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# Age dependency of estrogen responsiveness in the uterus and adipose tissue of aromatase-knockout (ArKO) mice

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ABSTRACT

Aging is often associated with weight gain caused by metabolic changes including an increase of body fat. In this study we assessed the impact of age on estrogen responsiveness in the uterus and adipose tissue (AT) in aromatase-knockout (ArKO) mice. ArKO mice at the age of three or twelve months respectively were treated s.c. with vehicle, $E_2$ (10 μg/kg BW/d) or genistein (15 mg/kg BW/d) for three days. In the ArKO mouse model we were able to demonstrate that estrogen treatment resulted in an age specific response pattern both on a physiological and molecular level. Assessment of basal gene expression levels revealed significant age dependent differences only for elevated *Esr1* levels in the uterus and leptin levels in infrarenal fat as well as lower levels of *Pparg* in the gonadal fat tissue. Investigating age dependency of estrogen responsiveness we were able to show that the $E_2$ and genistein resulted in age related pattern of regulation of expression of *Esr1* and *Lep* in infrarenal and gonadal AT as well as the uterine expression of *Pgr*, *Ltf* and *Pparg*. In conclusion, evidence is provided that aging has an impact on the effectiveness of estrogen regulated processes in uterus and AT of ArKO mice. It remains to be elucidated whether or not this is associated with weight gain caused by an increase in body fat mass.



## 1. Introduction

Aging is often associated with an increase in body fat mass [1], progressive development of insulin resistance and a number of general metabolic changes [1,2]. Besides the increased body weight these metabolic changes result in changes of serum parameters linked to energy and lipid metabolism [3], as well as to changes in gene expression in metabolically active tissues e.g. adipose tissue (AT) but also in reproductive organs like the uterus.

In the study presented here, we aimed to link metabolic changes resulting from estrogen deficiency to their impact on estrogen responsiveness. For this purpose we used aromatase-knockout (ArKO) mice as a model for estrogen deficiency since these animals have a functional disruption of the *Cyp19* (aromatase) gene. Jones et al. [2] have already published several phenotypical observations related to age as this mouse strain is characterized by a pronounced age-dependent increased obesity, with accumulation of abdominal AT [4] caused by estrogen deficiency. Phenotypically ArKO mice are therefore similar to estrogen receptor alpha knockout mice [5,6].

Darimont et al. showed a further connection between obesity and estrogen deficiency by demonstrating a decreased lipolysis in AT in rats following ovariectomy (ovx) [7]. On the other hand 17β-estradiol ($E_2$) treatment reduces fatty acid synthesis and increases lipolysis in fat cells [8]. In general, obesity is associated with insulin resistance and increased levels of circulating lipids. Metabolic changes like impaired glucose tolerance and age dependent increase of obesity have already been described in male and female ArKO mice [5]. Treatment with $E_2$ reverses these effects [4]. Contrary to intuition, Jones et al. could show that food intake is reduced in ArKO mice compared to wild type (WT) mice, ruling out enhanced food uptake as a reason for the increased body weight gain in ArKO mice [2]. In addition it was shown that cholesterol substitution prevents obesity in ArKO mice via a decrease in hypertrophy and hyperplasia in AT [9].

It is known that estrogen deficiency has an impact on lipid metabolism [2,7,8]. Permanently reduced estrogen levels during the climacteric transition are associated with increased adiposity resulting in a higher risk of developing a metabolic syndrome (MetS). It has been demonstrated that adult female ArKO mice are significantly heavier and have significantly larger gonadal AT and infrarenal fat pads than their WT littermates from three months

*Abbreviations:* vt, vehicle treated (DMSO and castor oil); ArKO, aromatase-knockout; AT, adipose tissue; MetS, metabolic syndrome; PCR, polymerase chain reaction; s.c., subcutaneous; ELISA, enzyme-linked immunosorbent assay; $E_2$, estradiol.

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of age onwards [1]. Four months old ArKO mice have circulating leptin levels which are two- to threefold higher than those of WT animals [2]. It has to be expected that life long absence of estrogens in addition to changes in general physiological parameters (described above) influences gene expression as well.

Whereas processes discussed above are clearly progressing with age, these changes are often not considered in an experimental situation. Studies on estrogen responsiveness often do not consider the potential impact of age of the animal on the outcome of the experiment. Furthermore, although estrogen deficiency in humans usually occurs at an advanced age, the majority of uterotrophic assays in rodent are carried out in young animals (first three months of life). Therefore age apparently is not only a crucial factor for comparability of the results from uterotrophic assays, but also important if data obtained from experimental models are interpreted regarding their relevance for the human situation [10].

We have previously investigated the differences of estrogen responsiveness related to inactivation of Cyp19 by comparing responses in ArKO mice with those in castrated WT mice at the age of three months [11]. We comparatively analyzed these mice in the presence of $E_2$ and under $E_2$-deficient conditions and found significant differences in body weight and gene expression pattern in AT. In the study presented here, we first evaluated the effect of age on the expression levels of estrogen responsive genes in ArKO mice without any estrogenic treatment. In addition we treated ArKO mice of different ages with $E_2$ and genistein to investigate whether their estrogen responsiveness is dependent on age. As a readout we investigated metabolic changes in physiological parameters like body-, uterus- and AT-weight as well as in regulation of gene expression. For gene expression analysis we chose lactoferrin (*Ltf*) as a known estrogen marker gene and as the major ER/$E_2$ response gene in the mouse uterus [12] and estrogen receptor alpha (*Esr1*), as well as estrogen receptor beta (*Esr2*) as mediators of estrogen responsiveness. The estrogenic regulation of genes known to be involved in mediating fat cell biology was investigated for example by the assessment of expression levels of leptin (*Lep*) and peroxisome proliferator-activated receptor gamma (*Pparg*). *Lep* represents a gene which is linked to the MetS [13] and *Pparg* which represents a mechanistic link to energy metabolism [14]. In addition to $E_2$ we included an experimental group treated with the phytoestrogen genistein because it is often advertised as a "natural" alternative for hormone therapy for treatment of menopausal symptoms [15]. Genistein is an isoflavone found mainly in soy beans, with a known estrogenic activity [16,17]. The estrogenicity of genistein has been explained by its affinity to the estrogen receptors (ER), particularly to Esr2 [18] caused by the structural similarity to $E_2$ [19–21].

The main objective of this study was to provide evidence for the change in estrogen responsiveness in the course of aging in the ArKO mouse model. Therefore we examined the consequence of estrogen substitution on AT depots as well as on the uterus in young adult and in aged adult female ArKO mice.

## 2. Materials and methods

### 2.1. Mice

ArKO mice were kindly provided by Prof. Dr. Evan Simpson (Melbourne, Australia). They were generated by disrupting the *Cyp19* gene as described by Fisher et al. [22]. Subsequently these mice have been crossed back by us to a C57BL/6J background for 10 generations thereby assuring that the studied animals are identical in their genetic variation with the exception of the knock out of the Cyp19 gene and that epigenetic variation is reduced. Females and males heterozygous for aromatase were bred and their homozygous female offspring was used for the experiments performed. In addition homozygous WT offspring were used for some comparative investigations. The genotype was analyzed by PCR as previously described [23]. Animals were maintained under controlled conditions of temperature ($20 \pm 1\,^{\circ}C$, relative humidity 50–60%), illumination (12 h light, 12 h dark), water *ad libitum* and phytoestrogen free diet (Harlan 2019 Rodent Breeding, Harlan Winkelmann, Borchen, Germany). All animal handling and all experimental conditions were licensed and carried out according to the Institutional Animal Care and Use Committee guidelines as regulated by the German federal law governing animal welfare.

### 2.2. Treatment

Adult female ArKO mice were randomly assigned to six experimental groups of seven animals each, as illustrated in Table 1. Three and twelve months old animals were studied, divided into the following treatment groups: vehicle treated (vt) (solvent and castor oil), treatment with $E_2$ (10 μg/kg BW/d) (Sigma–Aldrich, Munich, Germany) and with genistein (15 mg/kg BW/d) (Sigma–Aldrich, Munich, Germany). Treatment was performed by s.c. injections once daily for three consecutive days.

### 2.3. Tissue collection

Necropsy was carried out following cervical dislocation and blood collection. The blood was allowed to clot. In addition, serum and plasma was collected and stored at $-20\,^{\circ}C$. At necropsy, uterus and infrarenal AT were removed, weighed and immediately stored in liquid nitrogen for future use.

### 2.4. Measurement of plasma and serum leptin levels

The glucose oxidase method was used to determine plasma glucose concentrations using a quantitative colorimetric glucose determination kit (QuantiChrom™ Glucose Assay Kit, BioAssay Systems, Hayward, USA) at 630 nm. Furthermore, serum leptin concentrations were determined by an enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of mouse leptin (RayBio® Mouse Leptin ELISA Kit, (RayBiotec Inc., Norcross, USA).

### 2.5. RNA preparation and real-time PCR

Total RNA was isolated from 30 mg uterine tissue, gonadal AT and infrarenal AT using Qiagen RNeasy®Plus (Qiagen GmbH, Hilden, Germany). RNA from all animals of the same treatment group was pooled and DNA contaminations were enzymatically eliminated by digestion (Desoxyribonuclease 1, Roche Diagnostic GmbH, Mannheim, Germany). Oligo$(dT)_{18}$-primers and MMLV-Reverse Transcriptase (Roche Diagnostic GmbH, Mannheim, Germany) were used for the first-strand cDNA synthesis. Relative mRNA levels were detected by quantitative real-time PCR using SYBR green as an intercalating fluorescent dye in an iCycler Thermal Cycler with iQ real-time Detection System (Bio-Rad Laboratories GmbH, Munich, Germany) as previously described [24]. Ribosomal Protein S18 (*Rps18*) mRNA was used as an internal reference gene [25,26]. For comparing basal gene expression the following formula was used: $E = 2^{-(Ct(RPS18) - Ct(target\ gene))}$ with E representing the expression of a target gene in a sample relative to the expression of RPS18 in the same sample. Expression changes following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method which additionally normalizes against the vehicle control group represented in the diagrams with a horizontal line [27]. For qPCR the following primers have been used (product sizes are indicated in brackets): *Rps18*-fwd: 5′-AGGATGTGAAGGATGGGAAG-3′, *Rps18*-rev: 5′-TTGGATACACCCACAGTTCG-3′ (187 bp); estrogen receptor

**Table 1**
Experimental design.

| | Untreated mice ($n=7$) | Treated mice ($n=7$) | Treated mice ($n=7$) | Untreated mice ($n=7$) | Treated mice ($n=7$) | Treated mice ($n=7$) |
|---|---|---|---|---|---|---|
| Animals | ArKO mice | | | | | |
| Age | 3 months | 3 months | 3 months | 12 months | 12 months | 12 months |
| Treatment | Vehicle | 17β-Estradiol | Genistein | Vehicle | 17β-Estradiol | Genistein |
| Chow/duration | Phytoestrogen free/3 days s.c. | | | | | |

**Table 2**
Body weight, uterus and infrarenal AT wet weights of vehicle-, $E_2$- and genistein treated ArKO mice at the age of 3 and 12 months.

| Treatment – age | Body weight (g) | Uterus (ww, g/kg BW) | Infrarenal adipose tissue (ww, g/kg BW) |
|---|---|---|---|
| Vehicle – 3 months | 25.36 ± 2.40 | 0.51 ± 0.22 | 10.87 ± 6.74 |
| $E_2$ – 3 months | 25.97 ± 3.14 | 2.66 ± 0.94$^{+++}$ | 8.37 ± 4.61 |
| Genistein – 3 months | 25.10 ± 3.44 | 0.44 ± 0.28 | 5.43 ± 2.99 |
| Vehicle – 12 months | 32.85 ± 4.17$^{***}$ | 0.33 ± 0.14$^{*}$ | 10.54 ± 5.23 |
| $E_2$ – 12 months | 33.89 ± 9.45$^{*}$ | 2.29 ± 0.71$^{+++}$ | 10.64 ± 3.36 |
| Genistein – 12 months | 36.84 ± 6.60$^{***}$ | 0.41 ± 0.13 | 13.43 ± 5.67$^{**}$ |

Results are presented as mean ± standard deviation ($n=7$).
$^{*}$ $p<0.05$.
$^{**}$ $p<0.01$.
$^{***}$ $p<0.001$ to ArKO group at the age of *3 months*.
$^{+++}$ $p<0.001$ to respective vt *ArKO*.

alpha- (*Esr1*) fwd: 5′-TGTTTGCTCCTAACTTGCTCCC-3′, *Esr1*-rev: 5′-GGTGGATGTGGTCCTTCTCTT-3′ (222 bp); estrogen receptor beta- (*Esr2*) fwd: 5′-GCCAGGAAGCAGAGAGTAGC-3′, *Esr2*-rev: 5′-TCATGCTGAGCAGATGTTCC-3′ (189 bp); progesterone receptor- (*Pgr*) fwd: 5′-CTGGCATGGTCCTTGGAG-3′, *Pgr*-rev: 5′-TGGAAGT-GTCAGGCTTTGTG-3′ (246 bp); lactoferrin- (*Ltf*), fwd: 5′-GCAAAA-CCACATCGGAGAAG′-3′, *Ltf*-rev: 5′-GGGAGTGAGGAGACCAGATG-3′ (204 bp); clusterin- (*Clu*) fwd: 5′-GATTCCCTCCCAGACACTCC-3′, *Clu*-rev: 5′-GTGTCACTGAGGTGGTGGTG-3′ (196 bp); leptin- (*Lep*) fwd: 5′-AGATCCCAGGGAGGAAAATG-3′, *Lep*-rev: 5′-TGAAGCCC-AGGAATGAAGTC-3′ (217 bp); leptin receptor- (*Lepr*) fwd: 5′-TCG-ACAAGCAGCAGAATGAC-3′, *Lep*-rev: 5′-CTTGTGCCCAGGAACAA-TTC-3′ (159 bp); insulin receptor- (*Insr*) fwd: 5′-GAATGTGGGGATG-TCTGTCC-3′, *Insr*-rev: 5′-ATCAGGTTCCGAACAGTTGC-3′ (201 bp); and peroxisome proliferator-activated receptor gamma- (*Pparg*) fwd: 5′-TCATGACCAGGGAGTTCCTC-3′ *Pparg*-rev: 5′-CAGG TTGTC-TTGGATGTCCTC-3′ (200 bp).

### 2.6. Statistical analysis

Gene expression data are presented as mean ± SD for all PCR reactions. All PCR reactions were conducted in triplicates with three independent cDNA samples. Statistical analysis was performed for weight measurements by ANOVA (one-way) followed by Bonferronie post hoc test; PCR data were analyzed using Students *t*-test.

## 3. Results

### 3.1. Physiological parameters

Independent of treatment twelve months old ArKO mice had a significantly higher body mass than ArKO mice at the age of three months (Table 2). In vehicle treated (vt) WT mice of different ages no significant difference in BW between the age groups was observed (data not shown). On average an increase of body weight in aged vt WT mice of 1.2 g was detectable. In contrast, in aged vt ArKO mice we measured an average increase of bodyweight of 7.48 g compared to the 3 months old counterparts.

The uterus wet weight of the ArKO mice was significantly increased after $E_2$ treatment compared to that of untreated ArKO mice of the same age (Table 2). In addition, the uteri of younger vt ArKO mice were 64% heavier than the uteri of the older ones. Following genistein treatment, no changes were detectable compared to vt treated control animals (Table 2).

Furthermore, infrarenal AT weight increased significantly by 40% in the twelve months old animals compared to the three months old mice only following the three-day treatment with genistein (Table 2).

### 3.2. Serum leptin and plasma glucose levels

Regarding circulating serum levels of leptin a tendency towards a slight elevation was observed in ArKO mice at the age of twelve months compared to the three months old ArKO mice when comparing identical treatment groups. The serum leptin level was also significantly increased in response to genistein treatment if compared to the control animals (Table 3). This effect however, was exclusively detectable in the twelve months old ArKO mice (Table 3).

Plasma glucose levels did not change significantly in any treatment group (Table 3).

### 3.3. Gene expression

Estrogen responsiveness was comparatively assessed in the two different age groups of ArKO mice in uterus, gonadal and infrarenal AT following treatment with $E_2$ or genistein. As we observed a comparatively large biological variation we used standard deviations (SD) as measure of variation instead of the standard error of the mean (SEM), to explicitly depict the degree of variation.

**Table 3**
Serum leptin level and plasma glucose level of vehicle-, $E_2$- and genistein treated ArKO mice at the age of 3 and 12 months.

| Treatment – age | Serum-leptin-level in pg/mL | Plasma-glucose-level in mg/mL |
|---|---|---|
| Vehicle – 3 months | 176.8 ± 45.4 | 98.5 ± 23.7 |
| $E_2$ – 3 months | 114.5 ± 31.9 | 108.8 ± 28.3 |
| Genistein – 3 months | 72.2 ± 25.9 | 91.5 ± 23.2 |
| Vehicle – 12 months | 213.20 ± 142.5 | 108.8 ± 39.4 |
| $E_2$ – 12 months | 271.2 ± 161.4 | 110.3 ± 1.64 |
| Genistein – 12 months | 1246.2 ± 994.4$^{**,+}$ | 109.2 ± 38.8 |

Results are presented as mean ± standard deviation ($n=7$).
$^{**}$ $p<0.01$ to *ArKO Gen, 3 months*.
$^{+}$ $p<0.05$ to *vt ArKO, 12 months*.

*3.3.1. Age related basal gene expression levels*

Cases of age dependent, statistically significant changes in expression amongst the genes investigated were rare. As one would expect, an up-regulation of *Lep* expression in the infrarenal fat tissue with increasing age was detectable. In gonadal adipose tissue a down-regulation of basal levels of *Pparg* became apparent in the twelve months old animals (Figs. 1–3, panel A).

*3.3.2. Estrogen responsiveness of uterine gene expression*

To investigate whether aging has an impact on uterine gene expression in response to $E_2$ and genistein in ArKO mice, the gene expression levels of *Esr1*, *Esr2*, *Pgr*, *Ltf* and *Pparg* were determined (Fig. 1).

As shown in Fig. 1B the expression level of *Esr1* in response to $E_2$ was found to be increased in young adult animals while it was slightly decreased in aged animals. In contrast for *Esr2* the response to $E_2$, a decrease was found in young adult animals and an increase in aged animals. *Ltf*, as the major ER/$E_2$ response gene in the mouse uterus was found to be increased in response to estradiol in both age groups, but this effect was 10-fold more pronounced in young adult animals. The expression of *Ltf* was decreased in response to genistein in older ArKO mice but not in the younger age group. *Pgr* expression was not significantly affected by $E_2$ treatment regardless of age, but was found to be strongly decreased in young animals in response to genistein. Finally, *Pparg* was found to be decreased in young adult animals by both $E_2$ and genistein treatment, again a feature not recapitulated in old animals.

*3.3.3. Estrogen responsiveness of gonadal adipose tissue*

Comparing three and twelve month old mice gene expression levels of *Lep*, *Lepr* and *Pparg* decreased following $E_2$ as well as genistein treatment (Fig. 2B). Of particular note both treatments resulted in a considerable up-regulation of the *Lep* mRNA level in the younger animals, up to four-fold in the $E_2$ and about 30-fold in the genistein group (Fig. 2B).

*3.3.4. Estrogen responsiveness of infrarenal adipose tissue*

For infrarenal AT the expression of the same set of genes as in the gonadal AT (*Esr1*, *Esr2*, *Lep*, *Lepr* and *Pparg*) was investigated. Age dependency of estrogen responsiveness was found for *Esr1* and *Lep* expression. Following $E_2$ and genistein treatment for both genes the responsiveness is less pronounced in age animals if compared to the responsiveness of three months old ArKO mice. *Esr2*, *Lepr* and *Pparg* showed a similar pattern. The expression of all three genes was decreased in response to $E_2$ treatment at both ages. Although genistein treatment of the younger mice resulted in a down-regulation in *Esr2*, *Lepr* and *Pparg*, this effect was almost completely attenuated in older mice. An inversed pattern of expression between $E_2$ and genistein treatment was observed for *Esr2* expression which was up-regulated in aged ArKO mice in response to genistein treatment while it was down-regulated in response to $E_2$ of compared to vt group.

## 4. Discussion

This is the first study investigating the impact of age on the regulation of estrogen responsive genes in estrogen deficient ArKO-mice. We thereby aimed to comparatively assess the estrogen responsiveness of young adult and aged mice in response to $E_2$ and genistein in this specific mouse model.

*4.1. Physiological parameters*

As described previously the ArKO mice at the age of twelve months are significantly heavier than ArKO mice aged three months. This observation was also made by Jones et al. who described that the body weight gain increased with age of ArKO mice [1]. In a pilot study to the experiment described here we observed that adult WT mice of identical genetic background to the ArKO mice display no significant age dependent difference in body weight (data not shown). Uteri of three months old vt ArKO mice were heavier than those of the twelve months old vt mice and this effect was still evident in trend comparing the $E_2$-treated groups. The reduced uterus wet weight of twelve months old mice could be an indicator for long-term effects related to life-long estrogen deficiency. This life-long estrogen deprivation may impact on the epigenome, as aromatase expression itself is subject of epigenetic regulation [28] and the general resetting of the epigenome presumable is influenced by estrogens as studies with estrogenic endocrine disrupters suggest [29,30].

Interestingly, no change in uterine wet weight has been observed following treatment with genistein, an observation also made by Moller et al. [31] in castrated rats. Mechanistically estrogenic responses in the uterus are primarily mediated by *Esr1* which is higher expressed in uterus than *Esr2*. As a consequence estrogen treated animals respond to $E_2$ with an increased proliferation in the uterus [32]. In contrast genistein shows a binding preference to *Esr2* [18] which explains that following genistein treatment proliferation resides on a lower level than following $E_2$ treatment.

The increase in body weight seems to be attributable to a significant gain of infrarenal AT mass which could be observed in the 12 months old animals as compared to the younger ones. All other treatments resulted only in a statistically not significant trend towards heavier fat pads at the age of twelve months. A potential mechanistic clue to explain this observation may come from a report on increased adipogenesis and lipogenesis in white adipose tissue of ArKO mice [33]. We did not investigate marker genes for adipogenesis and lipogenesis, but compared to the WT animals a higher amount of adipose tissue is observable in ArKO mice.

*4.2. Metabolic parameters*

Jones et al. demonstrated that serum leptin levels are significantly elevated in ArKO mice, an observation which is supported by our findings in this investigation as well as in a previous study [2]. This observation was not surprising, given the highly increased body weight of ArKO mice compared to their WT siblings and the established association between an obese stature and an increased production of leptin in most obese human subjects and most animal models of obesity [1,2,34]. Additionally, we could demonstrate that older ArKO mice of all treatment groups had a higher, although statistically not significant serum leptin level if compared to younger ArKO mice of the same treatment groups. This is in line with previous observations showing that leptin regulates body fat predominantly by decreasing food intake [2]. In addition higher leptin levels were observed in obese subjects and were discussed in relation to a deficiency of leptin function in these obese individuals [34,35], as serum-leptin seems to be unable to bind and so the transport to the lateral hypothalamic area is disconnected.

In line with this conclusion, we also showed a tendency towards a higher leptin level in older ArKO mice. Therefore the question arises whether leptin indeed represents the main factor responsible for an increasing AT or which additional factors could account for this observation. Kloting et al. proposed that increase of visceral AT is associated with insulin resistance independent of BMI in humans and clearly related to dysfunction of circulating adipokines [36]. We did not investigate whether ArKO mice of both ages are insulin resistant, but no differences in plasma glucose levels could be observed which makes this possibility unlikely.

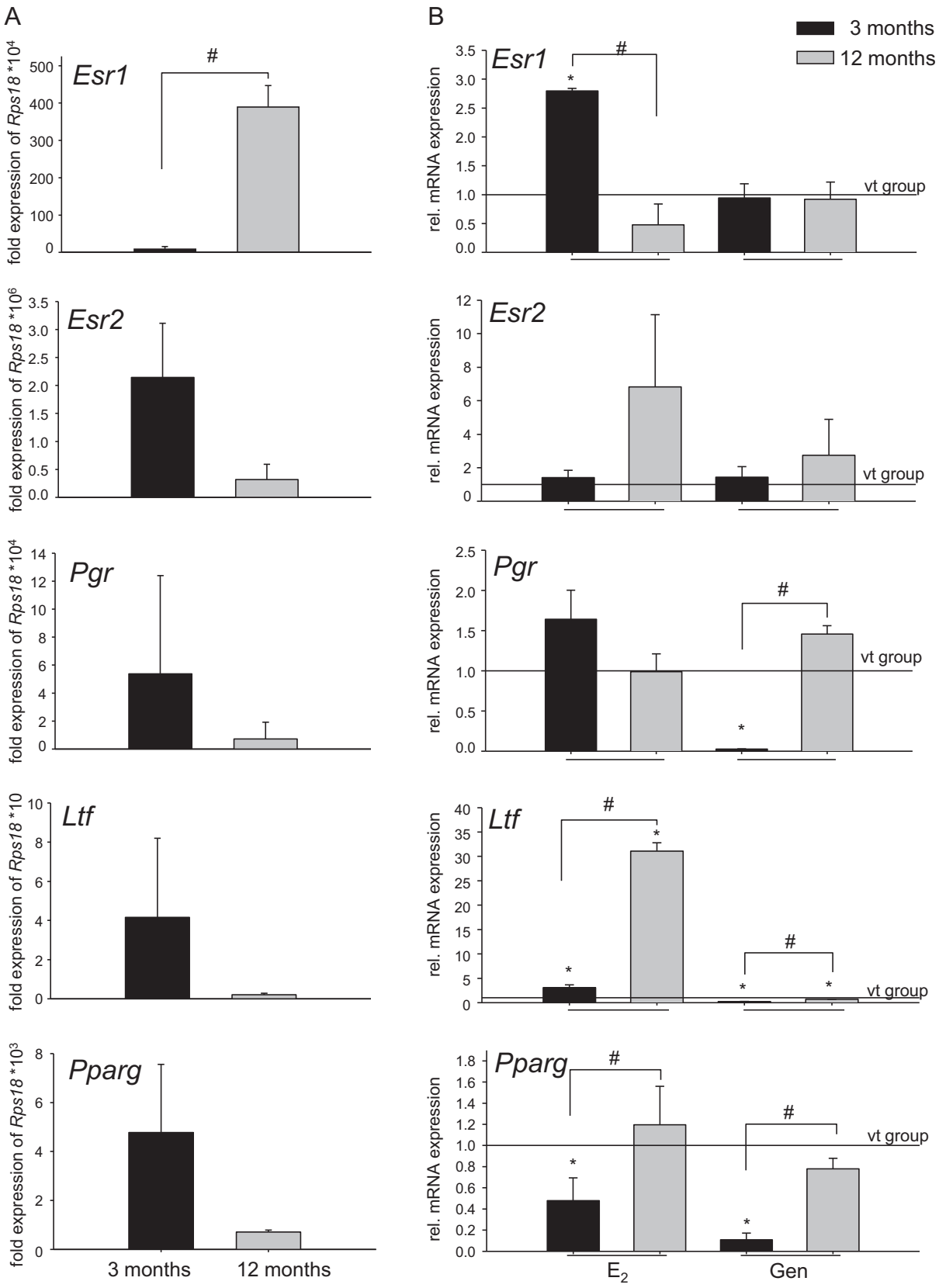


**Fig. 1.** Uterine. mRNA expression levels of *Esr1*, *Esr2*, *Pgr*, *Ltf* and *Pparg* in three and twelve months old ArKO mice. (A) Comparison of basal expression levels at both time points. (B) Expression levels following a three day treatment with either vehicle, $E_2$ or genistein. All gene expression levels were normalized against those measured in the vehicle treated group (black horizontal line). Data are presented as mean ± SD. Statistical analysis was performed using the Students *t*-test. * indicates a statistically significant difference ($p < 0.05$) compared to vehicle treated ArKO mice; # indicates a significant difference ($p < 0.05$) between indicated treatments.

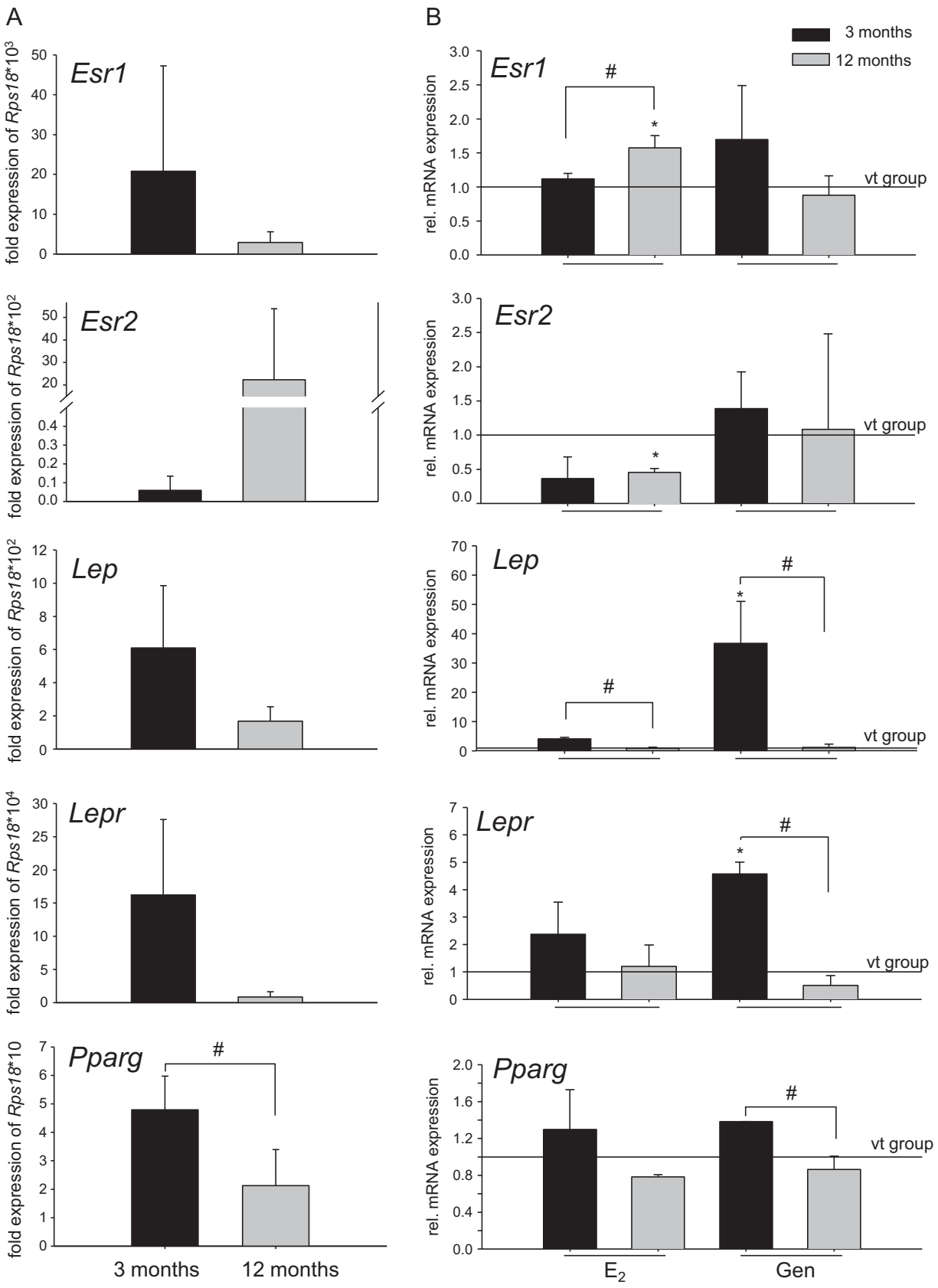


**Fig. 2.** Gonadal adipose tissue. mRNA expression levels of *Esr1*, *Esr2*, *Lep*, *Lepr* and *Pparg* in three and twelve months old ArKO mice. (A) Comparison of basal expression levels at both time points. (B) Expression levels following a three day treatment with either vehicle, $E_2$ or genistein. All gene expression levels were normalized against those measured in the vehicle treated group (black horizontal line). Data are presented as mean ± SD. Statistical analysis was performed using the Students *t*-test. * indicates a statistically significant difference ($p < 0.05$) compared to vehicle treated ArKO mice; # indicates a significant difference ($p < 0.05$) between indicated treatments.

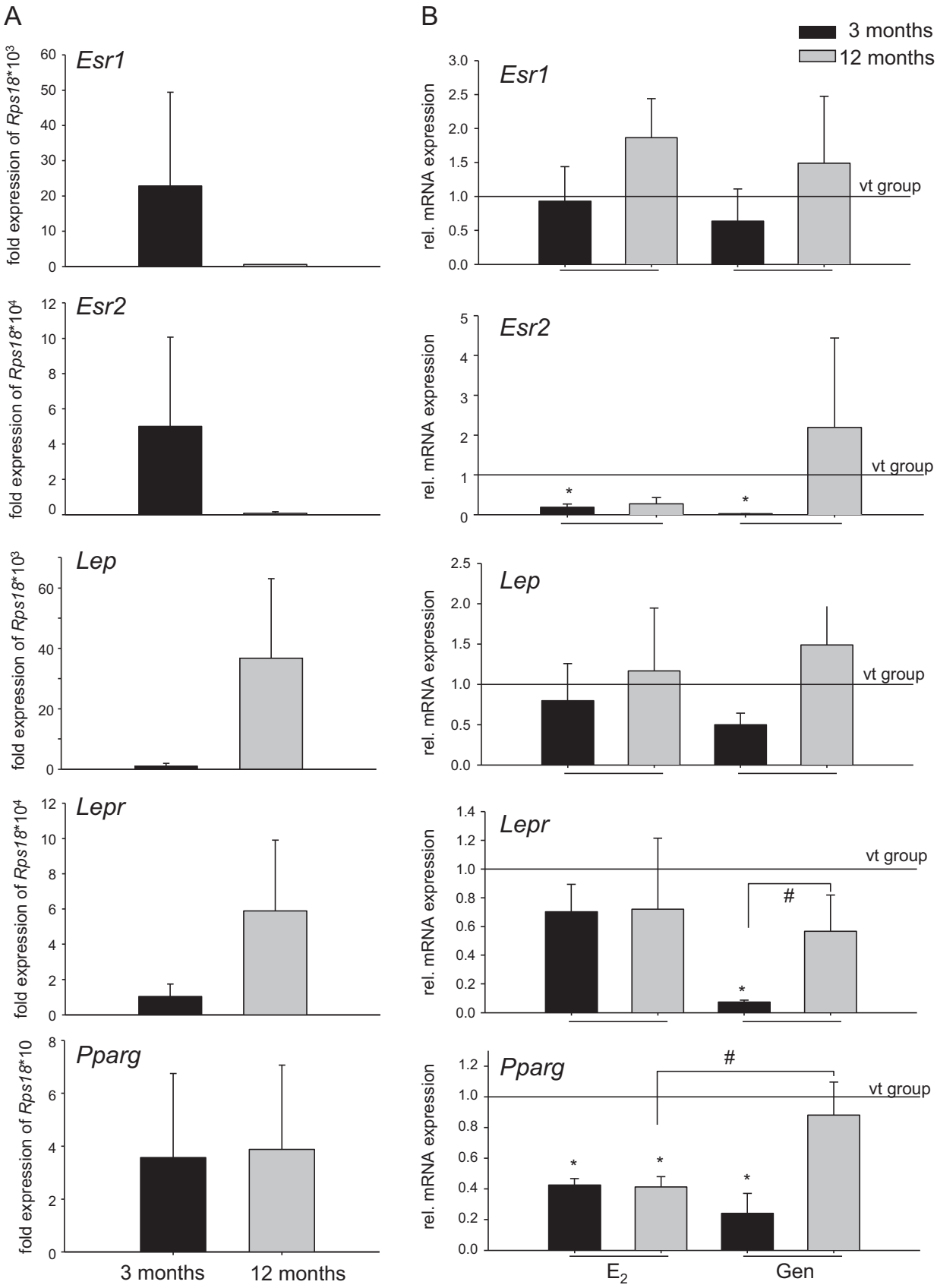


**Fig. 3.** Infrarenal adipose tissue. mRNA expression levels of *Esr1*, *Esr2*, *Lep*, *Lepr* and *Pparg* in three and twelve months old ArKO mice. (A) Comparison of basal expression levels at both time points. (B) Expression levels following a three day treatment with either vehicle, $E_2$ or genistein. All gene expression levels were normalized against those measured in the vehicle treated group (black horizontal line). Data are presented as mean ± SD. Statistical analysis was performed using the Students *t*-test. * indicates a statistically significant difference ($p < 0.05$) compared to vehicle treated ArKO mice. # indicates a significant difference ($p < 0.05$) between indicated treatments.

### 4.3. Gene expression data

In the study presented here, it became obvious that most of the investigated genes did not respond to estrogen treatment in a comparable manner (Figs. 1–3). However, the aging process already affected the basal levels of expression of the investigated genes. Furthermore, analyzing the responsiveness in groups treated with either $E_2$ or genistein age related changes in response to treatment could be detected in uterus and AT of ArKO mice.

In uterus, the estrogen response marker genes and *Esr2* showed an age related regulation pattern, resulting in a higher sensitivity to $E_2$ of three months old ArKO mice if compared to twelve months old ArKO mice. $E_2$ dependent regulation of *Ltf* in the mouse uterus has been previously established as the most sensitive marker for estrogenic responses [37]. For example, 4 h after three $E_2$ injections an increase of *Ltf* expression became detectable, which peaked at a 300-fold increase [37]. Our results suggest a substance specific regulation of expression of *Ltf* (increase after $E_2$ treatment, decrease after genistein treatment). This pattern was the same in both age groups but the extent of the change in responsiveness was different. It appears that regarding regulation of mRNA expression of this specific gene older ArKO mice react more sensitive to estrogen and genistein treatment than younger ArKO mice. This again demonstrates that animal age is a critical determinant for the outcome of an uterotrophic assay. An age dependent change in sensitivity in turn has critical implications for the interpretation and for the comparability of data obtained from these kinds of assays.

In gonadal AT we were able to demonstrate age related changes on basal gene expression, however only *Pparg* expression changes reached values of significance. Such age related changes of gene expression were also observed in $E_2$ and genistein treated ArKO mice. Here, the MetS related genes *Lep*, *Lepr* and *Pparg* showed a stronger regulation in younger mice compared to older mice. This means the estrogen responsiveness in younger mice is more pronounced than in aged animals. In the case of *Esr1* age dependent changes in the regulation of gene expression pattern are substance specific. The increase of *Esr1* expression following $E_2$ treatment was less pronounced in three months old animals than in twelve months old animals. Regarding genistein treatment the three months old mice display a more pronounced responsiveness. This leads to the conclusion that the substance used for treatment and the age of the ArKO-mice have a combined influence on the expression of estrogen response genes in gonadal AT.

For all four genes investigated age-dependent differences in estrogen responsiveness could be monitored in infrarenal AT. The findings in infrarenal AT also confirm our conclusion of the age dependent changes of estrogen responsiveness in gonadal AT in ArKO mice. These gene regulatory effects observed in infrarenal AT were even more pronounced in genistein treated animals. This observation is interesting in the perspective *Pparg* has been described as molecular target of genistein function thereby influencing the processes of AT remodelling [38].

In conclusion, using physiological parameters like organ and body weight, the evaluation of gene expression patterns and immunobiochemical assessment of serum parameters we were able to demonstrate a tissue and gene dependent change in $E_2$ responsiveness with increasing age in adult ArKO mice. Overall the aging process seems to attenuate the estrogen responsiveness in the investigated target tissues uterus, gonadal and infrarenal fat tissue, although exceptions were observed like for estrogenic regulation of expression of *Ltf* in the uterus. In conclusion, our results suggest that animal age, as shown in the experimental model of the ArKO-mouse, is a crucial determinant for estrogen responsiveness, and consequently also needs to be considered in the study design. This seems particularly important if experimental animal models are used to study treatment options for symptoms which in humans reflect physiological changes occurring as a consequence of the aging process like the menopausal transition.

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## References

[1] M.E. Jones, A.W. Thorburn, K.L. Britt, K.N. Hewitt, M.L. Misso, N.G. Wreford, J. Proietto, O.K. Oz, B.J. Leury, K.M. Robertson, S. Yao, E.R.l. Simpson, Aromatase-deficient (ArKO) mice accumulate excess adipose tissue, J. Steroid Biochem. Mol. Biol. 79 (1–5) (2001) 3–9.
[2] M.E. Jones, A.W. Thorburn, K.L. Britt, K.N. Hewitt, N.G. Wreford, J. Proietto, O.K. Oz, B.J. Leury, K.M. Robertson, S. Yao, E.R. Simpson, Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity, Proc. Natl. Acad. Sci. U.S.A. 97 (23) (2000) 12735–12740.
[3] K.L. Britt, A.E. Drummond, V.A. Cox, M. Dyson, N.G. Wreford, M.E. Jones, E.R. Simpson, J.K. Findlay, An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene, Endocrinology 141 (7) (2000) 2614–2623.
[4] I. Czajka-Oraniec, E.R. Simpson, Aromatase research and its clinical significance, Endokrynol. Pol. 61 (1) (2010) 126–134.
[5] P.A. Heine, J.A. Taylor, G.A. Iwamoto, D.B. Lubahn, P.S. Cooke, Increased adipose tissue in male and female estrogen receptor-alpha knockout mice, Proc. Natl. Acad. Sci. U.S.A. 97 (23) (2000) 12729–12734.
[6] J.M. Emmen, K.S. Korach, Estrogen receptor knockout mice: phenotypes in the female reproductive tract, Gynecol. Endocrinol. 17 (2) (2003) 169–176.
[7] C. Darimont, R. Delansorne, J. Paris, G. Ailhaud, R. Negrel, Influence of estrogenic status on the lipolytic activity of parametrial adipose tissue in vivo: an in situ microdialysis study, Endocrinology 138 (3) (1997) 1092–1096.
[8] F.M. Hansen, N. Fahmy, J.H. Nielsen, The influence of sexual hormones on lipogenesis and lipolysis in rat fat cells, Acta Endocrinol. (Copenh.) 95 (4) (1980) 566–570.
[9] M.L. Misso, K.N. Hewitt, W.C. Boon, Y. Murata, M.E. Jones, E.R. Simpson, Cholesterol feeding prevents adiposity in the obese female aromatase knockout (ArKO) mouse, Horm. Metab. Res. 37 (1) (2005) 26–31.
[10] M.E. Starr, B.M. Evers, H. Saito, Age-associated increase in cytokine production during systemic inflammation: adipose tissue as a major source of IL-6, J. Gerontol. A: Biol. Sci. Med. Sci. 64 (7) (2009) 723–730.
[11] M.I. Bader, J. Wober, G. Kretzschmar, O. Zierau, G. Vollmer, Comparative assessment of estrogenic responses with relevance to the metabolic syndrome and to menopausal symptoms in wild-type and aromatase-knockout mice, J. Steroid Biochem. Mol. Biol. (2011), doi:10.1016/j.jsbmb.2011.05.004.
[12] C.T. Teng, Lactoferrin gene expression and regulation: an overview, Biochem. Cell Biol. 80 (1) (2002) 7–16.
[13] L.K. Phillips, J.B. Prins, The link between abdominal obesity and the metabolic syndrome, Curr. Hypertens. Rep. 10 (2) (2008) 156–164.
[14] S.I. Anghel, W. Wahli, Fat poetry: a kingdom for PPAR gamma, Cell Res. 17 (6) (2007) 486–511.
[15] M. Evans, J.G. Elliott, P. Sharma, R. Berman, N. Guthrie, The effect of synthetic genistein on menopause symptom management in healthy postmenopausal women: a multi-center, randomized, placebo-controlled study, Maturitas 68 (2) (2011) 189–196.
[16] S.M. Boue, T.E. Wiese, S. Nehls, M.E. Burow, S. Elliott, C.H. Carter-Wientjes, B.Y. Shih, J.A. McLachlan, T.E. Cleveland, Evaluation of the estrogenic effects of legume extracts containing phytoestrogens, J. Agric. Food Chem. 51 (8) (2003) 2193–2199.
[17] P. Cos, T. De Bruyne, S. Apers, D. Vanden Berghe, L. Pieters, A.J. Vlietinck, Phytoestrogens: recent developments, Planta Med. 69 (7) (2003) 589–599.
[18] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (10) (1998) 4252–4263.
[19] T.F. Bovee, R.J. Helsdingen, I.M. Rietjens, J. Keijer, R.L. Hoogenboom, Rapid yeast estrogen bioassays stably expressing human estrogen receptors alpha and beta, and green fluorescent protein: a comparison of different compounds with both receptor types, J. Steroid Biochem. Mol. Biol. 91 (3) (2004) 99–109.
[20] S.O. Mueller, S. Simon, K. Chae, M. Metzler, K.S. Korach, Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells, Toxicol. Sci. 80 (1) (2004) 14–25.
[21] C.R. Sirtori, A. Arnoldi, S.K. Johnson, Phytoestrogens: end of a tale? Ann. Med. 37 (6) (2005) 423–438.
[22] C.R. Fisher, K.H. Graves, A.F. Parlow, E.R. Simpson, Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene, Proc. Natl. Acad. Sci. U.S.A. 95 (12) (1998) 6965–6970.
[23] K.M. Robertson, L. O'Donnell, M.E. Jones, S.J. Meachem, W.C. Boon, C.R. Fisher, K.H. Graves, R.I. McLachlan, E.R. Simpson, Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene, Proc. Natl. Acad. Sci. U.S.A. 96 (14) (1999) 7986–7991.

[24] A. Bliedtner, O. Zierau, S. Albrecht, S. Liebhaber, G. Vollmer, Effects of genistein and estrogen receptor subtype-specific agonists in ArKO mice following different administration routes, Mol. Cell. Endocrinol. 314 (1) (2010) 41–52.
[25] B.J. Deroo, S.C. Hewitt, S.D. Peddada, K.S. Korach, Estradiol regulates the thioredoxin antioxidant system in the mouse uterus, Endocrinology 145 (12) (2004) 5485–5492.
[26] E. Eisenberg, E.Y. Levanon, Human housekeeping genes are compact, Trends Genet. 19 (7) (2003) 362–365.
[27] J. Winer, C.K. Jung, I. Shackel, P.M. Williams, Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro, Anal. Biochem. 270 (1) (1999) 41–49.
[28] K.C. Knower, S.Q. To, E.R. Simpson, C.D. Clyne, Epigenetic mechanisms regulating CYP19 transcription in human breast adipose fibroblasts, Mol. Cell. Endocrinol. 321 (2) (2010) 123–130.
[29] A. Janesick, B. Blumberg, Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity, Birth Defects Res C Embryo Today 93 (2011) 34–50.
[30] R.L. Jirtle, M.K. Skinner, Environmental epigenomics and disease susceptibility, Nat. Rev. Genet. 8 (2007 Apr) 253–262.
[31] F.J. Moller, P. Diel, O. Zierau, T. Hertrampf, J. Maass, G. Vollmer, Long-term dietary isoflavone exposure enhances estrogen sensitivity of rat uterine responsiveness mediated through estrogen receptor alpha, Toxicol. Lett. 196 (3) (2010) 142–153.
[32] H.A. Harris, Estrogen receptor-beta: recent lessons from in vivo studies, Mol. Endocrinol. 21 (1) (2007) 1–13.
[33] M.H. Faulds, C. Zhao, K. Dahlman-Wright, J.A. Gustafsson, Regulation of metabolism by estrogen signaling, J. Endocrinol. (2011).
[34] R.V. Considine, M.K. Sinha, M.L. Heiman, A. Kriauciunas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, T.L. Bauer, et al., Serum immunoreactive-leptin concentrations in normal-weight and obese humans, N. Engl. J. Med. 334 (5) (1996) 292–295.
[35] T.A. Dardeno, S.H. Chou, H.S. Moon, J.P. Chamberland, C.G. Fiorenza, C.S. Mantzoros, Leptin in human physiology and therapeutics, Front. Neuroendocrinol. 31 (3) (2010) 377–393.
[36] N. Kloting, M. Fasshauer, A. Dietrich, P. Kovacs, M.R. Schon, M. Kern, M. Stumvoll, M. Bluher, Insulin-sensitive obesity, Am. J. Physiol. Endocrinol. Metab. 299 (3) (2010) E506–E515.
[37] C. Teng, Mouse lactoferrin gene: a marker for estrogen and epidermal growth factor, Environ. Health Perspect. 103 (Suppl. 7) (1995) 17–20.
[38] Z.C. Dang, V. Audinot, S.E. Papapoulos, J.A. Boutin, C.W. Lowik, Peroxisome proliferator-activated receptor gamma (PPARgamma) as a molecular target for the soy phytoestrogen genistein, J. Biol. Chem. 278 (2) (2003) 962–967.