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Spatiotemporal Expression Pattern of Gadd45g Signaling Pathway Components in the Mouse Uterus Following Hormonal Steroid Treatments

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Abstract

The uterus is a hormone-dependent organ under the control of estrogens, progestins and androgens. The actions of estradiol (E2) and progesterone on uterine physiology are well documented, while active androgens such as testosterone and dihydrotestosterone (DHT) are now also recognized to exert an important role to appropriately maintain the cyclical changes of the endometrium.

Based on our previous observations that DHT modulates GADD45g and the expression of specific cell cycle genes, the aim of this study was to identify the specific uterine cell types in which these cell cycle genes are modulated by hormonal steroids.

Using *in situ* hybridization and quantitative RT-PCR (QRT-PCR), the localization and measurement of mRNA expression of key cell cycle genes responding to E2 and DHT were performed in the uterus of ovariectomized mice. Interestingly, *in situ* hybridization experiments demonstrate that Gadd45g mRNA is detected early and strongly in stromal cells only, indicating that the early cell cycle arrest induced by DHT and E2 in the mouse uterus occurs in this compartment. On the other hand, the cell cycle stimulation represented by the late increase of Ccnb1, Cdc2a and Cdc25c gene expression is located exclusively in the glandular and luminal epithelial cells. QRT-PCR experiments confirm the regulation of mRNA expression observed following *in situ* hybridization. Surprisingly, both hormones appear to trigger effects in the same direction on cell cycle progression, with androgens inducing a lower modulation of gene expression levels. Additional experiments using the human uterine Ishikawa cell line confirmed the implication of the estrogen receptor in the GADD45g up-regulation following treatment with E2 while the regulation triggered by DHT is less clear. These observations illustrate clearly the stroma-epithelium interactions, which finely regulate the uterine physiology *via* paracrine mechanisms. Additional investigations are definitely needed to determine the exact role played by androgens in mouse uterine growth and differentiation.

Keywords: Uterus; Ovariectomized mice; Estrogens; Androgens; *in situ* hybridization; Gadd45g

Introduction

The uterus is a hormone-dependent organ under the control of estrogens, progestins and androgens. The actions of estradiol (E2) and progesterone on uterine physiology are well documented, while active androgens such as testosterone and dihydrotestosterone (DHT) are now also recognized to exert an important role to appropriately maintain the cyclical changes of the endometrium [1-6].

In both men and women, androgens are much more abundant than estrogens in terms of circulating steroids. In recent years, increasing evidence supports the use of androgens as hormone replacement therapy (HRT) in postmenopausal women to significantly improve their quality of life. Indeed, menopausal decline in androgens triggers side-effects influencing libido, vasomotor symptoms, mood, well-being, bone structure and muscle mass [7,8]. Moreover, the greater risk of breast cancer, deleterious effects on cardiovascular systems and other negative effects attributed to progestins [9,10] reinforces the use of a combination of estrogens and androgens as HRT in menopausal women [11-13]. In the mouse, while the estrogenic stimulation of proliferation of uterine epithelial and stromal cell growth and differentiation is well documented [14-20] the observation that DHT also exerts modulatory effects on these cells is known but not elucidated [1-4].

The signal sent by these hormones is transduced by their respective steroid receptors located in the different uterine compartments. Excluding some splicing variants, two major estrogen receptor (ER) forms, namely ERα and ERβ are expressed in both the epithelial and stromal cells of the rodent uterus [21-27]. Although ERβ is detected to a lower level and exclusively in the late phase of gestation, both forms are expressed throughout gestational development in a temporal-specific manner [5]. As for the androgen receptor (AR) it is expressed in both types of epithelial cells (glandular and luminal), in stromal and smooth muscle cells of the rat and mouse [27-29], and in the human uterus [30].

Few hypotheses have been suggested so far to explain the inhibitory action of androgens on the proliferative effect of estrogens, such as: 1) androgens can inhibit estrogenic effects by activating AR [31]; 2) the ligand-activated AR can interact with ERα and thus lowers its transcriptional activity [32] and; 3) androgens induce the down-regulation of ERα particularly in stromal and myometrial cells [33]. On the other hand it has been demonstrated that androgens can alter uterine weight and its morphology, and that AR induces an estrogen-like gene expression response which is required for growth and differentiation of stromal and epithelial cells [34]. Moreover,

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critical interactions between stromal and epithelial cells have been demonstrated to mediate the hormone effects on epithelial cells [35].

The major uterine cancer, referred to as endometrial cancer, first develops in the glandular cells of the lining. Later this cancer may spread in stromal cells and ultimately into myometrium and cervix. On the other hand sarcomas, representing about 5% of uterine cancers, arise in myometrial cells, grow rapidly and may also reach the endometrium [36].

In a previous study we have demonstrated a significant up-regulation of expression of Gadd45g and other key cell cycle genes (Rad51, Cdc2a, Ccnb1 and Cdc25c) following an androgenic treatment [4], and all these genes interact with each other in a common pathway. Gadd45g expression, like other Gadd45 family members (a and b), is induced in response to multiple environmental and physiological stress [37]. However, each member of the family likely has a unique function, Gadd45g being mainly induced as a primary response to IL-2 and IL-6 [38].

Despite the well-known inhibitory effect of DHT on estrogen-induced uterine epithelial cell proliferation, the mechanism by which a single DHT treatment exerts a residual estrogen-like action on cell proliferation is unknown. Therefore, based on our recent investigation demonstrating a stimulation of the cell cycle by DHT on mouse uterine cells [4], we undertook *in situ* and QRT-PCR experiments to assess the expression of key cell cycle components and discriminate their localization following estrogenic and androgenic treatments in the mouse uterus.

Materials and Methods

Animals and treatment

Ten to eleven week-old female C57BL/6 mice were received from Charles River (St-Constant, Canada) and were allowed to acclimate for 4 weeks. The animals were housed individually in an environmentally-controlled room (temperature: 22 ± 3°C; humidity: 50 ± 20%; 12-h light-12-h dark cycles, lights on at 07:15h). The mice had free access to tap water and a certified rodent feed (Lab Diet 5002 (pellet), Ralston Purina, St Louis, MO). The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals. Animals, weighing between 20 to 27g (mean 23.3g), were randomized according to their body weights and were assigned to 4 groups of 10 animals each as followed: 1) Gonadectomized (GDX) control; 2 to 4) GDX + DHT (0.1 mg/mouse) or GDX + E2 (0.05 µg/mouse). On day 1 of the study, animals were bilaterally ovariectomized under isoflurane anesthesia. Prior to the necropsy performed on day 8 of the study, mice received a single subcutaneous injection (0.2 ml/mouse) of the vehicle alone (5% ethanol-0.4% methylcellulose; group 1) or DHT/E2 (groups 2 to 4). The injection of vehicle was performed 24h prior to the necropsy for animals into group 1 while DHT/E2 was injected 3h (group 2), 12h (group 3) or 24h (group 4) prior to the necropsy. For RNA extraction, on day 8 of the study, 5 mice under isoflurane anesthesia were exsanguinated at the abdominal aorta followed by cervical dislocation. The uterus was collected and rapidly frozen in liquid nitrogen. For each group, the 5 uteri collected were pooled in the same tube. Tissues were kept at –80°C until RNA extraction. For *in situ* hybridization, 5 mice were anesthesized with ketamine/xylazine and perfused transcardially with 90 ml 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7·4). The uteri were excised and post-fixed in the same fixative for 24 h at 4°C. The tissues were placed in 15% sucrose in 0.1 M phosphate buffer before being quickly frozen in isopentane chilled in liquid nitrogen.

Quantitative Real –Time PCR (QRT-PCR)

Total RNA was purified using RNeasy MinElute Cleanup kit (QIAGEN, Valencia, CA) after a digestion step with DNase I (QIAGEN, Valencia, CA). The quality of RNA was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA). First-strand cDNA synthesis was accomplished using 5 µg of isolated RNA as reported previously [20]. The resulting products were purified with QIA quick PCR purification kits (QIAGEN, Valencia, CA). cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler Real-Time PCR apparatus (Roche Inc., Nutley, NJ). Reagents were obtained from the same company and used as described by the manufacturer. PCR amplification and reading of the fluorescence signal was performed as previously described [4,20,39]. Data calculation and normalization were performed using second derivative and double correction method as described previously [39], and using the housekeeping gene hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1). mRNA expression levels are expressed as number of copies/µg total RNA using a standard curve of Cp versus logarithm of the quantity. The standard curve was established using known cDNA amount of 10, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of Hprt1, and the Light Cycler 3.5 program provided by the manufacturer (Roche Inc.).

Experiments and data analysis related to the Ishikawa cell line were essentially performed as described above with the following modifications. Specific primers were designed to amplify a targeted portion of the human growth arrest and DNA-damage-inducible, gamma (GADD45g: NM_006705), alkaline phosphatase, placental-like 2 (ALPPL2: NM_031313) and androgen receptor (AR: NM_000044) transcripts. Five biological replicates (n=5) were performed for each sample and data analysis was carried out as previously described [39].

In situ hybridization

Frozen sections (10 µm thick) were serially cut and mounted onto gelatin- and poly-L-lysine-coated slides. Recombinant plasmid PCR® 2.1 (Invitrogen, Burlington, Ont. Canada) containing *Mus musculus* Gadd45g (485 bp, position 123-607 of NM_001817), Rad51 (821 bp, position 284-1104 of NM_011234), Ccnb1 (827 bp, position 428-1254 of NM_172301), Cdc2a (739 bp, position 207-945 of NM_007659.3) or Cdc25c (810 bp, position 674-1483 of NM_009860) fragments were used as DNA template for cRNA probe synthesis. These cDNA fragments were previously obtained by amplification using primer-specific polymerase chain reaction. *In situ* hybridization with antisense and sense ^{35}S-labeled cRNA probes was performed as previously described [40]. After hybridization, tissue sections were dehydrated and coated with liquid photographic emulsion (Kodak-NTB2; diluted 1:1 with water), and following 30–45 days of exposure, sections were processed and counterstained with haematoxylin.

Chemicals and cell culture experiments

E2 was obtained from Steraloids (Wilton, NH) while DHT was generously provided by Dr André Tchernof (Quebec, Canada). All media and supplements for cell culture were from Wisent Inc. (St-Bruno, QC), except for DMEM without phenol red, which was from Gibco (Burlington, ON). Fulvestrant and Casodex were purchased from Sigma Chemical Co (St. Louis, MO).

The human Ishikawa cell line derived from a well-differentiated endometrial adenocarcinoma [41] was kindly provided by Dr.

Chantal Guillemette (CHUQ Research Centre, Quebec, Canada). The Ishikawa cells were routinely maintained in DMEM containing 10% (v/v) FBS and supplemented with 100 units/ml penicillin, 100 µg/m1 streptomycin, 1% nonessential amino acids, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were plated in Falcon T75 flasks at a density of 1.5 X 10^6 cells at 37°C 5% CO2.

Twenty-four hours before the start of the experiment, Ishikawa cells were cultured to 60% confluency in a DMEM phenol red-free, all supplements mentioned above, and 0.25% FBS treated with dextran-coated charcoal to remove endogenous steroids. The day of the experiment, medium was replaced with fresh DMEM 0.25% FBS-charcoal containing the indicated concentrations of compounds (DHT: 1nM, Estradiol: 10nM, Fulvestrant: 1µM) in a final volume of 12 ml. Casodex at 1 µM was incubated with Ishikawa cells 1 h before the addition of androgens/estrogens wherever appropriate. It should be noted that doses of hormones or anti-hormones have been determined based on those commonly used in the literature. Cells were incubated at 37°C for 24 h, and half of the cells from a flask were homogenized in TRI Reagent solution (Ambion, Austin, TX) for RNA extraction.

Results

To further explore the mechanisms of the anti- and pro-proliferative effects of E2 and DHT on the mouse uterus, *in situ* hybridization and gene expression analyses (QRT-PCR) were performed on uterine tissue from the OVX mouse uterus treated either with E2 or DHT during 3h, 12h or 24h, using ^{35}S-labeled probes for Gadd45g (anti-proliferative effect) as well as for Rad51 (DNA repair gene allowing S-phase progression), Cdc2a, Ccnb1 and Cdc25c genes (pro-proliferative effect).

Regarding Gadd45g, a strong labeling was detected in the stromal cells of endometrial tissue, with a transient peak of signal intensity observed at a 3h time-interval with either E2 or DHT. Following treatment with E2, the labelling intensity declines at 12h (4-fold), and then decreases to the levels present in the control group at 24h (Figure 1). However, when DHT is administered, with the exception of the peak of expression at 3h, timepoints at 12h or 24 h do not reveal any significant increase in Gadd45g expression (Figure 2). DHT thus seems to exert a shorter effect on Gadd45g expression than E2. A weak staining is also observed in myometrial cells (data not shown), but no Gadd45g mRNA expression is observed in glandular and luminal epithelial cells for both hormones. The QRT-PCR experiments performed also support a peak of expression observed at 3h following both hormonal treatments. As illustrated in Figures 1 and 2, no significant autoradiographic reaction was observed following hybridization with the sense probe, which corresponds to the peak of expression, i.e. timepoint at 3h. Furthermore, as demonstrated by QRT-PCR experiments, E2 induces a much higher (30-fold) Gadd45g gene expression at 3h when compared to the control group, and over a longer time-interval (at 3h and 12h) than that observed following a DHT treatment (5-fold at 3h only).

Although non-significant, Rad51 expression showed a trend of up-regulation at 12h and 24h for both hormones (< 2-fold) (Figure 3A), and this expression is mainly detected in luminal and glandular epithelial cells (data not shown). Again Rad51 mRNA quantification is in agreement with the *in situ* hybridization experiments described above.

As for Ccnb1, Cdc2a and Cdc25c, they all interact with each other to control the G2/M phase transition. As observed for Rad51, *in situ* hybridization reveals that the increased expression of Ccnb1 (data not shown), Cdc2a (Figure 4 and Figure 5) and Cdc25c (data not shown) occur exclusively in the luminal and glandular epithelial cells (at 12h and/or 24h) while no significant change or no signal of mRNA expression is detected in stromal cells.

While Cdc2a mRNA expression increases gradually at 12h and 24h following a treatment with E2 (but to a lower level of induction with DHT) (Figures 4 and 5), the Cdc25c gene showed a clear induction of expression at 24h (E2: 3.2-fold), and also to a lower extent for DHT (2.4-fold) (Figure 3C). As for Ccnb1, E2 triggers a significant up-regulation of mRNA expression at 24h (2.3-fold), while only a modest increase is observed following an androgenic treatment (1.6-fold) (Figure 3B). Again, for all the genes tested involved in the promotion of the cell cycle (G2/M transition), hybridization with the radio labeled sense probe generated only a light, uniform background.

Specific histological changes are also triggered by both hormone treatments. As observed at the 12h and 24h time intervals, E2 administration induced an increase in the surface of endometrial components and muscle layer. The stroma cells appeared larger and less densely packed than those observed in untreated OVX animals (Figure 4). The epithelial cell height was also increased following E2

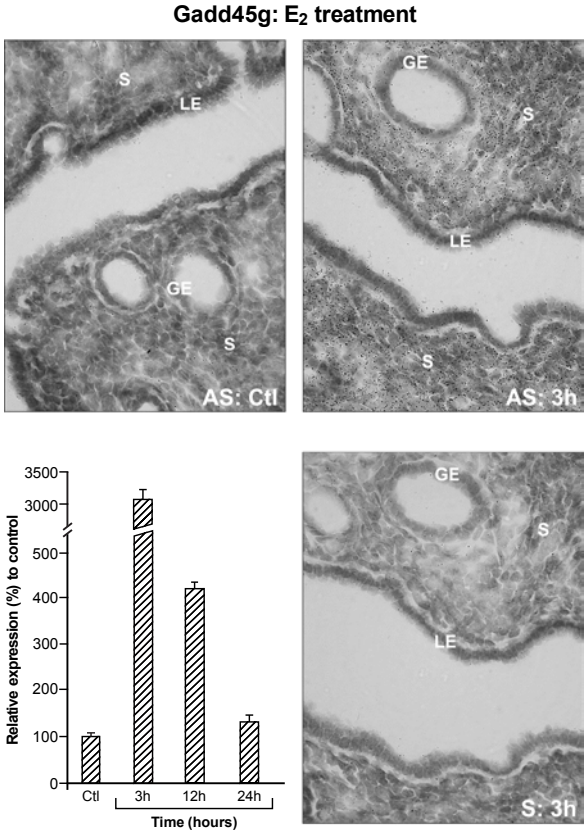

Figure 1: QRT-PCR and *In situ* hybridization showing cell type-specific expression of *Gadd45g* mRNA in the *Mus musculus* uterus following E2 treatment. The lower left panel displays the QRT-PCR mRNA measurements in the whole uterine tissue at 3h, 12h and 24h as well as in the control (Ctl: 24h vehicle-treated). Results are expressed in percent of the control (here defined as 100%) as mean and standard deviation (SD). The remaining panels display the mRNA labelling using the antisense probe of the section through the mouse uterus demonstrating the higher increase of *Gadd45g* expression as well as the corresponding control section (0h). AS: Antisense probe; S: Sense probe; LE: Luminal epithelial cells; GE: Glandular epithelial cells; S: Stromal cells. Using the sense probe, only a diffuse background is observed. Exposure, 30 days, × 600.

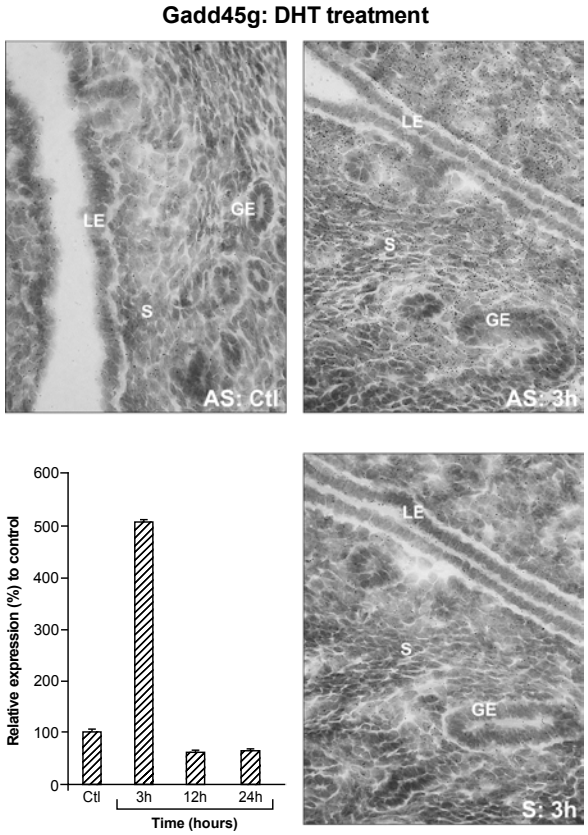

Figure 2: QRT-PCR and *In situ* hybridization showing cell type-specific expression of *Gadd45g* mRNA in the *Mus musculus* uterus following DHT treatment. The lower left panel displays the QRT-PCR mRNA measurements in the whole uterine tissue at 3h, 12h and 24h as well as in the control (Ctl: 24h vehicle-treated). Results are expressed in percent of the control (here defined as 100%) as mean and standard deviation (SD). The remaining panels display the mRNA labelling using the antisense probe of the section through the mouse uterus demonstrating the higher increase of *Gadd45g* expression as well as the corresponding control section (0h). AS: Antisense probe; S: Sense probe; LE: Luminal epithelial cells; GE: Glandular epithelial cells; S: Stromal cells. Using the sense probe, only a diffuse background is observed. Exposure, 30 days, × 600.

treatment (Figure 4). Similar changes, although less striking was also observed in DHT-treated animals (Figure 5).

Taken together, our results indicate that for the genes studied, both hormones induce effects in the same direction on cell cycle components expression, although estrogens (E2) exert a much stronger stimulation than androgens (DHT). Regarding the localization of the mRNA expression of these genes, both hormones seem to induce the expression in the same uterine compartments.

To establish whether AR and/or ER mediate the action of estrogens/androgens on GADD45g up-regulation, the ideal model would have been a mouse uterine cell line. However, to our knowledge no such cell line is available; therefore we used the human Ishikawa cell line which is routinely cultured in our laboratory to discriminate the role of both receptors in this regulation. As illustrated in Figure 6A, GADD45g mRNA expression is significantly increased following E2 treatment and this up-regulation is blocked in presence of the anti-estrogen fulvestrant demonstrating the direct implication of ER binding. On the other hand, DHT did not trigger any significant modulation of GADD45g expression. Panels B and C illustrate the regulation of estrogen- and androgen-responsive genes following androgen and estrogen treatments. As demonstrated, the expression of the estrogen-responsive gene ALPPL2 is strongly increased by E2 and this up-regulation is strongly but not completely inhibited in the presence of fulvestrant, while the selected androgen-responsive gene AR is significantly increased by E2 only. Given that GADD45g mRNA up-regulation could be linked to an increase of proliferation, we measured the mRNA levels of the proliferation gene PCNA. No significant change of mRNA regulation was observed following the estrogenic or androgenic treatment in Ishikawa cells (data not shown).

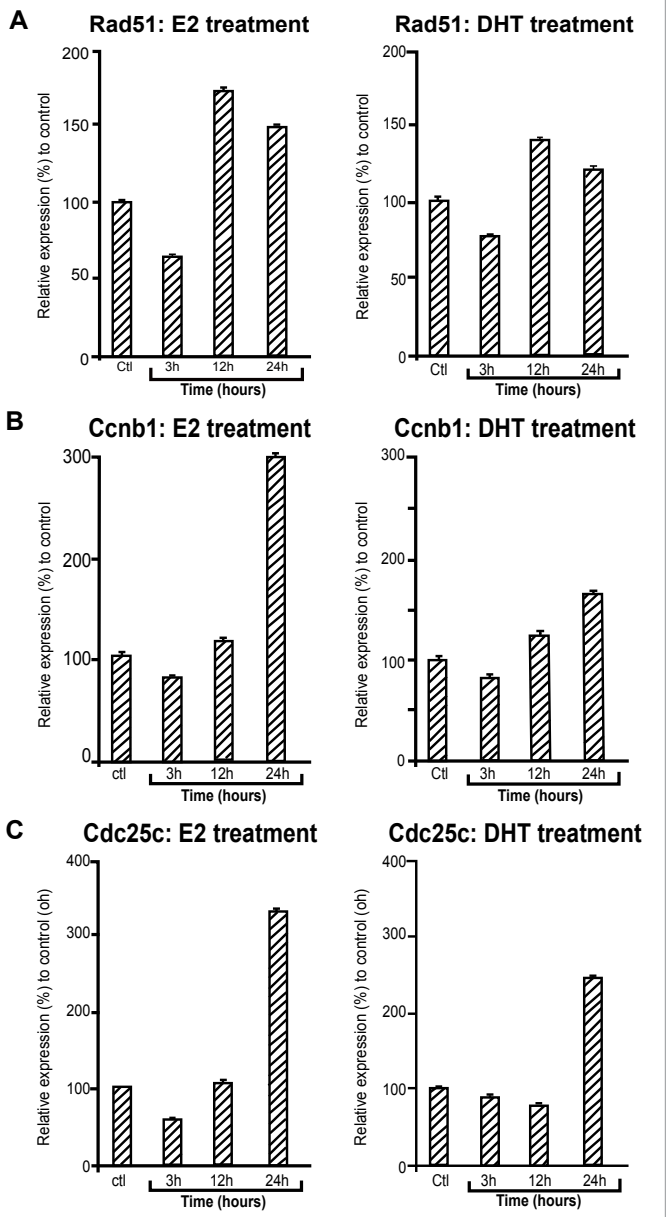

Figure 3: QRT-PCR showing cell-type specific expression of *Rad51* (panel A), *Ccnb1* (panel B) and *Cdc25c* (panel C) mRNA in the *Mus musculus* uterus following E2 (left panel) and DHT (right panel) treatments. The panels display the QRT-PCR mRNA measurements in the whole uterine tissue at 3h, 12h and 24h as well as in the control (Ctl: 24h vehicle-treated). Results are expressed in percent of the control (here defined as 100%) as mean and standard deviation (SD).

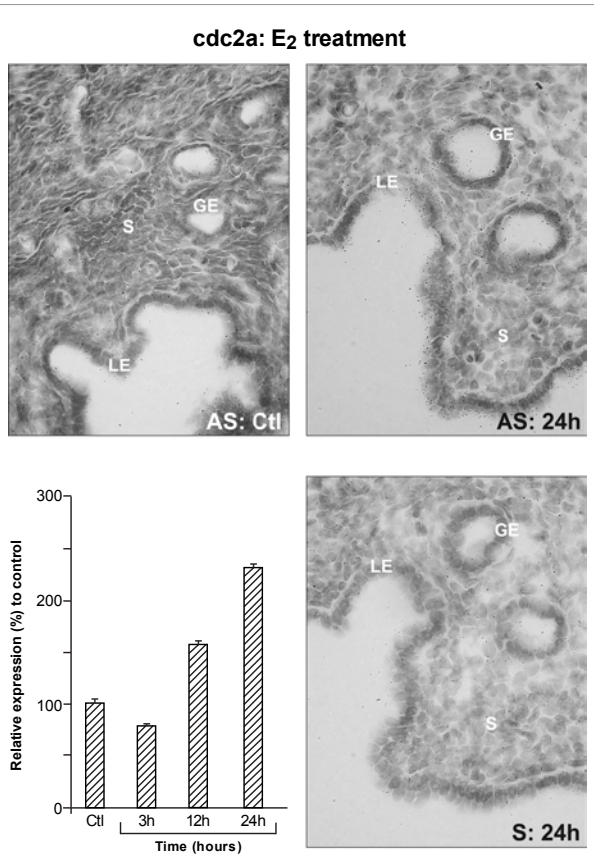

Figure 4: QRT-PCR and *In situ* hybridization showing cell type-specific expression of *Cdc2a* mRNA in the *Mus musculus* uterus following E2 treatment. The lower left panel displays the QRT-PCR mRNA measurements in the whole uterine tissue at 3h, 12h and 24h as well as in the control (Ctl: 24h vehicle-treated). Results are expressed in percent of the control (here defined as 100%) as mean and standard deviation (SD). The remaining panels display the mRNA labelling using the antisense probe of the section through the mouse uterus demonstrating the higher increase of *Cdc2a* expression as well as the corresponding control section (0h). AS: Antisense probe; S: Sense probe; LE: Luminal epithelial cells; GE: Glandular epithelial cells; S: Stromal cells. Using the sense probe, only a diffuse background is observed. Exposure, 30 days, × 600.

Discussion

Based on our previous microarray hybridization analyses indicating that androgens could stimulate the cell cycle progression to a certain level, here using QRT-PCR and *in situ* hybridization, we undertook a study on the localization as well as the quantification of key cell cycle components mRNA expression in mouse uterine tissue following androgenic (DHT) or estrogenic (E2) treatment.

The importance of the stromal-epithelial interactions has been well demonstrated using the tissue recombinant system, which allowed determining the role of steroid receptors in each tissue compartment. For example, it has been demonstrated that androgens cause embryonic mammary epithelial regression via the AR-positive mesenchyme, which in response to androgens is stimulated to condense around the epithelium, thus triggering epithelial regression [42]. Indeed, the effects of these hormones on female reproductive tract epithelium are mediated totally or in part through the corresponding receptors in stromal cells [43,44].

As observed following an estradiol treatment, androgens administration leads to a significant increase in endometrial and myometrial cross-sections area, which represents a clear trophic effect after 24h [3]. However, it has been demonstrated that E2 and DHT trigger distinct effects on the thickness and morphology of epithelial cells layer as well as on the edematous appearance of stromal cells [3]. Both hormones, particularly E2 after 4h, provoke a major influx of fluid into the uterus, and this increased vascular permeability is in part due to an increased mRNA expression of the vascular endothelial growth factor (VEGF) [18]. Indeed, in our previous studies VEGF mRNA expression was up-regulated by 6.7-fold and 3-fold at 3h following E2 or DHT administration, respectively [4,20], which supports its involvement in the regulation of vascular permeability.

In this study, DHT modulates cell cycle gene expression, although to a lower extent than with E2, and this expression occurs in the same compartments than those observed following E2 administration. Following either treatment with DHT or E2, the proliferative effect, represented by the induction of Ccnb1, Cdc2a and Cdc25c gene expression, is observed in the late phase of treatment (12h and 24h) in both glandular and luminal epithelial cells. This seems to correlate with the release of Gadd45g repression. Indeed, given that the Gadd45g expression increases dramatically during the early period of

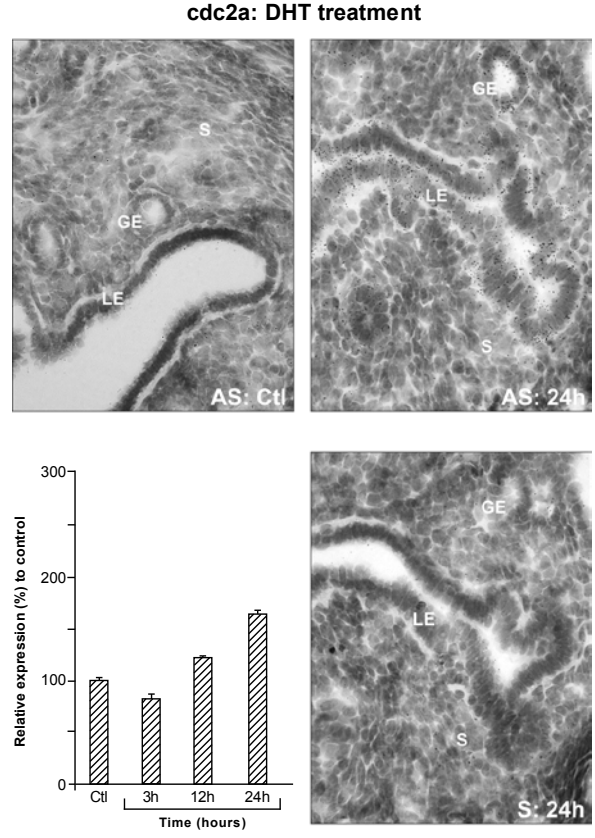

Figure 5: QRT-PCR and *In situ* hybridization showing cell type-specific expression of *Cdc2a* mRNA in the *Mus musculus* uterus following DHT treatment. The lower left panel displays the QRT-PCR mRNA measurements in the whole uterine tissue at 3h, 12h and 24h as well as in the control (Ctl: 24h vehicle-treated). Results are expressed in percent of the control (here defined as 100%) as mean and standard deviation (SD). The remaining panels display the mRNA labelling using the antisense probe of the section through the mouse uterus demonstrating the higher increase of *Cdc2a* expression as well as the corresponding control section (0h). AS: Antisense probe; S: Sense probe; LE: Luminal epithelial cells; GE: Glandular epithelial cells; S: Stromal cells. Using the sense probe, only a diffuse background is observed. Exposure, 30 days, × 600.

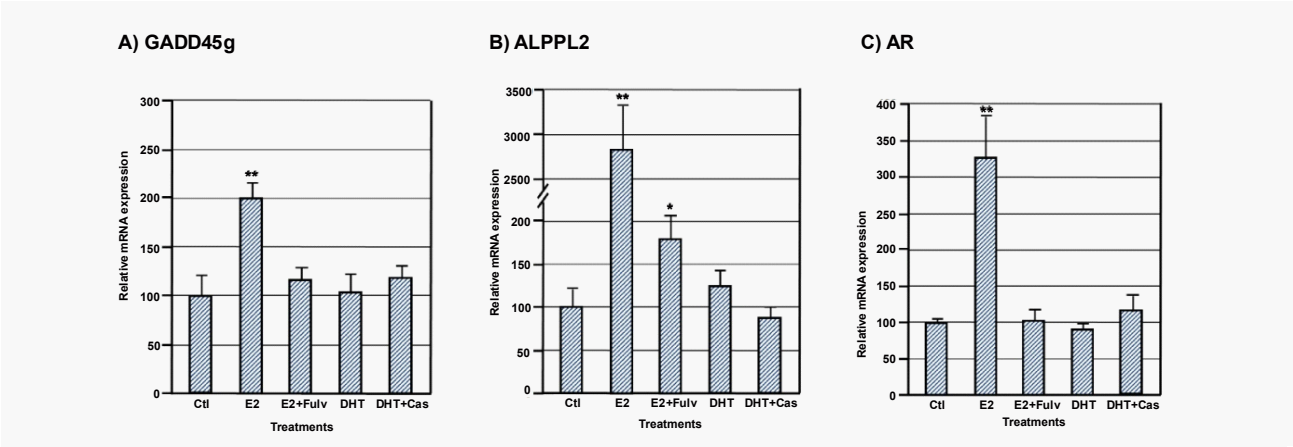

Figure 6: QRT-PCR showing expression of human GADD45g (Panel A), ALPPL2 (Panel B) and AR (Panel C) mRNA levels in the Ishikawa cell line following hormone treatments. Results are expressed in percent of the untreated control (here defined as 100%) as mean ± SEM. The asterisk indicates significant difference compared with the control (**P<0.01, *P<0.05).

hormonal treatment, and exclusively in stromal cells, this illustrates the importance of the stromal-epithelial compartment interactions, which regulate uterine cell growth and differentiation [35].

The Gadd45g gene product is known to play pivotal roles in G2/M arrest and induction of apoptosis signaling through the dissociation of the cyclinB/Cdc2 complex [45,46]. As both ER and AR are expressed in stromal cells, and given that the early induction of Gadd45g expression (3h) occurred exclusively in this compartment, this suggests a direct receptor-mediated action of both hormones leading to cell cycle arrest. However, previous observations have shown that Gadd45g is an up-regulated androgen-responsive gene with growth inhibitory activity in human prostate cancer cells, showing a rapid and transient dose-dependent stimulation followed by a decreased expression after 48h [47]. Moreover, this study supports that this up-regulation is sensitive to protein synthesis inhibition, suggesting an indirect effect of androgens on Gadd45g promoter. Indeed, E2 and DHT induce a rapid and transient expression of Gadd45 in the breast cancer cell line MCF7 [48] and in human prostate cancer cells [47], respectively, which is in agreement with the results described here.

It has also been suggested that an increased expression of the Gadd45 gene might not be directly linked to hormonal stimulation, but rather be caused by DNA damage induced by the hormone-treatment regimen [49]. In addition, Gadd45g expression has been shown to be induced by DNA damage in both a p53-dependent [50] and -independent [4,51] manner, and up-regulation of this gene could also be modulated by the oxidative stress [52]. Moreover, as it is the case for the expression of the p27 gene which is induced in stromal cells by E2 and TGFβ through paracrine mechanisms, Gadd45g gene expression is also induced by TGFβ, and both gene activation leads to cell cycle arrest, either at G1/S (p27) or G2/M phase (Gadd45g) [53-56]. Therefore one could speculate that the inhibition of cell cycle in both epithelial and glandular cells may be regulated in part via the stromal cells compartment.

Most studies regarding the cell cycle regulation by hormonal steroids mainly investigated genes involved in the G1 phase transition, namely Cyclin D and E as well as CDK inhibitors such as p21 and p27 [53,57]. On the other hand, the G2/M phase progression involves the activity of several cell-cycle regulatory proteins, such as Cyclin B1, Cdc2, Wee1, Cdc25C, and others [58-61]. Indeed, Ccnb1 expression would be stimulated as a consequence of DNA synthesis in rat uterine luminal epithelium rather than a direct response to estradiol treatment [62]. Although not statistically significant, an up-regulation of Ccnb1 gene expression has been reported in endometrial hyperplasia and endometrial cancer [63-66]. Indeed, G2 pathway alterations seem to be a moderately frequent event in endometrial cancer, and are thought to occur during carcinogenesis of the endometrium [64].

Despite the similar regulation and localization of key cell cycle regulators (such as Rad51, Gadd45g, Cdc2a, Cdc25c and Ccnb1) induced by both hormones, the results obtained in this study suggest that they are unlikely the major factors responsible for the residual estrogen-like effect observed on uterine cell proliferation triggered by an androgen treatment. It is likely that DHT regulates the trophic environment and architecture of the rodent uterus through a gene expression program, which is overlapping but distinct from the estrogen response [3]. However, the non-aromatizable androgenic hormone DHT can also be metabolized by 3β-hydroxysteroid dehydrogenase to 3βAdiol which has been shown to bind, although with a much lower affinity than E2, both ERα and ERβ [67]. During profound estrogen deprivation, 3βAdiol has been demonstrated to be estrogenic in the breast and reproductive tissues [68]. Indeed, 3βAdiol can induce the proliferation of breast cancer cells through direct activation of ERα [69]. Moreover, it has been reported that breast cancer cells can become hypersensitive to low concentrations of estrogens (3βAdiol) after long-term estrogen deprivation [70,71]. Thus, the androgen 3βAdiol resulting from DHT conversion could be involved in this residual estrogen-like effect observed in uterine tissues following a single DHT administration in OVX mice.

It was also of interest to determine the individual role of AR and ER in the regulation of GADD45g gene expression. Although the androgenic regulation in rodent and human endometrial cells is known to be different, we treated the only uterine cell line available, namely the Ishikawa cells with steroids and anti-hormones. Indeed, the androgens exert an uterotrophic action in mouse uterus [2-4,70,73] while in human endometrium, androgens are likely to be involved in the differentiation of human endometrial stromal cells into decidual cells as well as in the regulation of endometrial function pertaining to implantation and pregnancy [73]. As described in Figure 6, ER is likely to be involved in this regulation. However we could not confirm

the implication of AR, given the absence of significant up-regulation following DHT treatment. Indeed, there is no well-established androgen-responsive gene which has been reported in the literature to be significantly modulated by androgens in human uterus. Apparao et al. [74] described the up-regulation of AR proteins in Ishikawa cells following an androgenic treatment of 3-4 days , and Lovely's group reported a modest positive effect on AR binding activity and expression by Northern blot analysis [75]. However, in the current study we treated Ishikawa cells for 24h given that the increase of our gene of interest, GADD45g, was observed after a treatment of 3 hours in the mouse uterus, which could explain the absence of modulation of AR following DHT treatment. On the other hand, it is well known that estrogens up-regulate AR mRNA and protein expression in the well-differentiated human endometrial adenocarcinoma Ishikawa cell line [76,77]. Therefore, while we can confirm the involvement of ER in the up-regulation of GADD45g mRNA expression, we cannot exclude the participation of AR on the up-regulation observed previously in mouse uterus based exclusively on the results described above in human endometrial cells. In addition as described previously, the increase of GADD45g mRNA levels in Ishikawa cells following an estrogenic treatment of 24h could not be explained by cell proliferation given the absence of significant mRNA regulation of the proliferation gene PCNA. The results described above illustrate clearly that the stroma-epithelium interactions seem to finely regulate the uterine physiology *via* paracrine mechanisms. Other investigations are however definitely needed to identify additional players involved in the opposite actions (anti- and proliferative) induced by DHT in the presence and/or absence of estrogens, which could then further explain the exact role of androgens in mouse uterine growth and differentiation.

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