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Genistein Exposure Inhibits Growth and Alters Steroidogenesis in Adult Mouse Antral Follicles

Shreya Patel[a], **Jackye Peretz**[a], **Yuan-Xiang Pan**[b], **William G. Helferich**[b], and **Jodi A. Flaws**[a]

[a]Department of Comparative Biosciences, University of Illinois 2001 S. Lincoln Ave, Urbana, IL 61802

[b]Department of Food Science and Human Nutrition, University of Illinois 905 S. Goodwin, Urbana, IL 61801

Abstract

Genistein is a naturally occurring isoflavone phytoestrogen commonly found in plant products such as soybeans, lentils, and chickpeas. Genistein, like other phytoestrogens, has the potential to mimic, enhance, or impair the estradiol biosynthesis pathway, thereby potentially altering ovarian follicle growth. Previous studies have inconsistently indicated that genistein exposure may alter granulosa cell proliferation and hormone production, but no studies have examined the effects of genistein on intact antral follicles. Thus, this study was designed to test the hypothesis that genistein exposure inhibits follicle growth and steroidogenesis in intact antral follicles. To test this hypothesis, antral follicles isolated from CD-1 mice were cultured with vehicle (dimethyl sulfoxide; DMSO) or genistein (6.0 and 36 μM) for 18 – 96 hours (h). Every 24 h, follicle diameters were measured to assess growth. At the end of each culture period, the media were pooled to measure hormone levels, and the cultured follicles were collected to measure expression of cell cycle regulators and steroidogenic enzymes. The results indicate that genistein (36 μM) inhibits growth of mouse antral follicles. Additionally, genistein (6.0 and 36 μM) increases progesterone, testosterone, and dehydroepiandrosterone (DHEA) levels, but decreases estrone and estradiol levels. The results also indicate that genistein alters the expression of steroidogenic enzymes at 24, 72 and 96 h, and the expression of cell cycle regulators at 18 h. These data indicate that genistein exposure inhibits antral follicle growth by inhibiting the cell cycle, alters sex steroid hormone levels, and dysregulates steroidogenic enzymes in cultured mouse antral follicles.

Keywords

genistein; ovary; follicle; steroidogenesis

Corresponding author: Jodi A. Flaws, Ph.D., Professor, Department of Comparative Biosciences, University of Illinois, 2001 S. Lincoln Ave, Urbana, IL 61802, Phone: 217-333-7933, Fax: 217-244-1652, jflaws@illinois.edu.

E-mail addresses: Shreya Patel: Shreya.patel@214@gmail.com
Jackye Peretz: Jackye.peretz@gmail.com
Yuan-Xiang Pan: yxpan@illinois.edu
William Helferich: helferic@illinois.edu
Jodi Flaws: jflaws@illinois.edu

Introduction

Recent studies indicate that numerous people consume botanical compounds to prevent or treat a variety of medical conditions, including cancer, kidney disease, cardiovascular disease, neuronal injury, sexual dysfunction, inflammation, depression, and menopausal symptoms (Shibata, 2000; Ho and Jie, 2007; Andres *et al*., 2011; Chen *et al*., 2011; Khan *et al*., 2011). Many botanical compounds are known phytoestrogens, plant-derived chemicals that can bind to and signal through estrogen receptors (Kuiper *et al*., 1998; Khan *et al*., 2011; Yoon *et al*., 2014). Genistein is the predominant phytoestrogen in soy (*Glycine max*) and soy-derived products. It accounts for two thirds of soy isoflavone content and has been shown to bind both estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2), though it is thought to have a greater affinity for ESR2 than ESR1 (Kuiper *et al*., 1998; Khan *et al*., 2011; Yoon *et al*., 2014). Genistein is also a nutraceutical compound used as a chemo-preventive agent in women undergoing chemotherapy and as a treatment for menopausal symptoms (Khan *et al*., 2011; Yoon *et al*., 2014). Genistein is, therefore, considered to be one of the most relevant environmental estrogens in the human diet (Reinli and Block, 1996; Khan *et al*., 2011).

Although studies indicate that botanical compounds such as genistein may be useful in treating some adverse medical conditions (Khan *et al*., 2011; Yoon *et al*., 2014), the broader physiological impacts are less well understood. In particular, genistein exposure is a concern because it binds to estrogen receptors and can impact estrogen signaling pathways, such as steroidogenesis, which may have long-lasting adverse female health effects. Very little is known about the impact of botanical compounds on the ovary, the major site of estrogen receptors, steroidogenesis, and estradiol biosynthesis. Proper functioning of ovarian follicles is absolutely required for normal female fertility and estradiol biosynthesis. Further, low estradiol levels have been associated with reduced fertility, cardiovascular disease, mood disorders, adverse menopausal symptoms, and osteoporosis (Bush *et al*., 1987; Armamento-Villareal *et al*., 1992; Bagur and Mautalen, 1992; Christiansen, 1993; Everson *et al*., 1995; Cooper and Sandler, 1998; Mosca, 1998; Dennerstein *et al*., 1999; Hu *et al*., 1999). Thus, botanical compounds that target the ovary have the potential to adversely impact overall female health.

Unfortunately, studies on the effects of genistein on the ovary are limited and mainly focus on the impact of embryonic and neonatal exposures to genistein on the developing ovary (Chen *et al*., 2007; Jefferson *et al*., 2007; Jefferson *et al*., 2009). Such studies have shown that embryonic or neonatal exposures to genistein cause the development of multi-oocyte follicles, increase atresia (Medigovic *et al*., 2012), and reduce fertility in rodents (Britt *et al*., 2005; Jefferson *et al*., 2005; Chen *et al*., 2007; Jefferson *et al*., 2007; Jefferson *et al*., 2009; Cimafranca *et al*., 2010; Zhuang *et al*., 2010). In addition, one study indicates that exposure to genistein after weaning alters circulating estradiol levels in rats, with the lower dose (10 mg/kg for three weeks) increasing estradiol levels and the higher dose (100 mg/kg for three weeks) inhibiting estradiol levels (Zin *et al*., 2013).

Although the impacts of genistein on the developing and pre-pubertal ovary are important, it is also important to evaluate the effects of botanical compounds on the adult ovary. Women are often exposed to botanical compounds in their diet and as potential preventive agents and treatments for a variety of conditions, including cancer, neuronal loss, kidney disease, and menopausal symptoms (Khan *et al*., 2011). Unfortunately, epidemiological studies investigating the effects of botanicals, including genistein, on women's health are scarce. One study suggests that consumption of a soy-based diet is associated with decreased circulating estradiol levels in premenopausal women (Lu *et al*., 2001). Another study indicates that genistein inhibits steroidogenesis and steroidogenic enzymes in cultured human luteinized granulosa cells (Whitehead *et al*., 2002; Lacey *et al*., 2005; Rice *et al*., 2006). Interestingly, some studies using animal models suggest similar effects on steroidogenesis in isolated and cultured ovarian follicular cells. Genistein inhibits steroidogenesis or steroidogenic enzymes in cultured rat pre-antral follicles (Myllymaki *et al*., 2005), rat granulosa-luteal cells (Whitehead and Lacey, 2000), and porcine granulosa, theca, or luteal cells (Gregoraszczuk *et al*., 1999; Nynca and Ciereszko, 2006; Tiemann *et al*., 2007; Basini *et al*., 2010). Exposure to genistein also alters follicle growth (Zhuang *et al*., 2010), induces follicular atresia (Zin *et al*., 2013), and inhibits oocyte maturation (Chan, 2009). However, these studies have not examined the effects of genistein on the intact, adult antral follicle, the functional unit of the ovary. Further, they have not fully determined the mechanism by which genistein alters steroidogenesis in the adult ovary. Thus, the goal of these studies was to test the hypotheses that genistein inhibits 1) antral follicle growth and steroidogenesis in the adult mouse ovary and 2) steroidogenesis by inhibiting the levels of precursor hormones and the necessary steroidogenic enzymes.

Materials and Methods

Chemicals

Genistein (98% purified via HPLC, Botanical Research Center, University of Illinois) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) then diluted in DMSO to achieve final treatment concentrations of 1.6 and 9.6 μg of genistein per mL of culture media (6.0 and 36 μM). These concentrations were selected based on previous studies using a range of similar doses that show that genistein affects female reproductive function (Gregoraszczuk *et al*., 1999; Whitehead and Lacey, 2000; Jefferson *et al*., 2002). The concentrations are higher than observed *in vivo* after dietary consumption in rodents (Santell *et al*., 1997; Holder *et al*., 1999; Chang *et al*., 2000; Doerge *et al*., 2002), but serve as models for the mechanistic effects of genistein on the ovary.

Animals

Adult, cycling, female CD-1 mice were purchased from Charles River (Wilmington, MA). The mice were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility and were provided food (Harlan Teklad 8626) and water for *ad libitum* consumption. Temperature was maintained at 22 ± 1°C and animals were exposed to 12 hour light-dark cycles. The Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign approved all procedures concerning animal care, euthanasia, and tissue collection.

In vitro time-course follicle culture

Female CD-1 mice were euthanized on postnatal days (PND) 32–35 and their ovaries removed using aseptic technique. Antral follicles were mechanically isolated from the ovary based on relative size (250–400 μm), cleaned of interstitial tissue using fine watchmaker forceps, individually placed in wells of a 96-well culture plate, and covered with unsupplemented α-minimum essential media (α-MEM) prior to treatment. Follicles from 2–3 mice were isolated per experiment, providing approximately 20–40 antral follicles from each mouse. Each experiment contained a minimum of 8–12 follicles per treatment group.

Concentrations of vehicle control (DMSO) and genistein (6.0 and 36 μM) were individually prepared in supplemented α-MEM. Supplemented α-MEM was prepared with the following: 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml human recombinant follicle-stimulating hormone (FSH; Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor- UCLA Medical Center, Torrance, CA), 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) (Cortvrindt and Smitz, 2002; Miller *et al*., 2005; Gupta *et al*., 2006). Various stock concentrations of genistein were prepared (2.2 and 12.8 mg/mL) so that an equal volume of chemical could be added to each well to control for solvent concentration (0.75 μL of genistein or DMSO per mL of media).

Antral follicles were cultured from 18 to 96 h in an incubator supplying 5% CO_2 at 37°C. After each culture, follicles were pooled by treatment group and snap-frozen in liquid nitrogen and then subjected quantitative real time PCR (qPCR) as described below. Media were collected and stored at −80°C until subjected to hormone assays as described below.

Analysis of follicle growth

To evaluate follicle growth over time, follicles were measured along perpendicular axes every 24 h for 96 h. The diameters were recorded in microns, averaged among treatments groups per 24 h interval, and then converted to percent change at each time-point. Percent change was determined by dividing the average diameters of the follicles at each 24 h interval per treatment group by the initial average measurement (0 h) of each respective treatment group.

Analysis of hormone levels

Media were collected from the *in vitro* culture system at 24 h intervals, from at least three separate experiments, and subjected to enzyme-linked immunosorbent assays (ELISA) for measurement of progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, estrone, and estradiol levels. ELISA kits were purchased from DRG International Inc. (Springfield, NJ). Assays were run according to the manufacturer's instructions. Some samples were diluted to match the dynamic range of each ELISA kit. The samples were run in triplicate and had intra- and inter-assay coefficients of variability below 10%.

Analysis of gene expression by qPCR

Cultured antral follicles were collected at 24 h intervals and total RNA was extracted using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. Reverse transcriptase generation of cDNA was performed with 0.3–0.5 μg of total RNA using an iScript RT kit (Bio-Rad Laboratories, Hercules CA). qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) and accompanying software (CFX Manager Software) according to the manufacturer's instruction. qPCR was performed using 1 μL cDNA, 1 μL of gene specific primers (Integrated DNA Technologies, Inc., Coralville, IA; Table 1), 3 μL of molecular water, and 5 μL of a SsoFast EvaGreen Supermix qPCR kit (Bio-Rad laboratories, Hercules, CA) per sample. The qPCR program protocol was similar to that used in previous studies (Hannon *et al*., 2015). A standard curve was generated from five serial dilutions of a combination of samples to calculate the amplification efficiency of each primer and determine relative expression of each target gene to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). *Gapdh* was used as a reference gene for each sample because its expression did not differ between treatment groups.

Statistical Analyses

All data were analyzed using SPSS software (SPSS Chicago, IL) and expressed as mean ± the standard error of the mean (SEM). All normally distributed data were analyzed using a one-way analysis of variance test (ANOVA). A Dunnett's t post-hoc test was used for the follicle growth and gene expression analyses, and a Tukey's post-hoc test was used for hormone level data analysis. Any non-normally distributed data were analyzed using a Kruskal-Wallis test. Statistical significance was assigned at $p \leq 0.05$, $n = 3 - 5$ separate experiments. At 48 h, DHEA levels for the DMSO and genistein 1.6 μM groups were below the level of detection of our ELISA kits. Thus, to complete statistical analysis, we used the lowest detectable amount of DHEA provided by the kit.

Results

Effect of Genistein on Follicle Growth

Follicles treated with vehicle control (DMSO) grew significantly over time (Figure 1). However, exposure to genistein (36 μM) significantly inhibited antral follicle growth compared to DMSO, beginning at 24 h and continuing throughout the 96 h culture (Figure 1; $n=3-5$, $p \leq 0.05$). Exposure to a lower dose of genistein (6 μM) did not significantly inhibit follicle growth compared to DMSO at any time point (Figure 1).

Effect of Genistein on Apoptotic Factors

Because we observed inhibited growth with genistein exposure at 24 h of culture, we examined gene expression of factors that regulate atresia at an earlier time point (18 h) to determine if genistein was inhibiting follicle growth by causing atresia. We selected the anti-apoptotic factor B-cell lymphoma 2 (*Bcl2*) and the pro-apoptotic factor Bcl-2-associated X protein (*Bax*) because they are important factors in regulating apoptosis in the ovary (Kaipia and Hsueh, 1997). Our data indicate that genistein does not affect the expression of the ratio

of *Bax* to *Bcl2* (Figure 2), suggesting that genistein exposure at this early time point does not inhibit antral follicle growth by inducing atresia.

Effect of Genistein on Cell Cycle Regulators

Given that genistein does not affect growth by inducing atresia, we examined whether it inhibits growth by altering cell cycle regulation. Our data show that genistein significantly increases expression of the cell cycle inhibitor cyclin-dependent kinase inhibitor 1a (*Cdkn1a*) about 17-fold compared to control (Figure 2; n=4, $p \leq 0.05$). Genistein did not affect the expression of the cell cycle activators cyclin A2 (*Ccna2*), cyclin D2 (*Ccdn2*), and cyclin-dependent kinase 4 (*Cdk4*) (Figure 2), but it did slightly increase the expression of the cell cycle activators cyclin B1 (*Ccnb1*) and cyclin E1 (*Ccne1*) compared to control (Figure 2; n=4, $p \leq 0.05$).

Effect of Genistein on Estradiol Production

Given that growing antral follicles are major producers of estradiol (Hirshfield, 1991; Findlay *et al*., 2001) and that our data indicate that genistein inhibits follicle growth, we next examined whether genistein affects the ability of antral follicles to produce estradiol. At 24 and 48 h, genistein exposure did not significantly affect estradiol levels produced by antral follicles compared to controls (Figure 3). However, at 72 and 96 h both doses of genistein (6 and 36 μM) significantly decreased the levels of estradiol produced by antral follicles compared to controls (Figure 3; n= 3, $p \leq 0.05$).

Effect of Genistein on Metabolic Pre-Cursors to Estradiol

Production of estradiol is dependent partly on the availability of its metabolic precursors, estrone, testosterone, androstenedione, DHEA, and progesterone. Thus, inhibition of these sex steroid hormones would indirectly inhibit production and levels of estradiol. To test this possibility, we examined whether exposure to genistein alters the production of estrone, testosterone, androstenedione, DHEA, or progesterone by adult antral follicles. At 24 h, estrone levels in all treatment groups were below the level of detection of the ELISA kits (Figure 4). At 48 h, genistein exposure did not affect estrone levels compared to controls. However, at 72 and 96 h, both doses of genistein (6.0 and 36 μM) significantly decreased estrone levels compared to controls (Figure 4; n=3-5; $p \leq 0.05$).

At 24, 48, and 96 h, genistein exposure did not significantly affect testosterone levels compared to controls (Figure 5). At 72 h, both doses of genistein (6 and 36 μM) significantly increased the levels of testosterone produced by antral follicles compared to controls (Figure 5; n=3; $p \leq 0.05$). In contrast, genistein exposure did not significantly affect androstenedione levels compared to controls at any time-point (Figure 6).

At 24 h, DHEA levels in all treatment groups were below the level of detection of the ELISA kits (Figure 7). However, at 48 and 72 h, genistein exposure (36 μM) significantly increased DHEA levels compared to control (Figure 7; n=3-5; $p \leq 0.05$). At 96 h, both concentrations of genistein (6 and 36 μM) significantly increased DHEA levels compared to control (Figure 7; n=3-5; $p \leq 0.05$).

At 24 h, genistein exposure did not significantly affect progesterone levels produced by antral follicles compared to controls (Figure 8). At 48 and 72 h, however, both doses of genistein (6 and 36 μM) significantly increased the levels of progesterone compared to controls (Figure 8; n=3; $p \leq 0.05$). At 96 h, only the high dose of genistein (36 μM) significantly increased the levels of progesterone compared to controls (Figure 8; n=3; $p \leq 0.05$).

Effect of Genistein on the Expression of Steroidogenic Enzymes

Our data indicate that genistein increased testosterone, DHEA, and progesterone levels (Figures 5, 7, and 8), but decreased estradiol and estrone levels compared to control (Figures 3 and 4), suggesting that genistein may alter the ability of antral follicles to synthesize sex steroid hormones. Therefore, we investigated if genistein alters the expression of the following steroidogenic enzymes at each time-point in the culture.

Steroidogenic acute regulatory protein (Star)—STAR is a transport protein responsible for regulating cholesterol transfer into the mitochondria and is a rate-limiting step in steroidogenesis (Miller and Strauss, 1999; Strauss *et al*., 1999). At 24 and 48 h, genistein did not affect *Star* expression compared to control (Figure 9A). However, at 72 and 96 h, genistein (36 μM) significantly increased *Star* expression compared to controls (Figure 9A; n=3-5; $p \leq 0.05$).

Cytochrome P450 side chain cleavage (Cyp11a1)—CYP11A1 is another rate-limiting step in steroidogenesis and it is responsible for metabolizing cholesterol into pregnenolone, the required pre-cursor for progesterone (Miller and Strauss, 1999; Payne and Hales, 2004). At 24–72 h, genistein did not significantly alter the expression of *Cyp11a1* compared to controls (Figure 8B). However, at 96 h, it increased the expression of *Cyp11a1* compared to control (Figure 9B; n=3-5; $p \leq 0.05$).

3β-hydroxysteroid dehydrogenase 1 (Hsd3b1)—HSD3B1 is integral in metabolizing and converting Δ5-steroids (pregnenolone and DHEA) into Δ4-steroids (progesterone and androstenedione) (Readhead *et al*., 1983; Payne and Hales, 2004). At 24 h, genistein (36 μM) decreased the expression of *Hsd3b1* compared to controls (Figure 9C; n=3-5; $p \leq 0.05$), but it did not affect expression at any other time-point (Figure 9C).

Cytochrome P450 17a1 (Cyp17a1)—CYP17A1 is important for the synthesis of DHEA and androstenedione from pregnenolone and progesterone, respectively (Conley and Bird, 1997; Payne and Hales, 2004). At 24 and 96 h genistein (36 μM) significantly decreased *Cyp17a1* expression compared to controls. (Figure 9D; n=3-5; $p \leq 0.05$), but not at 48 and 72 h. At 72 h, there was a trend towards decreased expression with genistein exposure (36 μM) when compared to controls (Figure 9D).

17β-hydroxysteroid dehydrogenase 1 (Hsd17b1)—HSD17B1 is responsible for conversion of androstenedione to testosterone as well as estrone to estradiol (Armstrong, 1968; Payne and Hales, 2004). Genistein did not affect the expression of *Hsd17b1* compared to controls at any time-point (Figure 9E; n=3-5).

Cytochrome P450 19 (Cyp19a1)—CYP19A1 is responsible for the conversion of androstenedione to estrone as well as testosterone to estradiol (Armstrong, 1968; Payne and Hales, 2004). Genistein did not affect the expression of *Cyp19a1* compared to controls at any time-point (Figure 9F; n=3-5).

Effect of Genistein on the Expression of Estrogen Receptors

Genistein is known as an estrogenic compound and has the ability to bind and act through ESR1 and ESR2 (Kuiper *et al*., 1998). Thus, we examined the effect of genistein exposure on *Esr1* and *Esr2* expression in antral follicles. At 24, 72, and 96 h, genistein did not affect the expression of *Esr1* or *Esr2*. However, at 48h, genistein exposure increased the expression of *Esr1* (6 μM) and *Esr2* (36 μM) compared to controls (Figure 10; n=3, $p \leq 0.05$).

Discussion

Using an *in vitro* follicle culture system, we have shown that genistein inhibits growth of antral follicles, alters the expression of cell cycles regulators, disrupts the production of sex steroid hormones, and alters the expression of steroidogenic enzymes. Specifically, we observed that genistein exposure inhibits antral follicle growth as early as 24 h in culture (Figure 1). Our data are consistent with a previous *in vivo* study indicating that exposure to genistein (160 mg/kg/day) significantly inhibits follicle growth because it increases the percentage of primordial follicles, but decreases the number of total antral follicles in 4 month and 15 month old rats (Zhuang *et al*., 2010).

To determine the cause of genistein-induced antral follicle growth inhibition, we examined the expression of apoptotic factors *Bax* and *Bcl2* at 18 h of culture. We found that genistein did not affect apoptotic factors in antral follicles (Figure 2), suggesting that atresia is not the cause of genistein-induced inhibition of antral follicle growth. Further, when observed the genistein-treated follicles under a light microscope, we noticed that the follicles appeared to be healthy, but not growing (data not shown). These results differ from previous studies that indicate that genistein causes atresia of rat follicles. Medigovic *et al*. dosed immature female rats with 50 mg/kg/day genistein daily from postnatal days (PND) 18 to 20 and found decreased numbers of primordial, primary, and secondary follicles and increased atresia (Medigovic *et al*., 2012). Zin *et al*. also found that genistein treatment (10 or 100 mg/kg/day) from PND 22 to 42 increases atresia in Sprague-Dawley rats (Zin *et al*., 2013). Our results likely differ from these studies due differences in age, species, and doses of genistein.

The observation that genistein inhibits follicle growth, but does not induce atresia led us to examine the effects of genistein on cell cycle regulators at 18 h. Previous studies on other endocrine disruptors such as bisphenol A and phthalates show that they can affect follicle growth by altering expression of cell cycle regulators (Peretz et al., 2012; Craig et al., 2013). Our data indicate that genistein significantly increases the expression of the cell cycle inhibitor *Cdkn1a* about 17-fold compared to control (Figure 2). Further, our data indicate that although genistein also significantly increases the expression of the cell cycle activators *Ccnb1* and *Ccne1* (Figure 2), the change is minor in comparison to that of *Cdkn1a*, and is

likely a compensatory effect. Collectively, these data suggest that genistein inhibits follicle growth by inhibiting the cell cycle.

Previous studies indicate that estradiol is necessary for normal follicle growth (Channing*et al*., 1980; Findlay *et al*., 2001; Drummond, 2006; Chaffin and Vandevoort, 2013). Therefore, we initially hypothesized that genistein would inhibit antral follicle growth due to altered steroidogenesis. Instead, we found that genistein (36 μM) inhibits growth as early as 24 h (Figure 1), whereas genistein does not affect estradiol levels until 72 h (Figure 3). Additionally, the lower dose of genistein (6 μM) does not affect follicle growth at any time point (Figure 1), but still decreases estradiol levels beginning at 72 h (Figure 3). Thereby, it is unlikely that the genistein-induced inhibition of growth is due to decreased levels of estradiol. Instead, it is more likely that inhibition of follicle growth is the cause of decreased estradiol production.

Our data indicate that genistein exposure decreases estradiol and estrone levels in the media of cultured antral follicles. Estradiol and estrone are produced by converting precursor hormones in the theca and granulosa cells of the follicles (Armstrong, 1968). Thus, it is possible that the inhibition of antral follicle growth by genistein (36 μM) results in fewer granulosa cells being present in the follicle to produce estradiol. This is supported by our data indicating that genistein exposure (36 μM) inhibits antral follicle growth first at 24 h (Figure 1), whereas it inhibits estradiol levels beginning at 72 h (6 and 36 μM) (Figure 3). Another likely scenario is that genistein is affecting the metabolism of estradiol and estrone. It is possible that genistein increases estradiol and estrone metabolism by inducing cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1). Thus, future studies should determine whether genistein affects estradiol metabolism by inducing CYP1A1 and CYP1B1. Additionally, our data indicate that genistein exposure increases the expression of *Esr1* (6 μM) and *Esr2* (36 μM) (Figure 10). It is possible that this genistein-induced increase could lead to greater responsiveness of antral follicles to genistein and further result in decreased production of estradiol, but this needs to be examined in future studies.

Our data on the effects of genistein on estradiol levels are consistent with several, but not all previous studies. Specifically, we showed that genistein exposure reduced estradiol production from cultured antral follicles. Similarly, dietary genistein exposure decreased estradiol levels in male mice (8 mg/kg; (Ryokkynen *et al*., 2006)), cultured swine granulosa cells (185 μM; (Basini *et al*., 2010)), and Sprague Dawley rats (10 and 100 mg/kg/day; (Zin *et al*., 2013)). Additionally, daily consumption of soy products containing genistein reduced circulating levels of estradiol over the menstrual cycle in nine healthy regularly cycling women (Lu *et al*., 2001). Further, acute (4 h) and chronic (24 h) genistein exposure inhibited estradiol production in cultured, luteinized human granulosa cells (Whitehead *et al*., 2002). In contrast, neonatal genistein exposure (50 mg/kg/day) did not affect serum levels of estradiol when measured before puberty or during pregnancy in female mice (Jefferson *et al*., 2005). Further, genistein did not affect FSH-stimulated estradiol production in cultured porcine (0.5-50 μM; (Nynca and Ciereszko, 2006)) or immature rat granulosa cells (1–10 μM; (Myllymaki *et al*., 2005)). The reasons for these differences in results pertaining to the effects of genistein on estradiol levels are unclear, but likely stem from differences in

species (mice versus rats, humans, and pigs), and study design (doses of genistein, timing of treatment, age of animals, and *in vivo* versus *in vitro* methods). It is also possible that our *in vitro* results differ from other *in vitro* studies because we used an antral follicle culture system, which consists of the intact functional units of the ovary. Other studies used isolated granulosa cells (Myllymaki *et al*., 2005; Nynca and Ciereszko, 2006), which normally produce sex steroid hormones in conjunction with theca cells and, thus, may behave differently alone compared to intact antral follicles.

Our data indicate that genistein exposure also alters the levels of other precursor hormones (Figures 4–8). Specifically, we observed that genistein exposure increases testosterone, DHEA, and progesterone levels compared to control (Figures 5, 7, and 8). Similar to our results, one study indicates that genistein exposure (10–10,000 ng/mL) increases progesterone release by bovine granulosa cells and rabbit granulosa cells (100–10,000 ng/mL) (Makarevich *et al*., 1997). Additionally, genistein exposure (0.1–3 μM) increases FSH-induced progesterone levels in rat granulosa cells (Haynes-Johnson*et al*., 1999). This same study, however, shows that higher concentrations of genistein (30–100 μM) decrease FSH-induced progesterone levels (Haynes-Johnson *et al*., 1999), thereby indicating dose related differences in the effects of genistein on steroidogenesis.

Our findings on the effects of genistein on progesterone and testosterone differ from other previously conducted studies. One study indicates that neonatal genistein exposure (50 mg/kg/day) does not affect serum levels of progesterone or testosterone when measured before puberty and during pregnancy in mice (Jefferson *et al.*, 2005). Additionally, genistein exposure (1, 18.5, and 185 μM) inhibits FSH-stimulated and basal progesterone levels in cultured porcine granulosa cells (Nynca and Ciereszko, 2006; Basini *et al.*, 2010)), LH-stimulated progesterone secretion in porcine luteinized granulosa cells (0.5-50 μM; (Nynca *et al.*, 2015)), and prolactin-stimulated progesterone secretion in porcine thecal cells (45 μM; (Gregoraszczuk *et al.*, 1999)). Furthermore, genistein exposure (50 μM) inhibits the ability of primary porcine granulosa cells to produce progesterone (Tiemann *et al.*, 2007). Genistein exposure (1–100 μM) also decreases progesterone levels in human granulosa cells (Whitehead *et al.*, 2002; Lacey *et al.*, 2005). It is likely that our results differ from previous studies largely due to species differences in the effects of genistein on sex steroid hormone levels. Most previous studies were conducted with pigs (Gregoraszczuk *et al.*, 1999; Nynca and Ciereszko, 2006; Tiemann *et al.*, 2007; Basini *et al.*, 2010; Nynca *et al.*, 2015) or human cells (Whitehead *et al.*, 2002; Lacey *et al.*, 2005), whereas our study was conducted in mice. Further, our results likely differ from the other study conducted in mice (Jefferson *et al.*, 2005) because of the differences in study design, such as *in vitro* vs *in vivo* experiments and pre-pubertal vs adult, non-pregnant exposure timing, as well as the differences in genistein concentrations. Traditional toxicology dogma indicates that endocrine disrupting chemicals can have differing effects across a range of concentrations and that low dose effects may be different than those observed at higher doses (Vandenberg *et al.*, 2012).

Adequate estradiol biosynthesis is dependent on the availability of upstream precursor hormones and steroidogenic enzymes. In the estradiol biosynthesis pathway, StAR transports cholesterol from the outer membrane to the inner membrane of the mitochondria (Miller and Strauss, 1999; Strauss *et al.*, 1999). Cholesterol then is converted to

pregnenolone by CYP11A1 (Payne and Hales, 2004). Pregnenolone is then converted to estradiol via two pathways. In the first pathway, pregnenolone is converted to DHEA by CYP17A1 (Conley and Bird, 1997). DHEA is then converted to androstenedione by HSD3B1 (Armstrong, 1968; Readhead *et al.*, 1983). Androstenedione is converted to either estrone by CYP19A1 or testosterone by HSD17B1 (Armstrong, 1968; Payne and Hales, 2004). Finally, testosterone is converted to estradiol via CYP19A1 (Armstrong, 1968; Payne and Hales, 2004). In the second pathway, pregnenolone is converted to progesterone by HSD3B1 (Readhead *et al.*, 1983). The progesterone is converted to androstenedione by CYP17A1 (Conley and Bird, 1997), then testosterone, and finally estradiol (Armstrong, 1968; Payne and Hales, 2004).

Although the mechanism by which genistein alters testosterone and DHEA, remains unknown, our steroidogenic enzyme findings shed some light on why we observe a genistein-induced increase in progesterone levels. We observed a genistein-induced decrease in *Cyp17a1* as early as 24 h and it continued until 96 h (Figure 9D). Because CYPA17A1 is the enzyme responsible for progesterone metabolism, a decrease in its expression would decrease progesterone metabolism. Further, beginning at 72 h, we observed an increase in *Star* and *Cyp11a1* expression (Figures 9A and 9B), two enzymes that increase progesterone production. Collectively, the decrease in progesterone metabolism and increase in progesterone production led to unusually high levels of progesterone in response to genistein.

In conclusion, our results indicate that genistein exposure inhibits the growth of mouse antral follicles *in vitro*, likely by inhibiting the cell cycle. Our results also indicate that estradiol, a key factor involved in the growth of antral follicles, is significantly decreased in genistein-treated follicles compared to control. Additionally, our results show that genistein alters the levels of the estradiol precursor hormones estrone, testosterone, DHEA, and progesterone. Finally, our data indicate that genistein induces dysregulation of steroidogenic enzyme expression and this may be the cause of genistein-induced altered hormone levels in cultured mouse antral follicles. This study, as well as future ones that further elucidate how genistein alters steroidogenic enzymes and steroid hormone production, are important because genistein-induced altered hormone levels and inhibition of antral follicle growth could lead to numerous health risks and female infertility. Many people, both men and women, are exposed to genistein and other phytoestrogen-based botanicals regularly as part of their diet or as therapeutics for reproductive and non-reproductive diseases. Therefore, it is imperative to understand how genistein and other botanicals may impact overall public health.

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Highlights

- Genistein exposure inhibits antral follicle growth
- Genistein exposure alters expression of cell cycle regulators
- Genistein exposure alters sex steroid hormones
- Genistein exposure alters expression of steroidogenic enzymes
- Genistein exposure alters *Esr1* and *Esr2* expression

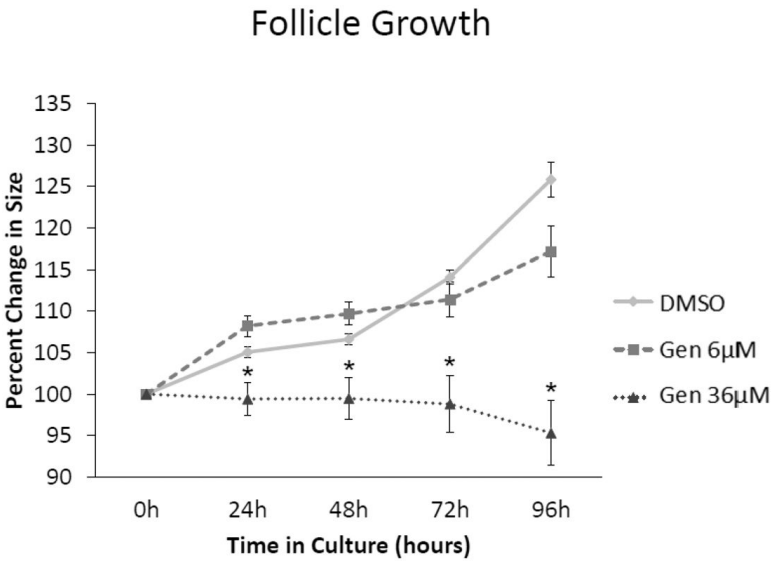

Figure 1. Effect of genistein on follicle growth over time

After isolation, antral follicle growth was measured daily along perpendicular axes and percent change in growth was determined from 24 to 96 h. The graph represents the means ± SEM of percent change in follicle growth from 3 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point ($n=3$; $p \leq 0.05$).

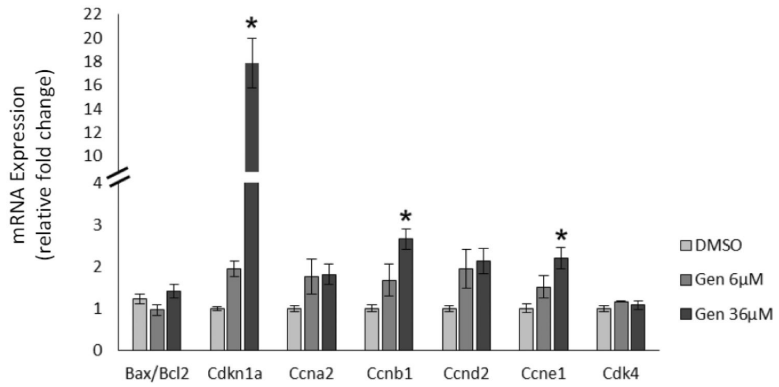

Figure 2. Effects of genistein on apoptotic factors and cell cycle regulators at 18 h

After culture of antral follicles for 18 h, follicles were collected and subjected to RNA extraction. The RNA was reverse transcribed to cDNA and used to measure gene expression of *Bax*, *Bcl2*, *Cdkn1a*, *Ccna2*, *Ccnb1*, *Ccnd2*, *Ccne1*, and *Cdk4* by quantitative polymerase chain reaction (qPCR). Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=4; $p \leq 0.05$).

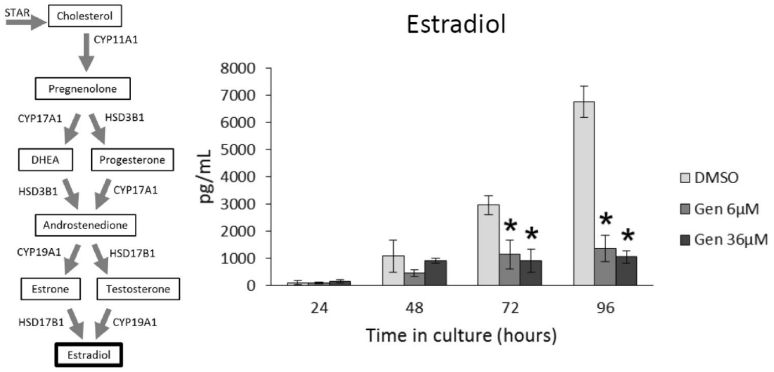

Figure 3. Effects of genistein on estradiol production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for estradiol. The graph represents the means ± SEM from 3 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3; $p \leq 0.05$).

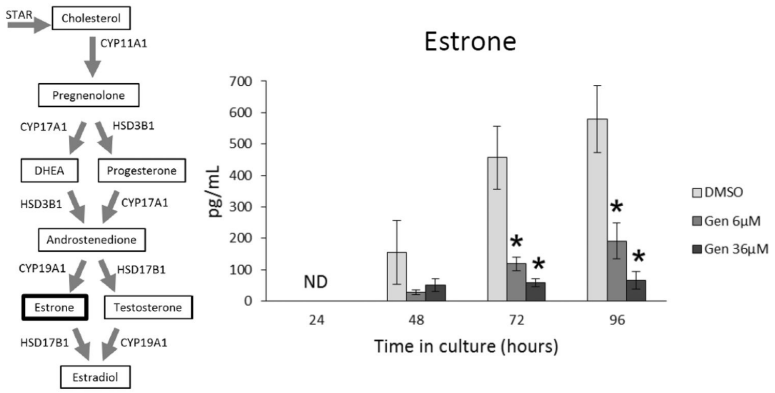

Figure 4. Effects of genistein on estrone production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for estrone. The graph represents the means ± SEM from 3-5 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3-5; $p \leq 0.05$).

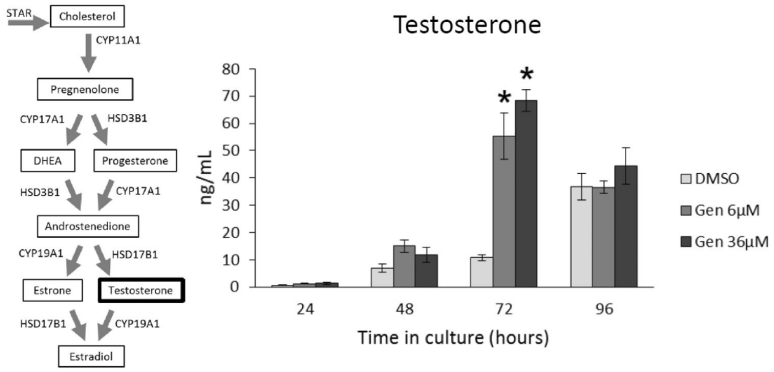

Figure 5. Effects of genistein on testosterone production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for testosterone. The graph represents the means ± SEM from 3 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3; $p \leq 0.05$).

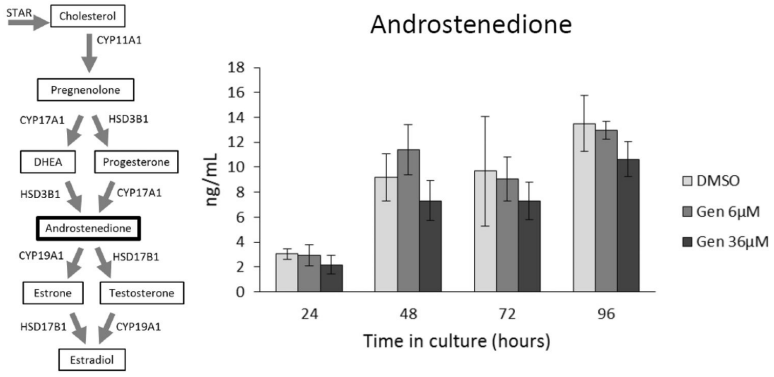

Figure 6. Effects of genistein on androstenedione production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for androstenedione. The graph represents the means ± SEM from 3 separate experiments.

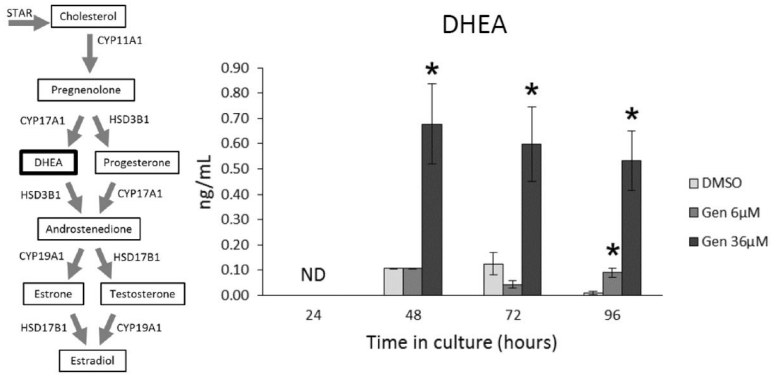

Figure 7. Effects of genistein exposure on DHEA production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for DHEA. The graph represents the means ± SEM from 3-5 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3-5; $p \leq 0.05$).

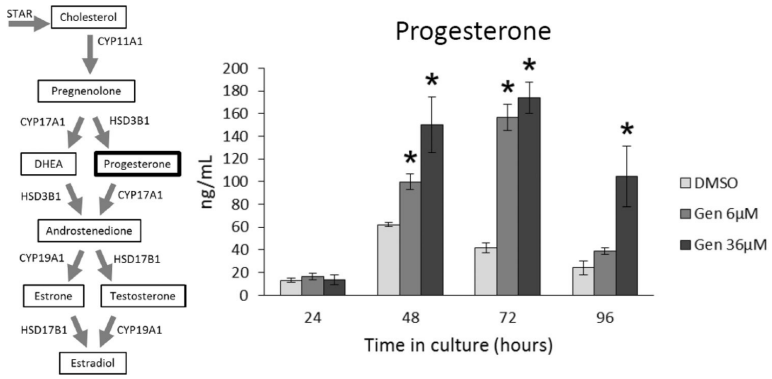

Figure 8. Effects of genistein on progesterone production over time

After culture of antral follicles for 24-96 h, media were collected and subjected to enzyme-linked immunosorbent assays for progesterone. The graph represents the means ± SEM from 3 separate experiments. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3; $p \leq 0.05$).

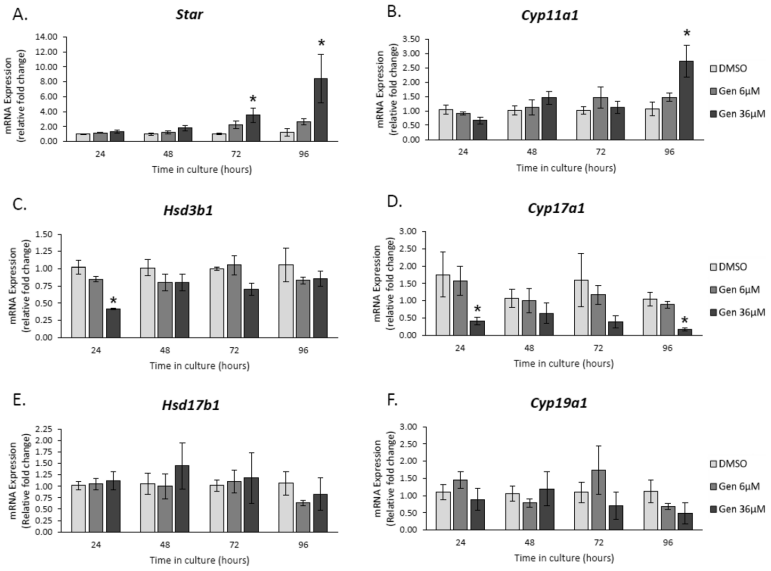

Figure 9. Effects of genistein on the gene expression of steroidogenic enzymes over time

After culture of antral follicles for 24-96 h, follicles were collected and subjected to RNA extraction. The RNA was reverse transcribed to cDNA and used to measure gene expression of key steroidogenic enzymes by qPCR. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3-5; $p \leq 0.05$).

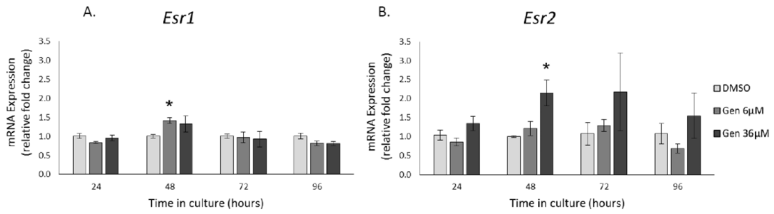

Figure 10. Effects of genistein on the gene expression of *Esr1* and *Esr2*

After culture of antral follicles for 24-96 h, follicles were collected and subjected to RNA extraction. The RNA was reverse transcribed to cDNA and used to measure gene expression of *Esr1* and *Esr2* by qPCR. Asterisks (*) indicate a significant difference between control groups and genistein at each time point (n=3-5; $p \leq 0.05$).

Table 1

Primers used in Quantitative Real-Time Polymerase Chain Reactions (qPCR)

Gene	Gene Symbol	Primer Sequence	
		Forward	Reverse
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
Steroidogenic acute regulatory protein	*StAR*	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG
Cytochrome P450 cholestrol side-chain cleavage	*Cyp11a1*	AGATCCCTTCCCCTGGCGACAATG	CGCATGAGAAGAGTATCGACGCATC
3b-Hydroxysteroid dehydrogenase 1	*Hsd3b1*	CAGGAGAAAGAACTGCAGGAGGTC	GCACACTTGCTTGAACACAGGC
Cytochrome P450 steroid 17-a-hydroxylase 1	*Cyp17a1*	CCAGGACCCAAGTGTGTTCT	CCTGATACGAAGCACTTCTCG
17b-Hydroxysteroid dehydrogenase 1	*Hsd17b1*	ACTGTGCCAGCAAGTTTGCG	AAGCGGTTCGTGGAGAAGTAG
Cytochrome P450 aromatase	*Cyp 19a1*	CATGGTCCCGCAAACTGTGA	GTAGTAGTTGCAGGCACTTC
Estrogen receptor 1 (alpha)	*Esr1*	CCGTGTGCAATGACTATGCC	GTGCTTCAACATTCTCCCTCCTC
Estrogen receptor 2 (beta)	*Esr2*	GGAATCTCTTCCCAGCAGCA	GGGACCACATTTTTGCACTT
Cyclin A2	*Ccna2*	GCTCTACTGCCCGGAGGCTGA	TGGCCTACATGTCCTCTGGGGAA
Cyclin B1	*Ccnb1*	TGCATTCTCTCAGTGCCCTCCACA	AGACAGGAGTGGCGCCTTGGT
Cyclin D2	*Ccnd2*	CCTTTGACGCAGGCTCCCTTCT	ACCCTGGTGCACGCATGCAAA
Cyclin E1	*Ccne1*	GGTGTCCTCGCTGCTTCTGCTT	CCGGCTAACCATGGCGAACGGA
Cyclin-dependent kinase 4	*Cdk4*	AGAAACCCTCGCTGAAGCGGCA	TGGGGGTGAACCTCGTAAGGAGA
Cyclin-dependent kinase inhibitor 1A (p21)	*Cdkn1a*	TTAGGCAGCTCCAGTGGCAACC	ACCCCCACCACCACACACCATA
B cell lymphoma 2	*Bcl2*	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC
Bcl2-associated X protein	*Bax*	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG