



HAL
open science

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-00529006>

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.

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.

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

3 **Anja Bliedtner*¹, Oliver Zierau¹, Steffen Albrecht², Stefanie Liebhaber¹, Günter**
4 **Vollmer¹**

5

6

7 ¹ Chair of Molecular Cell Physiology and Endocrinology, Institute for Zoology, Technische
8 Universität Dresden, Germany

9 ² Department of Gynaecology and Obstetrics, University Hospital Carl-Gustav-Carus
10 Technische Universität Dresden, Germany

11

12

13

14 DISCLOSURE STATEMENT: The authors have nothing to disclose.

15

16 * Corresponding author:

17

18 Anja Bliedtner

19 Technische Universität Dresden

20 Institut für Zoologie

21 Zellescher Weg 20b, Raum 249,

22 01217 Dresden

23 Germany

24 Phone: +49 351 463 34733

25 Fax: +49 351 463 31923

26 Email: anja.bliedtner@tu-dresden.de

27

28

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 α -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 μ g/ kg BW; po 50 μ 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 α and ER β -
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

60 Introduction

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

72 Estrogenic effects are mediated through the two estrogen receptors (ER α and ER β)
73 (Green et al., 1986, Greene et al., 1986, Kuiper et al., 1996, Mosselman et al., 1996).
74 They are encoded by two separate genes (*ESR1* and *ESR2*, respectively), but share
75 the common structural architecture of nuclear receptors. These transcription factors
76 exhibit an evolutionary conserved domain structure; reviewed by Heldring et al.
77 (2007). In the central DNA-binding domain, both estrogen receptors share the
78 highest sequence similarity (95%), which allows them to bind to identical DNA
79 response elements. Despite exhibiting merely 60% similarity, the C-terminal ligand-
80 binding domains bind E2 with equivalent affinities but also permit ER-subtype
81 specificity for different ligands.

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

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

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

101 However, the aromatase knock-out (ArKO) mice may be useful alternatives to study
102 estrogenic responses, because both ERs are still intact and functional (Simpson,
103 2004), while in difference to the ovariectomized rodent model the progestogens are
104 not compromised. In addition, the ovariectomy is not necessary, because these
105 animals are completely devoid of endogenous estrogen production due to their
106 defective estrogen biosynthesis resulting from disruption of the aromatase gene
107 (*CYP19a*) (Fisher et al., 1998). However, the responsiveness to exogenous
108 estrogens account for ER-subtype specificity studies, such as analyzing hormonal
109 potency of xeno- and phytoestrogens.

110 In the present study, we analyzed the effects in the uterus and the ovaries of four
111 reference substances following oral or subcutaneous application, the phytoestrogen
112 GEN and two synthetic ER-specific agonists (PPT and DPN) as well as EE and
113 compared these to the carrier-control group. GEN is a mild-selective agonist for ER β
114 with more than 7-fold higher binding affinity for ER β than ER α (Kuiper et al., 1997).
115 PPT has a 410-fold binding preference for ER α over ER β (Stauffer et al., 2000).
116 Accordingly, ER β is almost not bound and therefore not activated. DPN represents,
117 at least within a limited dosage range, agonistic properties for ER β with more than
118 70-fold higher binding preference for ER β than ER α (Meyers et al., 2001). EE is a
119 well-known orally active steroidal derivative of the natural 17 β -estradiol and an
120 approved reference ER-agonist (Kanno et al., 2001, Rider, 2002).

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

128 and Korach, 1999). The ER β is minorly present in all uterine cell types (Weihua et al.,
129 2000). Accordingly, the functional properties of ER β in the uterus are less obvious,
130 even though it provokes some estrogenic effects, for instance increasing
131 progesterone receptor (*PGR*) expression (Kurita et al., 2001) and modulating ER α
132 actions (Weihua et al., 2000).

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

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

148 **Materials and Methods**

149 *Substances*

150 17 α -ethynylestradiol (EE; 17 α -ethynyl-1,3,5(10)-estriene-3,17 β -diol;19-Nor-1,3,5(10),
151 17 α -pregnatrien-20yne-3,17-diol) and genistein (GEN; 4',5,7-trihydroxyisoflavone)
152 were purchased from Sigma-Aldrich (Deisenhofen, Germany), propylpyrazole-triol
153 (PPT; 4, 4',4'' - (4 -propyl-(H)-pyrazole-1 ,3 ,5 -triy)l) *trisphenol*) and diarylpropionitrile
154 (DPN; 2, 3-*bis* (4-hydroxyphenyl)-propionitrile) were purchased from Tocris
155 Bioscience (Bristol, UK).

156 *Animals*

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

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

168 *Treatment and experimental design*

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

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

191 24 hours after the last injection and accordingly after 72 h of exposure to
192 experimental chow, animals were sacrificed by cervical dislocation. The body weight

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

198 *Gonadotropin levels*

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

205 *Gene expression analyses*

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

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

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

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

234 *Histological analysis*

235 Formalin-fixed uterine and ovarian tissues of the CO and the experimental groups
236 were dehydrated and processed for paraffin embedding. Tissue cross sections
237 (3 μ m) were processed for hematoxylin-eosin staining to evaluate the
238 histopathological changes of the tissues. The height of the uterine epithelia was
239 determined using a microscope micrometer scale and the program *ImageJ*
240 (<http://rsbWeb.nih.gov/ij>). The ovarian cross sections were examined according to
241 their overall morphology and phenotype of follicles and interstitial cells. Unfortunately,
242 there were no tissue samples of the WT tissues available for histological
243 examinations.

244 *Immunohistochemistry (IHC) against PCNA Protein*

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

251 *Statistical analysis*

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

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

258 Furthermore, UWW data were presented in a box plot chart as median with a 10th to
259 90th percentile box for a more precise documentation. The statistical analysis of
260 UWW, gonadotropin levels and PCNA immunohistochemical staining was performed
261 using the *Mann-Whitney U*-test. A value of $p < 0.05$ was considered as significantly
262 different from CO.

263 Results

264 *Uterine wet weight and epithelia height*

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

270 As depicted in figure 1, the relative UWW of the WT littermates were 3.7 fold higher
271 ($p < 0.01$) than those of CO mice. Irrespective of the route of administration, the
272 treatment with 17 α -ethynylestradiol (EE) increased the UWW of ArKO mice even
273 above the WT level (po 7.1 ± 2.4 fold and sc 6.9 ± 1.7 fold vs. CO; both $p < 0.001$).
274 Analyzing the effects of the ER α -agonist PPT, the oral administration caused a
275 stronger weight increase (4.1 ± 0.9 fold vs. CO; $p < 0.001$) than the sc injection
276 (2.2 ± 0.7 fold vs. CO; $p < 0.01$). Neither genistein (GEN; po 0.9 ± 0.2 and sc
277 1.1 ± 0.4) nor ER β -agonist (DPN; po 1.3 ± 0.6 ; sc 0.8 ± 0.2) substitution resulted in
278 significant changes of UWW compared to CO.

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

287 *Serum gonadotropin levels*

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

297 *Uterine gene and protein expression*

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

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

309 In addition, we performed immunohistochemistry (IHC) on the uterine cross sections
310 with an antibody against PCNA protein to confirm the real-time PCR results (figure
311 3 C). The amount of the PCNA-positive stained uterine epithelial cells was clearly
312 enhanced following EE and PPT treatment. Additionally, the absence of PCNA-
313 positive cells following DPN treatment was detected as well as low numbers in the
314 genistein groups.

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

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

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

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

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

344 *Ovarian gene expression*

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

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

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

361 On the other hand, the gene expression levels of *ESR2* were clearly higher than the
362 respective *ESR1* as evaluated by ΔCt values (data not shown). In the ovaries of WT
363 mice, *ESR2* level was 3.1 fold above CO. As illustrated in figure 6, all substances
364 evidently increased *ESR2* level at the administered *per os* doses (PPT po & DPN po
365 $p < 0.05$, EE po $p = 0.06$ vs. CO), whereas subcutaneous doses did not cause
366 significant changes.

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

376 Furthermore, previous studies have demonstrated the emergence of male-like
377 somatic cells in the ovaries of estrogen-depleted mice (Britt et al., 2004, Couse et al.,
378 1999). They reported an influence of estrogen treatment on the gene expression of
379 *Sox9* (*Sry*-like homeobox transcription factor 9) and up- and down-stream factors,
380 which are shown to be related to male-like structures visible in ArKO ovaries (Britt et
381 al., 2004). In the present study, the expression of some of these nuclear transcription
382 factors were examined by real-time PCR: *Sf-1* (steroidogenic factor 1) and the
383 related *LRH-1* (liver receptor homologue 1), as well as *DAX-1* (the dosage-sensitive
384 sex reversal adrenal hypoplasia congenital critical region on the X-chromosome
385 gene 1) and *SOX-9*. In the ovaries of CO mice, the transcription levels of all these
386 genes were very close to detection limit. Regarding *DAX-1* and *SOX-9*, no evaluation

387 of expression profiles was possible because of very weak gene expressions (data not
388 shown). Nevertheless, in WT ovaries the gene expression levels of all analyzed
389 genes were obviously higher than in CO or treated ArKO mice ovaries. However, SF-
390 1 was significantly up-regulated ($p < 0.05$ vs. CO) in all per os groups, except for
391 GEN (figure 8A). Furthermore, sc administrations induced minor changes of SF-1
392 gene expression (EE and PPT sc $p < 0.05$). Regarding LRH-1 levels, all substances
393 clearly evoked up-regulations (figure 8 B).

394 Discussion

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

401 This study was intended to qualitatively examine the route of oral substance
402 administration, particularly regarding PPT and DPN treatment and additionally to
403 evaluate ER-specific reactions of ArKO mice. For us, it was important to access a
404 non-invasive route of substance administration which is able to reproduce the
405 consistent effects of subcutaneous injections. Furthermore, not much was initially
406 known about the quality of effects resulting from oral administration of PPT and DPN.
407 Therefore, in our experimental design we have scrutinized two administration groups
408 in parallel (sc and po) for every tested substance. We chose the well-known and well-
409 characterized synthetic estrogen 17α -ethynylestradiol (EE) as reference substance,
410 firstly for acting via both ER-subtypes and secondly for its oral bioavailability
411 (Inhoffen and Hohlweg, 1938, Kuiper et al., 1997). Regarding the ER-subtype
412 specific substances, we made use of PPT and DPN because they are well described
413 and commercially available. The ER α -specific agonist, propylpyrazole-triol (PPT) has
414 a 410-fold binding preference for ER α over ER β (Stauffer et al., 2000). The potency-
415 selective agonist for ER β diarylpropionitrile (DPN) has a more than 70-fold higher
416 binding preference for ER β than ER α (Meyers et al., 2001). For oral administrations,
417 we decided to use 10-fold higher doses because initially we expected oral effects of
418 PPT and DPN to be weaker due to metabolic aspects. Both were applied at doses of
419 0.5 mg / kg BW for sc administration and 5 mg / kg BW for per os administration at

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

430 **Uterine responses of ArKO mice**

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

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

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

447 In this aspect, GEN caused similar patterns like DPN. Additionally, GEN has
448 previously been characterized to have a higher preference for ERβ (Kuiper et al.,
449 1997). Still, also agonistic properties were described earlier for GEN with a faint
450 increase of UWW in OVX rats after 3 day treatment (Moller et al., 2009). However,
451 short term administration of GEN, even in very high doses, did not result in a steady
452 increase of UWW in OVX rat (Diel et al., 2004). Then again, GEN caused an
453 expansion of the luminal epithelial cell layer in ArKO mice (Toda et al., 2005). In

454 ArKO mice, it was shown previously that life-long intake of genistein enriched diets
455 increased uterine and ovarian weights, whereby an association with proliferation was
456 not clear (Britt et al., 2005). Our studies demonstrated an inability of GEN to
457 stimulate the UWW in ArKO mice in the here applied doses. Furthermore, this was
458 correlated with the inability to significantly induce uterine proliferation, which was
459 analyzed on mRNA and protein level. Apparently, there exists a variety of studies
460 including time- and dose-dependencies of uterine actions which indicated SERM-like
461 properties for GEN (Toda et al., 2005, Diel et al., 2001, Diel et al., 2004, Moller et al.,
462 2009). Furthermore, in the context of the biphasic pattern of uterotrophy induced by
463 estrogenic substances, a weak induction of UWW by genistein was previously
464 supposed as a result from imbibitions of water other than from induced proliferation
465 (Diel et al., 2004). In our study, genistein treatment clearly resulted in a reduction of
466 the gonadotropin levels, which is considered as a possibility for estrogen-related
467 effects. Nevertheless, details on proliferation modulation by phytoestrogens need
468 further investigations.

469 **Gene expression profiles of ArKO uteri**

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

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

488 and differentiation status of uterine tissue in more detail and to prove the ability of
489 ArKO mice's uteri to respond directly to estrogenic signals. ESR1 gene expression
490 levels in CO were similar to WT. Even though the ESR1 levels markedly exceeded
491 that of ESR2, we hardly observed an alteration following substance administration.
492 This stands in contrast to effects previously observed in rat uteri, where 17 β -estradiol
493 down-regulated both ESR1 and ESR2 expression (Zierau et al., 2008).
494 In this study, only oral administration of DPN significantly decreased ER α levels.
495 Mechanistically, it was shown previously that ER β could serve to inhibit ER α
496 signalling in target tissues (Frasor et al., 2003, Hall and McDonnell, 1999), maybe
497 this is realized also by influencing gene expression. Additionally, the low ER β gene
498 expression levels in the uterus were significantly increased upon EE (per os) and
499 PPT treatment, although never reaching ER α level. These observations were rather
500 unexpected and stand in contrast to various other studies, which reported a reduction
501 of both ER α and ER β expression upon estrogen treatments (Diel et al., 2004, Pillai et
502 al., 2002). According to Weihua and colleagues, ER β tends to down-regulate ER α
503 activity (Weihua et al., 2000). In the present study, it seems that ER α activation
504 increases ER β expression and ER β activation inhibits ER α expression. This may add
505 an interesting aspect to the complexity of estrogenic cross talk in regulating
506 networks, including feedback regulations of their own receptors.
507 Even though ER β expression was increased, the dominant role of ER α stayed
508 immanently superior in the uterus also in the estrogen-free background of ArKO
509 mice. Diel et al. described that a high ER α /ER β ratio was correlated with increased
510 proliferation (Diel et al., 2004). Here we observed that only upon stimulation of ER α
511 signaling in combination with a high ER α / ER β ratio proliferation was switched on,
512 whereas activating only ER β even with a high ER α /ER β ratio was not sufficient to
513 increase proliferation.
514 Adding to the understanding of potential molecular mechanisms of regulations in the
515 uterus, we have analyzed three estrogen-response genes: progesterone receptor
516 (PGR), lactotransferrin (LTF) and complement component 3, (C3). All three genes
517 contain estrogen-response elements in their 5'-regulatory regions (O'Lone et al.,
518 2004) and are described earlier to be directly regulated by estrogens (Toda et al.,
519 2001, Zierau et al., 2008). Here, all were clearly up-regulated by EE treatment in the
520 uteri of ArKO mice. In this experimental design, LTF was the most sensitive
521 estrogen-response gene with an expected increase of gene expression following

522 ER α activation. DPN added a more interesting aspect, because subcutaneous
523 administration led to a highly significant down-regulation of *LTF*. In accordance,
524 Couse et al. found no up-regulation of lactotransferrin as well as progesterone
525 receptor in the uteri of α ERKO upon estrogenic signals, but no down-regulation was
526 observed so far. It needs further investigations to analyze details of ER-specificity of
527 ERE-dependent mechanism and their responsibilities for the observed effect.
528 Another explanation might as well be alternative pathways of LTF gene regulation
529 involving activation of cAMP or growth factor response elements, which were found
530 in the promoter region of this gene (Shi and Teng, 1996, Teng et al., 1998). *C3*
531 revealed a similar but weaker profile in ArKO uteri, which was surprising, because in
532 the OVX rats *C3* is a very strong estrogenic-marker (Diel et al., 2004, Zierau et al.,
533 2008). Here, we could only speculate about species specificities or even side effects
534 resulting from ovariectomy, but no definite explanation can be given for that result.
535 PGR, which is an important steroid receptor in the uterus, revealed an interesting
536 seemingly ER-specific expression pattern in the uteri of differentially treated ArKO
537 mice. In WT mice and in ArKO mice following EE administration, *PGR* expression
538 levels were significantly elevated compared to CO. The ER α -agonist (PPT) did not
539 influence, while the ER β -agonist (DPN) significantly decreased *PGR* expression. It is
540 known, that *PGR* is transcribed under the regulation of estrogens (Toda et al., 2001),
541 although differential expression patterns are described in the literature regarding
542 effects following estrogen treatments (Waters et al., 2001, Adashi and Hsueh, 1982).
543 There might be dose-dependent or ER-subtype specific regulation patterns involved,
544 but also a time-dependent regulation has been demonstrated for *PGR* (Zierau et al.,
545 2008). Furthermore, the 5' region of *PGR* gene contains a lot of regulatory
546 sequences, including EREs, PREs, AP1-binding sites and others (Hagihara et al.,
547 1994) which implies a complex molecular regulation pattern. ArKO mice might add to
548 the understanding of regulations of *PGR* expression, because in contrast to OVX or
549 immature *in vivo* models, progesterone production is not compromised.

550 **Gene expression profiles of ArKO ovaries**

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

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

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

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

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

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

600 **Influence on gonadotropins**

601 In accordance with Fisher et al., we observed elevated gonadotropin levels in female
602 ArKO mice (Fisher et al., 1998). This reflects a disruption of negative feedback on the
603 hypothalamic-pituitary axis leading to elevated gonadotropin levels. In our study, we
604 observed a reversion of these levels back to or even below WT level by application of
605 ER α -agonists. However, the effects of estrogenic substances on serum LH and FSH
606 levels were complex, whereby the effects resulting from oral administration gave a
607 clearer pattern. PPT as well as EE strongly decreased LH and FSH back or even
608 below to WT level, respectively. Surprisingly, results from the EE sc did not show any
609 effect, which may be a dose effect. There is an importance of ER α signaling for LH
610 feedback suggested by Couse et al., who showed that LH levels were increased in
611 α ERKO and $\alpha\beta$ ERKO mice compared with Wt and β ERKO mice (Couse et al., 2003).
612 However, they observed a normal FSH regulation in α ERKO and $\alpha\beta$ ERKO females.
613 Our data showed a decrease of FSH level following ER α - as well as ER β -agonist
614 treatment, which indicates a role for ER β -dependent regulation, but also other
615 regulation mechanisms like those via inhibin/activin add to complexity (Couse et al.,
616 2003).

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

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

627 **Summary**

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

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

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

650 Regarding the administration routes, we were aiming to evaluate effects resulting
651 from oral application of PPT and DPN. We chose 10-fold higher doses on the oral
652 route, because we initially expected lower effects than finally observed. For reaching
653 a quantitative comparison of different administration routes equal doses should be
654 applied in further experiments. Overall, effects resulting from EE treatments were
655 less dose- and route-dependent, whereas for PPT, *per os* dose led to more obvious
656 effects than sc injections, for instance an increase of UWW or proliferation markers.
657 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

661 **Acknowledgements**

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

666 **Declaration of interest statement**

667 The authors have declared no conflict of interest.

668

Accepted Manuscript

668 **References**

- 669 Adashi, E. Y. & Hsueh, A. J. (1982) Estrogens augment the stimulation of ovarian
670 aromatase activity by follicle-stimulating hormone in cultured rat granulosa
671 cells. *J Biol Chem.* 257, 6077-6083.
- 672 Britt, K. L., Simpson, E. R. & Findlay, J. K. (2005) Effects of phytoestrogens on the
673 ovarian and pituitary phenotypes of estrogen-deficient female aromatase
674 knockout mice. *Menopause.* 12, 174-185.
- 675 Britt, K. L., Stanton, P. G., Misso, M., Simpson, E. R. & Findlay, J. K. (2004) The
676 effects of estrogen on the expression of genes underlying the differentiation of
677 somatic cells in the murine gonad. *Endocrinology.* 145, 3950-3960.
- 678 Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker, V. R., Davis, B. J. &
679 Korach, K. S. (1999) Postnatal sex reversal of the ovaries in mice lacking
680 estrogen receptors alpha and beta. *Science.* 286, 2328-2331.
- 681 Couse, J. F. & Korach, K. S. (1999) Estrogen receptor null mice: what have we
682 learned and where will they lead us? *Endocr Rev.* 20, 358-417.
- 683 Couse, J. F., Yates, M. M., Walker, V. R. & Korach, K. S. (2003) Characterization of
684 the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice
685 reveals hypergonadism and endocrine sex reversal in females lacking
686 ERalpha but not ERbeta. *Mol Endocrinol.* 17, 1039-1053.
- 687 Deroo, B. J., Hewitt, S. C., Peddada, S. D. & Korach, K. S. (2004) Estradiol regulates
688 the thioredoxin antioxidant system in the mouse uterus. *Endocrinology.* 145,
689 5485-5492.
- 690 Diel, P., Geis, R. B., Caldarelli, A., Schmidt, S., Leschowsky, U. L., Voss, A. &
691 Vollmer, G. (2004) The differential ability of the phytoestrogen genistein and of
692 estradiol to induce uterine weight and proliferation in the rat is associated with
693 a substance specific modulation of uterine gene expression. *Mol Cell*
694 *Endocrinol.* 221, 21-32.
- 695 Diel, P., Schmidt, S. & Vollmer, G. (2002) In vivo test systems for the quantitative and
696 qualitative analysis of the biological activity of phytoestrogens. *J Chromatogr B*
697 *Analyt Technol Biomed Life Sci.* 777, 191-202.
- 698 Diel, P., Smolnikar, K., Schulz, T., Laudenschlager, U., Michna, H. &
699 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., Pelto-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.

- 767 Mosselman, S., Polman, J. & Dijkema, R. (1996) ER beta: identification and
768 characterization of a novel human estrogen receptor. *FEBS Lett.* 392, 49-53.
- 769 Nimrod, A., Bedrak, E. & Lamprecht, S. A. (1977) Appearance of LH-receptors and
770 LH-stimulable cyclic AMP accumulation in granulosa cells during follicular
771 maturation in the rat ovary. *Biochem Biophys Res Commun.* 78, 977-984.
- 772 O'lonc, R., Frith, M. C., Karlsson, E. K. & Hansen, U. (2004) Genomic targets of
773 nuclear estrogen receptors. *Mol Endocrinol.* 18, 1859-1875.
- 774 Odum, J., Lefevre, P. A., Tittensor, S., Paton, D., Routledge, E. J., Beresford, N. A.,
775 Sumpter, J. P. & Ashby, J. (1997) The rodent uterotrophic assay: critical
776 protocol features, studies with nonyl phenols, and comparison with a yeast
777 estrogenicity assay. *Regul Toxicol Pharmacol.* 25, 176-188.
- 778 Pelletier, G. & El-Alfy, M. (2000) Immunocytochemical localization of estrogen
779 receptors alpha and beta in the human reproductive organs. *J Clin Endocrinol*
780 *Metab.* 85, 4835-4840.
- 781 Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time
782 RT-PCR. *Nucleic Acids Res.* 29, e45.
- 783 Pillai, S. B., Jones, J. M. & Koos, R. D. (2002) Treatment of rats with 17beta-estradiol
784 or relaxin rapidly inhibits uterine estrogen receptor beta1 and beta2
785 messenger ribonucleic acid levels. *Biol Reprod.* 67, 1919-1926.
- 786 Richards, J. S. (1975) Estradiol receptor content in rat granulosa cells during
787 follicular development: modification by estradiol and gonadotropins.
788 *Endocrinology.* 97, 1174-1184.
- 789 Rider, V. (2002) Progesterone and the control of uterine cell proliferation and
790 differentiation. *Front Biosci.* 7, d1545-1555.
- 791 Saarinen, N. M., Bingham, C., Lorenzetti, S., Mortensen, A., Makela, S., Penttinen,
792 P., Sorensen, I. K., Valsta, L. M., Virgili, F., Vollmer, G., Warri, A. & Zierau, O.
793 (2006) Tools to evaluate estrogenic potency of dietary phytoestrogens: A
794 consensus paper from the EU Thematic Network "Phytohealth" (QLKI-2002-
795 2453). *Genes Nutr.* 1, 143-158.
- 796 Shi, H. & Teng, C. (1996) Promoter-specific activation of mouse lactoferrin gene by
797 epidermal growth factor involves two adjacent regulatory elements. *Mol*
798 *Endocrinol.* 10, 732-741.
- 799 Simpson, E. R. (2004) Models of aromatase insufficiency. *Semin Reprod Med.* 22,
800 25-30.

- 801 Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J.,
802 Katzenellenbogen, B. S. & Katzenellenbogen, J. A. (2000) Pyrazole ligands:
803 structure-affinity/activity relationships and estrogen receptor-alpha-selective
804 agonists. *J Med Chem.* 43, 4934-4947.
- 805 Teng, C., Shi, H., Yang, N. & Shigeta, H. (1998) Mouse lactoferrin gene. Promoter-
806 specific regulation by EGF and cDNA cloning of the EGF-response-element
807 binding protein. *Adv Exp Med Biol.* 443, 65-78.
- 808 Toda, K., Hayashi, Y., Okada, T., Morohashi, K. & Saibara, T. (2005) Expression of
809 the estrogen-inducible EGFP gene in aromatase-null mice reveals differential
810 tissue responses to estrogenic compounds. *Mol Cell Endocrinol.* 229, 119-
811 126.
- 812 Toda, K., Takeda, K., Okada, T., Akira, S., Saibara, T., Kaname, T., Yamamura, K.,
813 Onishi, S. & Shizuta, Y. (2001) Targeted disruption of the aromatase P450
814 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-
815 oestradiol. *J Endocrinol.* 170, 99-111.
- 816 Waters, K. M., Safe, S. & Gaido, K. W. (2001) Differential gene expression in
817 response to methoxychlor and estradiol through ERalpha, ERbeta, and AR in
818 reproductive tissues of female mice. *Toxicol Sci.* 63, 47-56.
- 819 Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E. V., Warner, M. &
820 Gustafsson, J. A. (2000) Estrogen receptor (ER) beta, a modulator of ERalpha
821 in the uterus. *Proc Natl Acad Sci U S A.* 97, 5936-5941.
- 822 Winer, J., Jung, C. K., Shackel, I. & Williams, P. M. (1999) Development and
823 validation of real-time quantitative reverse transcriptase-polymerase chain
824 reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal*
825 *Biochem.* 270, 41-49.
- 826 Zierau, O., Kretschmar, G., Moller, F., Weigt, C. & Vollmer, G. (2008) Time
827 dependency of uterine effects of naringenin type phytoestrogens in vivo. *Mol*
828 *Cell Endocrinol.* 294, 92-99.
- 829
- 830

830 **Tables:**

831

832

Table 1 Experimental doses of estrogenic substances.

substance	doses in mg / kg BW / d	
	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

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

834

835

836

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

		LH in $\mu\text{IU} / \text{ml}$	FSH in mIU / 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*

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

842 * $p < 0.05$ 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 α	<i>ESR1</i>	fwd	TGTTTGCTCCTAACTTGCTCCT	222
		rev	GGTGGATGTGGTCCTTCTCTT	
estrogen receptor β	<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	TGAACAGCACCAGGAACTTG	
lactotransferrin	<i>LTF</i>	fwd	GCAAAACCACATCGGAGAAG	204
		rev	GGGAGTGAGGAGACCAGATG	
antigen identified by monoclonal antibody Ki 67	<i>MKI67</i>	fwd	CCAAGGGTAACTCGTGGAAC	242
		rev	TCCTTAAACTCAGGCAGTAGCAG	
proliferating cell nuclear antigen	<i>PCNA</i>	fwd	ACATTGGAGATGCTGTTGTGA	206
		rev	CAGTGGAGTGGCTTTTGTGA	
progesterone receptor	<i>PGR</i>	fwd	CTGGCATGGTCCTTGGAG	246
		rev	TGGAAGTGTCAAGCTTTGTG	
ribosomal protein S18	<i>RPS18</i>	fwd	AGGATGTGAAGGATGGGAAG	187
		rev	TTGGATACACCCACAGTTCCG	
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 μ 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 μ 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 β (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

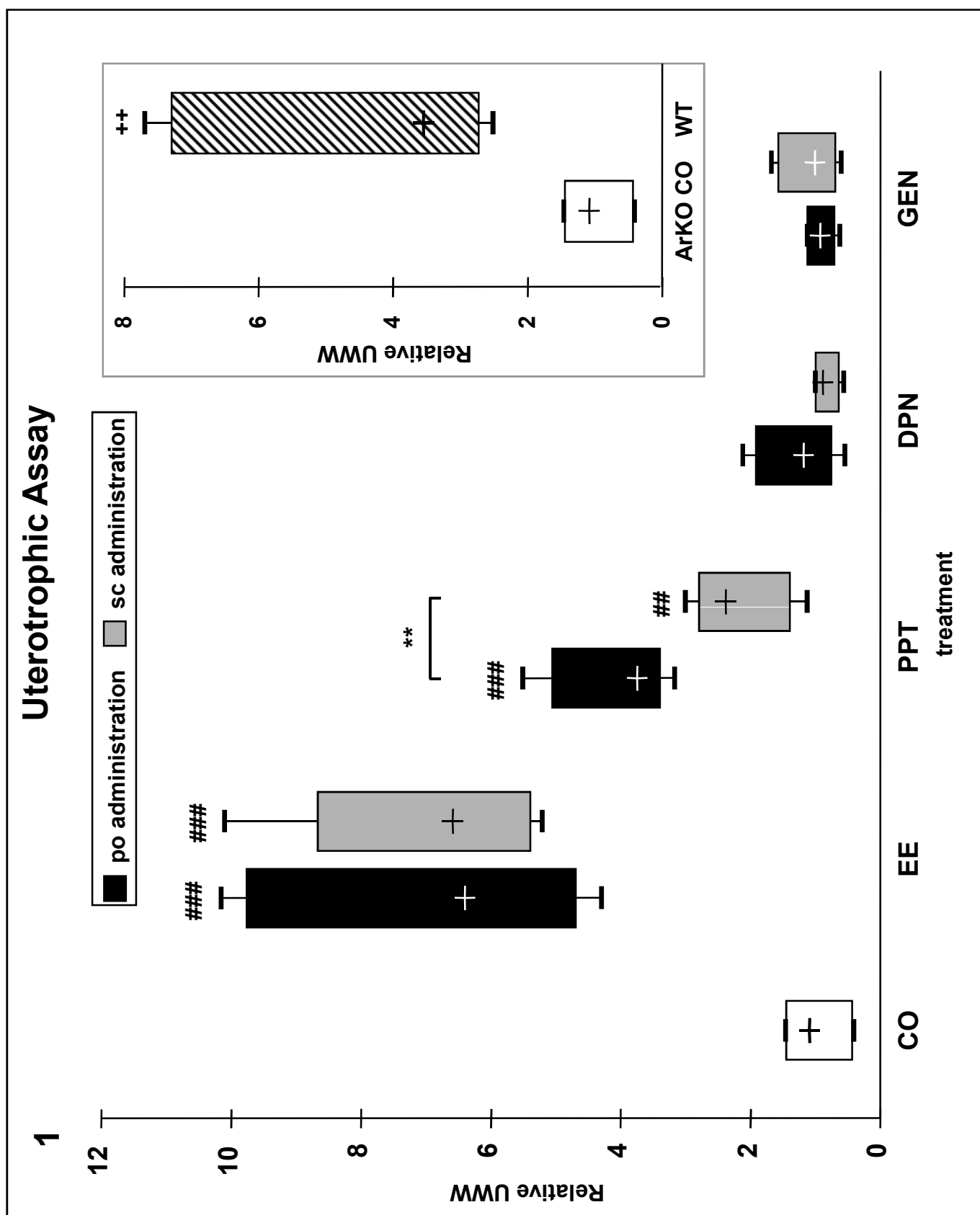


Figure 2

