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Segregated responses of mammary gland development and vaginal opening to prepubertal genistein exposure in *Bsc12*^{-/-} female mice with lipodystrophy

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Abstract

Berardinelli-Seip congenital lipodystrophy 2-deficient (*Bsc12*^{-/-}) mice recapitulate human BSCL2 disease with lipodystrophy. *Bsc12*-encoded seipin is detected in adipocytes and epithelium of mammary gland. Postnatal mammary gland growth spurt and vaginal opening signify pubertal onset in female mice. *Bsc12*^{-/-} females have longer and dilated mammary gland ducts at 5-week old and delayed vaginal opening. Prepubertal exposure to 500 ppm genistein diet increases mammary gland area and accelerates vaginal opening in both control and *Bsc12*^{-/-} females. However, genistein treatment increases ductal length in control but not *Bsc12*^{-/-} females. Neither prepubertal genistein treatment nor *Bsc12*-deficiency affects phospho-estrogen receptor α or progesterone receptor expression patterns in 5-week old mammary gland. Interestingly, *Bsc12*-deficiency specifically reduces estrogen receptor β expression in mammary gland ductal epithelium. In summary, *Bsc12*^{-/-} females have accelerated postnatal mammary ductal development but delayed vaginal opening; they display segregated responses in mammary gland development and vaginal opening to prepubertal genistein treatment.

Keywords

Bsc12/Seipin; lipodystrophy; mammary gland; vaginal opening; puberty; genistein

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Introduction

Mammary gland development and vaginal opening are estrogen-dependent processes and markers for pubertal onset in mice [1–5]. Mammary gland development is also an indicator of pubertal onset in most girls [6]. Pubertal mammary gland growth is characterized by branching morphogenesis to form a ductal tree filling the fat pad [7–9]. Estrogen receptor alpha (ER α)-deficiency leads to failed mammary gland development beyond prepubertal stage [9]; ER $\beta^{-/-}$ nulliparous mammary glands appear to have normal ductal growth but decreased side branching [10–12]. Estrogenic endocrine disruptors can affect both pubertal mammary gland development [1, 13] and vaginal opening, which is used as an external biomarker for pubertal onset in rodents [1, 4, 14–16].

Epidemiological studies have suggested that obesity may be causally related to earlier puberty in girls (reviewed in [17]). One study involving 135,223 girls (born between 1930 and 1969) indicates that heavier girls at age seven had earlier puberty; it also suggests that obesity epidemic is not solely responsible for the trend of younger age at puberty and that endocrine disruptors could contribute to this trend [18]. Indeed, we have demonstrated in mice that prepubertal exposure to endocrine disruptors accelerates pubertal onset [1, 4, 19, 20].

We hypothesized that both body fat and endocrine disruptors could affect pubertal onset and that body fat could influence the effect of endocrine disruptors on pubertal onset. This hypothesis was tested in *Bscl2*^{-/-} mice [21] that recapitulate human Beinaardinalli-Seipin Congenital Lipodystrophy type 2 (BSCL2) disease. *BSCL2/Bscl2* gene encodes seipin, an integral endoplasmic reticulum membrane protein that plays an essential role in adipose tissue development [21–27]. Seipin is highly expressed in the adipose tissue, brain and testis of both human and mouse [22, 28–30]. Mutations or deletion of *BSCL2/Bscl2* are associated with generalized lipodystrophy characterized by a near complete absence of adipose tissue and associated metabolic complications [21, 22, 25–27]. Although it was reported that among 45 patients (27 boys and 18 girls) with *BSCL2* mutations, one girl had precocious puberty [31], it is unknown whether seipin deficiency and related lipodystrophy affect pubertal onset. It is also unknown whether lipodystrophy affects the responsiveness to prepubertal exposure of endocrine disruptors. These two aspects were investigated in this study. Genistein was used as a testing endocrine disruptor. A dose of 500 ppm in the diet was chosen because it was found in some soy products, such as soy bacon [32], and it was previously demonstrated to be an effective dose to influence pubertal onset in mice [1]. Pubertal mammary gland development and vaginal opening were two end points for determining pubertal onset in this study.

Materials and Methods

Animals

Bscl2-deficient mice (*Bscl2*^{-/-}) on C57BL/6J background were derived from a colony at Georgia Regents University, which was originally derived from a colony at Baylor College of Medicine [21]. Mice with different genotypes were from *Bscl2*^{+/-} (Het) females X *Bscl2*^{+/-} (Het) males. They were genotyped as previously described [21]. All mice were

maintained on PicoLab mouse diet 20 with soybean as a main protein source. They were housed in polypropylene cages with free access to food and water on a 12 h light/dark cycle (0700–1900) at $23\pm 1^\circ\text{C}$ with 30–50% relative humidity at the College of Veterinary Medicine animal facility at the University of Georgia. All methods used in this study were approved by the University of Georgia IACUC Committee and conform to National Institutes of Health guidelines and public law.

Treatment

Bscl2^{+/+} (WT) and *Bscl2*^{+/-} (Het) mice had no difference in phenotypes and were both included in the genotype control for *Bscl2*^{-/-} (Hom) mice. At weaning (3 weeks old), control females and their *Bscl2*^{-/-} female littermates were randomly assigned into 0 ppm (vehicle control) or 500 ppm genistein diet groups, resulting in four groups: control mice, 0 ppm; control mice, 500 ppm; *Bscl2*^{-/-} mice, 0 ppm; and *Bscl2*^{-/-} mice, 500 ppm. The 0 ppm and 500 ppm genistein diets were prepared as described previously [1, 33]. Briefly, 0 g or 0.25 g genistein (LC Laboratories, Woburn, MA) was dissolved in 150 ml 70% ethanol, then mixed well with 500 g phytoestrogen-free AIN-93G diet (Bio-Serv, Frenchtown, NJ) in different glass bowls to obtain 0 ppm and 500 ppm genistein diets, respectively. Food pellets were hand squeezed, air dried at room temperature, and kept at 4°C . Fresh diets were prepared every two weeks. Body weight was measured at 3, 4, and 5 weeks old. At least 6 mice were included in each study group. Vaginal opening was evaluated daily from weaning until detection [1].

Tissue collection

All mice at 5 weeks old ± 1 day were dissected at estrus stage, which was determined by vaginal smear prior to dissection [1], except in *Bscl2*^{-/-}, 0 ppm group, in which 6 out of the 7 mice had vaginal opening on PND34 or 35, and they were dissected on the day of vaginal opening detection. Both vaginal opening and estrus stage occur after an estrogen increase [16, 34–36]. The day at vaginal opening and the day at estrus stage were chosen as relatively comparable stages ~5 weeks old between control and *Bscl2*^{-/-} females. The 4th inguinal mammary glands on both sides were collected, one was saved for whole mount analysis and the other was fixed in 10% formalin for histology and immunohistochemistry.

Mammary gland whole mount and quantification of mammary gland development

Mammary gland whole mount and quantification were done as previously described [1, 37]. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the size of each lymph node, the length of the longest duct from the nipple, the diameter of the widest mammary gland duct at the position near each lymph node, the occupied area of each mammary gland (approximated by a polygon area that covered all the ducts), the width of the ductal tree passing the lymph node, and the number of terminal end buds (TEB) with diameters larger than 70 μm .

Histology

Mammary gland histology was done as previously described [1].

Immunohistochemistry

Paraffin sections (5 μm) of the 4th inguinal mammary glands were used in immunohistochemistry as previously described [1, 38]. Seipin expression was detected in 5 weeks old *Bsc12*^{+/+} and *Bsc12*^{-/-} mammary glands as well as 3 months old *Bsc12*^{+/+} and *Bsc12*^{-/-} testes using our customized rabbit polyclonal anti-seipin antibody (1:1,000, 2.21 $\mu\text{g}/\text{ml}$, Thermo Scientific), which was raised against the C-terminal 17 amino acids of mouse seipin. *Bsc12*^{+/+} mammary gland sections and testis sections without primary antibody were also included. To determine the effects of *Bsc12*-deficiency and genistein treatment on the expression of phospho-estrogen receptor α (P-ESR1/P-ER α), estrogen receptor β (ESR2/ER β), and progesterone receptor (PR) in the mammary gland, mammary gland sections from three mice in each of the four groups (control mice, 0 ppm; control mice, 500 ppm; *Bsc12*^{-/-} mice, 0 ppm; and *Bsc12*^{-/-} mice, 500 ppm) were evaluated using immunohistochemistry for P-ER α (rabbit anti-phospho-ER α antibody, 1:100, 10 $\mu\text{g}/\text{ml}$, Abcam), ER β (anti-ER β antibody, 1:50, 20 $\mu\text{g}/\text{ml}$, Abcam), and PR (rabbit anti-PR antibody, 1:200, 6 $\mu\text{g}/\text{ml}$, Daco, Denmark) as previously described [1, 38, 39]. Sections were counterstained with Harris Hematoxylin.

Statistical analysis

Nonparametric Two-Sample Kolmogorov-Smirnov test was used to compare the ages at vaginal opening, the sizes of lymph nodes, the ductal lengths and the areas of mammary glands, the diameters of mammary gland ducts, the widths of ductal trees passing the lymph nodes, and the numbers of TEB. Two-tail unequal variance student's t-test was used to compare the weaning body weight. ANOVA with repeated measure was used to compare the body weight from 3 to 5 weeks old in different genotypes and treatments. Error bars represented standard deviation. The significance level was set at $p < 0.05$.

Results and Discussion

Body weight

Bsc12^{-/-} females had significantly lower body weight than the control mice only at weaning (3 weeks old) but not at 4 or 5 weeks old (Fig. 1A), in agreement with our previous report [21]. The average weaning weights were 10.87 ± 1.18 g (N=23) for control females and 9.69 ± 0.85 g (N=13, $P < 0.01$) for *Bsc12*^{-/-} females.

Interestingly, we noticed a dramatic difference in the weaning body weight of the control C57BL/6J females (10.87 ± 1.18 g, N=23) in this study compared to that of the control C57BL/6J females in our previous study, which was 7.22 ± 1.05 g (N=111, $P < 0.001$, two-tailed unequal variance t-test) [1]. The main difference in these two studies was the diet. In the previous study, our C57BL/6J colony was maintained on phytoestrogen-free AIN-93G diet (3.8 kcal/g), and the ancestors of the females used in the previous study were already on this diet for at least 2 generations. In this current study, all the female pups were from parents maintained on PicoLab mouse diet 20 (4.6 kcal/g).

Vaginal opening

Although it has been debated for its accuracy as a biomarker for puberty [40], vaginal opening has been used as a standard endpoint for assessing pubertal development by U.S. Environmental Protection Agency (EPA) (http://www.epa.gov/endo/pubs/pubertal_protocol_2007_v7.2c.pdf) and it has been used in many rodent studies to indicate pubertal onset [15, 41–44]. Analyses of the original data from our previous study [1] revealed a significant correlation between age at vaginal opening and age at first copulation following vaginal opening. In addition, other biomarkers for puberty, such as vaginal estrus, vaginal plug, and ovulation, also showed a consistent sequential pattern following vaginal opening [15, 40]. Therefore, vaginal opening can be used as an easily obtainable noninvasive biomarker for pubertal onset in rodents [15].

Genistein treatment significantly advanced vaginal opening in both control and *Bscl2*^{-/-} mice (Fig. 1B). Interestingly, without genistein treatment, *Bscl2*^{-/-} mice had significantly delayed vaginal opening compared to the control mice (Fig. 1B). The lower body weight in the newly-weaned *Bscl2*^{-/-} mice (Fig. 1A) might contribute to the delayed vaginal opening. This difference seemed to be erased by 500 ppm genistein treatment because no significant difference in the ages at vaginal opening was observed between genistein-treated control and *Bscl2*^{-/-} mice (Fig. 1B). These results indicated that although *Bscl2* deficiency delayed vaginal opening, it did not seem to affect the responsiveness to genistein treatment.

In our previous study [1], the age at vaginal opening in the vehicle control group was 32.2±2.8 days old (N=35). It was significantly younger in the vehicle control mice (28.5±2.9 days old, N=17, P<0.001) in this study. There could be two related potential explanations: body weight and diet. The body weight in the previous study [1] was significantly lower than that in the current study (Fig. 1A). Normally, higher body weight is correlated with earlier pubertal development (reviewed in [45]). Females in the previous study were not exposed to phytoestrogen-containing diet directly or indirectly from gestation to weaning because they were from a colony maintained on phytoestrogen-free AIN-93G diet [1]. Females in the current study were indirectly exposed to PicoLab mouse diet 20 from gestation to weaning. Soybean was a main protein source in this diet, and it contained phytoestrogens genistein and daidzein [46–48]. It has been reported that *in utero* and lactational exposure to phytoestrogens could promote pubertal onset [49, 50] and the phytoestrogen content in the diet could affect the age at vaginal opening [51].

Mammary gland

Since seipin-deficiency is associated with generalized lipodystrophy characterized by a near complete absence of adipose tissue [21, 22, 25–27], it was expected that adipocyte-rich mammary gland would have abnormalities in the *Bscl2*^{-/-} female mice. Indeed, several obvious differences were observed. Whole mount mammary glands showed enlarged lymph nodes (1.23±0.24 mm² (N=7) vs. 0.54±0.22 mm² (N=9) in the control, P<0.001), and longer and wider mammary gland ducts in the 5 weeks old *Bscl2*^{-/-} female mice (Figs. 2A, 2B). Histology of mammary gland confirmed enlarged ductal lumen and smaller adipocytes in these mice (Figs. 2C, 2D). The average diameters of the widest mammary gland ducts near the lymph nodes were 0.027±0.006 mm (N=9) for the control females and 0.063±0.007 mm

(N=7, $P<0.001$) for the *Bscl2*^{-/-} females. Interestingly, cells and patches of cells were often seen in the *Bscl2*^{-/-} mammary gland ductal lumen (Fig. 2D). It was possible that they were sloughed ductal epithelial cells.

Upon postweaning 500 ppm genistein treatment, the control mice had significantly increased ductal length and area of mammary gland at 5 weeks old (Figs. 3A, 3B, 3E, 3F). This effect was consistent with our previous study in C57BL/6J females derived from a colony maintained on phytoestrogen-free AIN-93G diet [1]. In *Bscl2*^{-/-} mammary glands, segregated responses upon 500 ppm genistein treatment were observed. Significantly increased mammary gland area but not mammary gland ductal length was observed in the 500 ppm genistein-treated *Bscl2*^{-/-} females (Figs. 3C–3F). This could be attributed to the accelerated ductal growth in the *Bscl2*^{-/-} females, which had significantly longer duct than the control females on either 0 or 500 ppm genistein diet (Figs. 3A–3E). It was possible that the ductal lengths in the *Bscl2*^{-/-} females had reached the maximum and 500 ppm genistein treatment could not further extend the ductal lengths. In support of this speculation, we found that the number of TEB (highly proliferative structures located at the tips of the invading ducts) in the *Bscl2*^{-/-} females on 0 ppm genistein diet was comparable to those in the control females and the *Bscl2*^{-/-} females on 500 ppm genistein diet (Fig. 3G).

However, there was still limited room to expand the mammary gland area in the *Bscl2*^{-/-} females. In the control females, the average increase of mammary gland area was ~200% upon genistein treatment; while in the *Bscl2*^{-/-} females, it was ~15% in response to 500 ppm genistein treatment (Figs. 3A~3D, 3F). Although the mammary gland ductal tree was supported by the surrounding fat tissue, which was not well developed in the *Bscl2*^{-/-} females (Fig. 2D), it did not seem to limit the ductal growth. However, the width of ductal tree passing the lymph node was narrower in the 500 ppm genistein-treated *Bscl2*^{-/-} females (2.91 ± 0.46 mm (N=6), Fig. 3D) compared to 500 ppm genistein-treated control mice (3.99 ± 0.37 mm (N=6), $P=0.026$, Fig. 3B). This observation could be supported by the longer ductal length in the *Bscl2*^{-/-} mammary gland (Fig. 3E) but comparable area of mammary gland between control and *Bscl2*^{-/-} mammary glands upon 500 ppm genistein treatment (Fig. 3F).

Expression of seipin in 5 weeks old mammary gland

Seipin has been demonstrated to be highly expressed in adipose tissue, brain and testis [22, 28, 29, 52]. Its expression in the mammary gland had not been previously reported. Immunohistochemistry showed seipin expression in the adipocytes and the ductal epithelial cells of 5 weeks old *Bscl2*^{+/+} mammary gland (Fig. 4A). However, there were still lower levels of staining in the *Bscl2*^{-/-} mammary gland (Fig. 4B) but no staining in the mammary gland without the primary antibody (Fig. 4C). Seipin was highly detected in the *Bscl2*^{+/+} spermatids but not in the *Bscl2*^{-/-} spermatids (Figs. 4D, 4E) as previously reported [29, 52]. However, compared to the testis section without the primary antibody (Fig. 4F), the *Bscl2*^{-/-} testis had background immunostaining throughout all the cell types (Fig. 4E). The expression of seipin in the mammary gland supported its potential role in mammary gland development and/or function.

ER and PR expression

ER α is critical for pubertal mammary gland development [9]. ER β seems to be important for side branching [10], presumably through its involvement in progesterone production from corpora lutea [10–12] because PR-mediated progesterone signaling is critical for side branching [53, 54]. It was previously demonstrated that postweaning genistein treatment did not influence P-ER α , ER β , and PR expression in C57BL/6 mammary gland at 5 weeks old [1]. Here we further examined P-ER α , ER β , and PR expression in 5 weeks old mammary gland to determine if any of these receptors had altered expression in the *Bscl2*^{-/-} mammary glands treated with 0 or 500 ppm genistein diets.

P-ER α was mainly detected in the nuclei of epithelial cells and adipocytes; there was no obvious difference in the P-ER α expression patterns among all four groups (Figs. 5A–5D). ER β was comparably highly expressed in the epithelial cells and adipocytes of the control mammary glands (both 0 and 500 ppm genistein-treated) (Figs. 5E, 5F). However, its expression was lower in the epithelial cells compared to that in the surrounding adipocytes of the *Bscl2*^{-/-} mammary glands (both 0 and 500 ppm genistein-treated) (Figs. 5G, 5H). The expression levels of ER β in the adipocytes of the control mammary gland and of the *Bscl2*^{-/-} mammary gland seemed to be comparable (Figs. 5E–5H). PR was mainly detected in the nuclei of mammary gland epithelial cells, and no obvious difference in the epithelial PR expression pattern was observed among all four groups (Figs. 5I–5L).

The mechanism of ER β downregulation in the *Bscl2*^{-/-} ductal epithelium and the consequence of such cell-type specific downregulation are unclear. It was demonstrated that glucocorticoid receptor (GR) deficiency (*GR*^{-/-}) could lead to accelerated pubertal mammary ductal growth and distention [55], similar to what was seen in the *Bscl2*^{-/-} mammary gland (Figs. 2, 3). Based on a mammary gland transplant study, the driving force for the *GR*^{-/-} mammary gland duct phenotypes was from the transplanted *GR*^{-/-} duct itself and not the host wild type fat pad [55]. ER β has an antiproliferative function in the uterus [8, 56]. It may also have an antiproliferative function in the mammary gland. With this assumption, reduced ER β expression specifically in the *Bscl2*^{-/-} ductal epithelium could lead to increased proliferation of the ductal epithelium, leading to longer and dilated *Bscl2*^{-/-} mammary gland duct (Figs. 2, 3).

Leptin and genistein on puberty

There is a leptin surge during the second postnatal week, preceding the developmental estrogen increase in the female mice [57]. Leptin might promote puberty by indirectly stimulating gonadotropin-releasing hormone (GnRH) production [58]. Leptin is synthesized in the adipocytes and leptin levels are positively correlated with fat mass [59]. Therefore, lipodystrophy is often accompanied with reduced leptin levels [60]. Indeed, our *Bscl2*^{-/-} mice were previously demonstrated to have greatly reduced leptin levels [21], which might cause the delayed pubertal onset indicated by delayed vaginal opening (Fig. 1B). However, prepubertal exposure to 500 ppm genistein significantly accelerated vaginal opening in the *Bscl2*^{-/-} females to a level comparable to that in the genistein-treated control females (Fig. 1B).

Genistein can induce GnRH pulsatile production in the prepubertal mice [61]. It advances pubertal onset possibly via suppressing inhibitory and activating stimulatory components of the GnRH network [62]. Leptin administration could restore pubertal onset in the leptin-deficient mice [63]. However, *in vitro* studies showed that genistein could decrease leptin production in adipocytes [64, 65], while an *in vivo* study on adult ovariectomized C57BL/6 mice treated with 1500 ppm genistein diet for 21 days did not show any effect of genistein on blood leptin levels [66]. These observations suggested that genistein advanced pubertal onset in the *Bscl2*^{-/-} females but it might not act through increasing leptin levels. It could possibly enhance leptin sensitivity in the brain in a way similar to what estrogen does [67]. It is also possible that genistein could circumvent the defect(s) leading to delayed pubertal onset in *Bscl2*^{-/-} females via an unknown pathway.

Fat and pubertal mammary gland development

Pubertal mammary gland growth is characterized by ductal morphogenesis with extensive epithelial cell proliferation, a process that is believed to be regulated by paracrine signaling [7–9, 68]. Adipocytes play a key role in this paracrine regulation of pubertal mammary gland development [69]. Ablation of adipocytes during puberty could inhibit pubertal mammary gland growth, and restoration of adipocytes could rescue it to a considerable extent [70]. A-ZIP/F1 transgenic mice without white adipose tissue have short and dilated mammary gland ducts and lack normal pubertal mammary gland growth, resulting from the lack of adipose tissue but not any defects in the ductal epithelial cells [71]. Although the *Bscl2*^{-/-} females also lack well-developed fat tissue [21], they have long and dilated mammary gland ducts at 5 weeks old (Figs. 3A–3E). It is possible that the longer mammary gland ducts in the *Bscl2*^{-/-} females (Fig. 3E) are caused by the local loss of seipin in the ductal epithelium (Fig. 4A), potentially involving downregulation of ER β in the ductal epithelium (Fig. 5G, 5H).

Summary

Both mammary gland development and vaginal opening are markers for pubertal onset in mice. These two processes are segregated in the *Bscl2*^{-/-} female mice with lipodystrophy, indicated by accelerated mammary gland ductal growth but delayed vaginal opening. *Bscl2*^{-/-} females are responsive to genistein treatment, indicated by accelerated vaginal opening and increased mammary gland area. Mammary gland development is not as responsive as vaginal opening upon genistein treatment in the *Bscl2*^{-/-} females. This can be explained by accelerated pubertal mammary gland ductal growth but limited mammary fat pad in the *Bscl2*^{-/-} females. Reduced expression of ER β may contribute to the phenotypes in the *Bscl2*^{-/-} mammary gland ducts.

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Highlights

Accelerated pubertal mammary gland ductal development in *Bscl2*^{-/-} mice.

Delayed vaginal opening in *Bscl2*^{-/-} mice.

Accelerated pubertal onset by postweaning dietary genistein exposure.

Segregated responses of mammary gland development and vaginal opening to prepubertal genistein exposure in *Bscl2*^{-/-} mice.

Decreased ER β expression in *Bscl2*^{-/-} mammary gland ductal epithelium.

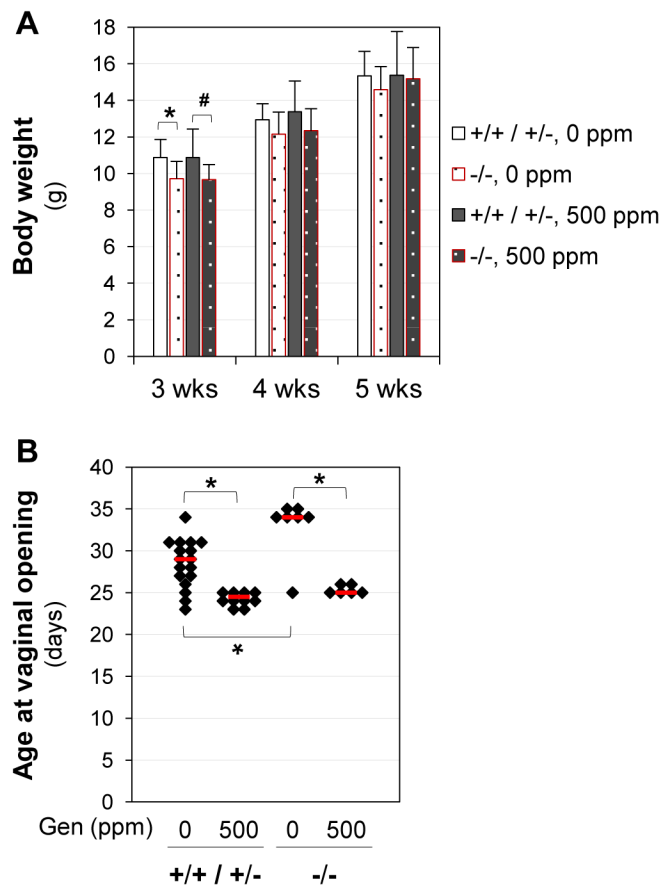


Figure 1.

Body weight and age at vaginal opening. A. Body weight from weaning (3 weeks old, 3wks) to dissection (5 wks). Body weight at weaning: two-tail unequal variance t-test; * $P=0.022$; # $P=0.086$. Body weight from 3 to 5 weeks old: ANOVA repeated measures; no treatment related difference ($P=0.645$). Error bars, standard deviations. $N=6-15$. B. Age at vaginal opening. * $p<0.05$; nonparametric Two-Sample Kolmogorov-Smirnov test. Black diamonds indicate data from individual mice; red lines indicate median in each group. $N=6-17$. +/+ / +/-, *Bscl2*^{+/+} (WT) and *Bscl2*^{+/-} (Het) mice as the genotype control for *Bscl2*^{-/-} (Hom) mice; -/-, *Bscl2*^{-/-} mice. Genistein (Gen) diets: 0 ppm or 500 ppm.

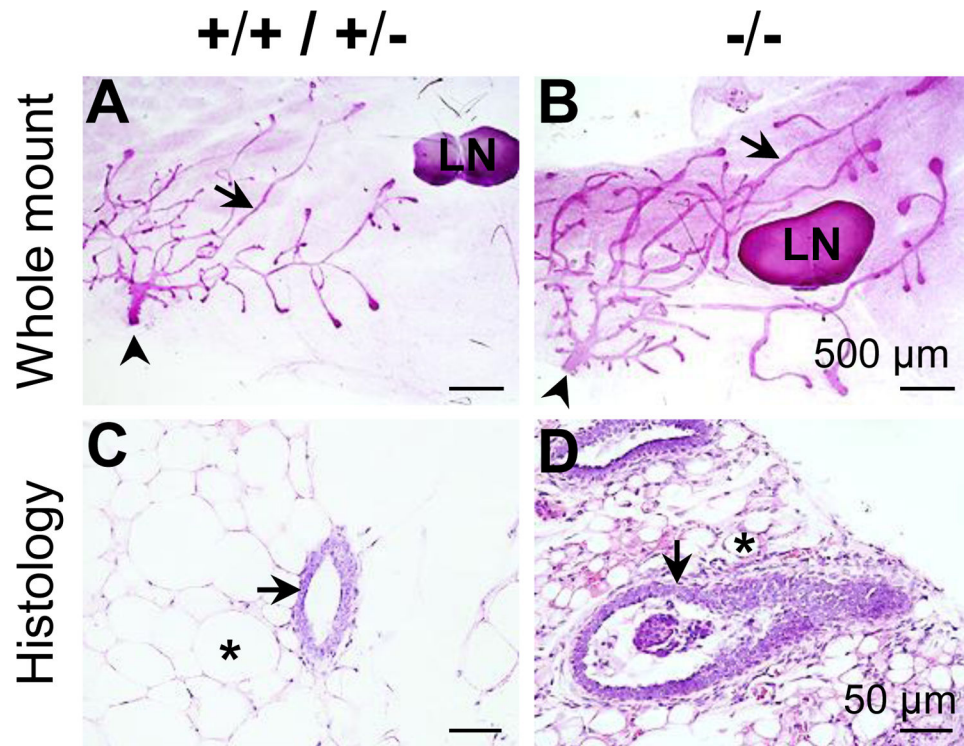


Figure 2. Representative images of whole mount and histology of mammary gland of females at 5 weeks old on vehicle control diet. A. Whole mount of *Bsc12*^{+/+} / *Bsc12*^{+/-} control mammary gland. B. Whole mount of *Bsc12*^{-/-} mammary gland. Scale bars in A & B, 500 µm. C. Histology of *Bsc12*^{+/+} / *Bsc12*^{+/-} control mammary gland. D. Histology of *Bsc12*^{-/-} mammary gland. H & E stain. Scale bars in C & D, 50 µm. A~D: arrows, mammary gland ducts; arrowheads, nipples; *, mammary gland adipocyte; LN, lymph node.

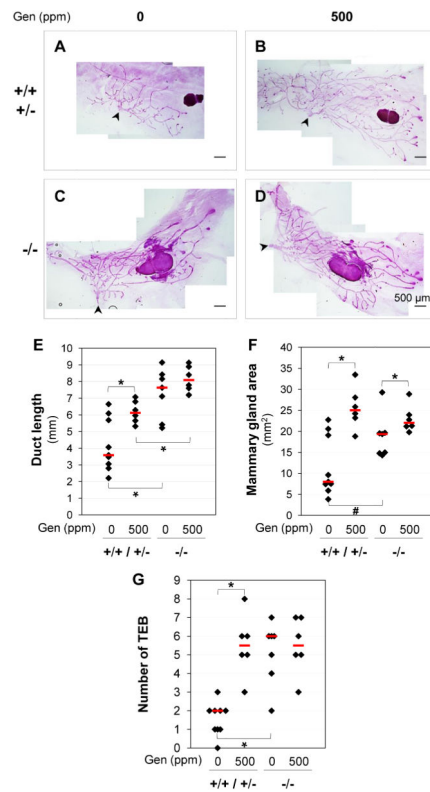


Figure 3.

Effects of *Bsc12*/seipin and genistein on mammary gland development. A~D. Representative images of whole mount mammary glands in *Bsc12*^{+/+} / *Bsc12*^{+/+}, 0 ppm genistein group (A), *Bsc12*^{+/+} / *Bsc12*^{+/-}, 500 ppm genistein group (B), *Bsc12*^{-/-}, 0 ppm genistein group (C), and *Bsc12*^{-/-}, 500 ppm genistein group (D) at 5 weeks old. E. Lengths of the longest mammary gland ducts. * P<0.05. F. Areas occupied by mammary gland ducts. * P<0.05; # P=0.092. G. Numbers of terminal end buds (TEB). * P<0.05. E–G: nonparametric Two-Sample Kolmogorov-Smirnov test. Black diamonds indicate data from individual mice; red lines indicate median in each group. N=6–9. +/+ / +/+, *Bsc12*^{+/+} and *Bsc12*^{+/-} control mice; -/-, *Bsc12*^{-/-} mice; Genistein (Gen) diets, 0 ppm or 500 ppm. A–D: scale bars, 500 μ m; arrowheads, nipples.

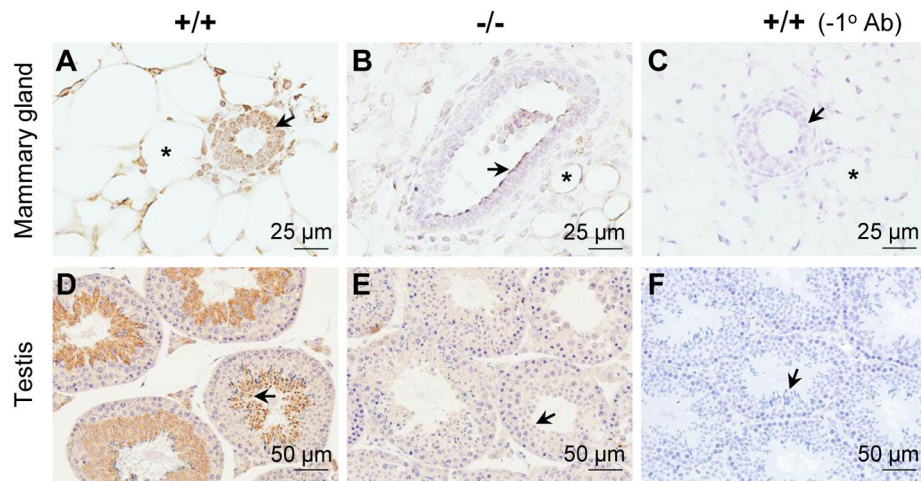


Figure 4. Immunohistochemistry detection of seipin expression in 5 weeks old mammary glands and 3 month old testes. A. Wild type (+/+) mammary gland with anti-seipin antibody. B. *Bsc12*^{-/-} mammary gland with anti-seipin antibody. C. Wild type (+/+) mammary gland without primary antibody (-1° Ab). A~C: arrows, mammary gland ductal luminal epithelial cells; *, mammary gland adipocytes; scale bars, 25 μm. D. Wild type (+/+) testis with anti-seipin antibody. E. *Bsc12*^{-/-} testis with anti-seipin antibody. F. Wild type (+/+) testis without primary antibody (-1° Ab). D~F: arrows, spermatids; scale bars, 50 μm. Brown signal, positive staining.

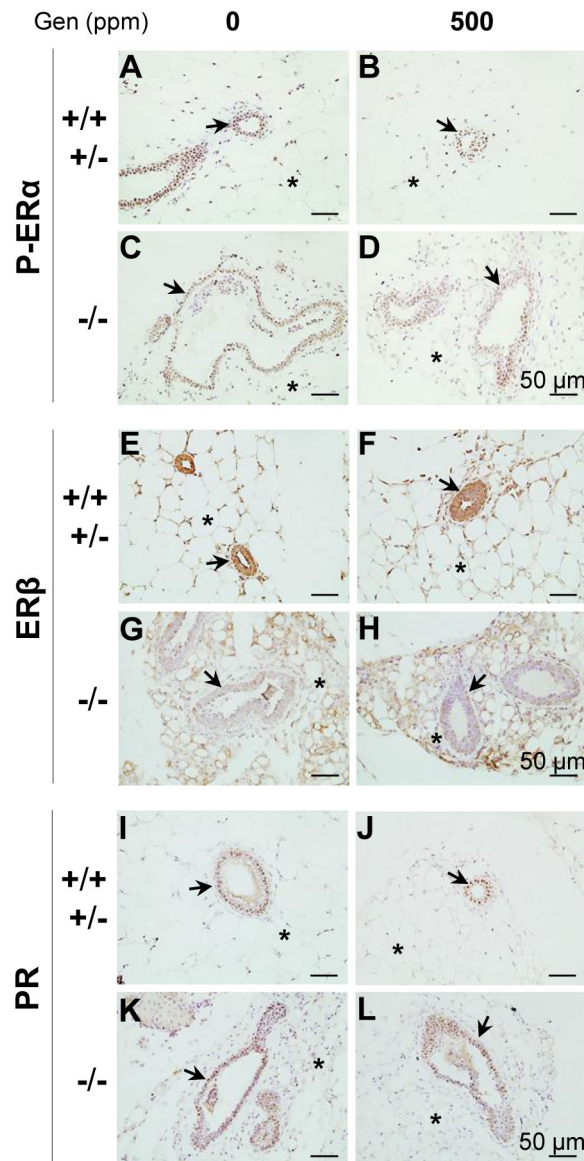


Figure 5.

Expression of phospho-estrogen receptor alpha (P-ERα/P-ESR1), estrogen receptor beta (ERβ/ESR2), and progesterone receptor (PR) in 5 weeks old mammary gland. Sections from 3 mice in each group were examined and representative images were shown. A. P-ERα, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 0 ppm genistein group. B. P-ERα, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 500 ppm genistein group. C. P-ERα, *Bsc12*^{-/-}, 0 ppm genistein group. D. P-ERα, *Bsc12*^{-/-}, 500 ppm genistein group. E. ERβ, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 0 ppm genistein group. F. ERβ, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 500 ppm genistein group. G. ERβ, *Bsc12*^{-/-}, 0 ppm genistein group. H. ERβ, *Bsc12*^{-/-}, 500 ppm genistein group. I. PR, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 0 ppm genistein group. J. PR, *Bsc12*^{+/+} / *Bsc12*^{+/-}, 500 ppm genistein group. K. PR, *Bsc12*^{-/-}, 0 ppm genistein group. L. PR, *Bsc12*^{-/-}, 500 ppm genistein group. Dark brown, immunostaining; purple-blue, counter staining with Harris Hematoxylin; no specific immunostaining in the minus primary

antibody negative control (Fig. 4C). Arrows, mammary gland ducts; *, mammary gland adipocytes.

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