



# Effects of Genistein and Estrogen Receptor Subtype-Specific Agonists in ArKO Mice Following Different Administration Routes

Anja Bliedtner, Oliver Zierau, Steffen Albrecht, Stefanie Liebhaber, Günter Vollmer

## ► To cite this version:

Anja Bliedtner, Oliver Zierau, Steffen Albrecht, Stefanie Liebhaber, Günter Vollmer. Effects of Genistein and Estrogen Receptor Subtype-Specific Agonists in ArKO Mice Following Different Administration Routes. *Molecular and Cellular Endocrinology*, 2009, 314 (1), pp.41. <10.1016/j.mce.2009.07.032>. <hal-00529006>

**HAL Id: hal-00529006**

**<https://hal.science/hal-00529006v1>**

Submitted on 24 Oct 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

## Accepted Manuscript

Title: Effects of Genistein and Estrogen Receptor Subtype-Specific Agonists in ArKO Mice Following Different Administration Routes

Authors: Anja Bliedtner, Oliver Zierau, Steffen Albrecht, Stefanie Liebhaber, Günter Vollmer



PII: S0303-7207(09)00404-3  
DOI: doi:10.1016/j.mce.2009.07.032  
Reference: MCE 7281

To appear in: *Molecular and Cellular Endocrinology*

Received date: 13-2-2009  
Revised date: 9-7-2009  
Accepted date: 31-7-2009

Please cite this article as: Bliedtner, A., Zierau, O., Albrecht, S., Liebhaber, S., Vollmer, G., Effects of Genistein and Estrogen Receptor Subtype-Specific Agonists in ArKO Mice Following Different Administration Routes, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2009.07.032

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Effects of Genistein and Estrogen Receptor Subtype-Specific  
Agonists in ArKO Mice Following Different Administration Routes**

**Anja Bliedtner\*<sup>1</sup>, Oliver Zierau<sup>1</sup>, Steffen Albrecht<sup>2</sup>, Stefanie Liebhaber<sup>1</sup>, Günter Vollmer<sup>1</sup>**

<sup>1</sup> Chair of Molecular Cell Physiology and Endocrinology, Institute for Zoology, Technische Universität Dresden, Germany

<sup>2</sup> Department of Gynaecology and Obstetrics, University Hospital Carl-Gustav-Carus Technische Universität Dresden, Germany

DISCLOSURE STATEMENT: The authors have nothing to disclose.

\* Corresponding author:

Anja Bliedtner  
Technische Universität Dresden  
Institut für Zoologie  
Zellescher Weg 20b, Raum 249,  
01217 Dresden  
Germany  
Phone: +49 351 463 34733  
Fax: +49 351 463 31923  
Email: anja.bliedtner@tu-dresden.de

28 **Keywords:** ArKO mice, estrogen receptors, ER-subtype-selective ligands,  
 29 uterotrophic assay, genistein

## 30 **Abstract**

31 We have scrutinized the effects of the phytoestrogen genistein and three synthetic  
 32 estrogen receptor agonists, 17 $\alpha$ -ethynylestradiol (EE), propylpyrazole-triol (PPT) and  
 33 diarylpropionitrile (DPN) in the completely estrogen-free background of aromatase  
 34 knockout (ArKO) mice by means of two routes of substance administration: oral via  
 35 diet (*per os*; po) or subcutaneous injection (sc) with the intention to evaluate the  
 36 ArKO mice as sensitive model organism for uterotrophic assays. Additionally, we  
 37 were aiming to qualitatively analyze effects resulting from oral administration path, in  
 38 particular for PPT and DPN. Therefore, we analyzed the resulting uterine wet weights  
 39 (UWW) and epithelial heights as physiological endpoints of function as well as the  
 40 gonadotropin levels. Moreover, the gene expression profiles of estrogen receptors as  
 41 well as important uterine and ovarian estrogen-response genes were investigated by  
 42 real-time PCR.

43 The uterus of ArKO mice responded very sensitive upon the substitution with EE (sc  
 44 5  $\mu$ g/ kg BW; po 50  $\mu$ g/ kg BW) in a proliferative manner. This was evaluated inter  
 45 alia by increased UWW and by up-regulation of the expression of proliferation-  
 46 associated and estrogen-response genes. It is important to note, that ER $\alpha$  and ER $\beta$ -  
 47 agonist, PPT and DPN respectively (po 5 mg / kg BW and sc 0.5 mg / kg BW), so far  
 48 have only been just for sc applications. Here, effects resulting from oral application  
 49 were qualitatively described evaluated for their applicability. The UWW and  
 50 expression of proliferation-associated genes were increased following both po and sc  
 51 treatment with PPT. DPN did not exert an increase of the UWW, but a significant  
 52 decrease of proliferation-associated gene and protein expression. Additionally, a  
 53 substantial hypoplasia was detectable in the uterine cross sections of DPN-treated  
 54 mice. On the other hand, the phytoestrogen genistein (sc 10 mg/ kg BW; po 70  
 55 mg/ kg BW) did not cause detectable uterotrophic responses or large changes of  
 56 uterine and ovarian gene expression profiles under the applied experimental  
 57 conditions, but significantly reduced the elevated gonadotropin levels of ArKO mice.  
 58 In summary, we showed the utility of ArKO mice to detect ER-specific effects, in  
 59 particular those of PPT and DPN also when applied orally.

60

## Introduction

Estrogens play an important role in development and maintenance of secondary sexual organs and in regulation of reproductive functions in the female organism. In particular, the uterine and the ovarian functions are tightly regulated and coordinated mainly by 17 $\beta$ -estradiol (E2) in liaison with progesterone. Female reproductive organs, such as the uterus, the ovaries and the mammary glands are primary estrogen target organs and therefore express high amounts of estrogen receptors (Pelletier and El-Alfy, 2000). The responsiveness of those organs upon estrogen treatment is utilized in so called uterotrophic assays and extensively studied in various *in vitro* and *in vivo* test systems mostly by evaluating the growth and the proliferation potential as well as determining alterations of gene expression (Diel et al., 2002).

Estrogenic effects are mediated through the two estrogen receptors (ER $\alpha$  and ER $\beta$ ) (Green et al., 1986, Greene et al., 1986, Kuiper et al., 1996, Mosselman et al., 1996). They are encoded by two separate genes (*ESR1* and *ESR2*, respectively), but share the common structural architecture of nuclear receptors. These transcription factors exhibit an evolutionary conserved domain structure; reviewed by Heldring et al. (2007). In the central DNA-binding domain, both estrogen receptors share the highest sequence similarity (95%), which allows them to bind to identical DNA response elements. Despite exhibiting merely 60% similarity, the C-terminal ligand-binding domains bind E2 with equivalent affinities but also permit ER-subtype specificity for different ligands.

There exists a variety of natural and synthetic substances influencing ER signaling. On the one hand, the specificity of substances to only one ER-subtype is useful for a pharmacological evaluation of the contribution of either receptor to the observed response, and therefore may lead to new therapeutic potentials or strategies. On the other hand, the substances themselves can be characterized by their potentials for activation or inhibition of specific ER functions.

Both ERs differ in their tissue distribution throughout the organism and play distinct physiological roles. These complex functional properties can not be fully assessed even if different *in vitro* test systems are combined. Whereas, the rodent uterotrophic assay is a validated and extensively used test system for evaluating estrogenic potency of substances (Kanno et al., 2001, Saarinen et al., 2006). There exist different protocols for investigating estrogenic-responses in estrogen-low or -deprived

animals, for instance immature females or adult ovariectomized rodents. Those are treated for three or more days with estrogenic compounds, followed by determination of total uterine weight and organ resection for tissue-specific gene expression analyses (Diel et al., 2002). According to Diel and Odum, the choice of rodent species does not seem to be a critical factor in this assay, since the sensitivity to various receptor ligands appears to be similar in rats and mice (Diel et al., 2002, Odum et al., 1997).

However, the aromatase knock-out (ArKO) mice may be useful alternatives to study estrogenic responses, because both ERs are still intact and functional (Simpson, 2004), while in difference to the ovariectomized rodent model the progestogens are not compromised. In addition, the ovariectomy is not necessary, because these animals are completely devoid of endogenous estrogen production due to their defective estrogen biosynthesis resulting from disruption of the aromatase gene (*CYP19a*) (Fisher et al., 1998). However, the responsiveness to exogenous estrogens account for ER-subtype specificity studies, such as analyzing hormonal potency of xeno- and phytoestrogens.

In the present study, we analyzed the effects in the uterus and the ovaries of four reference substances following oral or subcutaneous application, the phytoestrogen GEN and two synthetic ER-specific agonists (PPT and DPN) as well as EE and compared these to the carrier-control group. GEN is a mild-selective agonist for ER $\beta$  with more than 7-fold higher binding affinity for ER $\beta$  than ER $\alpha$  (Kuiper et al., 1997). PPT has a 410-fold binding preference for ER $\alpha$  over ER $\beta$  (Stauffer et al., 2000). Accordingly, ER $\beta$  is almost not bound and therefore not activated. DPN represents, at least within a limited dosage range, agonistic properties for ER $\beta$  with more than 70-fold higher binding preference for ER $\beta$  than ER $\alpha$  (Meyers et al., 2001). EE is a well-known orally active steroidal derivative of the natural 17 $\beta$ -estradiol and an approved reference ER-agonist (Kanno et al., 2001, Rider, 2002).

The uterus and the ovaries were chosen as two estrogen-target organs with clear differences in ER-distribution. ER $\alpha$  is the predominant ER-subtype expressed in the uterus acting as the major regulator of estrogenic signaling. ER $\alpha$  activation induces cell proliferation and primes for progesterone action, therefore preparing for decidualization and implantation (Frasor et al., 2003). Furthermore, studies of the ER $\beta$ -deficient mice demonstrate typical uterine ER $\alpha$ -effects, such as increasing wet weight, cell proliferation, water imbibition and hyperemia (Krege et al., 1998, Couse

and Korach, 1999). The ER $\beta$  is minorly present in all uterine cell types (Weihua et al., 2000). Accordingly, the functional properties of ER $\beta$  in the uterus are less obvious, even though it provokes some estrogenic effects, for instance increasing progesterone receptor (*PGR*) expression (Kurita et al., 2001) and modulating ER $\alpha$  actions (Weihua et al., 2000).

In female individuals, the ovaries are the main source of endogenous estrogen production. In addition, estrogens control follicular development in the ovaries with great importance for reproduction. These influences might be directly effected via ERs, which are located in the somatic cells of the ovaries, or indirectly as consequences of modulation of gonadotropin levels (Britt et al., 2004). Both ERs are expressed in the ovary with ER $\beta$  being the predominant subtype. Upon activation, ER $\beta$  influences the proliferation and development of granulosa cells (Drummond et al., 1999). However, the precise molecular mechanism of regulations is still ambiguous.

In this study, we were interested in the potentiality of PPT and DPN together with genistein following an oral application path in a totally estrogen-free animal model. Therefore, we examined their effects in the ArKO mice in qualitative similarity to the respective sc groups and compared all with the EE groups and carrier-control. Hence, we analyzed physiological parameters and additionally detailed molecular mechanisms in the uterus and the ovaries on mRNA and protein level.

## Materials and Methods

### *Substances*

17 $\alpha$ -ethynylestradiol (EE; 17 $\alpha$ -ethynyl-1,3,5(10)-estriene-3,17 $\beta$ -diol; 19-Nor-1,3,5(10), 17 $\alpha$ -pregnatrien-20yne-3,17-diol) and genistein (GEN; 4',5,7-trihydroxyisoflavone) were purchased from Sigma-Aldrich (Deisenhofen, Germany), propylpyrazole-triol (PPT; 4, 4', 4'' - (4 -propyl-(H)-pyrazole-1 ,3 ,5 -triyl) *trisphenol*) and diarylpropionitrile (DPN; 2, 3-*bis* (4-hydroxyphenyl)-propionitrile) were purchased from Tocris Bioscience (Bristol, UK).

### *Animals*

The ArKO mice and wild-type (WT) littermates used in this study were bred from the founder population kindly provided by Prof. Dr. Evan Simpson (Prince Henry's Institute of Medical Research, Clayton, Australia). The mice were housed under

controlled conditions of temperature ( $20 \pm 1$  °C, relative humidity 50 – 70 %) and illumination (12 h light / 12 h dark) and had free access to diet and water. The company Altromin (Lage, Germany) produced the standard and the experimental diets containing the test substances. An isoflavone-free diet with a 17% protein-content based on milk-protein was used as standard chow. All animal handling and experimental conditions were conducted in accordance to the principles and procedures of the German Federal Law for Animal Welfare and were approved by the Animal Care and Use Committee of the Technische Universität Dresden.

### *Treatment and experimental design*

The substances were administered either *per os* (po, orally) via diet or by subcutaneous (sc) injection according to a three-day uterotrophic assay. The ArKO mice of an average age of 8 weeks were randomly divided into experimental groups and the control group (designated as CO) (n = 5-8). Moreover, a group of female wild-type littermates (WT; n = 6) with the same genetic background (*C57BL/6J*) were included in the experimental setup as a reference group for physiologically normal mice. Mice were maintained on isoflavone-free diet, because it has been recently shown from our laboratory team, that long-term intake of dietary phytoestrogens influences the estrogenic responses in the organism (Moller et al., 2009).

The substance administrations were organized as follows. The mice of the po groups had 72 hours access to the experimental chow and the mice of the sc groups were injected at three time points (0 h, 24 h and 48 h). Subcutaneous injections consisted of castor oil with the substances pre-dissolved in DMSO. The mice of the ArKO control group (CO) and WT group were injected with the carrier solution (DMSO in castor oil) only. For the oral substitution, the substances were blended with the standard diet by the manufacturer in concentrations of 0.5 mg / kg diet (EE) and 50 mg / kg diet (PPT, DPN). The average amount of chow taken up by the mice was about 10 % of their bodyweight (BW) per day (data not shown). Therefore, the oral exposure was estimated to 0.05 mg / kg BW / d for EE and 5.0 mg / kg BW / d for the ER-agonists (PPT, DPN). The GEN-rich diet, containing 700 µg / g GEN, was custom-made by LC Laboratories (Woburn, USA). Table 1 summarizes the experimental doses applied in this study.

24 hours after the last injection and accordingly after 72 h of exposure to experimental chow, animals were sacrificed by cervical dislocation. The body weight



was measured and blood samples were taken. Uteri and ovaries were collected and weighed after removal of associated fat. For histological analyses, one uterine horn and one ovary per mouse were fixed in buffered formalin-solution (4%, pH 7.4). The remaining tissue was snap-frozen in liquid nitrogen for subsequent gene expression analyses.

### *Gonadotropin levels*

Mouse-specific immunoassay kits (USCN life science & technology Co., Wuhan, China) were used for *in vitro* quantitative determination of serum FSH and LH concentrations. Blood samples were taken post-mortem and allowed to clot before centrifugation (4 min at 3,000 x g) for serum preparation. Serum samples were diluted 1:10 and 1:100 for LH and FSH, respectively, in PBS buffer (pH 7.0 - 7.2) before proceeding according to manufacturer's protocol.

### *Gene expression analyses*

Total RNA was isolated from the frozen tissues using the RNeasy™ Mini Plus Kit (Qiagen) according to the manufacturer's instruction. Three microgram of total RNA of uterine samples and 0.6 µg ovarian samples were digested with DNase I (Promega) to eliminate DNA residues, which was checked by real-time PCR. MMLV Reverse Transcriptase (Promega) and oligo-d(T)<sub>18</sub> primer (MWG; Ebersberg, Germany) were used for cDNA synthesis. The resulting cDNA was amplified by real-time PCR in the iCycler (Biorad) using Platinum® Taq DNA polymerase (Invitrogen Life Technologies, Karlsruhe, Germany) and the Sybrgreen® detection system (Sigma). All PCR reactions were conducted in triplicates and repeated three times. Relative gene expression levels of target genes were corrected for the expression of the internal reference gene (*RPS18*; ribosomal protein S18) (Eisenberg and Levanon, 2003, Deroo et al., 2004). *RPS18* expression was not influenced by the treatments (data not shown). If not otherwise stated, data were calculated as fold change normalized to carrier-treated ArKO group (CO) using the  $\Delta\Delta$  cycle threshold ( $\Delta\Delta CT$ ) calculation method (Winer et al., 1999, Pfaffl, 2001).

### *Oligonucleotides used as primers for real-time PCR*

Based on the DNA sequences available at the UCSC Genome Browser database (<http://genome.ucsc.edu/>), the following primer pairs were designed to specifically amplify the cDNAs of interest (see Table 2). The primer selection was accomplished

using the software Primer3 (Whitehead Institute for Biomedical Research Cambridge, MA, USA) upon specific parameters. The specificity of the primers was confirmed with the *Blat*- and *in silico* PCR tools provided at the UCSC Genome Browser website. All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Sequences and amplicon sizes of the primer pairs are listed in Table 2. The efficiency of every PCR reaction was optimized for primer- and magnesium chloride concentrations. All PCR products exhibited a single peak in the melting curve analysis and were identified upon their appropriate amplicon sizes by gel electrophoresis on ethidium bromide agarose gels.

### *Histological analysis*

Formalin-fixed uterine and ovarian tissues of the CO and the experimental groups were dehydrated and processed for paraffin embedding. Tissue cross sections (3  $\mu$ m) were processed for hematoxylin-eosin staining to evaluate the histopathological changes of the tissues. The height of the uterine epithelia was determined using a microscope micrometer scale and the program *ImageJ* (<http://rsbweb.nih.gov/ij>). The ovarian cross sections were examined according to their overall morphology and phenotype of follicles and interstitial cells. Unfortunately, there were no tissue samples of the WT tissues available for histological examinations.

### *Immunohistochemistry (IHC) against PCNA Protein*

Paraffin cross sections of uterine tissues (1-2  $\mu$ m) were rehydrated starting with xylol via grading ethanol to double distilled water. For the IHC, the NOVA detect anti-rabbit, HRP/DAB kit (Dianova, Hamburg, Germany) was utilized. The IHC protocol was conducted according to the manufacturer's protocol. A specific primary antibody against PCNA (PAI-30254, Affinity BioReagents™) was diluted 1:200 in 5% milk powder solution and incubated for 2 hr at room temperature.

### *Statistical analysis*

Gene expression data are presented as statistical mean  $\pm$  SD of all PCR reactions, which were conducted in triplicates and repeated with three separate cDNAs. If not otherwise stated, relative gene expression levels were documented as fold change against CO, each corrected for the internal reference gene (*RPS18*). These data

were analysed using the Students  $t$ -Test indicating significant differences versus the CO group (\*  $p < 0.05$ ).

Furthermore, UWW data were presented in a box plot chart as median with a 10<sup>th</sup> to 90<sup>th</sup> percentile box for a more precise documentation. The statistical analysis of UWW, gonadotropin levels and PCNA immunohistochemical staining was performed using the Mann-Whitney U-test. A value of  $p < 0.05$  was considered as significantly different from CO.

## Results

### *Uterine wet weight and epithelia height*

The uterine wet weights (UWW) and epithelia heights were the main physiological endpoints utilized for the assessment of estrogenicity. The UWW were normalized against the bodyweight and calculated by fold change relative to the ArKO carrier-treated control group (CO;  $1 \pm 0.4$  fold) to obtain a more precise evaluation and a better comparability.

As depicted in figure 1, the relative UWW of the WT littermates were 3.7 fold higher ( $p < 0.01$ ) than those of CO mice. Irrespective of the route of administration, the treatment with 17 $\alpha$ -ethynylestradiol (EE) increased the UWW of ArKO mice even above the WT level (po  $7.1 \pm 2.4$  fold and sc  $6.9 \pm 1.7$  fold vs. CO; both  $p < 0.001$ ). Analyzing the effects of the ER $\alpha$ -agonist PPT, the oral administration caused a stronger weight increase ( $4.1 \pm 0.9$  fold vs. CO;  $p < 0.001$ ) than the sc injection ( $2.2 \pm 0.7$  fold vs. CO;  $p < 0.01$ ). Neither genistein (GEN; po  $0.9 \pm 0.2$  and sc  $1.1 \pm 0.4$ ) nor ER $\beta$ -agonist (DPN; po  $1.3 \pm 0.6$ ; sc  $0.8 \pm 0.2$ ) substitution resulted in significant changes of UWW compared to CO.

In addition, the morphologies of uterine epithelia for all treatment groups are depicted in figure 2. EE increased the uterine epithelial heights following both administration routes (po  $1.5 \pm 0.3$  fold and sc  $1.5 \pm 0.2$  fold vs. CO), which was the same for per os administration of PPT ( $1.5 \pm 0.2$  fold vs. CO). In the case of sc administration of PPT, the heights of the uterine epithelia did not change versus CO ( $0.9 \pm 0.1$  fold vs. CO) which was similar in the GEN- as well as the DPN-group, whereby it was noticeable that uteri of DPN-treated mice showed visible changes in overall size and morphology (figure 2 F & G).

### *Serum gonadotropin levels*

In ArKO mice, serum LH (luteinizing hormone) and FSH (follicle-stimulating hormone) were significantly elevated compared to WT littermates (table 3). The oral administration of the tested substances led to a significant decrease of the LH and FSH levels, whereas subcutaneous injections engendered a much weaker effect, except for DPN. The subcutaneous effects on the gonadotropin levels were less obvious in the PPT group and not observable for EE. Overall, DPN had only weak but significant influences on the LH and FSH levels. Genistein caused a significant reduction (i.e. back to WT level) of both LH and FSH serum levels following both administration paths,

### *Uterine gene and protein expression*

To investigate the molecular mechanisms regarding the sensitivity of ArKO mice uteri to exogenous estrogens, we performed real-time PCR of either genes, related to proliferation or sensitive estrogen-responsive genes.

Although there were obvious differences of relative UWW between CO and WT (figure 1) no significant differences of the proliferation index genes, PCNA and MKI67 (figure 3 A and B, respectively) were detected on mRNA level. Following EE and PPT treatment, the expression of proliferation-associated genes was increased either by trend in the case of PCNA or significantly regarding MKI67 ( $p < 0.05$ ) compared to CO. On the other hand, oral administration of DPN caused a significant down-regulation of PCNA ( $p < 0.01$ ) and MKI67 ( $p < 0.001$ ), even though sc treatment dose was not effective (figure 3 A & B). Furthermore, genistein only had minor influences.

In addition, we performed immunohistochemistry (IHC) on the uterine cross sections with an antibody against PCNA protein to confirm the real-time PCR results (figure 3 C). The amount of the PCNA-positive stained uterine epithelial cells was clearly enhanced following EE and PPT treatment. Additionally, the absence of PCNA-positive cells following DPN treatment was detected as well as low numbers in the genistein groups.

Detailed mechanisms of the observed estrogen-responses were first studied by evaluating the gene expression of ESR1 and ESR2 ( $ER\alpha$  and  $ER\beta$  genes, respectively) by real-time PCR. As expected, in the uterus ESR1 expression was on a markedly higher level than ESR2, as assessed from  $\Delta CT$  values. However, the tested substances did not significantly alter the ESR1 expression (figure 4 A). On the

other hand, the minorly expressed ER $\beta$  was strongly influenced by the specific treatments (figure 4 B). The oral administration of EE and PPT as well as sc administration of PPT significantly increased the transcription of ESR2 ( $p < 0.05$ ), while DPN and GEN caused minor changes only.

Progesterone receptor gene (PGR), the third steroid receptor analyzed, showed a different expression pattern. In the WT reference group and both EE groups (po and sc), PGR expression was significantly increased compared to CO, while PPT and GEN did not influence, but DPN significantly decreased PGR gene expression ( $p < 0.01$  po; figure 4 C).

Lactotransferrin gene (LTF), which is a powerful estrogen-responsive gene in the uterus of mice (Toda et al., 2001), was sensitively up-regulated following ER $\alpha$ -stimulation and down-regulated following ER $\beta$ -activation (figure 5 A) in ArKO uteri. In detail, LTF was about 30-fold up-regulated following EE administration (po and sc; both  $p < 0.05$ ) and about 20-fold enhanced following oral administration of PPT ( $p < 0.05$  vs. CO), thereby rising above the WT expression level (16.5 fold,  $p < 0.05$  vs. CO). By contrast, a highly significant reduction of LTF gene expression level was observed following sc injection of DPN (sc 0.3 fold  $p < 0.001$ ). Additionally, genistein treatment seemingly reduced the expression of LTF.

Complement component 3 (C3) was an additional estrogen-response gene studied in the uterus of ArKO mice (figure 5 B). In ArKO uteri, only EE treatment was able to strongly increase C3 expression (4 fold,  $p < 0.05$  EE sc vs. CO) even beyond WT level (2.3 fold vs. CO). On the other hand, the oral treatment with PPT or GEN exerted only a minor activation ( $p < 0.05$  and  $p < 0.07$  vs. CO, respectively), while DPN did not cause any changes.

### *Ovarian gene expression*

The number of analytical tools to clearly demonstrate effects of ER $\beta$ -selective substances is rather limited. This was the motivation to evaluate gene expression profiles of estrogen-regulated genes in the ovaries of ArKO mice. Therefore, we performed real-time PCR of ovarian genes related to either proliferation or differentiation as well as genes known to be influenced by estrogen-signaling in the ovaries.

Unexpectedly, there was no detectable ESR1 gene expression in the ovaries of carrier-treated ArKO mice (CO). Therefore,  $\Delta\Delta CT$  values of ESR1 real-time

experiments could not be calculated as fold change vs. CO. Instead, relative gene expressions were corrected for the internal control gene (*RPS18*) and analyzed by calculating  $2^{(-\Delta ct)}$  to obtain a reliable interpretation (Pfaffl, 2001, Winer et al., 1999). Nevertheless, in ovaries of mice that received estrogenic substances, *ESR1* gene was clearly expressed. Additionally, it is important to note that all substances possessed higher impacts on *ESR1* gene expression following the oral administration route than those of respective sc groups, as assessed from the  $\Delta CT$  values (data not shown).

On the other hand, the gene expression levels of *ESR2* were clearly higher than the respective *ESR1* as evaluated by  $\Delta CT$  values (data not shown). In the ovaries of WT mice, *ESR2* level was 3.1 fold above CO. As illustrated in figure 6, all substances evidently increased *ESR2* level at the administered *per os* doses (PPT po & DPN po  $p < 0.05$ , EE po  $p = 0.06$  vs. CO), whereas subcutaneous doses did not cause significant changes.

In addition, we performed real-time PCR analyses of *PCNA* and luteinizing hormone receptor (*LHR*) to evaluate the ovarian proliferation and differentiation status, respectively. *PCNA* gene expression was increased versus CO following oral substance administrations except for the GEN group (figure 7A). In WT ovaries, *LHR* was clearly higher expressed than CO, even though no statistical significance could be calculated due to high variances (figure 7B). Although, none of the treatments increased *LHR* gene expression up to WT level, significant up-regulations following PPT (po and sc  $p < 0.05$  vs. CO) or GEN ( $p < 0.01$  po vs. CO) treatment were detected (figure 7B).

Furthermore, previous studies have demonstrated the emergence of male-like somatic cells in the ovaries of estrogen-depleted mice (Britt et al., 2004, Couse et al., 1999). They reported an influence of estrogen treatment on the gene expression of *Sox9* (Sry-like homeobox transcription factor 9) and up- and down-stream factors, which are shown to be related to male-like structures visible in ArKO ovaries (Britt et al., 2004). In the present study, the expression of some of these nuclear transcription factors were examined by real-time PCR: *Sf-1* (steroidogenic factor 1) and the related *LRH-1* (liver receptor homologue 1), as well as *DAX-1* (the dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X-chromosome gene 1) and *SOX-9*. In the ovaries of CO mice, the transcription levels of all these genes were very close to detection limit. Regarding *DAX-1* and *SOX-9*, no evaluation

of expression profiles was possible because of very weak gene expressions (data not shown). Nevertheless, in WT ovaries the gene expression levels of all analyzed genes were obviously higher than in CO or treated ArKO mice ovaries. However, SF-1 was significantly up-regulated ( $p < 0.05$  vs. CO) in all per os groups, except for GEN (figure 8A). Furthermore, sc administrations induced minor changes of SF-1 gene expression (EE and PPT sc  $p < 0.05$ ). Regarding LRH-1 levels, all substances clearly evoked up-regulations (figure 8 B).

## Discussion

In the last decades, it became apparent that regulations and molecular mechanisms of estrogen and particularly estrogen-like substance actions are far more complex than believed initially. To achieve a better understanding of ER-subtype specific reactions, we analyzed ArKO mice as a totally estrogen-free animal model, which is responsive to exogenous estrogen signals because of both ERs being functional and receptive (Fisher et al., 1998, Toda et al., 2001).

This study was intended to qualitatively examine the route of oral substance administration, particularly regarding PPT and DPN treatment and additionally to evaluate ER-specific reactions of ArKO mice. For us, it was important to access a non-invasive route of substance administration which is able to reproduce the consistent effects of subcutaneous injections. Furthermore, not much was initially known about the quality of effects resulting from oral administration of PPT and DPN. Therefore, in our experimental design we have scrutinized two administration groups in parallel (sc and po) for every tested substance. We chose the well-known and well-characterized synthetic estrogen  $17\alpha$ -ethynylestradiol (EE) as reference substance, firstly for acting via both ER-subtypes and secondly for its oral bioavailability (Inhoffen and Hohlweg, 1938, Kuiper et al., 1997). Regarding the ER-subtype specific substances, we made use of PPT and DPN because they are well described and commercially available. The ER $\alpha$ -specific agonist, propylpyrazole-triol (PPT) has a 410-fold binding preference for ER $\alpha$  over ER $\beta$  (Stauffer et al., 2000). The potency-selective agonist for ER $\beta$  diarylpropionitrile (DPN) has a more than 70-fold higher binding preference for ER $\beta$  than ER $\alpha$  (Meyers et al., 2001). For oral administrations, we decided to use 10-fold higher doses because initially we expected oral effects of PPT and DPN to be weaker due to metabolic aspects. Both were applied at doses of 0.5 mg / kg BW for sc administration and 5 mg / kg BW for per os administration at

which they were described to be ER-specific (Frasor et al., 2003). In this experiment, *per os* doses of 50 µg EE per kg BW and sc doses of 5 µg EE / kg BW were applied to assess useful effects in ArKO mice. Finally, genistein (GEN) which is an extensively studied phytoestrogen of the class of isoflavones with mild selective-agonistic properties on ERβ (Kuiper et al., 1997) was included in this study to serve as a natural estrogen-like substance. For GEN treatment, sc doses of 10 mg / kg BW and *per os* doses of 70 mg / kg BW resulting from the experimental diet were administered to ArKO mice. All these substances were analyzed for their effects in comparison to carrier-treated ArKO mice (CO) and also in relation to untreated wild-type littermates (WT).

### **Uterine responses of ArKO mice**

In this study, the highly sensitive regulations of uterine and ovarian physiology and molecular parameters of ArKO mice were not studied within the light of reproductive biology, but as parameters for characterization of the ER subtype-specific substances. In particular, the uterus of estrogen-deprived animals is known to sensitively react upon activation of ERα signaling by triggering growth and proliferation (Diel et al., 2004).

In accordance with other groups, we observed that uteri of carrier-treated ArKO mice were dramatically diminished in uterine size, weight and volume compared to wt siblings (Fisher et al., 1998, Toda et al., 2001). Here, this estrogen-deprivation was mainly observed on dramatically decreased uterine wet weights (UWW), but also the epithelial heights were reduced. As expected, EE and PPT substitution increased both physiological parameters, whereby UWW reactions were more sensitive than epithelial heights, which differs from ovariectomized rats (Diel et al., 2002).

On the other hand, no significant alterations of UWW or epithelial heights could be observed following DPN and GEN administration. Moreover, the overall morphology of uteri cross sections of the DPN groups was observably hypoplastic.

In this aspect, GEN caused similar patterns like DPN. Additionally, GEN has previously been characterized to have a higher preference for ERβ (Kuiper et al., 1997). Still, also agonistic properties were described earlier for GEN with a faint increase of UWW in OVX rats after 3 day treatment (Moller et al., 2009). However, short term administration of GEN, even in very high doses, did not result in a steady increase of UWW in OVX rat (Diel et al., 2004). Then again, GEN caused an expansion of the luminal epithelial cell layer in ArKO mice (Toda et al., 2005). In



ArKO mice, it was shown previously that life-long intake of genistein enriched diets increased uterine and ovarian weights, whereby an association with proliferation was not clear (Britt et al., 2005). Our studies demonstrated an inability of GEN to stimulate the UWW in ArKO mice in the here applied doses. Furthermore, this was correlated with the inability to significantly induce uterine proliferation, which was analyzed on mRNA and protein level. Apparently, there exists a variety of studies including time- and dose-dependencies of uterine actions which indicated SERM-like properties for GEN (Toda et al., 2005, Diel et al., 2001, Diel et al., 2004, Moller et al., 2009). Furthermore, in the context of the biphasic pattern of uterotrophy induced by estrogenic substances, a weak induction of UWW by genistein was previously supposed as a result from imbibitions of water other than from induced proliferation (Diel et al., 2004). In our study, genistein treatment clearly resulted in a reduction of the gonadotropin levels, which is considered as a possibility for estrogen-related effects. Nevertheless, details on proliferation modulation by phytoestrogens need further investigations.

#### **Gene expression profiles of ArKO uteri**

We supported and specified the physiological data by analyzing gene expression levels of two marker genes for proliferation, *PCNA* and *MKI67*. Interestingly, in CO and WT uteri levels of both were on an equal level, even though the UWW were disparate. This could indicate an equilibrium state without exogenous induction. Following EE and PPT treatment, gene expression of both proliferation-associated genes was highly stimulated, in accordance with UWW results. In contrast, oral administration of DPN caused a significant reduction of proliferation markers on mRNA and protein level. Previously, Frasor et al. (2003) demonstrated that the ER $\beta$ -specific agonist DPN has a very limited ability to stimulate proliferation in the uterus as well as causing a 30% reduction of the PPT-stimulated UWW increase. It is widely accepted that ER $\beta$ -signaling is unable to induce uterine growth and proliferation, which was among others clearly demonstrated in the  $\alpha$ ERKO mice (reviewed by Couse and Korach, 1999). Nevertheless, the reported DPN effects in ArKO mice, which were detectable on mRNA and on protein level, were rather unexpected and need further confirmation.

We increased the analytical spectrum of the uterotrophic assay by including gene expression analyses of estrogen-responsive genes. First, we analyzed the estrogen receptor status and the progesterone receptor expression to itemize the proliferation

and differentiation status of uterine tissue in more detail and to prove the ability of ArKO mice's uteri to respond directly to estrogenic signals. ESR1 gene expression levels in CO were similar to WT. Even though the ESR1 levels markedly exceeded that of ESR2, we hardly observed an alteration following substance administration. This stands in contrast to effects previously observed in rat uteri, where 17 $\beta$ -estradiol down-regulated both ESR1 and ESR2 expression (Zierau et al., 2008).

In this study, only oral administration of DPN significantly decreased ER $\alpha$  levels. Mechanistically, it was shown previously that ER $\beta$  could serve to inhibit ER $\alpha$  signalling in target tissues (Frasor et al., 2003, Hall and McDonnell, 1999), maybe this is realized also by influencing gene expression. Additionally, the low ER $\beta$  gene expression levels in the uterus were significantly increased upon EE (*per os*) and PPT treatment, although never reaching ER $\alpha$  level. These observations were rather unexpected and stand in contrast to various other studies, which reported a reduction of both ER $\alpha$  and ER $\beta$  expression upon estrogen treatments (Diel et al., 2004, Pillai et al., 2002). According to Weihua and colleagues, ER $\beta$  tends to down-regulate ER $\alpha$  activity (Weihua et al., 2000). In the present study, it seems that ER $\alpha$  activation increases ER $\beta$  expression and ER $\beta$  activation inhibits ER $\alpha$  expression. This may add an interesting aspect to the complexity of estrogenic cross talk in regulating networks, including feedback regulations of their own receptors.

Even though ER $\beta$  expression was increased, the dominant role of ER $\alpha$  stayed immanently superior in the uterus also in the estrogen-free background of ArKO mice. Diel et al. described that a high ER $\alpha$  /ER $\beta$  ratio was correlated with increased proliferation (Diel et al., 2004). Here we observed that only upon stimulation of ER $\alpha$  signaling in combination with a high ER $\alpha$  / ER $\beta$  ratio proliferation was switched on, whereas activating only ER $\beta$  even with a high ER $\alpha$  /ER $\beta$  ratio was not sufficient to increase proliferation.

Adding to the understanding of potential molecular mechanisms of regulations in the uterus, we have analyzed three estrogen-response genes: progesterone receptor (PGR), lactotransferrin (LTF) and complement component 3, (C3). All three genes contain estrogen-response elements in their 5'-regulatory regions (O'Lone et al., 2004) and are described earlier to be directly regulated by estrogens (Toda et al., 2001, Zierau et al., 2008). Here, all were clearly up-regulated by EE treatment in the uteri of ArKO mice. In this experimental design, LTF was the most sensitive estrogen-response gene with an expected increase of gene expression following

ER $\alpha$  activation. DPN added a more interesting aspect, because subcutaneous administration led to a highly significant down-regulation of *LTF*. In accordance, Couse et al. found no up-regulation of lactotransferrin as well as progesterone receptor in the uteri of  $\alpha$ ERKO upon estrogenic signals, but no down-regulation was observed so far. It needs further investigations to analyze details of ER-specificity of ERE-dependent mechanism and their responsibilities for the observed effect. Another explanation might as well be alternative pathways of LTF gene regulation involving activation of cAMP or growth factor response elements, which were found in the promoter region of this gene (Shi and Teng, 1996, Teng et al., 1998). *C3* revealed a similar but weaker profile in ArKO uteri, which was surprising, because in the OVX rats C3 is a very strong estrogenic-marker (Diel et al., 2004, Zierau et al., 2008). Here, we could only speculate about species specificities or even side effects resulting from ovariectomy, but no definite explanation can be given for that result. PGR, which is an important steroid receptor in the uterus, revealed an interesting seemingly ER-specific expression pattern in the uteri of differentially treated ArKO mice. In WT mice and in ArKO mice following EE administration, *PGR* expression levels were significantly elevated compared to CO. The ER $\alpha$ -agonist (PPT) did not influence, while the ER $\beta$ -agonist (DPN) significantly decreased *PGR* expression. It is known, that *PGR* is transcribed under the regulation of estrogens (Toda et al., 2001), although differential expression patterns are described in the literature regarding effects following estrogen treatments (Waters et al., 2001, Adashi and Hsueh, 1982). There might be dose-dependent or ER-subtype specific regulation patterns involved, but also a time-dependent regulation has been demonstrated for *PGR* (Zierau et al., 2008). Furthermore, the 5' region of *PGR* gene contains a lot of regulatory sequences, including EREs, PREs, AP1-binding sites and others (Hagihara et al., 1994) which implies a complex molecular regulation pattern. ArKO mice might add to the understanding of regulations of *PGR* expression, because in contrast to OVX or immature *in vivo* models, progesterone production is not compromised.

#### **Gene expression profiles of ArKO ovaries**

Ovaries were examined because they represent a target organ with high ER $\beta$  distribution (Drummond et al., 2002) and may be useful to evaluate in particular the ER $\beta$ -specific actions of the tested substances. Unfortunately, the measured ovarian weights as well as the morphological analysis of ovarian cross sections were not convincing enough for a conclusive comparison of substance responses (data not

shown). Probably, the three-day treatment period was not sufficient to abundantly influence ovarian phenotypes. Nevertheless, we used the ovaries of differentially treated ArKO mice to isolate RNA and analyze estrogen-responsiveness by gene expression analysis. In the ovaries, ESR2 was highly expressed but hardly influenced by the treatments. Although, there were no transcripts of ESR1 detectable in ovaries of CO, ESR1 transcription was switched on following application of the substances, irrespective of the type of ligand. This shows the capacity of ArKO ovaries to respond directly via both ERs upon stimulation.

Additionally, we were examining the ovarian proliferation and differentiation status. Thereby, the LH-receptor status served as indicator for differentiation, because of its association with improved morphology (Britt et al., 2004) and its expression described to increase in antral follicles (Nimrod et al., 1977). The WT ovaries displayed the most differentiated profile according to high LHR gene expression, whereas in CO the lowest levels were detected. None of the treatments led to significant alterations compared to CO level. There could also be a connection to serum LH levels which are known to decrease LHR expression (Nimrod et al., 1977, Richards, 1975).

Furthermore, we investigated the ovarian proliferation status by analyzing the expression levels of PCNA. Identical to the findings in the uterus, CO and WT level did not significantly differ in their profile. Hence, significant induction of proliferation was detected following EE and PPT (po) and in tendency DPN administration. According to Britt et al., ovarian weights were dependant on specific treatment, which should be taken into account in further studies (Britt et al., 2005, Britt et al., 2004).

Since previous studies described the emergence of male somatic cells in the ovaries of estrogen-depleted mice and described an improvement by administration of estrogenic substances (Britt et al., 2004, Couse et al., 1999), we used these steroidogenic marker genes to evaluate their expression pattern in specifically treated ArKO mice. All these genes encode for nuclear transcription factors, which are naturally involved in the gonadal development. A high expression of Sox9 and changes of up- and down-stream regulating factors (SF-1, LRH-1 and DAX-1) are responsible for the male-like phenotype of the gonads (Britt et al., 2004). In CO, the gene expression level of all of these transcription factors were very close to the detection limit. In our experimental setup, these genes, which were regulated by Britt and colleagues, were not abundantly influenced in our three-day assay. Perhaps, the

treatment period was too short or the experimental dosages were too low for influencing ovarian mechanisms. It is also possible that 16-wk old mice were already too old for evaluating pattern of those development related genes. It is conversant that the microenvironment of the ovaries has a great importance because it is flooded with high doses of estrogenic substances. Furthermore, activated intraovarian auto-regulatory feedback mechanisms play a role in maturation of the ovaries (Adashi and Hsueh, 1982, Iida et al., 1991). In this study, we can not find a conclusive answer to the functional aspects. Nevertheless, it was clearly evident that the substances provoked a greater effect following the oral doses. Probably, the sc injection doses were too weak to possess effects in the ovaries lifespan.

#### **Influence on gonadotropins**

In accordance with Fisher et al., we observed elevated gonadotropin levels in female ArKO mice (Fisher et al., 1998). This reflects a disruption of negative feedback on the hypothalamic-pituitary axis leading to elevated gonadotropin levels. In our study, we observed a reversion of these levels back to or even below WT level by application of ER $\alpha$ -agonists. However, the effects of estrogenic substances on serum LH and FSH levels were complex, whereby the effects resulting from oral administration gave a clearer pattern. PPT as well as EE strongly decreased LH and FSH back or even below to WT level, respectively. Surprisingly, results from the EE sc did not show any effect, which may be a dose effect. There is an importance of ER $\alpha$  signaling for LH feedback suggested by Couse et al., who showed that LH levels were increased in  $\alpha$ ERKO and  $\alpha\beta$ ERKO mice compared with Wt and  $\beta$ ERKO mice (Couse et al., 2003). However, they observed a normal FSH regulation in  $\alpha$ ERKO and  $\alpha\beta$ ERKO females. Our data showed a decrease of FSH level following ER $\alpha$ - as well as ER $\beta$ -agonist treatment, which indicates a role for ER $\beta$ -dependent regulation, but also other regulation mechanisms like those via inhibin/activin add to complexity (Couse et al., 2003).

Finally, genistein possessed its most obvious effects in ArKO mice by the reduction of LH and FSH levels. This effect was previously described by Britt et al., whereby Gen+ diet significantly reduced FSH levels in ArKO mice, although the exact mechanisms has not been defined (Britt et al., 2005). There might be an induction of estrogenic effects from GEN by influencing gonadotropin release. The doses used for GEN treatments were previously described to induce specific effects in OVX rats (Diel et al., 2004, Moller et al., 2009). In ArKO mice, they need to be further

investigated, because they were inefficient, which could be due to species specificity or due to different estrogenic background levels and developmental histories of the animal model.

### **Summary**

In summary, we could demonstrate that ArKO mice respond very sensitively and reliably upon supplementation of estrogenic substances on both administration routes and were fulfilling the expectations of the classical uterotrophic assay. PPT induced typical estrogenic responses in the uterus on analyzed physiological endpoints and on expression of estrogen-response genes with stronger effects upon oral administration. The weaker sc effects probably resulted from a sub-optimal sc dosage versus an efficient oral dosage. For a quantitative comparison of oral versus subcutaneous effects equal doses need to be used.

We observed some novel and interesting expression profiles following DPN treatments, which were not related to a modulation of ER $\alpha$  effects but seemingly ER $\beta$  effects only. There was no uterotrophy recognized, UWW remained at control level. Furthermore, proliferation markers were significantly inhibited by DPN. Additionally, in the uterus estrogen-response genes were regulated in the opposite direction as by ER $\alpha$  signaling. We reported decreasing levels of *PGR* and *LTF* gene expression. In addition, *C3* expression was not altered at all by DPN treatment. Those results clearly exemplify so called anti-estrogenic properties in the uterus of ArKO mice which are seemingly ER $\beta$ -related effects. Finally, we can state that DPN doses used in this experiment were sufficient to activate ER $\beta$ -specific actions which need to be confirmed for their reliability in further experiments, but not ER $\alpha$ -specific effects.

GEN treatment did not exhibit strong effects under the utilized experimental conditions and hardly effected the expression of the analyzed estrogen-response genes, but clearly influenced the release of gonadotropins.

Regarding the administration routes, we were aiming to evaluate effects resulting from oral application of PPT and DPN. We chose 10-fold higher doses on the oral route, because we initially expected lower effects than finally observed. For reaching a quantitative comparison of different administration routes equal doses should be applied in further experiments. Overall, effects resulting from EE treatments were less dose- and route-dependent, whereas for PPT, *per os* dose led to more obvious effects than sc injections, for instance an increase of UWW or proliferation markers. This might indicate that sc doses of 500  $\mu\text{g} / \text{kg}$  BW are suboptimal to observe

658 reliable effects in ArKO mice in a three day assay, whereas the higher per os doses  
659 were efficient and might be a basis for further experiments in ArKO mice, but still  
660 need to be confirmed for long-term usage.

661

Accepted Manuscript

## **Acknowledgements**

The authors are very grateful to Dena Amer for critical reading and editing the manuscript. We would like to thank the team of the Institute for Pathology (Technische Universität Dresden) for their help during histological slides preparations. This work was supported by the Deutsche Krebsforschungsfoundation.

## **Declaration of interest statement**

The authors have declared no conflict of interest.



## References

- Adashi, E. Y. & Hsueh, A. J. (1982) Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. *J Biol Chem.* 257, 6077-6083.
- Britt, K. L., Simpson, E. R. & Findlay, J. K. (2005) Effects of phytoestrogens on the ovarian and pituitary phenotypes of estrogen-deficient female aromatase knockout mice. *Menopause.* 12, 174-185.
- Britt, K. L., Stanton, P. G., Misso, M., Simpson, E. R. & Findlay, J. K. (2004) The effects of estrogen on the expression of genes underlying the differentiation of somatic cells in the murine gonad. *Endocrinology.* 145, 3950-3960.
- Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker, V. R., Davis, B. J. & Korach, K. S. (1999) Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science.* 286, 2328-2331.
- Couse, J. F. & Korach, K. S. (1999) Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev.* 20, 358-417.
- Couse, J. F., Yates, M. M., Walker, V. R. & Korach, K. S. (2003) Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. *Mol Endocrinol.* 17, 1039-1053.
- Deroo, B. J., Hewitt, S. C., Peddada, S. D. & Korach, K. S. (2004) Estradiol regulates the thioredoxin antioxidant system in the mouse uterus. *Endocrinology.* 145, 5485-5492.
- Diel, P., Geis, R. B., Caldarelli, A., Schmidt, S., Leschowsky, U. L., Voss, A. & Vollmer, G. (2004) The differential ability of the phytoestrogen genistein and of estradiol to induce uterine weight and proliferation in the rat is associated with a substance specific modulation of uterine gene expression. *Mol Cell Endocrinol.* 221, 21-32.
- Diel, P., Schmidt, S. & Vollmer, G. (2002) In vivo test systems for the quantitative and qualitative analysis of the biological activity of phytoestrogens. *J Chromatogr B Analyt Technol Biomed Life Sci.* 777, 191-202.
- Diel, P., Smolnikar, K., Schulz, T., Laudénbach-Leschowski, U., Michna, H. & Vollmer, G. (2001) Phytoestrogens and carcinogenesis-differential effects of

- 700 genistein in experimental models of normal and malignant rat endometrium.  
701 Hum Reprod. 16, 997-1006.
- 702 Drummond, A. E., Baillie, A. J. & Findlay, J. K. (1999) Ovarian estrogen receptor  
703 alpha and beta mRNA expression: impact of development and estrogen. Mol  
704 Cell Endocrinol. 149, 153-161.
- 705 Drummond, A. E., Britt, K. L., Dyson, M., Jones, M. E., Kerr, J. B., O'donnell, L.,  
706 Simpson, E. R. & Findlay, J. K. (2002) Ovarian steroid receptors and their role  
707 in ovarian function. Mol Cell Endocrinol. 191, 27-33.
- 708 Eisenberg, E. & Levanon, E. Y. (2003) Human housekeeping genes are compact.  
709 Trends Genet. 19, 362-365.
- 710 Fisher, C. R., Graves, K. H., Parlow, A. F. & Simpson, E. R. (1998) Characterization  
711 of mice deficient in aromatase (ArKO) because of targeted disruption of the  
712 cyp19 gene. Proc Natl Acad Sci U S A. 95, 6965-6970.
- 713 Frasor, J., Barnett, D. H., Danes, J. M., Hess, R., Parlow, A. F. & Katzenellenbogen,  
714 B. S. (2003) Response-specific and ligand dose-dependent modulation of  
715 estrogen receptor (ER) alpha activity by ERbeta in the uterus. Endocrinology.  
716 144, 3159-3166.
- 717 Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P. & Chambon, P.  
718 (1986) Human oestrogen receptor cDNA: sequence, expression and  
719 homology to v-erb-A. Nature. 320, 134-139.
- 720 Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. & Shine, J. (1986)  
721 Sequence and expression of human estrogen receptor complementary DNA.  
722 Science. 231, 1150-1154.
- 723 Hagihara, K., Wu-Peng, X. S., Funabashi, T., Kato, J. & Pfaff, D. W. (1994) Nucleic  
724 acid sequence and DNase hypersensitive sites of the 5' region of the mouse  
725 progesterone receptor gene. Biochem Biophys Res Commun. 205, 1093-  
726 1101.
- 727 Hall, J. M. & McDonnell, D. P. (1999) The estrogen receptor beta-isoform (ERbeta) of  
728 the human estrogen receptor modulates ERalpha transcriptional activity and is  
729 a key regulator of the cellular response to estrogens and antiestrogens.  
730 Endocrinology. 140, 5566-5578.
- 731 Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague,  
732 M., Strom, A., Treuter, E., Warner, M. & Gustafsson, J. A. (2007) Estrogen

- 733 receptors: how do they signal and what are their targets. *Physiol Rev.* 87, 905-  
734 931.
- 735 Iida, K., Imai, A. & Tamaya, T. (1991) Stimulatory effects of estrogen on  
736 gonadotropin-releasing hormone-induced phosphoinositide turnover in  
737 granulosa cells. *J Steroid Biochem Mol Biol.* 38, 583-586.
- 738 Inhoffen, H. H. & Hohlweg, W. (1938) Neue per os-wirksame weibliche  
739 Keimdrüsenhormon-Derivate: 17-Aethinyl-oestradiol und Pregnen-in-on-3-ol-  
740 17 *Naturwissenschaften.* 26, 96.
- 741 Kanno, J., Onyon, L., Haseman, J., Fenner-Crisp, P., Ashby, J. & Owens, W. (2001)  
742 The OECD program to validate the rat uterotrophic bioassay to screen  
743 compounds for in vivo estrogenic responses: phase 1. *Environ Health*  
744 *Perspect.* 109, 785-794.
- 745 Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar,  
746 M., Korach, K. S., Gustafsson, J. A. & Smithies, O. (1998) Generation and  
747 reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl*  
748 *Acad Sci U S A.* 95, 15677-15682.
- 749 Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. &  
750 Gustafsson, J. A. (1997) Comparison of the ligand binding specificity and  
751 transcript tissue distribution of estrogen receptors alpha and beta.  
752 *Endocrinology.* 138, 863-870.
- 753 Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S. & Gustafsson, J. A. (1996)  
754 Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl*  
755 *Acad Sci U S A.* 93, 5925-5930.
- 756 Kurita, T., Lee, K., Saunders, P. T., Cooke, P. S., Taylor, J. A., Lubahn, D. B., Zhao,  
757 C., Makela, S., Gustafsson, J. A., Dahiya, R. & Cunha, G. R. (2001)  
758 Regulation of progesterone receptors and decidualization in uterine stroma of  
759 the estrogen receptor-alpha knockout mouse. *Biol Reprod.* 64, 272-283.
- 760 Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S. &  
761 Katzenellenbogen, J. A. (2001) Estrogen receptor-beta potency-selective  
762 ligands: structure-activity relationship studies of diarylpropionitriles and their  
763 acetylene and polar analogues. *J Med Chem.* 44, 4230-4251.
- 764 Moller, F. J., Zierau, O., Hertrampf, T., Bliedtner, A., Diel, P. & Vollmer, G. (2009)  
765 Long-term effects of dietary isoflavones on uterine gene expression profiles. *J*  
766 *Steroid Biochem Mol Biol.* 113, 296-303.

- Mosselman, S., Polman, J. & Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* 392, 49-53.
- Nimrod, A., Bedrak, E. & Lamprecht, S. A. (1977) Appearance of LH-receptors and LH-stimulable cyclic AMP accumulation in granulosa cells during follicular maturation in the rat ovary. *Biochem Biophys Res Commun.* 78, 977-984.
- O'lone, R., Frith, M. C., Karlsson, E. K. & Hansen, U. (2004) Genomic targets of nuclear estrogen receptors. *Mol Endocrinol.* 18, 1859-1875.
- Odum, J., Lefevre, P. A., Tittensor, S., Paton, D., Routledge, E. J., Beresford, N. A., Sumpter, J. P. & Ashby, J. (1997) The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regul Toxicol Pharmacol.* 25, 176-188.
- Pelletier, G. & El-Alfy, M. (2000) Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. *J Clin Endocrinol Metab.* 85, 4835-4840.
- Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45.
- Pillai, S. B., Jones, J. M. & Koos, R. D. (2002) Treatment of rats with 17beta-estradiol or relaxin rapidly inhibits uterine estrogen receptor beta1 and beta2 messenger ribonucleic acid levels. *Biol Reprod.* 67, 1919-1926.
- Richards, J. S. (1975) Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins. *Endocrinology.* 97, 1174-1184.
- Rider, V. (2002) Progesterone and the control of uterine cell proliferation and differentiation. *Front Biosci.* 7, d1545-1555.
- Saarinen, N. M., Bingham, C., Lorenzetti, S., Mortensen, A., Makela, S., Penttinen, P., Sorensen, I. K., Valsta, L. M., Virgili, F., Vollmer, G., Warri, A. & Zierau, O. (2006) Tools to evaluate estrogenic potency of dietary phytoestrogens: A consensus paper from the EU Thematic Network "Phytohealth" (QLKI-2002-2453). *Genes Nutr.* 1, 143-158.
- Shi, H. & Teng, C. (1996) Promoter-specific activation of mouse lactoferrin gene by epidermal growth factor involves two adjacent regulatory elements. *Mol Endocrinol.* 10, 732-741.
- Simpson, E. R. (2004) Models of aromatase insufficiency. *Semin Reprod Med.* 22, 25-30.

- Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S. & Katzenellenbogen, J. A. (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor- $\alpha$ -selective agonists. *J Med Chem.* 43, 4934-4947.
- Teng, C., Shi, H., Yang, N. & Shigeta, H. (1998) Mouse lactoferrin gene. Promoter-specific regulation by EGF and cDNA cloning of the EGF-response-element binding protein. *Adv Exp Med Biol.* 443, 65-78.
- Toda, K., Hayashi, Y., Okada, T., Morohashi, K. & Saibara, T. (2005) Expression of the estrogen-inducible EGFP gene in aromatase-null mice reveals differential tissue responses to estrogenic compounds. *Mol Cell Endocrinol.* 229, 119-126.
- Toda, K., Takeda, K., Okada, T., Akira, S., Saibara, T., Kaname, T., Yamamura, K., Onishi, S. & Shizuta, Y. (2001) Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17 $\beta$ -oestradiol. *J Endocrinol.* 170, 99-111.
- Waters, K. M., Safe, S. & Gaido, K. W. (2001) Differential gene expression in response to methoxychlor and estradiol through ER $\alpha$ , ER $\beta$ , and AR in reproductive tissues of female mice. *Toxicol Sci.* 63, 47-56.
- Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E. V., Warner, M. & Gustafsson, J. A. (2000) Estrogen receptor (ER)  $\beta$ , a modulator of ER $\alpha$  in the uterus. *Proc Natl Acad Sci U S A.* 97, 5936-5941.
- Winer, J., Jung, C. K., Shackel, I. & Williams, P. M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem.* 270, 41-49.
- Zierau, O., Kretzschmar, G., Moller, F., Weigt, C. & Vollmer, G. (2008) Time dependency of uterine effects of naringenin type phytoestrogens in vivo. *Mol Cell Endocrinol.* 294, 92-99.

**Tables:****Table 1** Experimental doses of estrogenic substances.

	doses in mg / kg BW / d	
substance	sc injection	Av. <i>per os</i> substitution
EE	0.005	0.05
PPT	0.5	5.0
DPN	0.5	5.0
GEN	10.0	70.0

Details for the substance administrations are described in material and methods.

**Table 3** Effects of estrogenic substances on gonadotropin levels in ArKO mice following either oral (po) or subcutaneous (sc) administration in comparison to female WT littermates.

		LH in $\mu\text{IU} / \text{ml}$	FSH in $\text{mIU} / \text{ml}$
ArKO		94 $\pm$ 6	44 $\pm$ 9
WT		47 $\pm$ 22 **	29 $\pm$ 2 *
EE	po	47 $\pm$ 25 **	26 $\pm$ 9 *
	sc	91 $\pm$ 39	40 $\pm$ 5
PPT	po	30 $\pm$ 13 *	17 $\pm$ 6 **
	sc	46 $\pm$ 24 *	39 $\pm$ 10
DPN	po	73 $\pm$ 52	23 $\pm$ 3 **
	sc	63 $\pm$ 18 **	32 $\pm$ 8
GEN	po	30 $\pm$ 14 **	25 $\pm$ 2 **
	sc	28 $\pm$ 14 **	30 $\pm$ 4*

Data are expressed as mean  $\pm$  SD (in  $\mu\text{IU} / \text{ml}$  and  $\text{mIU} / \text{ml}$  for LH (luteinizing hormone) and FSH (follicle-stimulating hormone), respectively. (IU meaning international units)

\*  $p < 0.05$  and \*\*  $p < 0.01$  versus ArKO control according to *Mann-Whitney-U*-test.

**Table 2** Oligonucleotides used as primers for the real-time PCR to analyse uterine and ovarian gene expression profile are listed in alphabetical order.

protein	gene		sequence (5'-3')	amplicon size (bp)
complement component 3	C3	fwd	CCAGCAGGTCATCAAGTCAG	185
		rev	ACGTGTCCTTCCCAATGATG	
dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X-chromosome gene 1	<i>DAX-1</i> ( <i>Nr0b1</i> )	fwd	TCAGGAAGAGCGAGAGGTG	186
		rev	CTGGCGTTGGTGAGCATAG	
estrogen receptor $\alpha$	<i>ESR1</i>	fwd	TGTTTGCTCCTAACTTGCTCCT	222
		rev	GGTGGATGTGGTCCTTCTCTT	
estrogen receptor $\beta$	<i>ESR2</i>	fwd	GCCAGGAAGCAGAGAGTAGC	189
		rev	TCATGCTGAGCAGATGTTCC	
luteinizing hormone/choriogonadotropin receptor	<i>LHR</i>	fwd	TCAATGGGACGACGCTAATC	205
		rev	CTGGAGGGCAGAGTTTTTCAG	
liver receptor homologue 1	<i>LRH-1</i> ( <i>Nr5a2</i> )	fwd	ATGGGAAGGAAGGGACAATC	183
		rev	TGAACAGCACCAGGAAGTTG	
lactotransferrin	<i>LTF</i>	fwd	GCAAAACCACATCGGAGAAG	204
		rev	GGGAGTGAGGAGACCAGATG	
antigen identified by monoclonal antibody Ki 67	<i>MKI67</i>	fwd	CCAAGGGTAACTCGTGGAAC	242
		rev	TCCTTAACTCAGGCAGTAGCAG	
proliferating cell nuclear antigen	<i>PCNA</i>	fwd	ACATTGGAGATGCTGTTGTGA	206
		rev	CAGTGGAGTGGCTTTTGTGA	
progesterone receptor	<i>PGR</i>	fwd	CTGGCATGGTCCTTGGAG	246
		rev	TGGAAGTGTGAGGCTTTGTG	
ribosomal protein S18	<i>RPS18</i>	fwd	AGGATGTGAAGGATGGGAAG	187
		rev	TTGGATACACCCACAGTTCC	
steroidogenic factor 1	<i>SF-1</i> ( <i>Nr5a1</i> )	fwd	GAGTTCGTCTGTCTCAAGTTCC	179
		rev	ACCTCCACCAGGCACAATAG	
sry-like homeobox transcription factor 9	<i>SOX-9</i>	fwd	CGGAGGAAGTCGGTGAAG	201
		rev	GTCGGTTTTGGGAGTGGTG	

**Figure captions:****Figure 1. Relative uterine wet weights (UWW) of the differentially treated groups of ArKO mice.**

Mice were treated according to the experimental design as described in material and methods and results are normalized against the carrier-treated CO group. The box plot shows the 90th to the 10th percentiles (box), the median (+) in addition to the minimum and maximum values. Statistical analyses were performed using the Mann Whitney *U*-test. The hash signs indicate the significant differences to CO, with  $p < 0.01$  (##) and  $p < 0.001$  (###). The highly significant difference between the CO and normal WT is indicated by '++'. The significant effects of *per os* versus the subcutaneous administration is indicated by \*\* ( $p < 0.01$ ).

**Figure 2. Light micrographs showing representative uteri cross sections (3  $\mu$ m) of ArKO mice in differential experimental groups**

(A) CO; (B) EE po; (C) EE sc; (D) PPT po; (E) PPT sc; (F) DPN po; (G) DPN sc; (H) GEN po; (I) GEN sc. The histological sections were stained with hematoxylin-eosin and documented at a magnification of 200x (black bar = 50  $\mu$ m).

**Figure 3. Gene and protein expression profiles of proliferation-associated genes in the uterus of differentially treated ArKO mice are analyzed upon two administration routes, *per os* and subcutaneous.**

Relative gene expression levels of (A) *PCNA* and (B) *MKI67* are normalized against the CO group (black horizontal line). Data are presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test,  $p < 0.05$  \*;  $p < 0.01$  \*\*. Figure 3 C shows the results from immunohistochemical staining against PCNA protein in uterine epithelial cells. The percentage of PCNA-positive cells is presented as mean  $\pm$  SD. Statistical analyse was performed using the *Mann-Whitney-U*-test,  $p < 0.01$  \*\*;  $p < 0.001$  \*\*\*.

**Figure 4. Gene expression profiles of relevant steroid receptor genes in the uterus of differentially treated ArKO mice are analyzed upon two administration routes, *per os* and subcutaneous.**



Relative gene expression levels of (A) ESR1, (B) ESR2 as well as (C) PGR are normalized against the CO group (black horizontal line). Data are presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test,  $p < 0.05$  \*;  $p < 0.01$  \*\*.

**Figure 5. Gene expression profiles of estrogen-response marker genes in the uterus of differentially treated ArKO mice are analyzed upon two administration routes, per os and subcutaneous.**

Relative gene expression levels of (A) LTF and (B) C3 are normalized against the CO group (black horizontal line). Data are presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test,  $p < 0.05$  \*;  $p < 0.01$  \*\*.

**Figure 6. Gene expression profile of estrogen receptor  $\beta$  (ESR2) gene in the ovary of differentially treated ArKO mice is analyzed upon two administration routes, per os and subcutaneous.**

Relative gene expression levels are normalized against the CO group (black horizontal line). Data are presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test ( $p < 0.05$  \*).

**Figure 7. Gene expression profiles of a proliferation-associated gene (PCNA; A) and an ovarian specific differentiation marker gene (LHR; B) in the ovary of differentially treated ArKO mice are analyzed upon two administration routes, per os and subcutaneous.**

Data is normalized against the CO group (black horizontal line) and presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test,  $p < 0.05$  \*.

**Figure 8. Gene expression profiles of estrogen-responsive nuclear factors in the ovary of differentially treated ArKO mice are analyzed upon two administration routes, per os and subcutaneous.**

Relative gene expression levels of (A) SF-1 and (B) LRH-1 are normalized against the CO group (black horizontal line). Data are presented as mean  $\pm$  SD. Statistical analyses were performed using the Student's *t*-test ( $p < 0.05$  \* and  $p < 0.01$  \*\*).

Figure 1

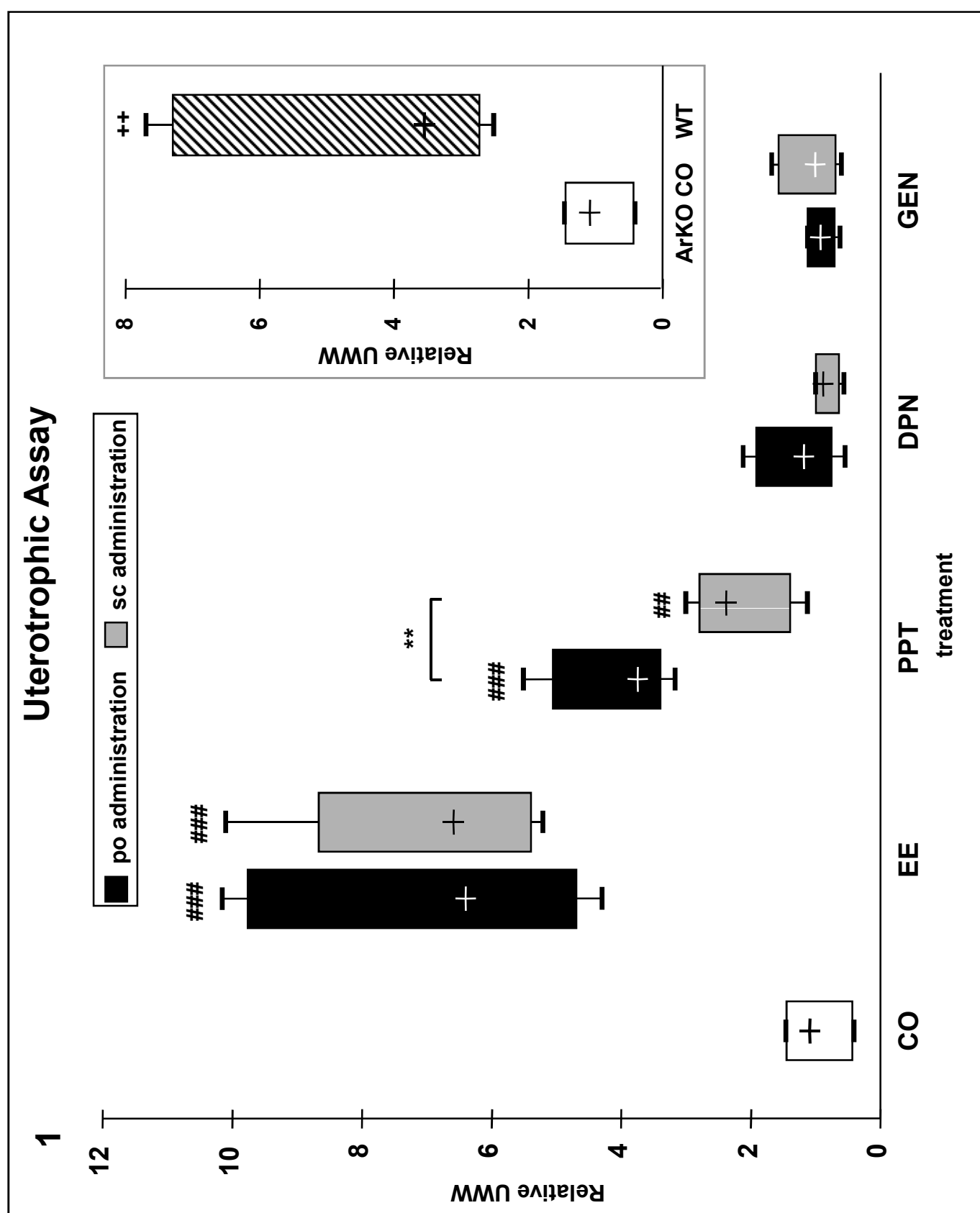


Figure 2

