

Prolactin/Signal Transducer and Activator of Transcription 5 (STAT5) signaling plays a central

role in milk production ability in MECs [30, 31]. Prolactin binds to the prolactin receptor in MECs, and STAT5 is then phosphorylated through Janus Activating Kinase 2 (JAK2). Phosphorylated STAT5 forms dimers that translocate into the nuclei to regulate transcription of genes related to milk production. In lactating mammary glands, prolactin/STAT5 signaling is maintained in an active state for milk production. In contrast, prolactin/STAT5 signaling is inactivated along with the reduction of milk production after weaning or in mastitis [32, 33].

Estrogen upregulates the expression of STAT5 and prolactin receptors (PRLRs) in mammary epithelium [34, 35]. The milk production status of alveolar MECs is maintained via prolactin/STAT5 signaling, and the inactivation of the pathway by inflammation shuts down the milk production [32, 36, 37]. These reports indicate that estrogen influences in milk production ability of MECs through prolactin/STAT5 signaling. The isoflavones have estrogenic activity with other various physiological activities. Moreover, blood levels of isoflavones are increased by eating legumes [9, 10, 38]. However, it remains unclear whether isoflavones and their metabolites influence milk production ability in MECs. This study aims to reveal the direct influence of each isoflavone on milk production ability in MECs by using a previously reported cell culture model [39].

2 Materials and Methods

2.1 Animal

Virgin female ICR mice were purchased from Sankyo Labo Service Corporation (Shizuoka, Japan) and were housed under a 12-hour light-dark interval at 24°C. MECs were isolated from the fourth mammary gland of virgin mice (9-14 weeks). All experiments were approved by the Animal Resource Committee of Hokkaido University (permission number: 14-0005) and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

2.2 Materials

The following antibodies were used as primary antibodies for immunological studies: rabbit polyclonal antibodies against STAT5 (Cell Signaling Technology, Danvers, MA), phosphorylated-STAT5 (pSTAT5, Tyr694, Cell Signaling Technology), and GM130 (Abcam, Cambridge, UK); mouse monoclonal antibody against β-actin (Sigma-Aldrich, St. Louis, MO); and goat polyclonal antibody against β-casein (Santa Cruz Biotechnology, Santa Cruz, CA). Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated donkey anti-goat, Alexa Fluor 546-conjugated donkey anti-rabbit secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA). The secondary horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat antibodies for western blotting analysis were purchased from Sigma-Aldrich.

Formononetin and p-ethylphenol were purchased from Sigma-Aldrich. Coumestrol and genistein were purchased from ENZO Life Sciences, Inc. (Farmingdale, NY). Biochanin A and daidzein were purchased from LKT Laboratories, Inc. (St. Paul, MN). Equol was purchased from Santa Cruz Biotechnology.

2.3 Cell culture

In this study, we were used a previously reported cell culture model [39]. After washing mammary glands isolated from virgin mice with PBS, lymph node and connective tissue were removed from the mammary glands. The mammary glands were then minced and incubated in RPMI 1640 medium (Sigma-Aldrich) containing 0.75 mg/ml collagenase (Wako, Osaka, Japan) for 2 hours at 37°C while shaking horizontally. After centrifugation (645 *g*, 1 min), the pellet was resuspended with RPMI 1640 medium containing 0.2% trypsin (Thermo Fisher Scientific) for 5 min at room temperature. After centrifugation (645 *g*, 1 min), the pellet was resuspended with 60% fetal bovine serum (FBS; Thermo Fisher Scientific) in RPMI 1640 medium and then centrifuged (3 *g*, 5 min). Trypsin treatment and centrifugation with FBS were repeated for isolation of MECs without unnecessary cells such as fibroblasts and myoepithelial cells.

MECs were cultured in RPMI 1640 medium supplemented with 5% FBS, 5 μg/ml insulin (Sigma-Aldrich) and 10 ng/ml epidermal growth factor (EGF, BD Biosciences, San Diego, CA) for 6 days. Subsequently, MECs were cultured with differentiation medium containing 1% FBS, 10 μg/ml insulin, 10 ng/ml EGF, 0.5 U/ml prolactin from sheep pituitary (Sigma-Aldrich) and 1 μM dexamethasone (Sigma-Aldrich) in the standard RPMI 1640 medium for 3 days. MECs were then treated with isoflavones for 2 days in RPMI 1640 medium containing 1% FBS, 10 μg/ml insulin, 10 ng/ml EGF, 0.5 U/ml prolactin and 1 nM dexamethasone. Control samples were cultured in the same manner without isoflavone treatment. For immunostaining, the cells were cultured on cover glasses coated with 0.1 mg/ml collagen (Cellmatrix type 1A; Nitta Gelatin, Osaka, Japan).

2.4 Immunostaining

MECs cultured on cover glasses were fixed in methanol for 10 min at -20°C and then in 4% formaldehyde in PBS for 10 min at 4°C. After being washed with PBS containing 0.05% Tween-20 (PBS-T), MECs were immersed in PBS-T containing 5% BSA (Sigma-Aldrich) for 1.5 hours at room temperature and incubated overnight at 4°C with primary antibodies diluted in PBS-T containing 2.5% BSA. The cells were then washed in PBS-T and incubated with secondary antibodies diluted with PBS-T containing 2.5% BSA for 1 hour at room temperature. Images of the cells were obtained using a confocal laser-scanning microscope (TCS SP5) and LAS AF software (Leica, Mannheim, Germany).

2.5 Western blotting

MECs and the conditioned medium were lysed in SDS-solubilizing buffer and then heated for 15 min at 70°C. The samples were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were immersed in the blocking solution (PBS-T containing 4% skim milk) for 1.5 hours at room temperature. In the case of β-casein, the blocking solution was PBS-T containing 2% fish gelatin (Sigma-Aldrich) and 0.5% BSA. After blocking, the membranes were incubated overnight at 4°C with primary antibodies diluted in PBS-T containing 2.5% BSA. The membranes were then washed in PBS-T and reacted for 45 min at room temperature with secondary antibodies coupled to horseradish peroxidase in the blocking solution. Immunosignals were detected using Luminate Forte Western HRP Substrate (Millipore, Billerica, MA). The images of the protein bands were obtained using a ChemiDoc™ EQ densitometer and analyzed using Quantity One software (Bio-Rad).

2.6 Quantitative PCR

Total RNA from cells was extracted using ISOGEN (Wako). Reverse transcription (RT) was performed using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). The quantitative PCR was conducted on a Light Cycler 480 (Roche Applied Science, Indianapolis, IN) with Thunderbird® SYBR® qPCR Mix (Toyobo). The cycling conditions were 95°C for 1 min followed by 40 cycles at 95°C for 15 sec and 58°C for 1 min. The primers are shown in Supporting Information Table 1. *Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* was used as an internal control.

2.7 Statistical analysis

The data are expressed as means ±SD. Significance values were calculated using Student's t-test (Fig. 2, 3) or using one-way ANOVA followed by a Dunnett's test (Fig. 1, 4). Differences were considered significant at p-values <0.05, indicated by asterisks. All experiments were performed on at least four different samples.

3 Results

3.1 Isoflavones differently influence in β-casein expression and secretion

The milk production ability of MECs was induced by treatment with prolactin and dexamethasone as described previously [39, 40]. MECs were then treated with isoflavones (coumestrol, biochanin A, genistein, p-ethylphenol, formononetin, daidzein and equol) at 0.1, 1.56 and 25 μM for 2 days. The amounts of intracellular and secreted β-casein in MECs treated with isoflavones were

detected by western blotting, and the relative amounts of β-casein were analyzed by densitometry (Fig. 1). Treatment coumestrol, biochanin A, genistein or formononetin at 25 μM decreased the levels of intracellular and secreted β-casein. Formononetin at 1.56 μM produced a decrease in secreted β-casein. On the other hand, no tested concentrations of daidzein, equol and p-ethylphenol significantly influenced the β-casein production. The intracellular localization patterns of β-casein in MECs treated with isoflavones at 25μM were observed by immunostaining (Supporting Information Fig. 1). In the controls without isoflavone treatment, most MECs were positive for β-casein and showed condensed localization of β-casein at the Golgi body, as indicated by the localization of GM130, which is a Golgi marker. Similar localization patterns were observed in MECs treated with p-ethylphenol, formononetin, daidzein or equol. Many of the cells treated with coumestrol, biochanin A or genistein were negative for β-casein. Moreover, coumestrol and genistein caused abnormal accumulations of β-casein in some MECs. These results show that different isoflavones produced the distinctly different effects on intracellular and secreted β-casein.

3.2 Isoflavones have different effects on the ability to produce milk components

To evaluate the influence of isoflavones on the synthesis of several milk components, mRNA expression of milk proteins (*Csn2, Wap, Ltf*), lactose synthesis-related genes (*Glut1, Ugp2, Lalba*) and triglyceride synthesis-related genes (*Slc27a3, Fabp3, Srebp1*) were investigated via quantitative PCR.

Regarding the mRNA expression of milk proteins, MECs treated with coumestrol showed decreases to less than 3% of control cells in the levels of *Csn2, Wap* and *Ltf* (Fig. 2A). Treatment with biochanin A decreased the mRNA expression of *Csn2* and *Ltf*, and genistein treatment downregulated *Wap* and *Ltf*. Treatment with either biochanin A or genistein reduced *Ltf* expression to less than half of the level in control cells. Treatment with p-ethylphenol, formononetin, daidzein or equol increased *Wap* expression to 1.8-3.0 times that of control cells; moreover, treatment with daidzein or equol increased *Csn2* expression over 2.5 times that of control. Isoflavones were divided into two types: those with promotive effects (p-ethylphenol, formononetin, daidzein and equol) and those with inhibitory effects (coumestrol, biochanin A and genistein).

Regarding lactose synthesis-related genes, compared with control cells, MECs treated with coumestrol showed a decrease in *Lalba* expression to approximately 10% of control cells and a significant decrease in *Ugp2* expression (Fig. 2B). Treatment with biochanin A or genistein also decreased the expression of *Lalba* by almost 50%. In contrast, treatment with p-ethylphenol, daidzein or equol increased the expression of *Lalba*. Moreover, daidzein and equol significantly increased expression of *Glut1*. Formononetin had no significant effects on the expression of lactose synthesis-related genes.

Regarding triglyceride synthesis-related genes, expression of *Slc27a3* was increased by treatment with both coumestrol and genistein (Fig. 2C). *Fabp3* was increased by coumestrol treatment but decreased by genistein treatment. On the other hand, *Srebp1* was decreased by coumestrol treatment but increased by genistein treatment. MECs treated with formononetin, daidzein or equol showed increased expression of *Srebp1*. Expression of *Fabp3* was decreased by treatment with daidzein and equol.

3.3 Isoflavones have different effects on PRLR/STAT5 signaling

PRLR/STAT5 signaling is indispensable for the induction of milk production in MECs [30]. The expression levels of *Prlr* and *Stat5a* in MECs treated with isoflavones were evaluated by quantitative PCR (Fig. 3A). MECs treated with coumestrol showed decreases in the *Prlr*. MECs treated with genistein showed decreases in the *Prlr* and *Stat5a*. On the other hand, treatment with biochanin A, formononetin, daidzein and equol increased the expression of the *Prlr*. Moreover, both biochanin A and daidzein increased *Stat5a* expression.

Activation of PRLR/STAT5 signaling is detected as phosphorylation of STAT5 and nuclear localization of pSTAT5 [31]. The amounts of pSTAT5 were evaluated by western blot analysis. Treatment with coumestrol decreased pSTAT5 to one-tenth of the level in control cells, associated with a significant decrease in STAT5 (Fig. 3B, C). MECs treated with biochanin A showed significantly decreased pSTAT5 levels without altered expression of STAT5. Treatment with genistein or formononetin decreased pSTAT5 in parallel with a decrease in STAT5 expression. Compared with control cells, treatment with genistein decreased the level of pSTAT5 by 50%, and formononetin decreased pSTAT5 by 30%. Daidzein treatment increased STAT5 expression in MECs, and the pSTAT5/STAT5 ratio in MECs treated with equol increased to 1.2 times of that in control cells.

The localization of pSTAT5a in MECs was observed via immunostaining (Fig. 3D). Most of the control MECs showed nuclear localization of pSTAT5a, with some differences in immunostaining intensity. Such nuclear localization of pSTAT5a was also observed in MECs treated with p-ethylphenol, formononetin, daidzein or equol, with additional localization of pSTAT5a in cytoplasm. In contrast, treatment with biochanin A weakened the immunostaining intensity of pSTAT5a in the nucleus. Treatment of coumestrol and genistein decreased the number of pSTAT5a-positive cells and weakened the immunostaining intensity of pSTAT5a in MECs. These results revealed that each isoflavone influenced PRLR/STAT5 signaling in different manners.

3.4 Soy isoflavone mixture influences in β-casein production and STAT5 activation.

Finally, we investigated the influences of a mixture of multiple isoflavones based on the intake of legume foods. For a soy isoflavone model, we prepared a mixture of equol, p-ethylphenol, and

genistein at a ratio of 2:2:1 based on blood levels of isoflavones in lactating cows after eating soy [41].

At 10 and 20 μM, the soy isoflavone mixture significantly decreased secreted β-casein to approximately 80% of that in the controls (Fig. 4A, B). Based on the immunostaining images, β-casein was accumulated in the cytoplasm after treatment with the soy isoflavone mixture at 20 μM (Fig. 4C). Moreover, the soy isoflavone mixture weakened the immunostaining intensity of pSTAT5a in MECs compared with that in the controls.

4 Discussion

In this study, we investigated the influence of isoflavones on the milk production ability of MECs. The results showed that isoflavones could be classified into a promotive group (p-ethylphenol, daidzein and equol) and an inhibitory group (coumestrol, biochanin A, genistein and formononetin) in the context of PRLR/STAT5 signaling. The high concentrations of coumestrol, biochanin A, genistein and formononetin reduced intracellular and secreted β-casein, which is a representative milk-specific protein. On the other hand, p-ethylphenol, daidzein and equol did not significantly influence β-casein production at any concentration. Coumestrol, genistein and formononetin seemed to inhibit secretion of β-casein from the result of immunostaining and western blotting. Coumestrol, biochanin A and genistein also down-regulated the mRNA expression of milk proteins such as the lactose synthesis-related gene *Lalba*. In contrast, p-ethylphenol, daidzein and equol up-regulated *Csn2* and/or *Wap* as well as *Lalba*. Activation of PRLR/STAT5 signaling has been reported to induce milk protein production in MECs. [30, 31]. In this study, the amount of PRLR and/or activated STAT5 (pSTAT5) was decreased after treatment with coumestrol, biochanin A, genistein or formononetin. In contrast, treatment with p-ethylphenol, daidzein or equol did not inhibit PRLR/STAT5 signaling. The ratio of activated STAT5 to total STAT5 decreased by treatment of coumestrol and increased by treatment of genistein and equol. These results indicate that the effect of isoflavones on milk protein production ability in MECs is regulated by PRLR/STAT5 signaling. STAT5 activation was also consistent with the expression of *Lalba* required for lactose synthesis, suggesting that isoflavones regulate lactose synthesis through PRLR/STAT5 signaling. However, individual isoflavones showed distinctly different influences on the mRNA expression of triglyceride synthesis factors, inconsistent with the activation of STAT5. Triglyceride synthesis may be regulated by isoflavones through other signaling pathways, such as peroxisome proliferator-activated receptor [42].

Isoflavones are called phytoestrogens because of their estrogen-like structure and physiological activity [16-18]. Therefore, the inhibitory effects of coumestrol, biochanin A, genistein and formononetin may be partially mediated by estrogen signaling. However, coumestrol and genistein reduced milk production ability in MECs strongly. In particular, coumestrol and

genistein down-regulated mRNA expression of *Prlr* and *Stat5a*, although biochanin A and formononetin up-regulated those genes. This suggests that coumestrol and genistein have an additional physiological activity affecting the expression of PRLR and STAT5. For example, coumestrol and genistein have been reported to induce apoptosis [43, 44]. Genistein, which is known as an inhibitor of tyrosine kinases, reduce prolactin signaling in the presence of insulin [45, 46]. Inhibitory effects of isoflavones may be mediated by various pathways in an isoflavone-type-specific manner.

Isoflavones are absorbed into the body after metabolic conversion by enteric bacteria [6]. Formononetin and daidzein are metabolized to equol, and biochanin A and genistein are metabolized to p-ethylphenol [7, 8]. In this study, the mRNA expression levels of *Wap* and *Lalba* mRNA were increased by treatment with formononetin, daidzein or equol but decreased by treatment with biochanin A or genistein. Thus, isoflavones in the same metabolic pathway showed the same effects on milk production ability in MECs. Moreover, the upstream isoflavones tended to inhibit milk protein production more than the downstream isoflavones. For example, p-ethylphenol and equol are the downstream metabolites of biochanin A and formononetin, respectively. Treatment with p-ethylphenol or equol did not influence milk production ability or PRLR/STAT5 signaling, although biochanin A and formononetin had inhibitory effects on those parameters. Taken together, these results suggest that inhibitory actions of the upstream isoflavones may be rendered ineffective through metabolic conversion by enteric bacteria.

We generally take in multiple isoflavones at the same time because each legume contains some types of isoflavones in a legume-specific manner [3, 47, 48]. Moreover, some isoflavones are converted into different isoflavones or isoflavone metabolites by isoflavone-metabolizing enteric bacteria [49]. Therefore, MECs are predicted to be exposed to multiple isoflavones at the same time *in vivo*. In this study, we prepared an isoflavone mixture model containing p-ethylphenol, equol and genistein (in a ratio of 2:2:1) as the soy isoflavone model. At 10 and 20 μM, the soy isoflavone model decreased secreted β-casein. In contrast, treatment with genistein alone at 1.56 μM did not produce those effects. In addition, single treatment of p-ethylphenol and equol also did not show the inhibitory effect on β-casein production at any concentration. However, the soy isoflavone mixture containing 1-2 μM genistein inhibited β-casein secretion. Thus, p-ethylphenol and equol in the soy isoflavone mixture are suggested to regulate β-casein production together with genistein. Multiple isoflavones may induce more complex effects on milk production ability in MECs because each isoflavone has different and multiple physiological activities [9, 10]. In this study, milk production ability of MECs was down-regulated by single treatment of isoflavone at a high concentration. However, simultaneous intake of multiple isoflavones by consumption of legumes may cause adverse effects on milk production ability of MECs at low concentrations. Furthermore, deliberate intake of multiple isoflavones by selective consumption of legumes may

induce better milk production ability because some isoflavones showed promotive effects on milk production ability in MECs in this study.

Isoflavones are a class of polyphenolic compounds contained in legumes. In this study, we focused on the direct influences of isoflavones in milk production ability by MECs. The results showed that the effects of isoflavones in milk production ability were distinctly different based on the isoflavone type. The inhibitory effects of some types of isoflavones were predicted to be mediated by PRLR/STAT5 signaling. Furthermore, treatment with multiple isoflavones produced different effects from single treatment with each isoflavone. The isoflavone compositions of legumes are distinctly different, and the isoflavone composition in the blood is altered by metabolic ability of enteric bacteria. The metabolic ability of isoflavones depends on enteric bacteria composition and the health conditions [41]. Thus, when eating legumes, it is necessary to be careful regarding the types and contents of isoflavones.

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Figure legend

Fig. 1

Different effects of isoflavones on the amounts of intracellular and secreted β-casein.

MECs were treated with isoflavones at 0.1, 1.56 or 25 μM for 2 days. The bands from western blotting show intracellular (black columns) and secreted β-casein (gray columns) in MECs and the culture medium, respectively. The graphs show the results for the densitometry analysis for intracellular and secreted β-casein. β-Actin was used as an internal control. The data are the expression level relative to those of control without isoflavone treatment and are presented as the mean ± SD (n=4-8). Asterisks show significant differences ($p<0.05$ versus control).

Fig. 2

Isoflavones induce promotive or adverse effects on the expression of mRNAs related to the synthesis of milk components.

Untreated MECs (control) and MECs treated with coumestrol (Co), biochanin A (Bi), genistein (Ge), p-ethylphenol (Pe), formononetin (Fo), daidzein (Da) or equol (Eq) at 25 μM for 2 days. Expression levels of milk protein (A; *Csn2*, *Wap*, *Ltf*), lactose synthesis-related genes (B; *Glut1*, *Ugp2*, *Lalba*), and triglyceride synthesis-related genes (C; *Slc27a3*, *Fabp3*, *Srebp1*) were quantified using real-time PCR. *Gapdh* was used as an internal control. The data are the expression level relative to those of control without isoflavone treatment and are presented as the mean ± SD (n=4-6). Asterisks show significant differences ($p<0.05$ versus control).

Fig. 3

Coumestrol, biochamin A, genistein and formononetin down-regulate prolactin/STAT5 signaling.

MECs were treated with isoflavones at 25 μM for 2 days. (A) mRNA expression of *Prlr* (black columns) and *Stat5a* (gray columns) were quantified by real-time PCR. *Gapdh* was used as an internal control (n=4-6). (B) The bands show pSTAT5 and STAT5 by western blotting. (C) The graphs show the results of the densitometry analysis of the bands for pSTAT5 (black columns), STAT5 (gray columns) and pSTAT5/STAT5 (white columns). β-Actin was used as an internal control (n=4-8). (D) Immunostaining images of pSTAT5a (green) with DAPI (blue). Scale bars

are 15 μm. Co: coumestrol, Bi: biochanin A, Ge: genistein, Pe: p-ethylphenol, Fo: formononetin, Da: daidzein, Eq: equol. The data are the expression level relative to those of control without isoflavone treatment and are presented as the mean ± SD. Asterisks show significant differences ($p<0.05$ versus control).

Fig. 4

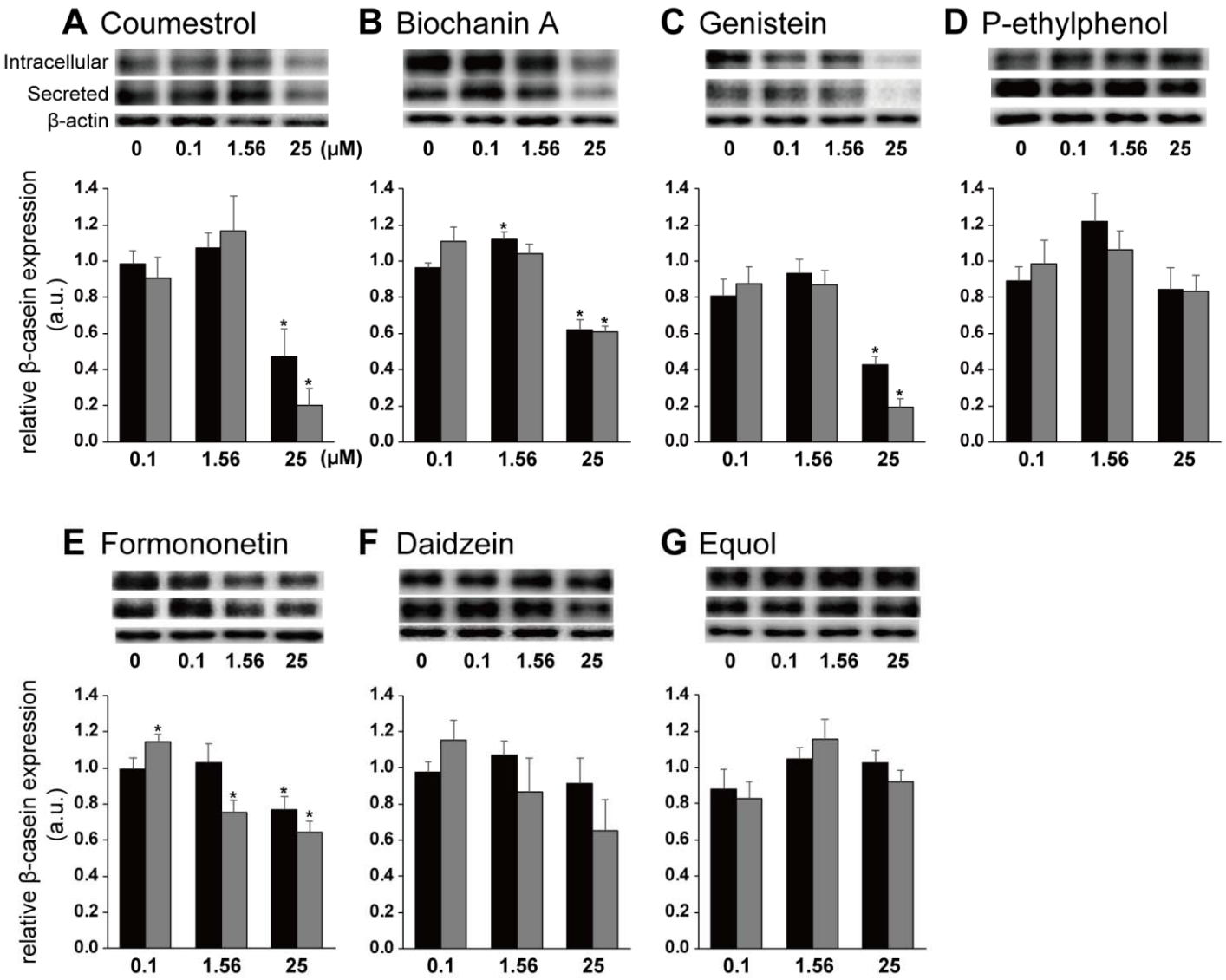
The multiple isoflavone mixture inhibits the milk component synthesis ability on MECs.

MECs were treated for 2 days with the soy isoflavone mixture composed of p-ethylphenol, equol and genistein (the ratio 2: 2: 1) at a total concentration of 1, 5, 10 or 20 μM. (A, B) the bands show intracellular β-casein (black columns), secreted β-casein (gray columns) and pSTAT5 (white columns) by western blotting. The data are the expression level relative to those of control without isoflavone treatment and are presented as the mean ± SD (n=6). Asterisks show significant differences ($p<0.05$ versus control). (C) Immunostaining images of β-casein (green; left) and pSTAT5a (green; right) with GM-130 (red; left) and DAPI (blue). Scale bars are 15 μm.

Supplementary Fig. 1

The different effects of isoflavones on β-casein localization.

The upper images show phase-contrast images of MECs. MECs formed cobblestone-like epithelial sheets without detectable effects of 2 days of treatment with isoflavone at 25 μM. The lower images show the localization of β-casein (green), DAPI (blue; nuclei) and GM130 (red; Golgi apparatus) in MECs. Scale bars are 50 μm in phase-contrast images and 15 μm in immunostaining images.



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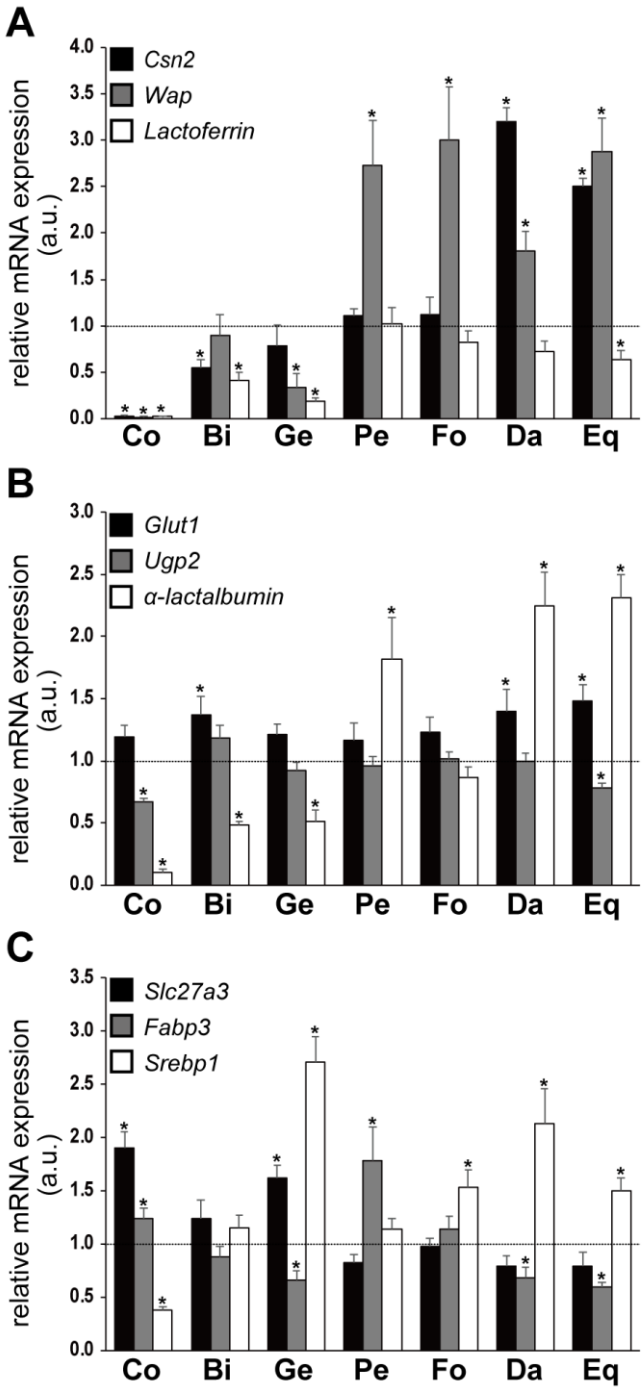

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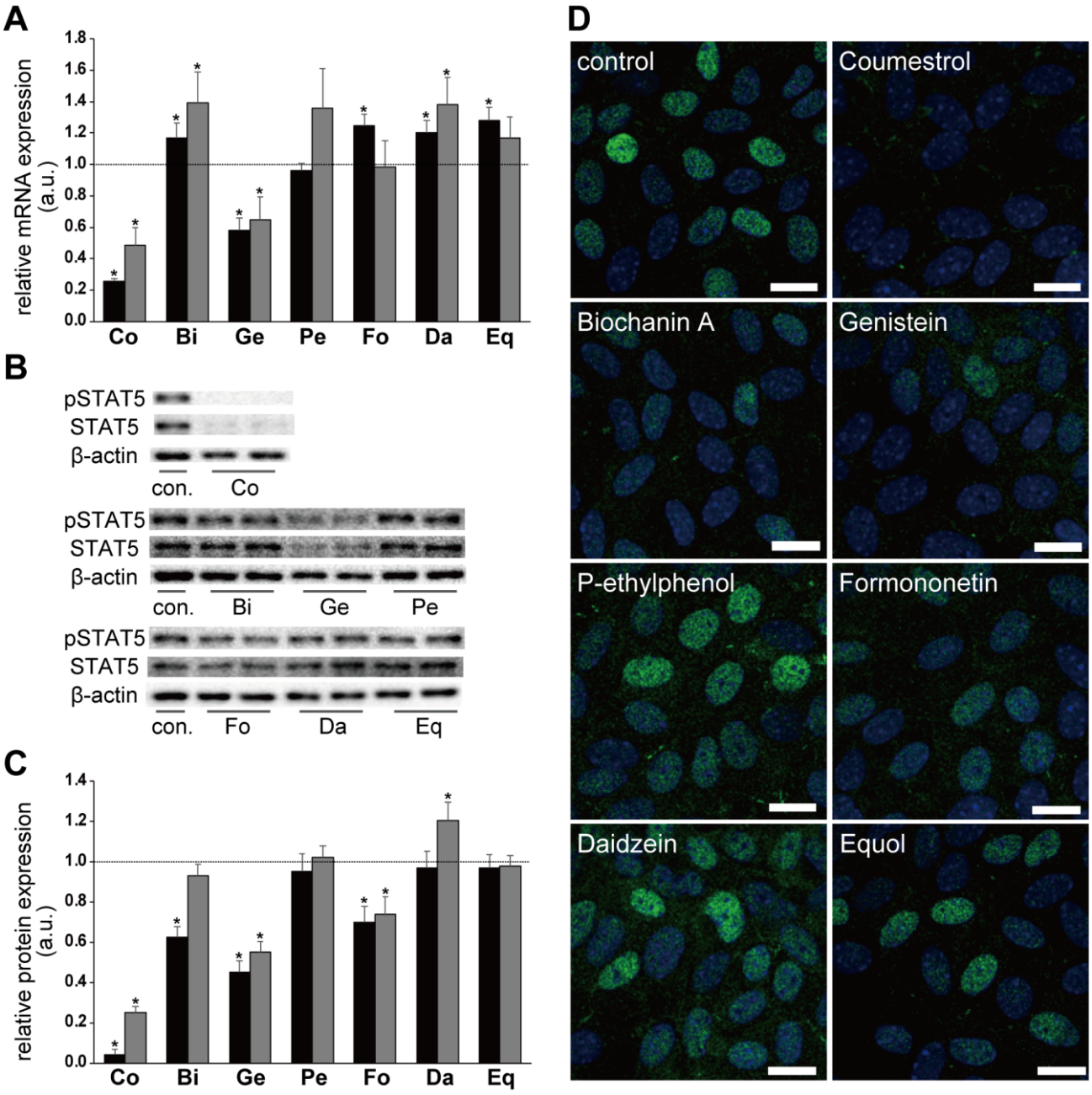

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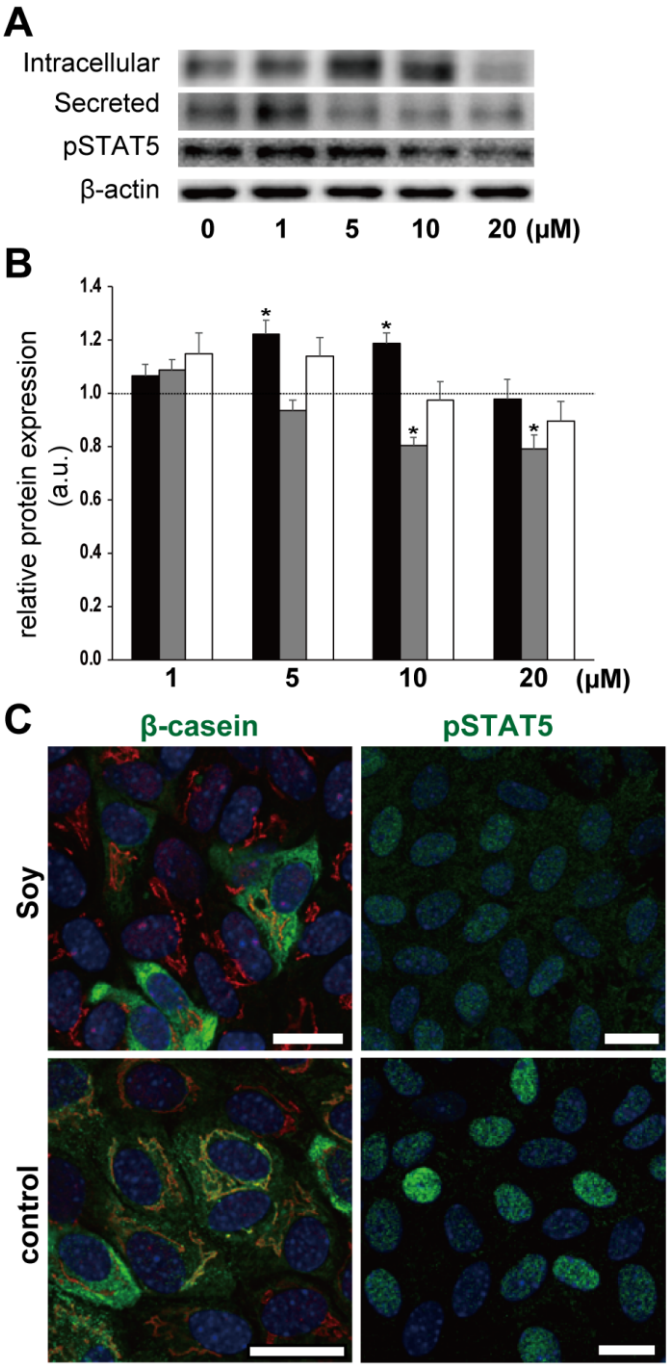

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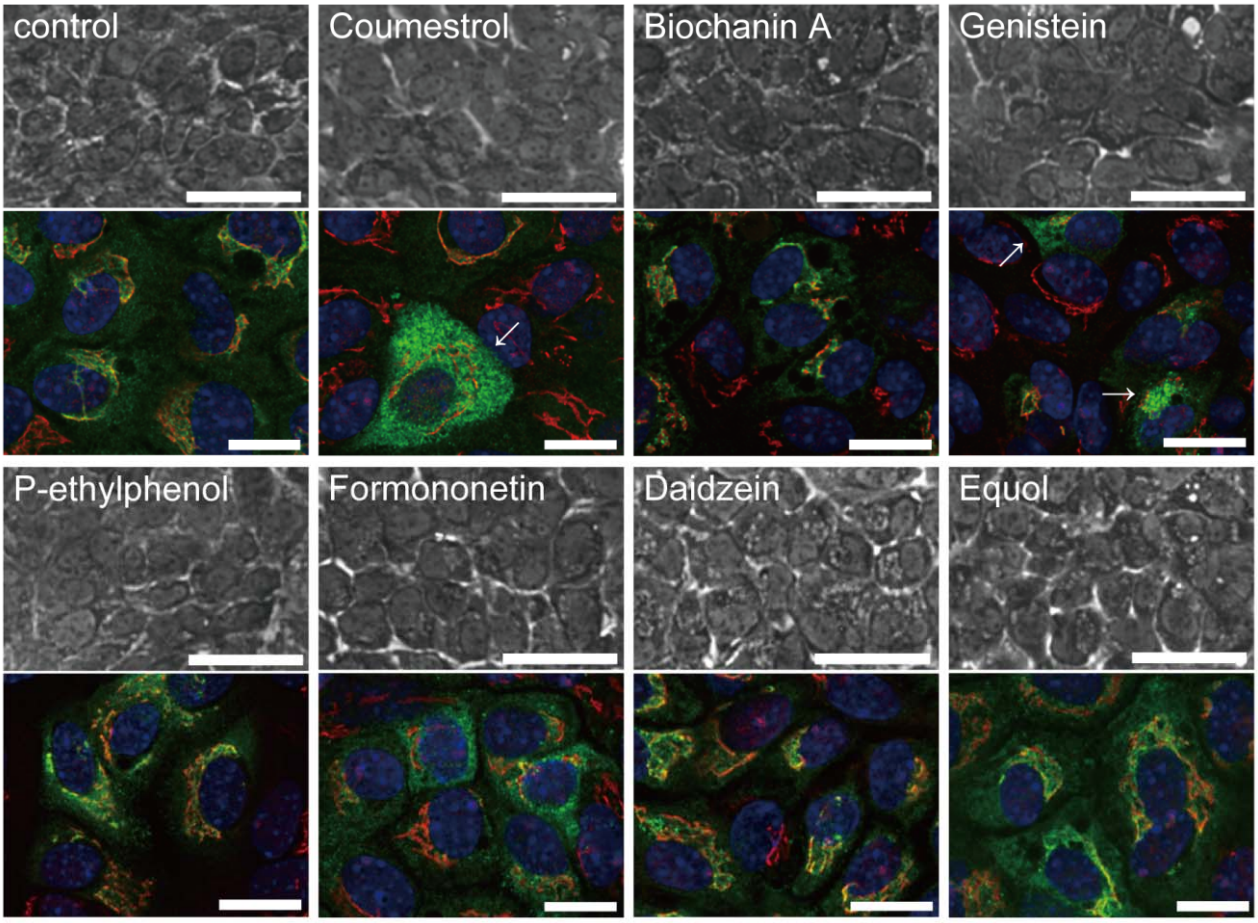

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Table 1 Primer sequences for real-time PCR

Gene	Accession number	Primer Forward	Primer Reverse	Product size
Csn2	NM_009972	cttcagaaggtgaatctcatggg	cagattagcaagactggcaagg	330
Wap	NM_011709	aacattggtgttccgaaagc	agggttatcactggcactgg	179
Lactoferrin	NM_008522	ggctgagaaggcaggaaatg	tttggggctatggctaggtg	183
Glut1	NM_011400	gcttcctgctcatcaatcgt	gccgaccctcttctttcatc	117
Ugp2	NM_139297	tcacaaacaaaacacgagcaga	cacttgagcgatttccacca	89
α-lactalbumin	NM_010679	accagtggctacgacacac	cggggaactcactacttttacac	106
Slc27a3	NM_011988	tctgggacgattgccagaaac	caagcgcaccttatggtcacac	116
Fabp3	NM_010174	agtcactggtgacgctggacg	aggcagcatggtgctgagctg	230
Srebp1	NM_011480	gtcagcttgtggcagtggag	tctgagggtggaggggtaag	90
Prlr	NM_011169.5	ttcttctcagagacacgcgg	agcgttctttagttctgctgga	246
Stat5a	NM_011488	gcaaaggggatggcagactt	oggtggaggctgttacttctaa	198
Gapdh	NM_008084	gagcgagaccccactaacatc	gcggagatgatgacccttttt	144