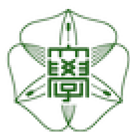

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Title: Adverse Effects of Coumestrol and Genistein on Mammary Morphogenesis and Future Milk Production Ability of Mammary Epithelial Cells

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Abstract

Isoflavones are a class of flavonoids present in legumes and are called phytoestrogens because of their estrogen-like activity. Endogenous estrogen is well known to regulate mammary gland morphogenesis during pregnancy. Each isoflavone also has different physiological activities. However, it is difficult to investigate the direct effect of each isoflavone in mammary morphogenesis *in vivo* because isoflavones are metabolized into different isoflavones by enteric bacteria. In this study, we investigated the direct influences of coumestrol, daidzein, and genistein on mammary structure development and future milk production ability of mammary epithelial cells (MECs) using *in vitro* culture models. Mouse MECs were cultured in Matrigel with basic fibroblast growth factor and epidermal growth factor to induce ductal branching and alveolar formation, respectively. Coumestrol and genistein inhibited ductal branching and alveolar formation by affecting the proliferation and migration of MECs with the induction of apoptosis. Daidzein hardly influenced mammary structure development. Furthermore, pretreatment with coumestrol adversely affected the induction of milk production ability of MECs. These results suggest that each isoflavone differentially influences mammary morphogenesis and future milk production by affecting MEC behaviors. These results

also suggest that the culture models are effective to study mammary epithelial

morphogenesis *in vitro*.

1. Introduction

The mammary gland is an exocrine gland that produces milk for suckling infants. During pregnancy, the epithelial ducts extend into the mammary fat pad with a series of branching to develop dendritic ductal networks.[1, 2] The alveolar structures are then formed in the terminal branches during late pregnancy. After parturition, alveolar mammary epithelial cells (MECs) synthesize milk components and then secrete them into the alveolar lumen. The milk is ejected from the alveolar lumen by contraction of myoepithelial cells in response to oxytocin stimulation and transferred through the mammary ducts to supply milk for suckling infants. Therefore, a well-developed network of mammary ducts and mature formation of the alveolar structure are indispensable for appropriate breastfeeding. Endogenous estrogen is well known to regulate mammary gland morphogenesis during pregnancy.[3]

Exogenous isoflavones are called phytoestrogens because they exhibit an estrogen-like structure and affect estrogen signaling by binding to estrogen receptors.[4] Various types of isoflavones are contained abundantly in legumes, with a diversity of isoflavone compositions among species. For example, soybean contains an abundance of genistein and daidzein, whereas alfalfa mainly contains coumestrol. [5, 6] These isoflavones each

have various and distinct physiological activities against MECs or breast cancer cells, such as the inhibition of protein tyrosine kinase by genistein and the anti-cancer effects of coumestrol.[7-10] In addition, lifelong exposure to isoflavone results in distinct dose-dependent effects on proliferation, gene expression, and DNA methylation in rat mammary glands[11]. However, it remains unclear how each type of isoflavone directly affects ductal branching and alveolar formation during pregnancy.

Recently, we have reported that each isoflavone individually influences the milk production ability of MECs through prolactin/STAT5 signaling in different manners by using *in vitro* culture models of lactation.[12] Each isoflavone also shows different effects on the expression patterns of tight junction (TJ) proteins and the barrier functions of TJs in lactating MECs.[13] The mammary gland requires sufficient cell growth for ductal branching and alveolar formation during pregnancy. Several isoflavones are also known to regulate cell migration, proliferation and apoptosis in breast cancer cells.[14-16] However, differences in the effects of each isoflavone on the behaviors of nontumor MECs remain unclear.

Blood levels of isoflavones are increased by eating legumes.[17] Asian people have a large intake of soybean-derived isoflavones such as daidzein and genistein.[18, 19] However,

it is difficult to investigate the direct effect of each isoflavone in mammary morphogenesis *in vivo* because isoflavones are metabolized into different isoflavones by enteric bacteria in the gastrointestinal tract.[20] In addition, the metabolic ability of isoflavones changes depending on the composition of enteric bacteria and health conditions.[21, 22] We have previously reported that coumestrol, daidzein and genistein directly influence the milk production ability of lactating MECs in different manners using a two-dimensional (2D) cell culture model *in vitro*.[12] In this study, we prepared 3D cell culture models to induce ductal branching and alveolar structure formation to investigate the direct effects of each isoflavone on mammary morphogenesis *in vitro*. The direct effects of isoflavone on future milk production ability of MECs were also investigated using *in vitro* culture models of lactation. The aims of this study were to reveal the direct influence of each isoflavone on milk production after parturition by affecting mammary morphogenesis and maturation of MECs during pregnancy independently of isoflavone metabolism by enteric bacteria.

2. Results

2.1. Isoflavones differentially influence ductal branch structure formation

The organoids of MECs were cultured in Matrigel in the presence of basic fibroblast growth factor (bFGF) for 5 days to induce ductal branching.[23] The organoids were also treated with isoflavones (coumestrol, genistein, and, daidzein) at 0.1, 0.5, 2.5, and 12.5 μM for the final 4 days. Phase-contrast microscopy revealed the gradual development of a branching structure in organoids in the absence of isoflavone (Figure 1A). The distal regions of the branched structure swelled similar to the alveolar buds *in vivo*[2]. Immunostaining images showed that MECs expressing claudin-3 (CLDN3) lined the luminal space with localization of occludin (OC), which is a TJ protein localized in the apical-most regions of the lateral membrane in the mammary epithelium.[24] CLND3 was localized in the cytoplasm and the basolateral membranes. The organoids treated with 12.5 μM coumestrol barely developed the branching structure. The number of branches per organoid decreased in response to coumestrol treatment in a dose-dependent manner. (Figure 1B). In contrast, daidzein treatment showed no obvious differences at any concentration. Genistein treatment at 12.5 μM weakly inhibited the formation of the ductal branching structure with a lumen, whereas organoids treated with 0.1, 0.5, or 2.5 μM genistein developed a branching structure similar to that in the untreated condition.

2.2. Isoflavones differentially influence alveolar structure formation

To induce alveolar structure formation *in vitro*, the organoids of MECs were embedded in Matrigel and then cultured in medium containing epidermal growth factor (EGF) for 5 days.[25] Coumestrol, genistein, and, daidzein were added to the medium at a final concentration of 0.1, 0.5, 2.5, and 12.5 μM at 6 h after cultivation. In the organoids without isoflavone treatment, MECs gradually lined the organoid surface, and a luminal structure was formed in central regions of the organoids (Figure 2A). Lined MECs also showed a positive reaction with CLDN7 in the cytoplasm and the basolateral membranes. CLDN7 is a marker for luminal cells.[24] Alveolar-like organoids containing luminal space were observed in approximately 90% of the organoids 5 days after cultivation in the control (Figure 2B). Coumestrol treatment caused inhibition of alveolar lumen formation in most of the organoids in a dose-dependent manner. DAPI-positive cells were observed in the central regions of the organoids. The ratio of the alveolar-like organoids was less than 30% and 10% in organoids treated with coumestrol at 2.5 and 12.5 μM for 5 days, respectively (Figure 2B). The high concentration of coumestrol treatment also reduced the organoid size to less than half of that of the untreated organoids (Figure 2C). In contrast, daidzein treatment showed no obvious differences at any concentration, except

that some organoids became distorted in shape (Figure 2A). Genistein treatment at 12.5 μM inhibited alveolar formation, although 0.1, 0.5, and 2.5 μM genistein had no influence. The size of the organoids was significantly reduced by 12.5 μM genistein treatment.

2.3. Effects of isoflavones on the proliferation, migration and apoptosis of MECs

To investigate how coumestrol and genistein inhibited morphological development in organoids, the influence of each isoflavone on cell proliferation was evaluated by WST-8 assays in mono-layered MECs. Coumestrol at 12.5 μM significantly inhibited the proliferation of MECs (Figure 3A), whereas daidzein had no effect at any concentration. Genistein stimulated proliferation at 0.1 and 0.5 μM, although MECs treated with 12.5 μM genistein showed reduced proliferation activity. In addition, proliferating MECs in the organoids were detected by immunostaining for Ki-67, which is a marker of proliferating cells. In the ductal branching model, Ki-67-positive cells were mainly observed in the branched-distal regions of the organoids that were untreated and treated with 12.5 μM daidzein (Figure 3B). The proliferating MECs were irregularly localized in the organoids treated with coumestrol. In organoids treated with genistein, proliferating MECs were mainly observed in the branched regions not but in the central regions of the

organoids. In contrast, proliferating MECs were observed in the MEC layer lining the luminal surface in the alveolar formation model regardless of isoflavone treatment, although the luminal structure of the organoids treated with coumestrol was defective (Figure 3C).

The influence of each isoflavone on cell migration was measured by a scratch assay. In MECs without isoflavone treatment, the area covered with MECs gradually expanded, and the scratch area had closed within 48 h (Figure 4A). MECs treated with coumestrol at 0.1 and 0.5 μM showed barely any effect on MEC migration, whereas 2.5 and 12.5 μM coumestrol caused a significant decrease in the area covered with MECs at 32 and 48 h after the scratch treatment (Figure 4B). Daidzein had no influence on MEC migration at any concentration. Genistein treatment at 12.5 μM caused a significant decrease in the area covered with MECs at 48 h after the scratch treatment.

Focal adhesions are multi-protein structures that form mechanical links between intracellular actin filaments for cell migration.[26] Vinculin is a key player in focal adhesion formation. The localization patterns of vinculin and actin filaments in MECs covering the scratch regions were observed by immunostaining for vinculin and by phalloidin staining for F-actin. Co-stained images of vinculin with F-actin showed focal

adhesions connected to the actin stress fibers in migrating MECs (Figure 4C). Abnormal localization of vinculin and disrupted actin stress fibers were observed in some MECs treated with coumestrol and genistein.

Apoptotic cells were detected by immunostaining of cleaved caspase-3 in the alveolar formation models. Weak positive reactions were observed in the culture models without isoflavone treatment and treatment with daidzein at 12.5 μM (Figure 5A). The ratio of organoids containing cleaved caspase-3-positive cells was less than 10% (Figure 5B). The organoids treated with coumestrol showed large numbers of cleaved caspase-3-positive cells, some of which were observed in central regions of the organoids. More than 60% of the organoids were positive for cleaved caspase-3. Apoptotic cells that were positive for cleaved caspase-3 were also observed in 40% of the organoids treated with genistein.

2.4. Effects of isoflavones on Akt signaling pathway

Akt signaling pathway regulates diverse cellular functions including cell proliferation migration, and apoptosis.[27] The amounts of phosphorylated Akt (p-Akt) and total Akt (t-Akt) were examined by western blotting and densitometry analysis of the bands (Figure 6). The ductal branching model treated with coumestrol for 4 days but not 1 day

significantly increased the p-Akt/t-AKt ratio. The increase in the p-Akt/t-AKt ratio was also observed in the alveolar formation model treated with coumestrol for 1 day. In the alveolar formation model treated with coumestrol for 4 days, p-Akt and the p-Akt/t-AKt ratio increased to 2.6 times of those in control. Daidzein and genistein hardly influenced on the levels of p-Akt, t-Akt or p-Akt/t-AKt ratio in the ductal branching and alveolar formation models.

2.5. Effects of isoflavones on the arrangement of myoepithelial and luminal cells

Myoepithelial cells, which localize on the outer surface of the mammary epithelial structure, are cytokeratin 14 (CK14)-positive in pregnant mammary glands.[28] In contrast, CK18-positive cells line the luminal surfaces of ducts and alveoli. In the ductal branching models, CK14-positive myoepithelial cells were mainly detected in proximal regions of the branched structure of the organoids without isoflavone treatment, as previously reported (Figure 7A).[29] CK18-positive cells were mainly observed in the distal swollen structure of the branched organoids. Similar localization patterns were observed in the organoids treated with daidzein and genistein (Figure 7B). In the organoids treated with coumestrol, CK14-positive cells were observed on the outer surface of the organoids, and

CK18-positive cells lined the ductal lumen.

Expression levels of CK14 and CK18 were measured by western blotting and densitometry analysis of the bands. The decrease in CK14 was caused by all types of isoflavone treatment for 5 days (Figure 7B, C). Coumestrol treatment for 5 days also decreased the expression levels of CK18 by half compared with the control.

2.6. Pretreatment with isoflavones adversely affects the induction of milk production ability in MECs

To investigate the direct influences of isoflavone exposure during late pregnancy on future milk production ability of alveolar MECs after parturition, the organoids in the alveolar formation model were treated with isoflavones for 3 days. After removal of isoflavones from the medium by washing, the organoids were then cultured in medium containing prolactin and dexamethasone for 2.5 days to induce milk production in MECs. Phase-contrast microscopy revealed the gradual accumulation of secreted components in the alveolar lumen in the organoids without and with pretreatment with daidzein and genistein (Figure 8A). The organoids with coumestrol pretreatment formed the luminal structure 1 day after washout, although the accumulation of secreted components was

barely observed for 2.5 days.

Immunostaining images of the organoids revealed the accumulation of β-casein, one of the major milk proteins, in the alveolar lumen. In the organoids without isoflavone pretreatment or with pretreatment with daidzein, complete accumulation of β-casein was detected in the alveolar lumen (Figure 8B). Conversely, the organoids pretreated with coumestrol and genistein showed sparse localization of β-casein in the alveolar lumen as well as some leakage of this protein (white arrows in Figure 8B). The ratio of the organoids with β-casein leakage was 53%, 6% and 32% in the culture models pretreated with coumestrol, daidzein and genistein, respectively (Figure 8C).

MECs synthesize some types of caseins containing α_{s1}-, β- and κ-caseins. UGP2 and FABP3/SREBP1 are involved in the synthesis of lactose and triglyceride, respectively.[30-32] Pretreatment with coumestrol downregulated the expression of *Csn1s1* (*α_{s1}-casein*) and *Csn2* (*β-casein*) in MECs (Figure 8D). *Fabp3* was downregulated following pretreatment with coumestrol and genistein. The expression levels of *Csn3* (*κ-casein*) and *Srebp1* remained unchanged in response to isoflavone pretreatment.

2.7. Effects of soy isoflavone mixture on ductal branch structure formation

Finally, we investigated the direct influences of a mixture of multiple isoflavones based on the intake of soy foods. For a soy isoflavone model, we prepared a mixture of genistein, daidzein, and equol at a ratio of 6:3:1 based on blood levels of isoflavones in Japanese women.[18]

The soy isoflavone mixture at 12.5 μM significantly inhibited the formation of the ductal branching structure (Figure 9A, C, D). The organoids treated with the soy isoflavone mixture 2.5 μM developed a branching structure with a lumen similar to that in the untreated condition although the size of the organoids was observed somewhat small (Figure 9B). Immunostaining images showed that MECs expressing CLDN3 lined the luminal space with localization of OC in the organoids treated with the soy isoflavone mixture at 2.5 and 12.5 μM. In control, CLND3 was localized in the cytoplasm and the basolateral membranes. In the organoids treated with the soy isoflavone mixture, CLND3 was localized near the OC-positive regions with weak positive reactions to the cytoplasm and the basolateral membranes.

3. Discussion

Exogenous isoflavones are called phytoestrogens because they have an estradiol-like

structure and bind to estrogen receptors. Endogenous estrogen signaling orchestrates mammary epithelial morphogenesis by regulating the proliferation and migration of MECs during pregnancy.[33] Each isoflavone also has additional distinct physiological activities that affect cell behavior in a non-estrogen-dependent manner, such as via Akt, NF-κB, and insulin-like growth factor signaling.[34-36] However, isoflavones are converted into different isoflavones by enteric bacteria *in vivo*.[22] Therefore, we investigated the direct influence of isoflavones on the morphogenesis of the mammary epithelial structure by employing ductal branching and alveolar formation models in this study. The results revealed that coumestrol and genistein inhibited ductal branching and alveolar structure formation. Furthermore, we investigate the influences of isoflavone exposure during late pregnancy on future milk production ability after parturition using the alveolar formation model *in vitro*. The organoids, which were pretreated with isoflavones, were cultured without isoflavones in medium containing prolactin and dexamethasone to induce milk production. The results showed pretreatment with coumestrol and genistein adversely affect the induction of milk production ability in MECs in the alveolar formation model. The inhibitory effects of coumestrol were comprehensively higher than those of genistein. In contrast, daidzein minimally influenced mammary morphogenesis and induction of

milk production ability. The mammary gland develops a mammary ductal network and then forms alveolar structures at the tip of the ducts during pregnancy.[1, 2] The alveolar MECs initiate the production of various milk components such as casein, lactose, and triglyceride after parturition.[37] Thus, coumestrol and genistein exposure during pregnancy is suggested to have an adverse effect on future milk production in mammary glands during lactation by inhibiting both morphogenesis of the mammary epithelial structure and induction of the milk production ability of MECs. Furthermore, these findings also suggest that the culture models of ductal branching and alveolar formation enables us to investigate the direct influences of isoflavones independently of isoflavone metabolism by enteric bacteria.

Cell proliferation and migration are indispensable for morphogenesis of the mammary epithelial structure.[3] The number of MECs influences the milk yield in lactating mammary glands.[38] Therefore, appropriate growth of MECs in association with mammary epithelial structure development is indispensable for sufficient milk production after parturition. In this study, coumestrol and genistein reduced cell proliferation and migration with the activation of apoptosis. Coumestrol is known to inhibit cell proliferation and induces apoptosis in ER-negative breast cancer cells.[8] Coumestrol also

inhibits cell migration and differentiation via regulation of Akt.[34, 39] Akt is a major intracellular signaling kinase that regulates cell cycle progression, survival, migration, proliferation, and apoptosis. In this study, coumestrol treatment induced activation of Akt signaling in the ductal branching and alveolar formation models. Coumestrol treatment may inhibit mammary morphogenesis by affecting cell behaviors via Akt signaling. In addition, genistein has been observed to stimulate and inhibit cell proliferation at low and high concentrations, respectively. Genistein, which is a kind of tyrosine kinase inhibitor, reduces proliferation and migration activities in various types of cells.[40-42] However, the administration of genistein at a low concentration (0.5 mg/kg/day) facilitates mammary gland development with increased ductal elongation, although a high concentration of genistein (50 mg/kg/day) leads to decreases in ductal branching.[43] Genistein has also been reported to stimulate cell proliferation with increases in epithelial branching in prepubertal rats.[44, 45] These findings suggest that genistein regulates mammary epithelial morphogenesis as a stimulator and as an inhibitor depending on its concentration.

The mammary gland forms multiple alveolar buds at the terminal end of the ductal tree during late pregnancy.[2] In this study, an alveolar bud-like structure was observed in the peripheral regions of the branched organoids in the ductal branching model without

isoflavone treatment or with genistein and daidzein treatment. The alveolar bud-like structure was composed of CK18-positive cells. CK18 is a specific marker for alveolar epithelial cells, whereas CK14 is a specific marker for basal and myoepithelial cells.[3] Coumestrol caused abnormal CK14 localization. CK14-positive myoepithelial cells are indispensable for appropriate ductal branching and alveolar formation.[46] Coumestrol also inhibited the formation of the alveolar lumen in the alveolar formation model. A high concentration of genistein also partially inhibited alveolar formation. These findings suggest that coumestrol and genistein have adverse effects on the development of the alveolar structure during late pregnancy. Furthermore, pretreatment with coumestrol and genistein caused downregulation of milk component-related genes and leakage of β-casein from the alveolar lumen. Recently, we have reported that coumestrol and genistein inhibit milk production and cause disruption of TJs in lactating MECs.[12, 13] The results of the present study additionally suggest that exposure to coumestrol and genistein during late pregnancy has adverse effects on the induction of the milk production ability of MECs after parturition.

In this study, coumestrol, daidzein and genistein showed distinct effects on mammary morphogenesis and future milk production ability *in vitro*. The culture models of ductal

branching and alveolar formation enabled us to strictly control the concentration of each isoflavone. In contrast, eating legumes results in the intake of multiple isoflavones simultaneously because each legume contains certain types of isoflavones in a legume-specific manner.[47-49] Isoflavones are also converted into different isoflavones or isoflavone metabolites by isoflavone-metabolizing enteric bacteria *in vivo*.[22] For example, daidzein and genistein are metabolized to equol and p-ethylphenol, respectively.[50, 51] The metabolic ability of isoflavones depends on the composition of enteric bacteria and the health conditions.[21] Blood levels of each isoflavone after eating legumes are altered depending on the metabolite activity *in vivo*.[4, 17, 52] Thus, MECs are predicted to be exposed to multiple isoflavones simultaneously *in vivo*. In this study, we prepared an isoflavone mixture model containing genistein, daidzein, and equol at a ratio of 6:3:1 based on blood levels of isoflavones in Japanese women.[18] At 12.5 µM, the soy isoflavone mixture inhibited the formation of the ductal branching structure. The soy isoflavone mixture at 12.5 µM contains 7.5 µM genistein, 3.75 µM daidzein, and 1.25 µM equol. Genistein and daidzein in blood plasma 6h after ingestion of 60 g baked soybean powder (kinako) are 2.5 µM and 1.5 µM, respectively.[53] Isoflavone contents of soybean powder are nearly equol to those of soy milk.[54] Therefore, excessive intake of

soy products may have adverse effects in mammary morphogenesis during pregnancy.

4. Conclusion

In summary, we observed that coumestrol and genistein had inhibitory effects on mammary ductal branching and alveolar formation by directly affecting proliferation, migration and cell death in MECs. Daidzein hardly influenced mammary structure development. Furthermore, pretreatment with coumestrol had inhibitory effects on the induction of milk production ability of alveolar MECs *in vitro*. These results suggest that each isoflavone differentially influences mammary morphogenesis and future milk production by affecting MEC behaviors. These results also suggest that the culture models of ductal branching and alveolar formation are effective to study of mammary epithelial morphogenesis *in vitro*. However, it remains unclear whether coumestrol and genistein affect mammary morphogenesis during pregnancy and milk production after parturition in human, cows and other mammals. Further investigations are required to reveal the influences of each type of isoflavones on mammary glands during pregnancy and after lactation *in vivo*.

5. Experimental Section

5.1. Animals

Virgin (9–14 weeks) ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained under a 12-hour light-dark interval at 22–25°C. The mice were decapitated, and then the fourth mammary glands were collected for isolation of MECs. All experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University (#14-0005) and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

5.2. Materials

DMEM/F-12, ITS-X, collagenase, and penicillin-streptomycin solution were obtained from Wako (Osaka, Japan). EGF and bFGF were obtained from BD Biosciences (Bedford, MA). Matrigel (growth factor reduced type) was purchased from Corning (Corning, NY). Dexamethasone and bovine pituitary extracts containing prolactin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from GIBCO-BRL (Grand Island, NY).

The following served as primary antibodies for immunostaining: rabbit polyclonal

antibodies against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, # 9664, 1:400), claudin-3 (CDLN3; Thermo Fisher Scientific, Waltham, MA, # 34-1700, 1:300), CLDN7 (Thermo, # 34-9100, 1:200), phospho-Akt (p-Akt; Cell Signaling Technology, # 4060, 1:1000), Akt (Cell Signaling Technology, # 4691, 1:1000); mouse monoclonal antibodies against occludin (OC; Thermo, # 33-1500, 1:800), keratin 14 (CK14, Thermo, # MA5-11599, 1:200), CK18 (Progen, Heidelberg, Germany, # 61028, 1:200), β-actin (1:100,000, # MA1-140, Thermo), vinculin (Chemicon International Inc, Temecula, CA, # CBL233, 1:600); a rat monoclonal antibody against Ki-67 (Dako, #GP40, 1:500); and a goat polyclonal antibody against β-casein (Santa Cruz Biotechnology, # M7249, 1:200). The secondary antibodies (an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody, Alexa Fluor 546-conjugated goat anti-mouse IgG antibody, Alexa Fluor 546-conjugated rabbit anti-goat IgG antibody, and Alexa Fluor 546-conjugated goat anti-rat IgG antibody) were purchased from Life Technologies (Gaithersburg, MD). The secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies for western blotting were purchased from Sigma-Aldrich.

5.3. 3D Cell culture models

MECs were isolated from the fourth mammary gland of virgin ICR mice. The minced mammary glands were incubated in DMEM/F-12 medium containing collagenase at 0.75 mg/mL for 2 h at 37°C with shaking at 70 rpm, followed by treatment with 0.25% trypsin in DMEM/F-12 with gentle pipetting for 5 min at room temperature. After centrifugation, the pellet was resuspended in 50% FBS in RPMI-1640 medium and then centrifuged at 100 rpm for 5 min to separate the epithelial fragments to obtain single cells including adipocytes and macrophages. The epithelial fragments were then treated with 0.25% trypsin and 2 mM EDTA in PBS for 10 min for dispersion into single MECs. To form organoids by aggregation of MECs, MECs were seeded on a dish coated with 12% polyacrylamide gel to inhibit cell-dish adhesion at a concentration of 1.0×10^6 cells/ml in DMEM/F-12 containing 10% FBS, 5 μg/ml ITS-X, 2.5 nM EGF, and 1 μM dexamethasone. They were then cultured for 16 h at 37°C to induce the formation of organoids.

Organoids were suspended into 20 μl of Matrigel at a concentration of 6-12 organoids/μl and cultured for 5 days. The culture medium used was DMEM/F-12 containing 5 μg/ml ITS-X and 2.5 nM bFGF or 2.5 nM EGF for the culture models of ductal branching or alveolar formation, respectively, as reported previously.[23, 25] Each

isoflavone was added to the culture medium 6 h and 1 day after cultivation in the alveolar formation and ductal branching models, respectively.

Organoids in the alveolar formation models, which were cultured in the presence of isoflavones for 3 days, were elaborately washed with DMEM/F-12. The organoids were then cultured with DMEM/F-12.containing 1.25 nM EGF, 1% FBS, 0.45 mg/ml bovine pituitary extract, and 1 μM dexamethasone for 2.5 days to induce milk production ability in MECs[55].

5.4. Immunofluorescence staining

Matrigel containing organoids in the culture models and cultured MECs on dishes were fixed with 4% formaldehyde in PBS for 20 min at 4°C. After treatment with 0.2% Triton X-100 in PBS for 20 min at 4°C, the fixed organoids and MECs were incubated with PBS containing 2.5% bovine serum albumin (BSA; Sigma-Aldrich) to block nonspecific interactions with antibodies and then with the primary antibody diluted in blocking solution overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), organoids and MECs were exposed to secondary antibodies diluted in blocking solution for 1 h at 4°C and then for 1 h at room temperature. Actin filament was stained with Acti-

stain 555 phalloidin (Cytoskeleton Inc., Denver, CO). Control samples were processed in the same manner, except in the absence of primary antibody. Immunofluorescence staining images were obtained with a confocal laser-scanning microscope (TCS SP5; Leica, Mannheim, Germany).

5.5. Histomorphometric analysis

Quantitative histomorphometry was conducted using ImageJ software (National Institutes of Health) and LAS AF Lite (Leica) to assess the number of branches, alveolar size, the coverage of MECs in the scratch area, ratio of alveoli containing luminal space, and with β-casein leakage. At least 16 individual images originating from four different culture models were used for the statistical analysis. The immunostaining images were obtained using the same settings of the confocal laser-scanning microscope.

5.6. Western blotting analysis

The samples of MECs were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h with PBST containing 4% nonfat dried milk

and then incubated overnight at 4°C with primary antibodies: CK14, CK 18, p-Akt, Akt, and β-actin diluted in PBST containing 2% BSA. Subsequently, the membranes were washed with PBST and incubated for 40 min at room temperature with the appropriate secondary HRP-conjugated antibodies diluted in PBST containing 3% nonfat dried milk. The immunoreactive bands were detected using Luminate Forte Western HRP substrate (Millipore, Billerica, MA). Images of the protein bands were obtained with a Bio-Rad ChemiDoc™ EQ densitometer and Bio-Rad Quantity One® software (Bio-Rad).

5.7. Quantitative PCR

Total RNA from the organoids of MECs was extracted using ISOGEN II (Wako). Reverse transcription (RT) was performed using ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR was conducted on a Light Cycler 480 (Roche Applied Science, Indianapolis, IN) with Thunderbird® SYBR® qPCR Mix (Toyobo). We used the following cycling conditions: 95°C for 1 min followed by 40 cycles at 95°C for 15 sec and 58°C for 1 min. The primer information is provided in Table 1. *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) served as an internal control.

5.8. Statistical analysis

Statistical analyses were performed using the Analyse-It™ add-in (version 1.73, Analyse-it Software, Ltd., Leeds, United Kingdom) for Microsoft Excel (Microsoft Corporation, Redmond, WA). The data are expressed as means (standard error of the mean [SEM]). Significance values were calculated using the Kruskal-Wallis and Steel-Dwass tests because the data did not follow a Gaussian distribution. Differences were considered significant at p-values <0.05 and are indicated by asterisks or different letters. All experiments for statistical analysis were performed a minimum of four times using MECs originating from different Matrigel-containing organoids.

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Conflict of Interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

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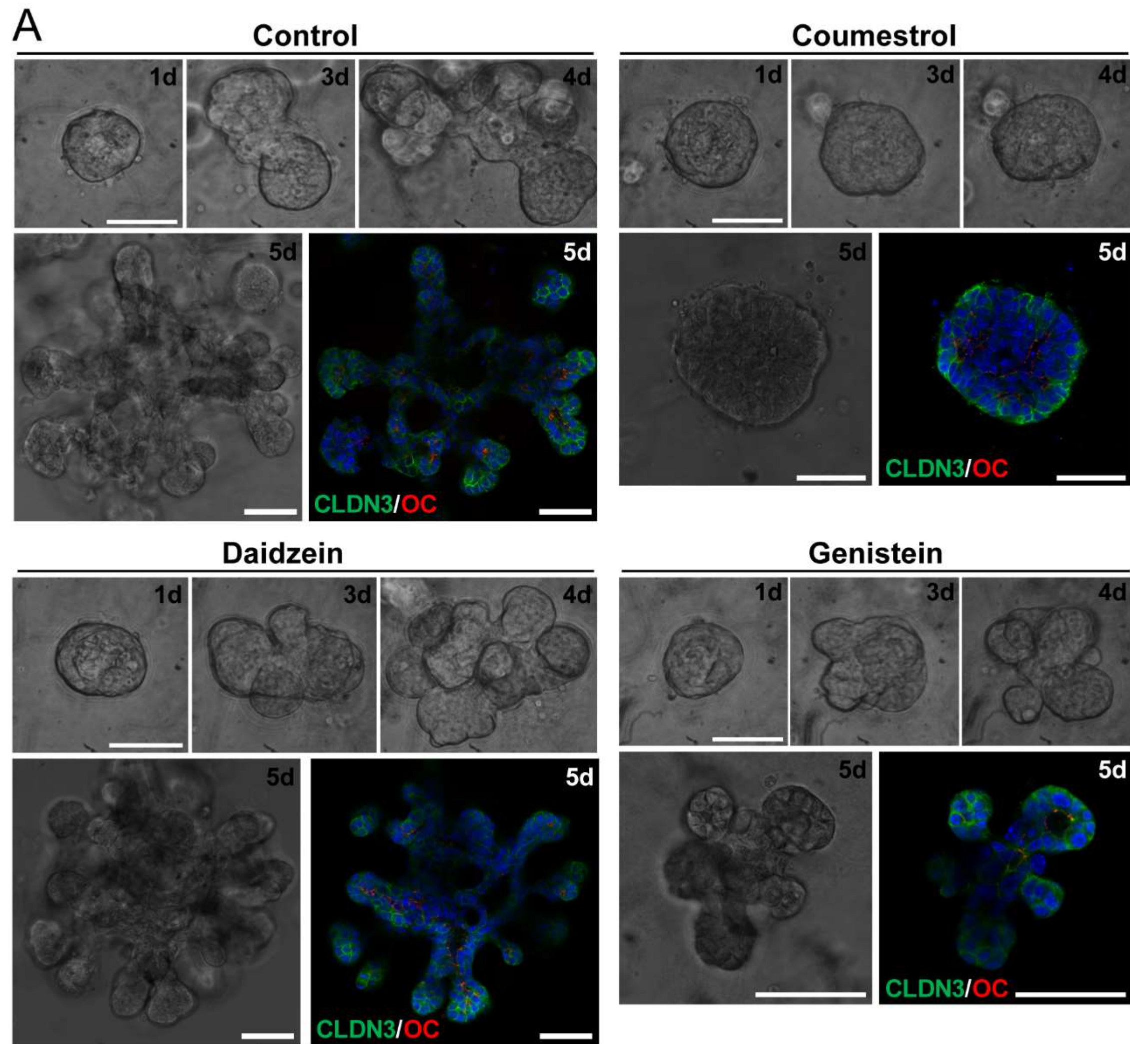

A
Control
1d
3d
4d
5d
5d
CLDN3/OC
Coumestrol
1d
3d
4d
5d
5d
CLDN3/OC
Daidzein
1d
3d
4d
5d
5d
CLDN3/OC
Genistein
1d
3d
4d
5d
5d
CLDN3/OC

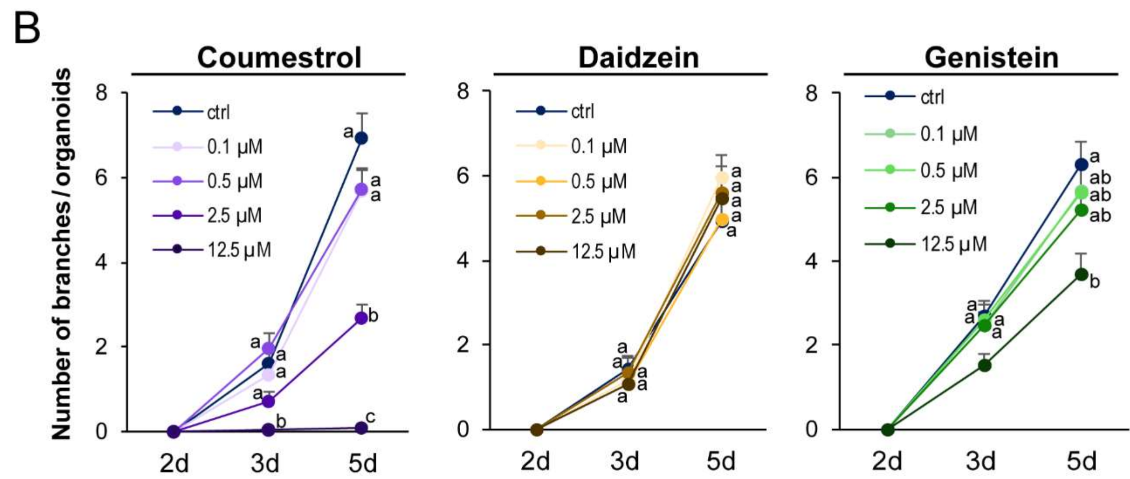

B
Coumestrol
Daidzein
Genistein
Number of branches / organoids
ctrl
0.1 μM
0.5 μM
2.5 μM
12.5 μM
2d
3d
5d

Figure 1

The effects of isoflavones on ductal branching.

The organoids of MECs were cultured for 5 days in the presence of bFGF to induce ductal branching. MECs also treated with each isoflavone at 0.1, 0.5, 2.5, and 12.5 μM for last 4 days. (A) Phase-contrast microscopic images of the organoids cultured with 12.5 μM isoflavones. The organoids of MECs were immunostained with CLDN3 (green) and OC (red) after treatment with each isoflavone at 12.5 μM for 4 days. Blue indicates nuclei stained with DAPI. Scale bars are 50 μm. (B) The graphs show the number of branched regions per organoid 2, 3, and 5 days after cultivation. The data are presented as means ± SEM (n = 24). Different letters indicate significant differences ($p<0.05$).

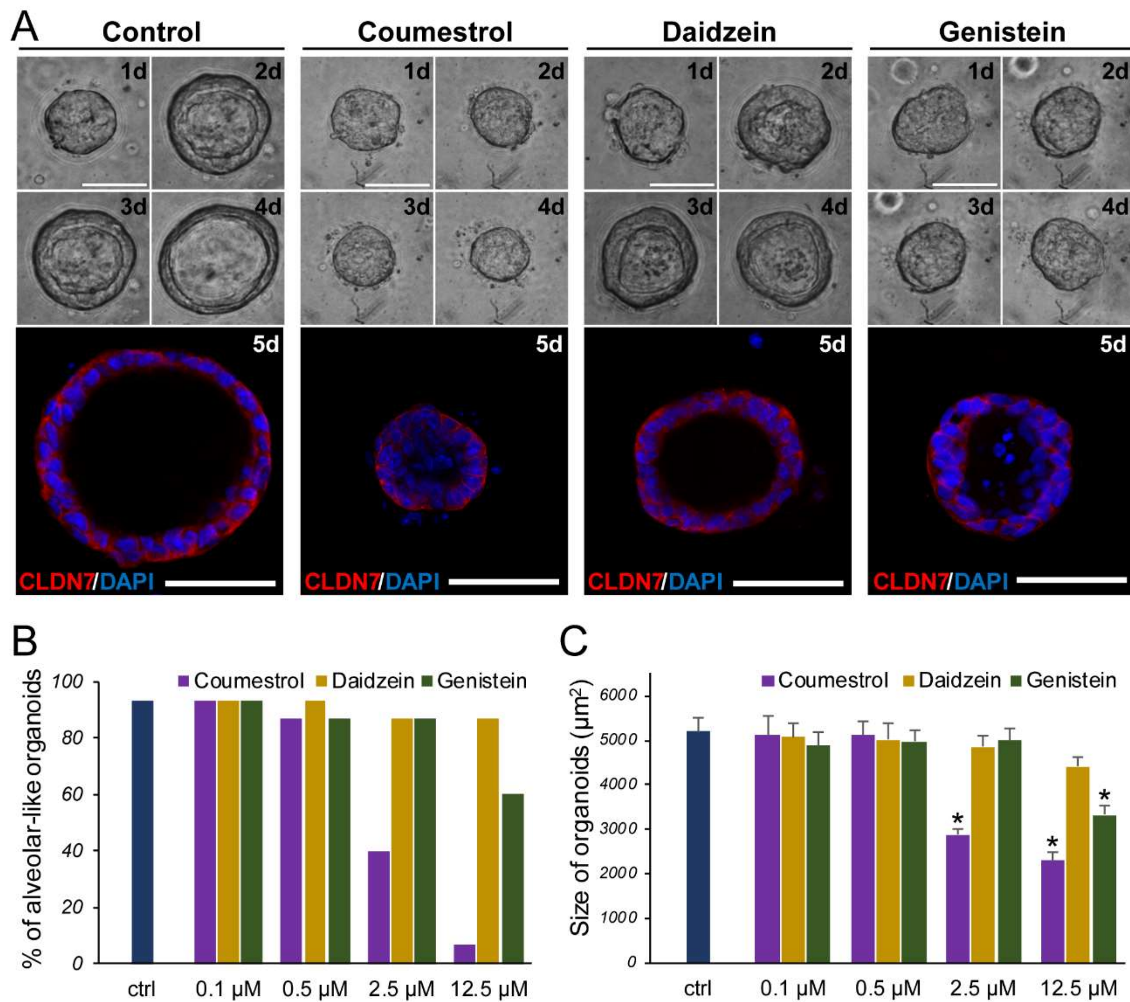


Figure 2

The effects of isoflavones on alveolar structure formation.

The organoids of MECs were cultured or 5 days in the presence of EGF to induce alveolar structure formation. MECs were also treated with each isoflavone at 0.1, 0.5, 2.5, and 12.5 μM 6 h after cultivation. (A) Phase-contrast microscopic images of the organoids cultured with 12.5 μM isoflavones. The organoids of MECs were immunostained with

CLDN7 after treatment with each isoflavone at 12.5 μM for 5 days. Blue indicates nuclei stained with DAPI. Scale bars are 50 μm. (B) The graphs show the ratio of the organoids with the luminal structure per total organoids cultured for 5 days in the presence of each isoflavone. (C) The graphs show the size of organoids cultured for 5 days in the presence of isoflavones. The data are presented as means ± SEM (n = 15). Asterisks show significant differences ($p<0.05$ versus control).

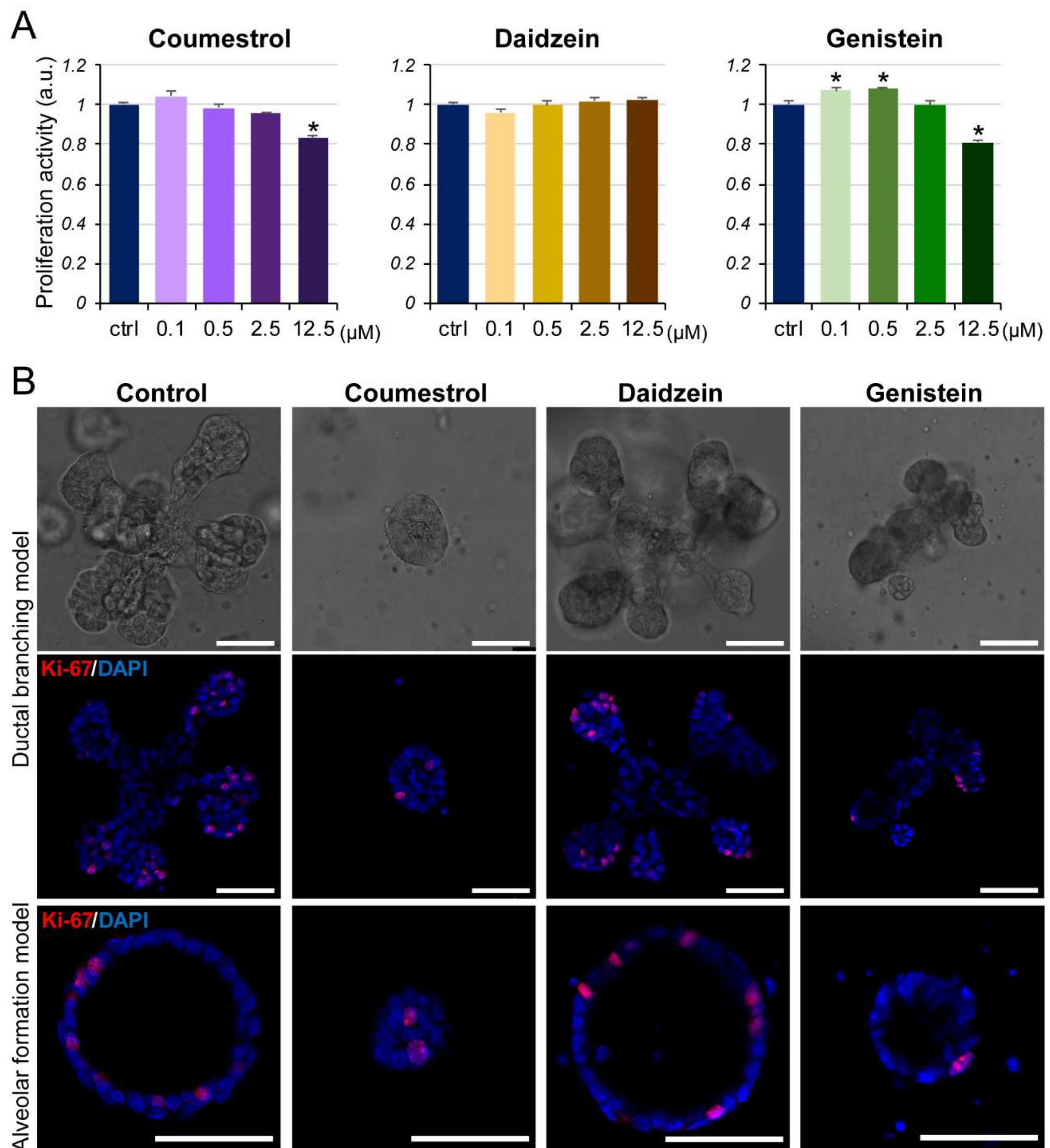


Figure 3

The effects of isoflavones on the proliferation of MECs.

(A) The proliferation activity of MECs, which were seeded in 96-well plates, was measured using the WST-8 assay after treatment with coumestrol, daidzein, and genistein at 0.1, 0.5, 2.5, and 12.5 μM for 3 days. The data are presented as means ± SEM (n = 7).

Asterisks show significant differences ($p<0.05$ versus control). (B) The organoids of MECs in the ductal branching and alveolar formation models were immunostained with Ki-67 (red) after treatment with each isoflavone at 12.5 μM for 4 days. Blue indicates nuclei stained with DAPI. Scale bars are 50 μm.

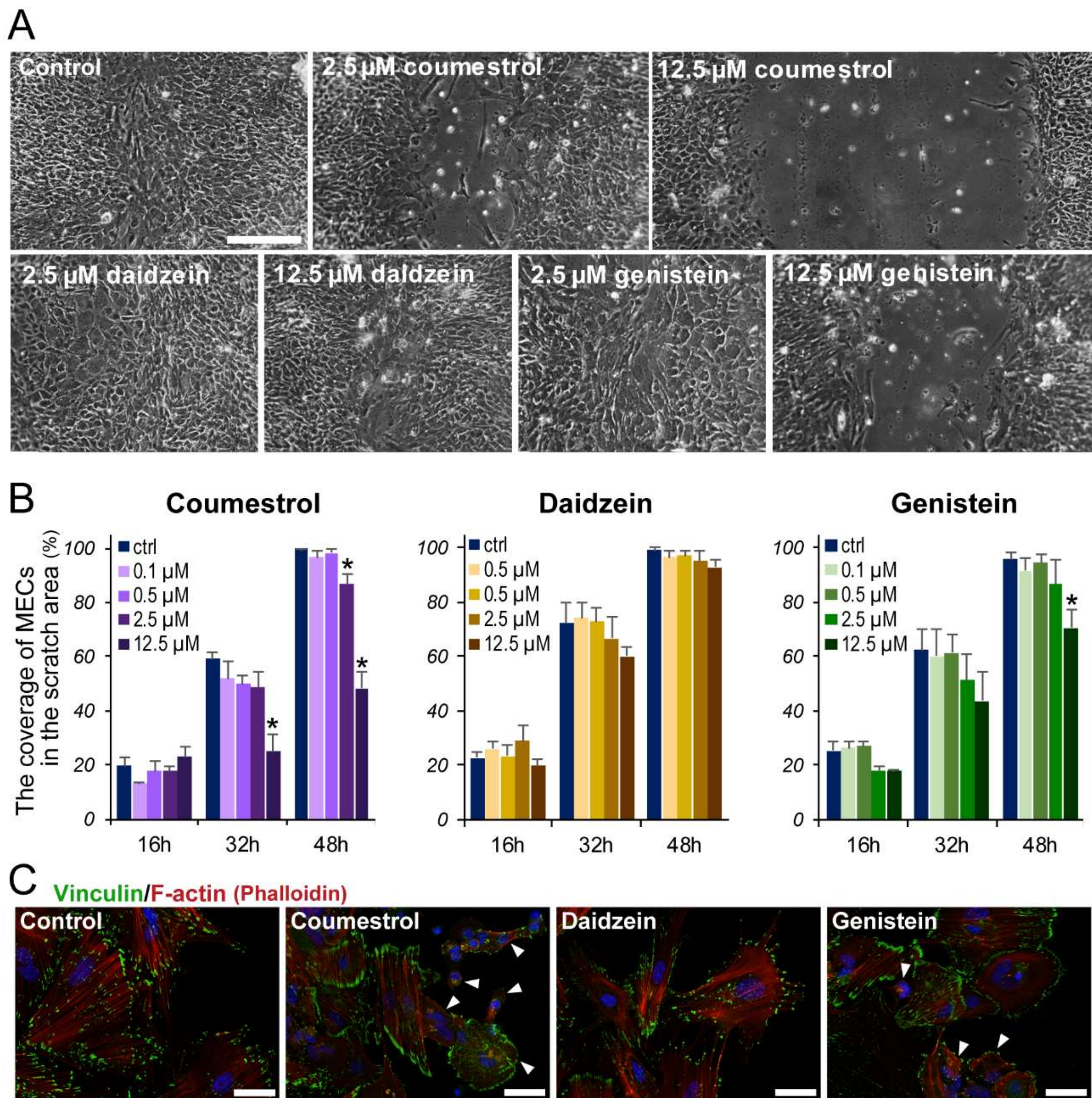


Figure 4

The effects of isoflavones on the migration of MECs.

The influence of each isoflavone on cell migration was assessed by the scratch assay. Confluent MEC layers were scratched at a width of 500 µm and then cultured with each isoflavone at 0.1, 0.5, 2.5, and 12.5 µM for 2 days. The covered area was measured 16, 32, and 48 h after the scratch treatment. (A) Phase-contrast microscopic images show the

MECs covering the scratch regions 48 h after the scratch treatment in the presence of isoflavones. Scale bars are 100 μm. (B) The graphs show the coverage of MECs in the scratch area. The data are presented as means ± SEM (n = 16). Asterisks show significant differences ($p<0.05$ versus control). (C) The MECs covering the scratch regions 24 h after the scratch treatment were immunostained with vinculin (green). Red and blue indicates actin filaments and nuclei stained with phalloidin and DAPI, respectively. Scale bars are 20 μm.

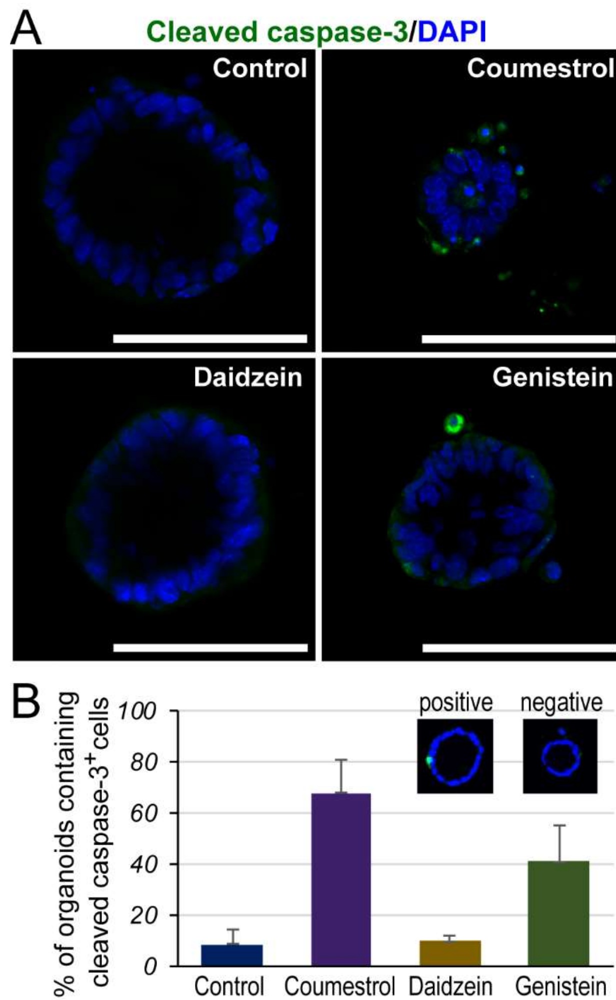


Figure 5

The effects of isoflavones on apoptosis in the organoids of the alveolar model.

(A) Apoptotic cells were detected by immunostaining of cleaved caspase-3 (green) in the organoids of the alveolar models treated with each isoflavone at 12.5 μM for 3 days. Blue indicates nuclei stained with DAPI. Scale bars are 50 μm. (B) The graph shows the ratio of cleaved caspase-3-positive cells per total cells determined by DAPI-positive nuclei. The data are presented as means ± SEM (n = 3).

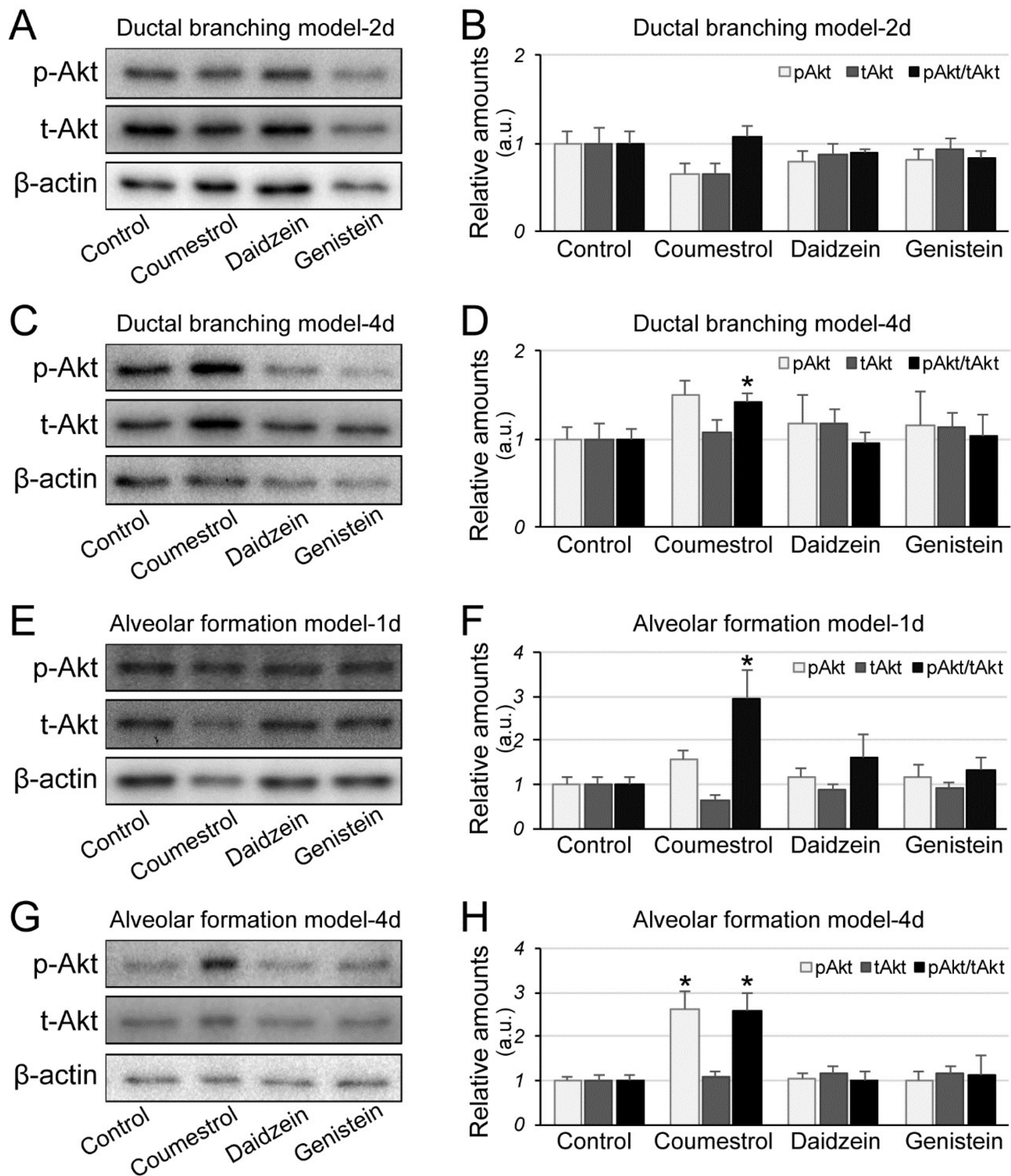


Figure 6

The effects of isoflavones on Akt signaling pathway.

The bands show phosphorylated-Akt (p-Akt) and total Akt (t-Akt) by western blotting in the organoids of the ductal branching model treated with each isoflavone at 12.5 μM

for 2 days (A) and 4 days (C) and in the organoids of the alveolar formation model treated with each isoflavone at 12.5 μM for 1 days (E) and 4 days (G). (B, D, F, H) The graphs show the results of the densitometry analysis of the bands for p-Akt and t-Akt. β-Actin was used as an internal control. The data are presented as means ± SEM (n = 6). Asterisks show significant differences ($p<0.05$ versus control).

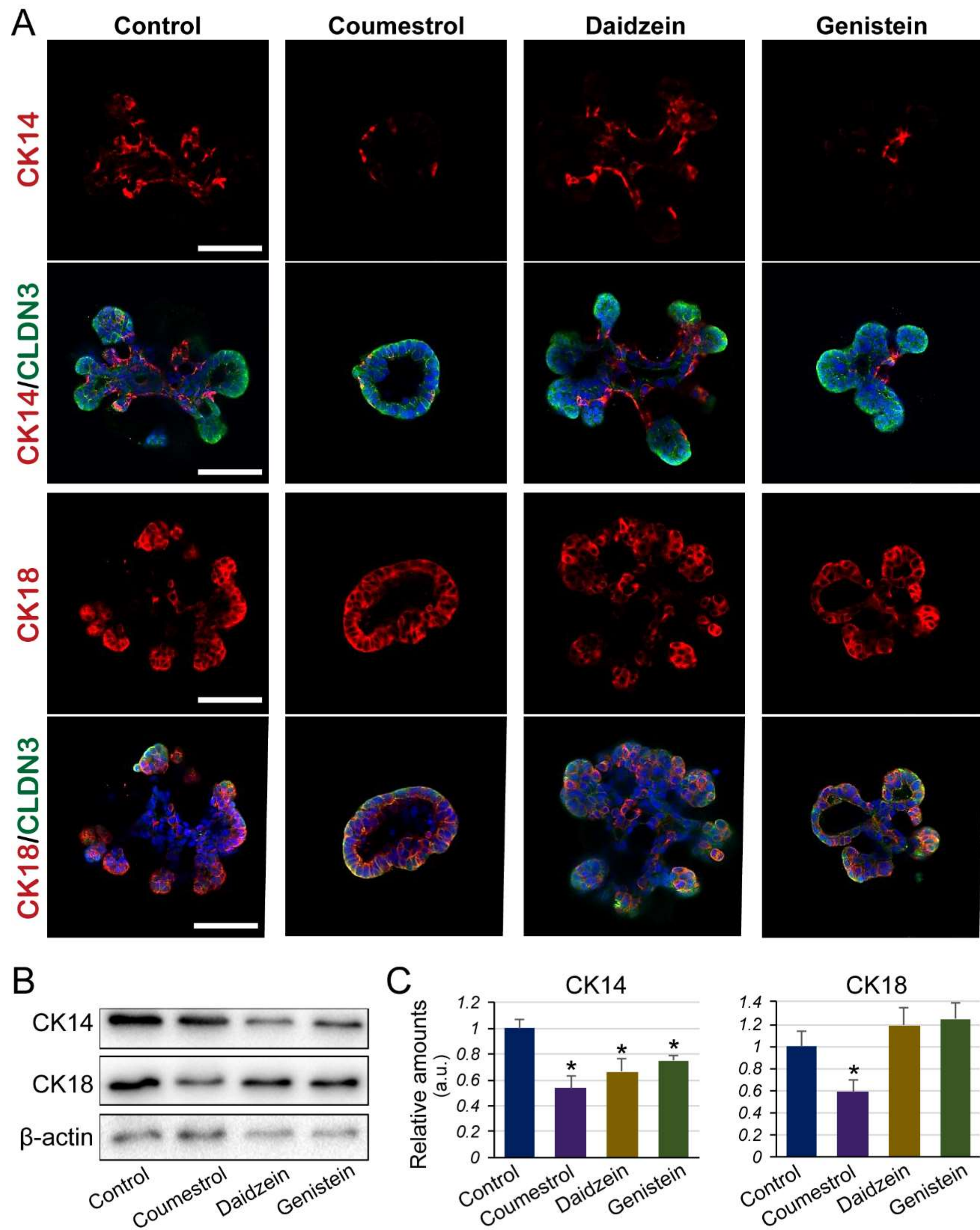


Figure 7

The effects of isoflavones on the expression pattern of CK14 and CK18.

(A) The myoepithelial and luminal cells in the organoids of the ductal branching model

treated with each isoflavone at 12.5 μM for 4 days were detected by immunostaining for CK14 (red) and CK18 (red), respectively. Green and blue indicates CLDN3 and nuclei stained with DAPI. Scale bars are 50 μm. (B, C) The bands show CK14 and CK18 by western blotting in the organoids of the ductal branching model treated with each isoflavone at 12.5 μM for 4 days. The graphs show the results of the densitometry analysis of the bands for CK14 and CK18. β-Actin was used as an internal control. The data are presented as means ± SEM (n = 6). Asterisks show significant differences ($p<0.05$ versus control).

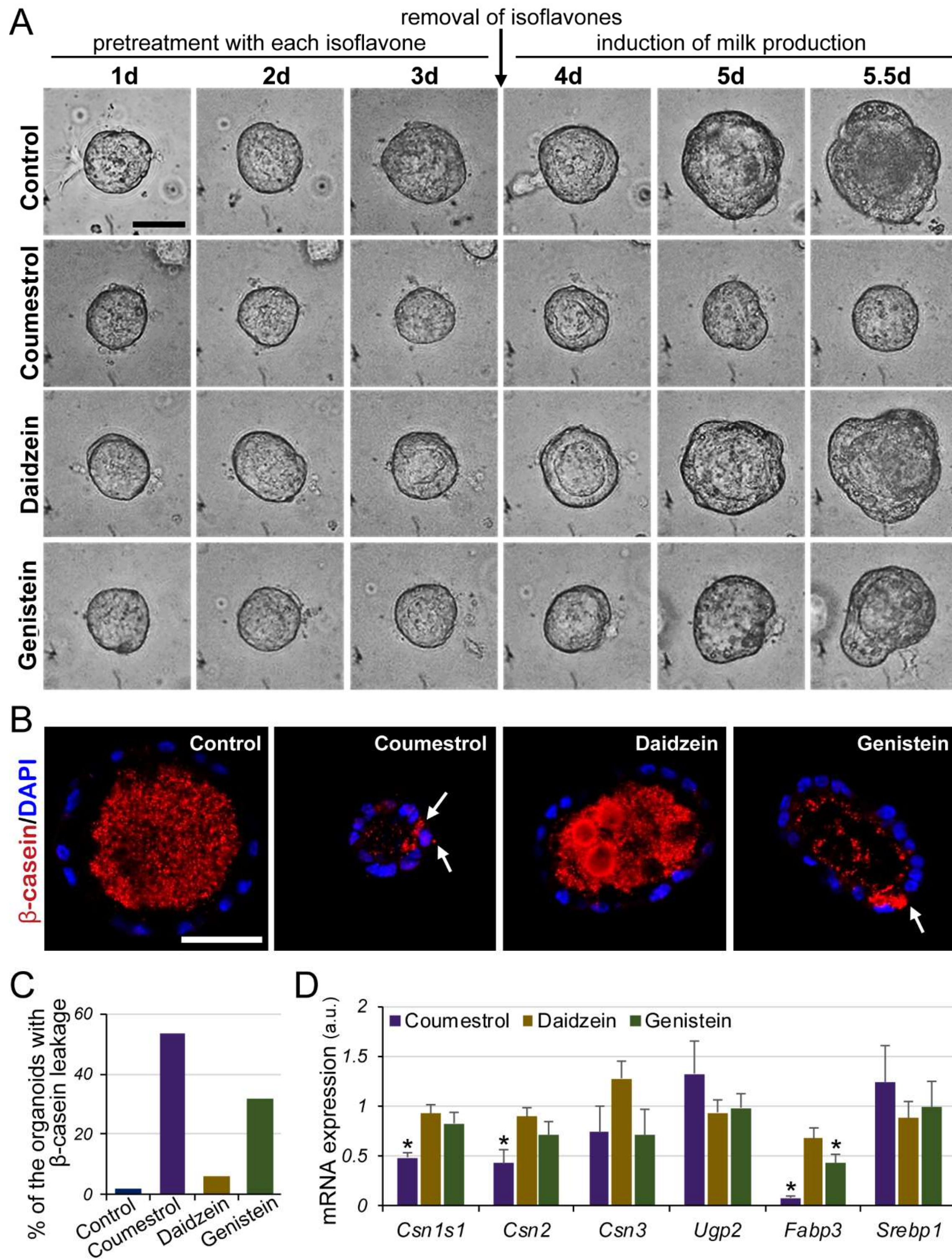


Figure 8

The effects of isoflavone-pretreatment on milk production in MECs.

The organoids of alveolar models pretreated with 12.5 µM isoflavone for 3 days were cultured in differentiation medium to induce milk production ability in MECs for 2.5 days. (A) Phase-contrast microscopic images of the organoids. (B) Immunostaining images show the localization of β-casein (red) in organoids cultured for 2.5 days with the differentiation medium without isoflavones. Blue indicates nuclei stained with DAPI. Scale bars are 50 µm. White arrows indicate the β-casein leakage. (C) The graph shows the ratio of the organoids, revealing the β-casein leakage, per total organoids. (D) Expression levels of caseins (A; *Csn1s1, Csn2, Csn3*), lactose synthesis-related genes (*Ugp2*), and triglyceride synthesis-related genes (*Fabp3*, *Srebp1*) were quantified by real-time PCR. *Gapdh* served as an internal control. The data are presented as means ± SEM (n = 4). Asterisks show significant differences ($p<0.05$ versus control).

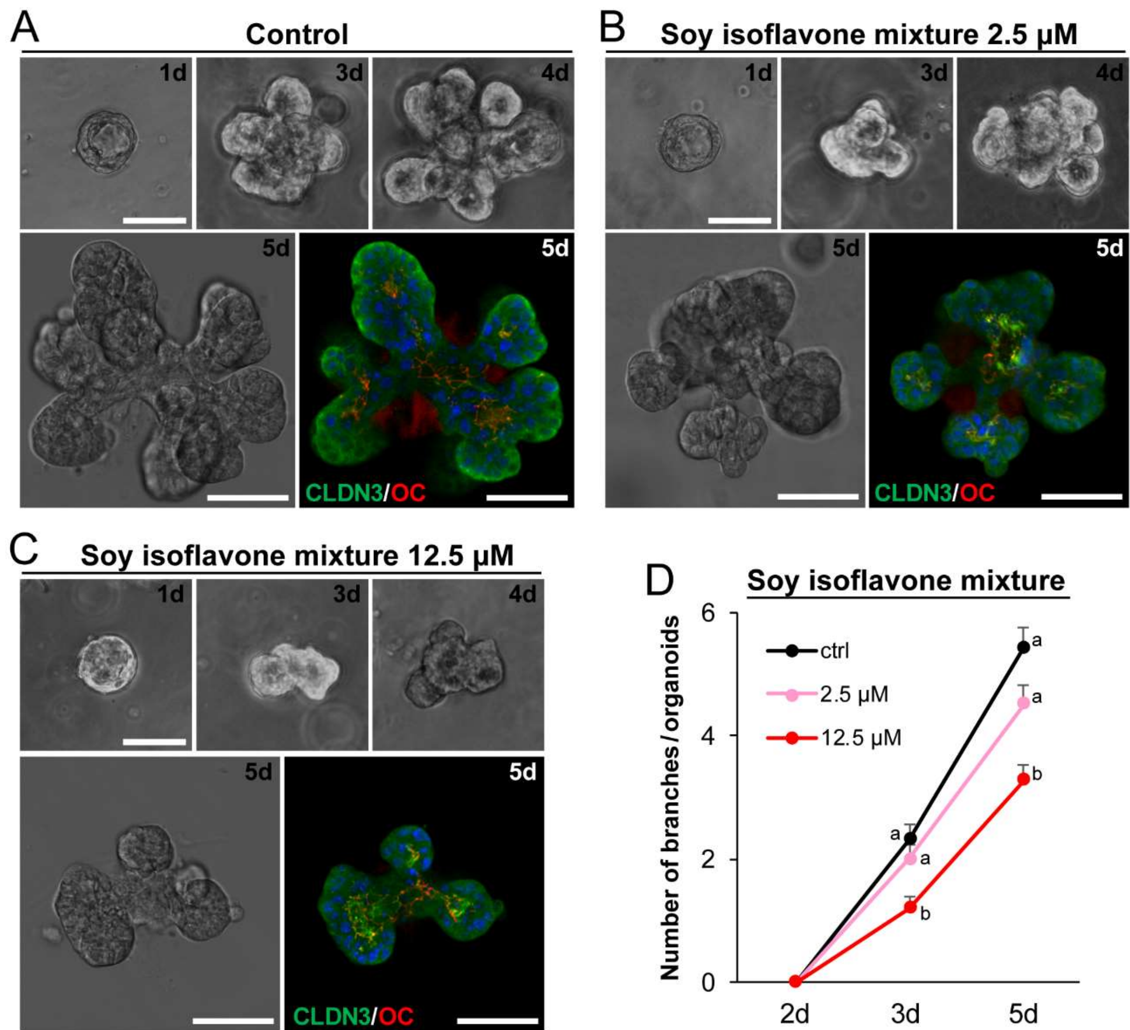


Figure 9

The effects of a soy isoflavone mixture on ductal branching.

The organoids of MECs were cultured for 5 days in the presence of bFGF to induce ductal branching. MECs also treated with the soy isoflavone mixture composed of genistein, daidzein, and equol (the ratio 6: 3: 1) at a total concentration of 2.5 and 12.5 μM for last 4 days. Phase-contrast microscopic images of the organoids untreated (A) and treated with the soy isoflavone mixture at 2.5 μM (B) and 12.5 μM (C). The organoids of

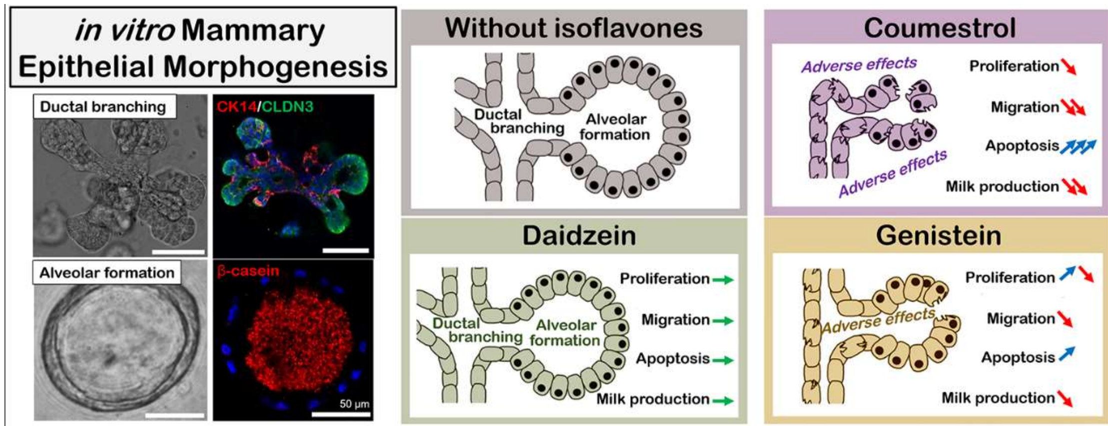
MECs were immunostained with CLDN3 (green) and OC (red) after treatment with the soy isoflavone mixture for 4 days. Blue indicates nuclei stained with DAPI. Scale bars are 50 μm. (D) The graphs show the number of branched regions per organoid 2, 3, and 5 days after cultivation. The data are presented as means ± SEM (n = 25). Different letters indicate significant differences ($p<0.05$).

Table 1

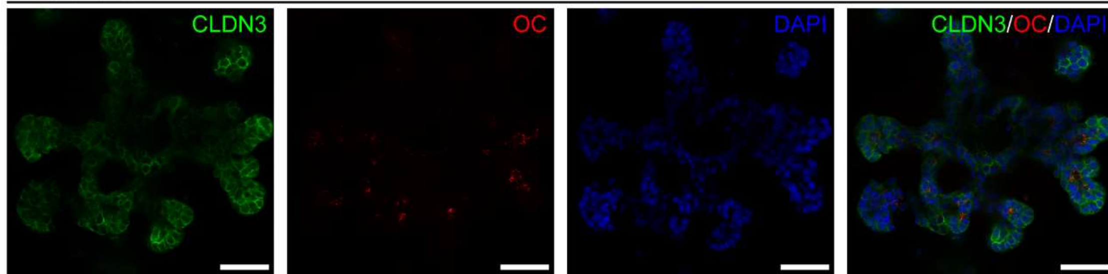
Primer Sequences for real-time PCR

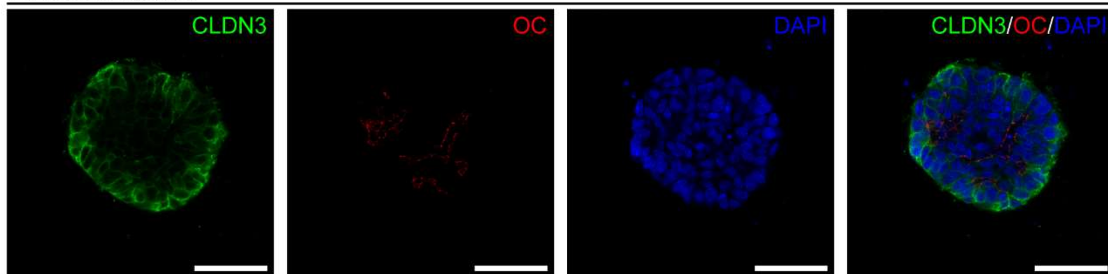
Gene	Accession number	Primers		product
		Forward	Reverse	size
Csn1s1	NM_007784	cctttcccctttgggcttac	tgaggtggatggagaatgga	193
Csn2	NM_009972	cttcagaaggtgaatctcatggg	cagattagcaagactggcaagg	330
Csn3	NM_007786	tcgaccccattactcccattgtgt	tgtaaaaggtaagggaagacgagaaagat	289
Ugp2	NM_139297	tcacaaacaaaacacgagcaga	cacttgagcgatttccacca	89
Fabp3	NM_010174	agtcactggtgacgctggacg	aggcagcatggtgctgagctg	230
Srebp-1	NM_011480	gtcagcttgtggcagtggag	tctgagggtggaggggtaag	90
Gapdh	NM_008084	gagcgagaccccactaacatc	gcggagatgatgaccctttt	144

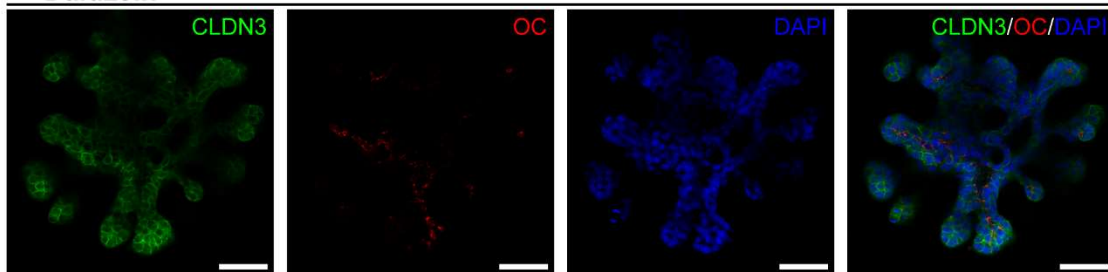
Graphic Abstract

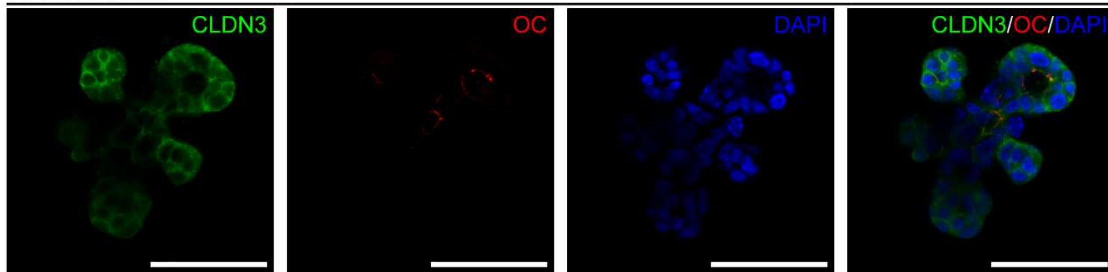


Supporting Information









Supplemental Figure 1

The effects of isoflavones on localization patterns of CLDN3 and OC in ductal branching models.

The organoids of MECs were cultured for 5 days in the presence of bFGF to induce ductal branching (A). MECs also treated with coumestrol (B), daidzein (C) and genistein (D) at 12.5 μM for last 4 days. The organoids of MECs were immunostained with CLDN3 (green) and OC (red). Blue indicates nuclei stained with DAPI. Scale bars are 50 μm.