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Daidzein-rich isoflavone aglycones inhibit cell growth and inflammation in endometriosis

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Highlights

- We proposed the efficacy of DRIAs and dietary supplement on endometriosis.
- DRIAs inhibit cell proliferation in human endometriotic stromal cells.
- DRIAs reduce inflammatory cytokines and exhibit ERβ-mediated activity.
- DRIAs reduce the extent of endometriosis-like lesions in a mouse model.
- DRIAs might be a potential therapeutic option for management of endometriosis.

Abstract

Endometriosis is an estrogen-dependent disease, and isoflavones interact with estrogen receptors. The purposes of this study are to investigate the *in vitro* and *in vivo* effects of daidzein-rich isoflavone aglycones (DRIAs), dietary supplements, on cellular proliferation in endometriosis. Stromal cells isolated from ovarian endometrioma (OESCs) and normal endometrium (NESCs) were cultured with DRIAs, i.e.*i.e.*, each of the DRIA components (daidzein, genistein, or glycitein), or isoflavone glycosides (IG; DRIA precursors). A mouse model of endometriosis was established by transplanting donor-mouse uterine fragments into recipient mice. Our results showed that DRIAs (0.2–20 (0.2–20 μM) inhibited the proliferation of OESCs ($P<0.05$ for 0.2 μM; $P<0.01$ for 2 and 20 μM) but not of NESCs. However, daidzein, genistein, glycitein, and IG did not inhibit their proliferation. DRIA-induced suppression was reversed by inhibition of the estrogen receptor (ER)β by an antagonist, PHTPP, or by ERβ siRNA ($P<0.05$), but not by MPP, an ERα antagonist. In OESCs, DRIAs led to reduced expression

of IL-6, IL-8, COX-2, and aromatase, as well as reduced aromatase activity, serum glucocorticoid-regulated kinase levels, and PGE_2 levels (P<0.05). Western blot and immunofluorescence assays revealed that DRIAs inhibited TNF-α-induced IκB phosphorylation and p65 uptake into the nuclei of OESCs. In the mouse model, a DRIA-containing feed significantly decreased the number, weight, and Ki-67 proliferative activity of endometriosis-like lesions compared to in mice fed with an IG-containing feed and the control feed (P<0.01). In conclusion, DRIAs inhibit cellular proliferation in endometriosis, thus representing a potential therapeutic option for the management of endometriosis.

Abbreviations: DRIAsdaidzeinrich isoflavone aglycones; Iso40Isoflavone40; OESCsovarian endometrioma stromal cells; NESCsnormal endometrium stromal cells; ILinterleukin; ERestrogen receptor

Keywords: aglyconeAglycone; isoflavoneIsoflavone; endometriosisEndometriosis; estrogenEstrogen receptor β; nuclearNuclear factor (NF)-κB

1 Introduction

Endometriosis, a common disease that affects 5–10% of women of reproductive age, is characterized by endometrium-like lesions growing outside of the uterine cavity [1]. Among patients with endometriosis, approximately 50% have major pelvic pain and 40–50% have fertility problems [2]. The substantial improvement in these symptoms after the onset of menopause suggests that estrogen has indispensable roles in the development and maintenance of endometriosis [3]. Estrogen acts mainly viavia estrogen receptor (ER)α and ERβ. Biological actions of estrogen, synthesized by locally elevated aromatase, mediated mainly by ERβ, are crucial for the establishment and maintenance of this disease, as ERβ is highly expressed in local endometriotic lesions [4]. Estrogen has been directly linked with the promotion of inflammation, as evidenced by the estrogen-mediated induction of various cytokines in endometriotic stromal cells (SCs) [5]. These cytokines, including interleukin (IL)-6, IL-8, cyclooxygenase-2 (COX-2), and $PGE_2,$ can stimulate hormone synthesis and regulate the activation of pro-survival signaling pathways, resulting in a feed-forward mechanism that promotes the persistence of lesions [6]. Therefore, endometriosis is considered an estrogen-dependent inflammatory disease [7].

Endocrine preparations currently recommended for the treatment of endometriosis include GnRH agonists, oral contraceptives, and progestogens. Despite the benefits of these therapies, they have undesirable side effects. For example, because these hormonal therapies suppress ovulation, they are not suitable for patients who are trying to conceive. Accordingly, a novel therapeutic strategy with fewer side effects in reproductive-aged patients with endometriosis is needed. Isoflavones are plant-derived nonsteroidal compounds that possess weak estrogenic activity. Structural similarities to estradiol (E2) allow isoflavones to bind to ERs and exert estrogen-like activity. Although the estrogenic activity of isoflavones is very weak, i.e.i.e., 1/1,000 to 1/,000–1/10,000 that of estradiol, some

isoflavones exert anti-estrogenic effects in reproductive-aged women with high estrogen levels [8,9]. Previous studies have shown that isoflavone intake may reduce the risk of breast, prostate, and colon cancer development [10] and improve climacteric disorder symptoms, such as hot flashes and osteoporosis in perimenopausal women [11,12]. Several clinical studies have demonstrated that isoflavones may induce luteal phase support in patients undergoing *in vitro* fertilization-embryo transfer, contributing to elevated rates of successful implantation and pregnancy [13]. However, isoflavones are associated with an increased risk of uterine endometrial hyperplasia and cancer development [14,15].

Isoflavones are present in two forms: aglycones and glycosides. Genistin and daidzin are representative isoflavone glycosides. Although they are abundant in soybeans, isoflavone glycosides have high molecular weights and need to be degraded by intestinal bacteria in order to be absorbed [16]. Isoflavone aglycones, which are metabolites obtained by the removal of sugar chains from glycosides, can be rapidly and efficiently absorbed, without intestinal bacterial degradation [17]. Genistein and daidzein are the major isoflavone aglycones obtained from genistin and daidzin, respectively. Genistein and daidzein show approximately 20-fold and 5-fold higher affinities toward ERβ than ERα [18]. Genistein stimulates tumor growth and attenuates the inhibitory effects of tamoxifen on estrogen-dependent MCF-7 human breast cancer cells [19,20]. Similarly, Kayisli et al. reported that genistein induces cell proliferation in endometrial stromal and cancer cells [21]. Daidzein suppresses tumor growth for MCF-7 xenografts in mice and induces tamoxifen anti-tumor effects [22,23]. These findings indicate that the type of isoflavone determines the effects on targeted cells.

Several studies have investigated the effects of isoflavones on endometriosis. Puerarin and parthenolide, a flavonoid, have been shown to inhibit the proliferation of ovarian endometrioma (OE) cells [24,25]. Genistein caused regression of endometriotic implants in the rat model [26]. However, the effects of isoflavones on endometriosis are still unclear. In this study, we investigated the inhibitory effects of daidzein-rich isoflavone aglycones (DRIAs) on endometriosis using SCs derived from OE (OESCs) and the normal endometrium (NESCs) as well as a mouse endometriosis-like lesion model.

2   Materials and ~~Methods~~methods

2.1   *Isoflavones and the isoflavone metabolite*

AglyMax, a DRIA-containing extract prepared by soybeangerm fermentation using Koji fungus (*Aspergillus awamori*), followed by ethanol/water extraction and purification, was obtained from Nichimo Biotics (Tokyo, Japan). AglyMax contains isoflavone aglycones, including daidzein, genistein, and glycitein at a

ratio of 7:1:2 [27,28]. Isoflavone-40 (Tama Biochemicals, Kawasaki, Japan), an isoflavone glycoside mixture, is a precursor of AglyMax. Daidzein, genistein, glycitein, and equol, an isoflavone metabolite, were purchased from Nacalai Tesque (Kyoto, Japan).

2.2 *Patients and samples*

OE samples were obtained from 24 women of reproductive age (median age, 38.0 years; range, 23–44 years) who underwent surgery for OE. The endometriosis stages of subjects were III (n = 14) and IV (n = 10), according to the revised American Society for Reproductive Medicine classification of endometriosis [29]. Normal endometrium samples were obtained from 12 subjects without endometriosis (NE) (median age, 37.0 years; range, 22–41 years) who underwent surgery for ovarian dermoid cysts. None of the women received hormone treatment for at least 6 months before the surgery. All patients were at the proliferative phase of the regular menstrual cycle. OE and NE samples were used for isolating OESCs and NESCs, respectively.

2.3 *Cell preparation and ribonucleic acid transfection*

Cells were isolated as described previously [30]. Briefly, tissues were minced, digested with 2.5% type I collagenase (Sigma-Aldrich) and 15 IU/mL deoxyribonuclease (DNase) I (Takara Shuzo), and filtered through a nylon cell strainer. The filtrate was centrifuged using Histopaque-1077 (Sigma-Aldrich) to remove red blood cells. Purity (>95%) of all 24 OESC preparations was confirmed by positive CD10 staining and negative staining for cytokeratin (Supplementary Fig. 1 Fig. 1, Table 1 Table 1). Subconfluent primary cultured stromal cells (SCs) were collected after treatment with 0.1% trypsin, resuspended in phenol red-free Dulbecco's modified Eagle medium (DMEM) (Nacalai Tesque) supplemented with 10% dextran-coated, charcoal-treated fetal bovine serum (FBS), and 1% penicillin and streptomycin (100 μg/mL), and subcultured in 96-well plates for the cell proliferation assay and the CytoTox-Fluor cytotoxicity assay, Six-well plates for RNA extraction and in the culture supernatant for Enzyme-linked immunosorbent assay, cover glass for the immunofluorescence cytochemistry, 10-cm dishes for the western blot analysis, 12-well plates for the aromatase enzyme assay. Cells were treated with or without DRIAs (20 μM) for 72 h and tumor necrosis factor (TNF)-α (1 ng/mL) was added to the medium for immunofluorescence cytochemistry and western blot analysis. The test compounds were dissolved in dimethyl sulfoxide and added to the culture medium at a final concentration of 0.1%. ERβ knockdown was performed using RNAiMAX (Invitrogen, Carlsbad, CA, USA) and ERβ short interfering RNA (siRNA; s4826, Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

2.4 *Cell proliferation assay*

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h, the cells were treated with or without methyl-piperidino-pyrazole (MPP) and/or PHTPP (Abcam, Cambridge, UK). The cells were treated with or without DRIAs, Iso-40, daidzein, genistein, glycitein, and equol. WST-8 proliferation activity was determined using SF reagent (Nacalai Tesque) after a 72-h incubation period using a multiwell spectrophotometer multiplex 9120 (Bio-Rad, Hercules, CA, USA) at a wavelength of 450/655 nm.

2.5 *Cytotoxicity determination*

Cytotoxicity was evaluated using the CytoTox-Fluor Cytotoxicity Assay (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. Cells were treated with DRIAs after 24 h. Fluorescence was measured 72 h after treatment with DRIAs at excitation/emission wavelengths of 485/520 nm using a SpectraMax M2e Instrument (Molecular Devices, Sunnyvale, CA, USA).

2.6 *RNA extraction, cDNA preparation, and real-time PCR*

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations were determined using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was prepared using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and GeneAmp PCR 9700 (Applied Biosystems). Quantitative real-time PCR was conducted using Thunderbird Master Mix (Toyobo) and the CFX Connect Real-Time PCR System (Bio-Rad). The PCR mixture contained cDNA samples and 0.3 μM each target gene primer, including those for interleukin (IL)-6, IL-8, aromatase, cyclooxygenase-2 (COX-2), and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All PCR procedures were followed by a melting curve analysis [31].

2.7 *Immunofluorescence cytochemistry*

The cells, fixed with 4% paraformaldehyde in Tris buffered saline (TBS), were then blocked with 5% normal goat serum in TBS containing 0.3% Triton X-100 for 1 h and incubated overnight at 4 °C with primary rabbit polyclonal anti-p65 antibody (Table 1) in TBS containing 1% BSA and 0.3% Triton X-100. Cells were incubated with Alexa-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h and mounted using VECTASHIELD Mounting Medium with Propidium Iodide (Vector Laboratories, Burlingame, CA, USA). An FV1000 confocal laser scanning microscope and FLUOVIEW software (Olympus, Tokyo, Japan) were used for image capture and the cell cycle distribution analysis.

2.8 *Western blot analysis*

Proteins were transferred to polyvinylidene fluoride membranes. The membranes were blocked with blocking buffer (5% skimmed milk in TBS containing 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4 °C with the appropriate primary antibody diluted in blocking buffer. The blots were incubated with secondary antibodies and signals were detected using the Chemi-Lumi One (Nacalai Tesque) and ChemiDoc XRSþ systems (Bio-Rad). Primary antibodies against phosphorylated inhibitor κB (p-IκB), total-inhibitor kB (t-IκB), ERβ, and GAPDH were used (Table 1).

2.9 *Enzyme-linked immunosorbent assay for PGE$_2$*

The concentration of PGE$_2$ in the culture supernatant was determined using the PGE$_2$ High-sensitivity Enzyme Immunoassay (EIA) Kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's instructions.

2.10 *Aromatase enzyme assay*

Aromatase enzyme activity was measured using a tritiated water release method as described previously [30]. Briefly, the cells were incubated for 6 h with serum-free medium containing 100 nM [1β-^{3}H] androstenedione (Perkin Elmer, Waltham, MA, USA) and 500 nM progesterone (Sigma-Aldrich, St. Louis, MO, USA) with or without DRIAs (20 μM) after washing twice with phosphate-buffered saline. Then, steroids were extracted with charcoal. An aliquot was mixed with Clear-sol I (Nacalai Tesque) and radioactivity was measured using a scintillation counter (Beckman Coulter, Brea, CA, USA).

2.11 *Animal experiment*

Female mice (6 weeks of age, BALB/c) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mouse endometriosis model was generated according to the methods described by Takai et al. [25], with some modifications. Recipient mice were ovariectomized and E2 valerate was injected (0.5 mg per mouse per week; Mochida Pharmaceutical Co., Tokyo, Japan) in sesame oil once per week for 6 weeks. Two weeks later, the uterus was removed from the donor mice (n = 10) and minced. The average weight of the minced uterine fragment was 55 ± 3 mg per mouse. A total of 25 mg with 500 mL of saline was injected into the peritoneal cavity of each recipient mouse. One week later, recipient mice were divided into three treatment groups. Each group was given normal food *ad libitum* with or without 0.6% DRIA food or 0.65% Iso-40 (Oriental Kobo Kogyo, Tokyo, Japan) (n = 5–8 mice per group). After 3 weeks, mice were evaluated, and the formation of endometriosis-like lesions in the peritoneal cavity was examined. The endometriosis-like lesions in the whole peritoneal cavity were confirmed by three authors (O.T., T.M., and A.K.). Endometriosis-like lesions were carefully removed from the peritoneal cavity and then weighed successively using a SHIMAZU AUX 120 weighing machine (Kyoto, Japan). Additionally, the effects of DRIAs and Iso-40 on the formation of endometriosis-like lesions were assessed using a microscope. Formalin-fixed specimens were embedded in paraffin and stained with hematoxylin and eosin

according to standard procedures. Anti-Ki-67 primary antibody (Table 1) was used to evaluate cell proliferation. Staining was assessed by three independent observers, who counted Ki-67-positive and -negative nuclei of all stromal cells at 400 × magnification. The average of values obtained in three fields for each lesion was considered the Ki-67-positive ratio.

2.12 *Dose estimation of isoflavones*

DRIAs are a dietary supplement and are often taken orally at a daily dose of 30 mg. The estimated concentration in the blood for this dose of DRIAs is approximately 2 μM, consistent with the concentration examined in this *in vitro* study. To increase the efficacy of this compound in OESCs, 20 μM DRIAs was used in subsequent experiments. Furthermore, to evaluate the effect of DRIAs and Iso-40 on endometriosis-like lesions *in vivo*, we used a mouse model of endometriosis. DRIAs, but not Iso-40, significantly and specifically inhibited the formation and cell proliferation of endometriosis-like lesions in the mouse model, consistent with our *in vitro* results. Based on measurements of food consumption, the estimated average isoflavone intake per mouse was 0.19 g/kg, which was equivalent to 0.23 g/day for a human (60 kg/body) after correction based on the difference in isoflavone metabolism between humans and mice. This dose for mice was approximately seven-fold higher than the daily dose of oral human intake and also comparable with the 2–20 μM DRIAs applied in our *in vitro* study.

2.13 *Statistical analysis*

The experiments with two conditions were analyzed by Mann-Whitney tests. All assays except them were performed using the repeated measures ANOVA, followed by multiple comparisons using Dunnett's procedure. All assays were performed in triplicate. Data are presented as means ± SEM. $P < 0.05$ was considered statistically significant.

3 Results

3.1 *DRIAs, but not isoflavone glycosides, suppressed the proliferation of OESCs*

First, we examined the effects of DRIAs on cell growth in OESCs. DRIAs significantly inhibited the proliferation of OESCs in a concentration-dependent manner (P <0.05 for 0.2 μM DRIAs; P <0.01 for 2 and 20 μM). However, DRIAs did not inhibit the proliferation of NESCs (Fig. 1A). To clarify the difference between aglycone and glycoside, we examined Iso-40, a precursor of AglyMax, on cell growth in OESCs. Iso-40 did not inhibit the proliferation of either OESCs or NESCs (Fig. 1B). Additionally, we confirmed that there was no increase in cytotoxicity when 20 μM DRIAs was applied (Fig. 1C); therefore, we used this concentration for subsequent *in vitro* experiments. Daidzein, genistein, and glycitein are DRIAs constituents. Equal is a representative metabolite of isoflavones. To investigate the inhibitory effect of DRIAs, we performed a cell proliferation assay using each compound as a treatment. However, these compounds did not affect OESC proliferations. (Fig. 1D).

3.2 *DRIAs decreased IL-6, IL-8, COX-2, and aromatase mRNA levels, PGE$_2$ protein levels, and aromatase enzyme activity*

Next, we assessed the IL-6, IL-8, COX-2, and aromatase mRNA levels in OESCs. As shown in Fig. 2 Fig. 2A, IL-6 mRNA expression was suppressed by 30% in OESCs treated with DRIAs than in non-treated OESCs. The mRNA levels of IL-8 in DRIA-treated OESCs were approximately 70% lower than that in the non-treated OESCs. COX-2 and aromatase mRNA levels were significantly lower in OESCs than in the non-treated OESCs (51% and 57% lower, respectively; P <0.01). However, no differences in the mRNA levels of these genes between OESCs and non-treated OESCs were observed after treatment with Iso-40 (Fig. 2A). PGE$_2$ levels were significantly lower in cells treated with DRIAs than in non-treated OESCs (P <0.05; Fig. 2B). In addition to the suppression of aromatase mRNA expression, DRIAs significantly inhibited aromatase activity in OESCs than in non-treated OESCs (P <0.05; Fig. 2C).

3.3 *DRIAs suppressed ~~TNF-α induced~~tnf-α-induced ~~IκB~~iκb expression and p65 uptake in cell nuclei*

We next investigated the effects of DRIAs on intracellular signaling pathways. We confirmed that TNF-α induces the phosphorylation of IκB in OESCs as previously reported [23,24]. Additionally, DRIAs considerably suppressed the phosphorylation of IκB (Fig. 3 Fig. 3A). To confirm these results, we assessed the localization of p65, a representative NF-kB subunit, by immunofluorescence staining. Together with the suppression of IκB phosphorylation, DRIA treatment led to the inhibition of p65 uptake in the nuclei of OESCs (Fig. 3B).

3.4 *DRIAs exhibited ~~ERβ mediated~~erβ-mediated activity in OESCs*

We investigated the mechanisms underlying the ER-mediated effects of DRIAs on endometriotic cells. Proliferation assays demonstrated that the addition of MPP, a specific ERα antagonist, did not affect the DRIA-induced response in OESCs (Fig. 4 Fig. 4A). However, the addition of PHTPP, a specific ERβ antagonist, to the culture medium reversed the DRIA-induced inhibition of cell proliferation (Fig. 4B). We further conducted ERβ knockdown experiments and confirmed the inhibition of ERβ expression at the mRNA and protein levels in OESCs (Figs. 4C and 4D). ERβ knockdown suppressed the DRIA-induced inhibition of proliferation in these cells (Fig. 4E). SGK1 was recently identified as a transcriptional target of ERβ [32]. DRIA treatment resulted in a significant suppression of SGK1 expression in OESCs compared with the expression levels in control cells (P <0.05). Furthermore, E2 treatment alone induced SGK1 expression, whereas the combined treatment with E2 and DRIAs resulted in significant inhibition of SGK1 expression in OESCs, compared with the observed expression following treatment with E2 alone (P <0.01; Fig. 4F).

3.5 *DRIAs reduced the extent of endometriosis-like lesions in a mouse model*

Endometriosis-like lesion samples were obtained from the abdominal cavities of all investigated mice. No mice died during the experiments. Lesion sizes ranged from approximately 2 to 10 mm in diameter (Fig. 5 Fig. 5A). Mice were classified into three groups: those fed with normal food, DRIA-containing DRIAs, or food supplemented with Iso-40. We used DRIAs and Iso-40 in the mouse model to clarify the difference between aglycones and glycosides. The total number of lesions and total weight in the DRIA-fed group were significantly lower than those of the other groups (P <0.01). However, there was no reduction in the total weight or number of lesions in the Iso-40-fed group compared with the control group (Fig. 5B and C). Additionally, there were no differences in the uterine weight or body weight among the three groups (Fig. 5D and E). Based on immunohistochemical analyses, endometriotic lesions were composed of epithelial and stromal cells, as evidenced by CD10, cytokeratin, and vimentin expression in these cells (data not shown). The epithelial cell monolayer lining the cyst was analyzed by Ki-67 immunostaining, and the percentage of Ki-67-stained cells was determined. In endometrial stroma, we demonstrated that the percentage of Ki-67-positive cells decreased significantly following DRIA treatment (P <0.01; Fig. 5F).

4 **Discussion**

We determined that DRIAs suppress cell proliferation in OESCs, but not in NESCs, at clinically feasible concentrations, without increasing cytotoxicity *in vitro,* demonstrating their potential for the treatment of endometriosis. However, the isoflavone-glycoside mixture Iso-40, containing DRIA precursors, did not have inhibitory effects. The observed difference between the effects of DRIA and Iso-40 on OESCs was attributed to the inherent differences between aglycones and

glycosides. This result was supported by our analyses of genistein, daidzein, glycitein, and equol, representative isoflavone aglycones and a metabolite, which had no effects on the proliferation of SCs. Our results indicate that the combination of compounds in DRIAs might be an effective treatment for endometriosis. Endometriosis is an estrogen-dependent inflammatory disease. Bulun [33] proposed a positive feedback cycle of estrogen-dependent growth in local endometriosis tissues. Abnormally elevated aromatase-induced overproduction of E2 in local tissues stimulates the development of endometriosis and upregulates COX-2 expression, which leads to the excessive production of PGE_2, in turn stimulating aromatase expression and activity. Our results indicate that DRIAs could interrupt the harmful cycle of endometriosis growth ~~via~~*via* the inhibition of estrogen synthesis and could relieve endometriosis-associated pelvic pain ~~via~~*via* the inhibition of COX-2 and PGE_2 production.

Previous studies have shown the aberrant secretion of inflammatory cytokines in local endometriotic lesions [34]. In this tissue, activated peritoneal macrophages secrete cytokines, such as IL-6 and IL-8 [35], which stimulate OESC proliferation in a concentration-dependent manner [36]. Therefore, targeting IL-6 and IL-8 expression and activity may help control not only inflammation, but also cell proliferation in endometriosis. Here, we demonstrated that DRIAs inhibit the expression of IL-6 and IL-8, contributing to the suppression of endometriosis development.

We further examined the mechanisms underlying the DRIA-induced suppression of inflammation and cell proliferation. NF-κB is a key regulator of various pathologic and inflammatory responses in endometriosis; in OESCs, it stimulates inflammation and cell proliferation, but inhibits apoptosis [37]. In the cytoplasm, NF-κB consists of subunits of Rel family proteins, including p65, assembled into dimers. NF-κB binds to IκB, an inhibitor of NF-κB, forming an NF-κB -IκB complex in the inactive state. In response to various stimuli, IκB is phosphorylated, and the activated NF-κB is translocated into the nucleus [38]. A recent study demonstrated that activation of the NF-κB -IκB complex could contribute to the elevation of inflammatory cytokines, IL-6 and IL-8 in endometriosis [37]. Our findings suggest that DRIAs may exert anti-inflammatory effects by suppressing both the phosphorylation of IκB and the uptake of p65 into the nucleus.

We analyzed the effects of DRIAs on ER activity in OESCs. Xue et al. [39] showed that ERβ expression levels are higher in endometriotic tissues than in NESCs. The binding affinity of daidzein, a major isoflavone found in DRIAs, to ERβ is five-fold higher in comparison to its binding affinity to ERα [18]. A recent report demonstrated that ERβ-selective antagonists might serve as novel therapeutics for the treatment of endometriosis [2,40]. Monsivais et al. [32] found that SGK1 is transcriptionally regulated by ERβ activation and that it may contribute to an increase in endometriotic cell survival.

Additionally, they demonstrated that E2 and DPN, selective ERβ agonists, induce the expression of SGK1. We demonstrated that DRIAs decrease SGK1 expression, irrespective of E2 treatment. These results suggest that DRIAs possess ERβ antagonist activity in OE tissues. Zhang et al. [41] showed that SGK1 enhances the activity of NF-κB by associating with and activating IκB kinase (IKK), and induces its ability to phosphorylate IκB, which leads to the degradation of IκB and NF-κB activation. Our results demonstrated that the inhibitory effect of DRIAs on OESC proliferation is suppressed by an ERβ antagonist, PHTPP, and that ERβ knockdown significantly inhibits DRIA effects. However, the ERα antagonist MPP did not reverse the effects of DRIAs, demonstrating that DRIA activity is mediated by ERβ, but not ERα, in OESCs. Furthermore, we showed that DRIA treatment leads to a decrease in SGK1 expression and TNF-α-induced IκB phosphorylation, most likely by interacting with ERβ, and these compounds have anti-inflammatory activity.

Several studies have investigated the effects of isoflavones on endometriosis. Yavuz et al. previously reported that genistein caused a regression of

endometriotic implants in a rat model [26], which contradicts our findings from human endometriotic stromal cells and the mouse model. Soukup et al.

previously demonstrated that the major metabolites of genistein in female rat were predominantly 7-glucuronides (81-93%)(81–93%), while those in human

were 7-sulfo-4'4'-glucorunides (39-49%) and diglucuronide (34%) and those in mice were monosulfates (33-41%)[42]. The remarkable differences in

isoflavones metabolites between species might explain the contradiction between our findings and the previous report. Next, to clarify the

difference between aglycones and glycosides, we used Iso-40, isoflavone glycoside, as well as the control group in the mouse model. Interestingly,

Iso-40 did not reduce the total number of endometriosis-like lesions. Soukup et al. also reported that the conversion rate was low in mice and much

lower in humans [42]. Thus, orally administered Iso-40 did not reach or affect the endometriotic-like lesions. There have been some studies comparing the

bioavailability of isoflavones aglycones with that of glucosides. Izumi et al. reported that the highest serum concentration after aglycones intake was more than two-times greater than that after glucosides in humans [16]. The concentration of DRIAs and Iso-40 in blood stream is still unknown in both humans and mice. However, Okabe et al. proved that aglycones possessed higher bioavailability than glycosides when administered orally [43]. In addition, the representative aglycones used in our experiments were synthetic one of which activity was still unknown. From our results, we could consider that synthetic aglycones have less activity than aglycones in DRIAs extracted from natural soybeans. Therefore, one hypothesis that explains the discrepancy between Iso-40 and DRIAs in this study is that isoflavone aglycones could be absorbed faster and in greater amounts than glycosides [16,43]. Taken together, the inconsistencies in bioactivity that we noted between DRIAs, Iso-40, and the constituents might be involved in the differences of the metabolism of isoflavones. The driving metabolites to affect the endometriosis should be further elucidated.

The main limitation of this study was the use of *in vitro* and mouse experiments. In the *in vitro* study, our conclusions are supported by the observation that the parameters were only altered in OESCs and not in NESCs. In contrast, the mouse model was applicable to peritoneal endometriosis but not ovarian

endometriosis because we used ovariectomized mice. Furthermore, in our mouse model, uterine tissue was implanted instead of endometriotic tissue. However, previous evidence showed this ~~in vivo~~*in vivo* model was useful for models for endometriosis [25].

In conclusion, our results indicate that DRIAs, a dietary supplement, exert inhibitory effects on OESC proliferation. This effect is most likely mediated by the inhibition of NF-κB activation and PGE$_2$ formation ~~via~~*via* the inhibition of aromatase activity and COX-2 expression. Additionally, DRIAs inhibit the formation of endometriosis-like lesions *in vivo*, indicating that DRIAs may be useful to treat patients with endometriosis and may contribute to pain relief. The results of this study suggest that DRIAs are a potential therapeutic option for the management of endometriosis. Endocrine preparations for the treatment of endometriosis have undesirable side effects; as a non-hormonal preparation with fewer side effects, DRIAs could be widely used as a new therapeutic target for endometriosis. Further investigation of the molecular mechanisms underlying the observed effects of DRIAs may lead to a better understanding and improved treatment of endometriosis. Clinical trials are necessary to clarify the effects of DRIAs in patients with endometriosis.

5 Declaration of interests

O.T., T.M., F.I., A.K., Y.T., H.K., H.O., I.K., and J.K. have nothing to disclose. S.S. and T.A. are employed by Nichimo Biotics Company Co., Ltd.

Appendix A   Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.jsbmb.2018.04.004.

Appendix B   [{(Appendix A)}] Supplementary data

The following is Supplementary data to this article:

mmc1

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Fig. 1   DRIAs suppressed proliferation of ovarian endometrioma stromal cells. (A) WST-8 assay of ovarian endometrioma stromal cells (OESCs) and normal endometrial stromal cells (NESCs) treated with DRIAs. (B) WST-8 assay of OESCs and NESCs treated with Iso-40. (C) Viability assay of OESCs. (D) WST-8 assay of OESCs treated with daidzein, genistein, glycitein, or equol. *P <0.05, **P <0.01 versusversus the control. V indicates the vehicle only. Solid and open bars indicate OESCs and NESCs, respectively.

Fig. 2   DRIAs decreased IL-6, IL-8, COX-2, and aromatase mRNA levels, PGE$_2$ protein levels, and aromatase enzyme activity. (A) Quantitative reverse transcription PCR analysis of interleukin (IL)-6, IL-8, COX-2, and aromatase mRNA levels for OESCs treated with DRIAs and Iso-40, or non-treated OESCs (vehicle only). (B) PGE$_2$ production in OESCs was detected using enzyme-linked immunosorbent assay (ELISA). (C) Aromatase activity was measured using

the tritiated water release method. *P <0.05, **P <0.01 ~~versus~~*versus* non-treated OESCs (V). Hatched bars indicate DRIAs, dotted bars indicate Iso-40, and horizontal striped bars indicate the non-treated OESCs.

Fig. 3 DRIAs suppressed TNF-α-induced IκB expression and p65 uptake in cell nuclei. (A) Western blot analysis to detect TNF-α-induced phosphorylation of IκB in OESCs. (B) Immunofluorescence cytochemistry of p65 uptake by OESC nuclei stimulated by TNF-α.

Fig. 4 DRIAs exhibited ERβ-mediated activity in ovarian endometrioma stromal cells. (A) WST-8 assay after DRIA treatment of OESCs with (bars with oblique lines) and without (solid bars) MPP, an ERα antagonist and (B) PHTPP (bars with double oblique lines), an ERβ antagonist. (C) The efficiency of ERβ knockdown was evaluated by real-time PCR and (D) western blot analysis. (E) WST-8 assay of OESCs after transfection with negative control siRNA (siNeg) or ERβ siRNA (siERβ). (F) Quantitative reverse transcription PCR to evaluate the effect of DRIAs and E2 on SGK1 expression. *P <0.05, **P <0.01. V indicates vehicle only. NS indicates not significant.

Fig. 5 DRIAs reduced the extent of endometriosis-like lesions in a mouse model. (A) Representative endometriosis-like cystic lesions in the abdominal cavity of recipient mice fed with normal food (Cont), DRIA-containing food (DRIAs), or Iso-40-containing food (Iso-40). Comparison of lesions with respect to total number (B), weight (C), and uterine weight (D) between the control (open bars), DRIA (solid bars), and Iso-40 treatments (dotted bars). (E) Comparison of body weight between the control (solid circles), DRIAs (solid triangles), and Iso-40 (solid squares) treated groups. (F) Ratio of Ki-67-immunostained stromal compartments in endometriosis-like lesions of control and DRIAs-treated groups. The average of values obtained in three fields for each lesion was considered for calculating the Ki-67-positive rate. **P <0.01. NS indicates not significant.

Table 1 List of antibodies used in this study.

Name of the antibody	Manufacturer, catalogue #.	Peptide/ protein target	Species raised, monoclonal,	Dilution used

	batch #		polyclonal, antigen purified	
Anti-Human CD-10	Dako Clone 56C6 Code M7308	Human CD10	Monoclonal Mouse	1:80
Anti-cytokeratin	Sigma-Aldorich C2526 074M4759V	Cytokeratin from human epidermis.	Monoclonal Mouse	1:200
Phospho-IκB (Ser32)(14D4)	Cell Signaling Technology #2859	Human IkB	Monoclonal Rabbit	1:1000
IκB (44D4)	Cell Signaling Technology	Human IkB	Monoclonal Rabbit	1:1000

	#4812			
ERβ	Santa Cruz Biotechnology Sc-373853	ERβ of human origin	Monoclonal Mouse	1:1000
GAPDH (14C10)	Cell Signaling Technology #2118	Human GAPDH	Monoclonal Rabbit	1:1000
NFκB p65 (C-20)	Santa Cruz Biotechnology Sc-372	NFκB p65 of human origin	Polyclonal Rabbit	1:50
Anti-Human Ki-67	Dako Clone MIB-1 Code M7240	Human Ki-67	Monoclonal Mouse	1:150