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Difference in signalling between various hormone therapies in endometrium, myometrium and upper part of the vagina

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BACKGROUND: Combined hormone treatments in post-menopausal women have different clinical responses on uterus and vagina; therefore, we investigated differences in steroid signalling between various hormone therapies in these tissues. METHODS: A total of 30 post-menopausal women scheduled for hysterectomy were distributed into four subgroups: control-group ($n = 9$), Tibolone-group ($n = 8$); estradiol (E_2)-group ($n = 7$); E_2 + medroxyprogesterone acetate (MPA)-group ($n = 6$). Medication was administered orally every day for 21 days prior to removal of uterus and upper part of the vagina. Tissue RNA was isolated, and gene expression profiles were generated using GeneChip technology and analysed by cluster analysis and significance analysis of microarrays. Apoptosis and cell proliferation assays, as well as immunohistochemistry for hormone receptors were performed. RESULTS: 21-days of treatment with E_2, E_2 + MPA or tibolone imposes clear differential gene expression profiles on endometrium and myometrium. Treatment with E_2 only results in the most pronounced effect on gene expression (up to 1493 genes differentially expressed), proliferation and apoptosis. Tibolone, potentially metabolized to both estrogenic and progestagenic metabolites, shows some resemblance to E_2 signalling in the endometrium and, in contrast, shows significant resemblance to E_2 + MPA signalling in the myometrium. In the vagina the situation is entirely different; all three hormonal treatments result in regulation of a small number (4–73) of genes, in comparison to signalling in endometrium and myometrium. CONCLUSION: Endometrium and myometrium differentially respond to the hormone therapies and use completely different sets of genes to regulate similar biological processes, while in this experiment the upper part of the vagina is hardly hormone responsive.

Keywords: endometrium; myometrium; vagina; estradiol tibolone; gene expression

Introduction

The uterus and the vagina originate from the same cotyledon: the paramesonephric ducts (Mullerian ducts), which develop into fallopian tubes, uterus and upper vagina (Yin and Ma, 2005). The uterus consists of a thick wall of smooth muscle (myometrium) and an internal stroma-epithelial lining (endometrium). Both functional layers of the uterus express estrogen and progesterone receptors (ER and PR) after birth, but steroid signalling has only been investigated in detail in the endometrium during the menstrual cycle (Ponnampalam *et al*., 2004; Mirkin *et al*., 2005; Punyadeera *et al*., 2005). For myometrium, molecular analyses mostly describe changes in gene expression that occur during pregnancy and labour (Esplin *et al*., 2005; Otun *et al*., 2005) or during development or treatment of leiomyoma (Levens *et al*., 2005; Mangioni *et al*., 2005). The vagina, known to respond to estrogen treatment (ET), expresses ER and PR, and gene regulation has been studied, though not very extensively, during development (Miyagawa *et al*., 2004; Yin and Ma, 2005; Masui *et al*., 2006) and during menopause when the vagina becomes atrophic and when local treatment with estriol (Seidlova-Wuttke *et al*., 2006), or oral hormone treatment is applied (Sagsoz *et al*., 2005).

During the first part of the menstrual cycle (follicular phase), estrogen signalling is responsible for build-up of the endometrial layer. Subsequently, progesterone levels increase during the second half of the menstrual cycle (luteal phase) and induce differentiation of the endometrium and thus inhibit estrogen-induced proliferation (Talbi *et al*., 2006). A decline in progesterone levels at the end of the luteal phase (progesterone withdrawal) results in menstrual shedding.

After the fertile age, when the ovaries cease to synthesize estrogens and progestagens, the uterus involutes and the vagina becomes atrophic. In addition, other positive effects of estrogens are declining such as temperature control and bone homeostasis. ET may solve these problems, but prolonged or unopposed estrogen signalling is not a favourable situation because it has been associated with an increased incidence of hyperplasia and endometrial cancer (Horwitz and Feinstein, 1986). Therefore, when ET is used for the treatment of symptoms associated with menopause and osteoporosis, in women who still have their uterus intact, estrogen signalling is counteracted by the addition of a progestagenic compound.

Combined hormone treatments in post-menopausal women have quite different clinical responses on uterus and vagina. The endometrium remains largely atrophic and the vagina is stimulated. This indicates that the uterus and vagina, containing both ER and PR, must have differences in their signalling pathways. In order to elucidate this, we have compared the effects of estradiol (E_2) treatment alone with those of E_2 + medroxyprogesterone acetate (E_2 + MPA) and tibolone treatment on signalling pathways in the endometrium, myometrium and vagina. Tibolone, an alternative for classical combined hormone treatment has, besides estrogenic and progestagenic properties, additional estrogen inactivating mechanisms in the endometrium (Morris *et al*., 1999), thus preventing stimulation of the endometrium. In the vagina, tibolone stimulates the maturation index similar to ET (Morris *et al*., 1999).

Materials and Methods

Patient treatments

In total, 30 healthy post-menopausal women who visited our clinics for treatment of endometrial prolapse, were included in this study. 'Post-menopausal' was defined as amenorrheic $\geq$1 year before screening, or amenorrheic $\geq$6 months before screening with a serum E_2 concentration of $\leq$20 pg/ml and a serum FSH concentration of $\geq$40 IU/l at screening. This study was designed as a controlled clinical trial. The trial was registered with the National Institutes of Health, registry number NCT00294463, at: http://www.clinicaltrials.gov, under the name of: effects of Tibolone Treatment on the Endometrium. Patients were excluded in case of histologic diagnosis of the endometrial biopsy as proliferative, secretory or menstrual type endometrium, endometrial metaplasia, endometrial or endocervical polyp(s), endometrial hyperplasia, cancer or any other histological abnormality. Double-layer endometrium thickness $>$4 mm measured by transvaginal ultrasound was also an exclusion criterion. A full description of the inclusion and exclusion criteria (Klaassens *et al*., 2006) and the histological (Klaassens *et al*., 2006), biochemical (Verheul *et al*., 2007) and molecular findings (Hanifi-Moghaddam *et al*., 2007) in the endometrium were reported previously. The study groups were: control-group (nine subjects, no hormonal treatment), Tibolone-group (eight subjects, 2.5 mg of tibolone administered orally every day, starting 21 days prior to surgery); E_2-group (nine subjects, 2 mg E_2 administered orally every day, starting 21 days prior to surgery); E_2 + MPA-group (six subjects, 2 mg E_2 + 5 mg MPA administered orally every day, starting 21 days prior to surgery). Investigations were conducted in accordance with the guidelines in The Declaration of Helsinki, and the local ethics committees of the participating hospitals approved the study protocol. Informed consent was obtained from all participants in the study.

Isolation of tissue, RNA isolation and gene expression profiling

For the endometrium, this methodology has been described (Hanifi-Moghaddam *et al*., 2007). In short, after hysterectomy, part of the uterus and the upper part of the vagina were dissected and tissue was snap-frozen. Cryostat sections (5 μm) of 100% pure endometrial and myometrial tissue, or vaginal submucosal/mucosa tissue, were homogenized by sonography and RNA was isolated using Trizol (Invitrogen, Life Technologies, Philadelphia, PA, USA). Purity of the tissues in the cryostat sections was assessed by microscopic evaluation of each first and last section in batches of ten. This stringent quality control has resulted in the loss of some tissues for further analysis (the exact tissues used in the current analysis are indicated at the top of Fig. 1). About 1 μg of total RNA was used to prepare antisense biotinylated RNA (www.affymetrix.com). The level and quality of complementary RNA (cRNA) was measured on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). The cRNA was fragmented with the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). Hybridization to Affymetrix U133plus2 GeneChips (54614 probe sets, representing ~47 000 transcripts), staining, washing and scanning procedures were carried out as described (Affymetrix).

Data analysis

Data were normalized according to the quantile method. After normalization, the intensity values below 30 were set at 30, since our method reliably identifies samples with an average intensity value of 30 or more but does not reliably discriminate values between 0 and 30.

Cluster analysis

For each probe set, the geometrical mean of the hybridization intensities to that probe set in all endometrial, myometrial and vaginal samples was calculated. The level of expression of each probe set in every sample was determined relative to this geometrical mean and logarithmically transformed (on a base 2 scale). Genes whose level of expression differed at least 3-fold, in at least one sample, from the geometrical means of all treated endometrial, myometrial and vaginal samples (reflecting up- or down-regulation) were selected for the initial unsupervised cluster analysis using the visualization tool of Omniviz (Ominviz, Maynard, MA, USA) (version 3.8).

SAM analysis

We used significance analysis of microarrays (SAM) (Tusher *et al*., 2001), implemented in Omniviz to determine genes with significantly differential expression between the treatment groups for all three tissues. SAM is a statistical method for identifying differentially expressed genes while controlling the overall false discovery rate (FDR). FDR is the percentage of genes identified by chance. We have chosen the settings for this analysis in such a way that the total number of falsely identified differential expressed genes for each tissue was $\leq$1.

Functional and biological classification of genes

All significantly differentially regulated genes were classified in predefined biological function-categories. The complete GO-classification data on the regulated genes in endometrium and myometrium can be reviewed at: http://www2.eur.nl/fgg/rede/hanifi_moghaddam. After reviewing this distribution of regulated genes, three significantly regulated biological functions were selected for a more detailed analysis: cell death, cell cycle and signal transduction.

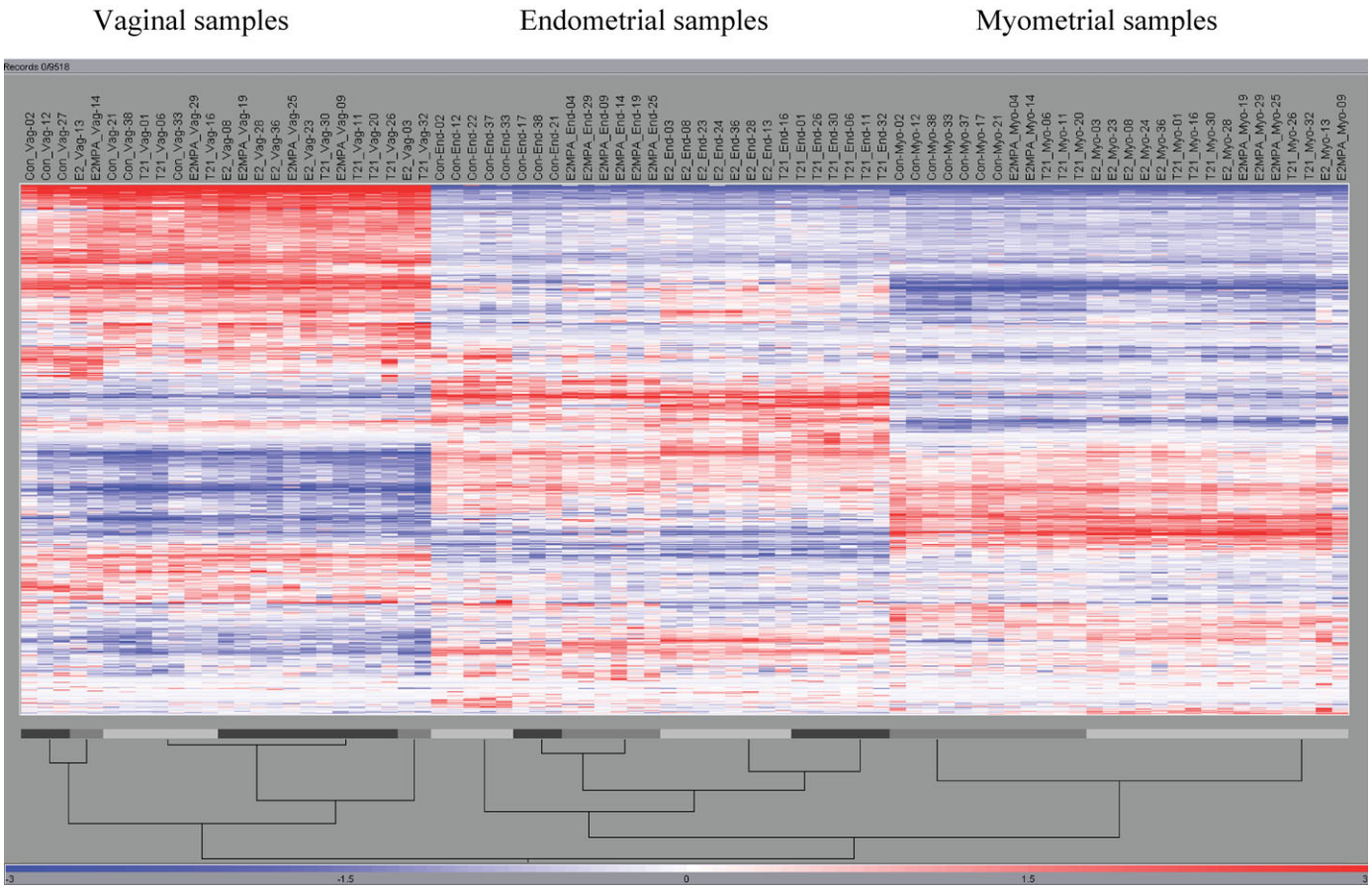

Figure 1: Unsupervised cluster analysis of differentially expressed genes between endometrial (End), myometrial (Myo) and vaginal (Vag) gene expression profiles

Those genes whose level of expression differed at least 3-fold, in at least one sample, from the geometric means of all treated endometrial, myometrial and vaginal samples (reflecting up- or down-regulation) were selected for this cluster analysis. In total, 9519 probesets, representing ~6000 genes, were used. Unsupervised clustering was performed using the visualization tool of Omniviz (version 3.8). At the top of the figure the treatments [control (Con), E_2 treatment alone, E_2 + MPA and T21 (tibolone)] and tissues followed by the individual patient numbers are indicated. At the bottom of the figure the main clusters and subclusters are indicated. Red indicates genes with a higher expression relative to the geometrical means; blue indicates genes with a lower expression relative to the geometric means

Real-time quantitative PCR of vaginal parameters

Real-time quantitative PCR (qPCR) (Deng *et al*., 2003) was performed on two selected vaginal genes: kRT2A and GREB1. The sequences of primers and probes used in this study are available upon request.

Immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay

Ki67 (marker of proliferation), ERα, PR (A and B) and androgen receptor (AR) staining was performed on 3 μm uterus tissue sections as described by Klaassens *et al*. (2006). Glycogen production was measured after Periodic Acid Schiff staining (Claver *et al*., 1984). Apoptosis was assessed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) was performed as described by de Vries *et al*. (2005) and adapted by us. Briefly, 3 μm paraffin embedded tissues sections were deparaffinized in xylene, rehydrated and washed in phosphate-buffered saline (PBS). The slides were heated in a microwave oven (two times 4 min at 700 W) in 0.01 M citrate buffer pH 6.0. After cooling and rinsing in milliQ water, endogenous peroxidases were quenched by a 5-min incubation in 2% H_2O_2 in PBS. Slides were then preincubated for 5 min in TdT-buffer (Promega, Madison, WI, USA) at 37°C and incubated for 1 h at 37°C in TdT-buffer containing 0.01 mM Biotin-16-dUTP (Boehringer Mannheim) and 0.3 U/μl TdT-enzyme (Promega). Slides were incubated in stop/wash buffer (Promega) for 15 min, followed by StreptABComplex (DAKO, Glostrup, Denmark), according to the manufacturer's protocol. Staining was developed with diaminobenzidine/concentrated metal complex (Pierce, Rockford, IL, USA) and counterstaining was carried out with haematoxylin. Evaluation of the Ki67 and apoptosis staining was performed with a computer-assisted imaging system Image J 1.32j (Wayne Rasband, National Institutes of Health, USA). Any nuclear staining regardless of intensity was considered positive. Positive nuclei were counted in one field of vision at ×10 magnification. Scoring was defined as the total number of positive nuclear cells per 1 mm^2 of epithelium, stroma or myometrium. Statistical analysis was performed using the Student's *t*-test (two-sided). $P < 0.05$ was considered to be statistically significant. Results are expressed as mean ± SEM.

Results

Earlier work performed by our group describes the histological, molecular and biochemical response of the endometrium to 3-week tibolone, E_2 or E_2 + MPA treatment compared with non-treated controls (Klaassens *et al*., 2006; Hanifi-Moghaddam

et al., 2007; Verheul *et al*., 2007) (hormone levels of individual patients are posted at: http://www2.eur.nl/fgg/rede/hanifi_moghaddam). Here, the endometrial gene expression data (Hanifi-Moghaddam *et al*., 2007) are reanalysed together with array data obtained from myometrial and (upper) vaginal tissues from the same patients. All raw array data were normalized together and SAM analysis was performed to find significantly differentially regulated genes.

Unsupervised cluster analysis

In total, 9519 probe sets (representing ~6000 different known genes) were identified that displayed at least a 3-fold change in expression in one or more patient samples relative to the geometrical means of all samples. Because of large differences between the tissues, this methodology primarily resulted in the identification of genes that were differentially regulated between endometrium, myometrium and vagina. Not surprisingly, cluster analysis revealed the formation of three main clusters: a vaginal sample cluster, an endometrial sample cluster and a myometrial sample cluster (Fig. 1).

Upon reviewing these three clusters more carefully, the endometrial cluster separated in three subclusters: one containing endometrial profiles only from control patients, another containing endometrial profiles from control and E_2 + MPA treated patients, and a third containing endometrial profiles from tibolone and E_2 treated patients. These results were in accordance with our earlier findings (Hanifi-Moghaddam *et al*., 2007).

Within the myometrial cluster there is a subdivision in two subclusters. There is a subcluster containing myometrial profiles from all control patients together with three tibolone and two E_2 + MPA treated patients, whereas the other subcluster contains all profiles from E_2 treated patients and the remainder of the profiles from tibolone and E_2 + MPA treated patients.

For the vaginal profiles subclusters can also be recognized. These subclusters, however, do not separate on the basis of different treatments, but presumably on the basis of other, unknown differences between patients, indicating that 21-day treatment with E_2, E_2 + MPA and tibolone does not have a specific impact on vaginal gene expression.

SAM analysis

SAM assigns a gene as significantly differentially expressed if the average expression of that gene in a treatment group significantly differs from another treatment group or from a control group (these data can be reviewed at: http://www2.eur.nl/fgg/rede/hanifi_moghaddam). In Table I, SAM analyses are summarized showing the number of significantly differentially expressed genes between the treatments or between treatments and controls. A number of interesting observations need to be mentioned.

First, both in the endometrium and in the myometrium E_2 only treatment has the highest impact on the gene expression profiles, whereas very few genes (16) are regulated in the vagina by E_2.

Secondly, when we compare tibolone signalling to E_2 + MPA signalling in the endometrium, 326 genes are found to be differentially regulated between these two treatment groups. This indicates a significant difference in signalling in the endometrium between these two hormone therapies. However, when we perform the same comparison in the myometrium, only four genes are found to be differently regulated, indicating that tibolone signalling is remarkably similar to E_2 + MPA signalling in the myometrium.

Third, treatment-induced signalling in vaginal tissue is very limited compared with signalling in the endometrium and myometrium, indicating that the upper part of the vagina, in this relatively short-term experiment, is not very hormone sensitive.

Biological functions in the vagina

To verify that the upper vagina, despite its reduced responsiveness, could potentially respond to the hormonal treatments, we have evaluated the presence of ERα, PR (A and B) and AR and compared the results with those in the endometrium and myometrium. In vagina, clear staining of both PR and ERα was observed; AR staining, however, was less clear (Fig. 2). In the endometrium, staining of ERα and PR was very obvious (as shown previously (Klaassens *et al*., 2006)) and is in line with the estrogen and progesterone responsiveness of this tissue. The myometrium also contains considerable amounts of ER and the PR. The AR expression in the endometrium and myometrium, however, is low.

The presence of both ER and PR in the upper vagina is not in line with the low gene signalling in the vagina after the hormonal treatments. Therefore, we have evaluated the vaginal tissues for glycogen production and epithelial thickness. Glycogen production was not enhanced by any of the treatments and vagina-epithelial thickness was also not changed by the 21-day treatments (data not shown). Furthermore, real-time

Table I. Number of significantly differentially expressed genes between the different treatments [control (Con), E_2 treatment alone, E_2 + MPA) and Tib (tibolone)] in endometrium, myometrium and vagina.

	E_2/Con	E_2 + MPA/Con	Tib/Con	E_2 + MPA/E_2	Tib/E_2	Tib/E_2 + MPA
Endometrium	1493	404	328	685	257	326
Myometrium	1047	257	257	325	192	4
Vagina	16	73	4	16	29	45

Using SAM (Tusher *et al*., 2001), implemented in Omniviz (version 3.8), the number of differentially expressed genes between two treatment groups, mentioned at the top of the figure, in one tissue was calculated. SAM is a statistical method for identifying differentially expressed genes while controlling the overall FDR. FDR is the percentage of genes identified by chance. We have chosen the settings for this analysis in such a way that the total number of falsely identified differentially expressed genes in a tissue was less than one. With $P < 0.00029$, for the endometrial analysis in total 3493 differentially expressed genes were found, for the myometrium 2082 differentially expressed genes were found with $P < 0.00048$ and for the vagina, 183 differentially expressed genes were found with $P < 0.0055$.

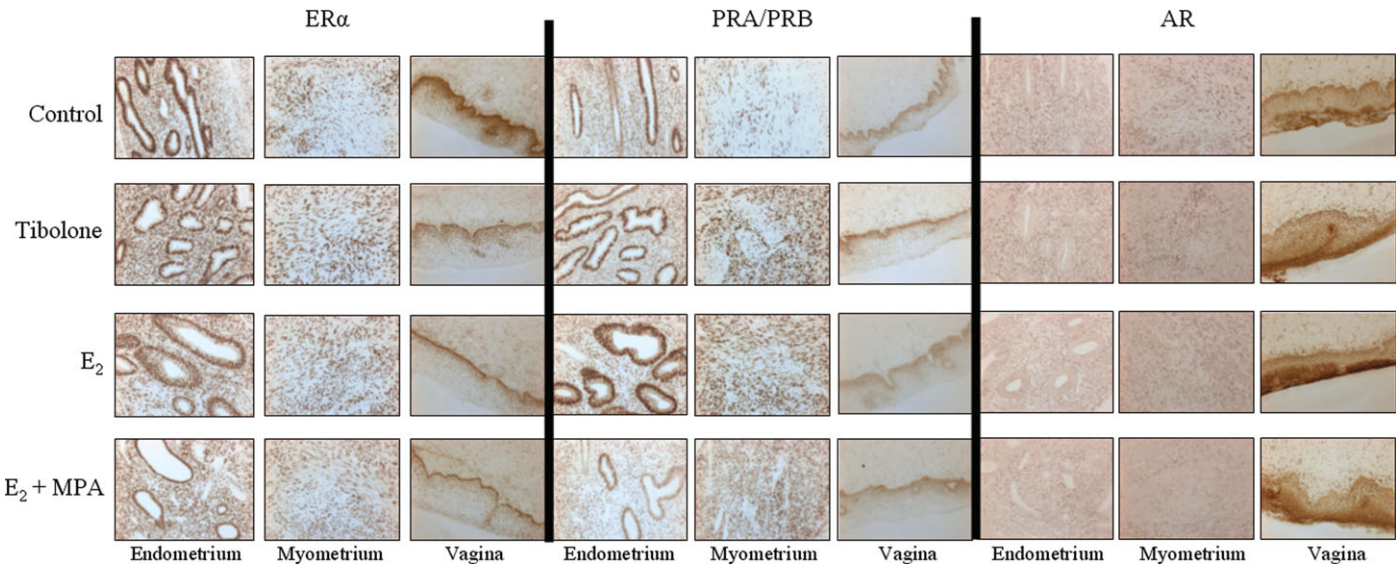

Figure 2: Immunohistochemical staining for ERα, PRA and PRB and AR in endometrial, myometrial and vaginal tissue after no treatment (control), and treatment with E_2, E_2 + MPA or tibolone
Representative images are shown for all different stainings and all treatment groups. Magnification is 200-fold

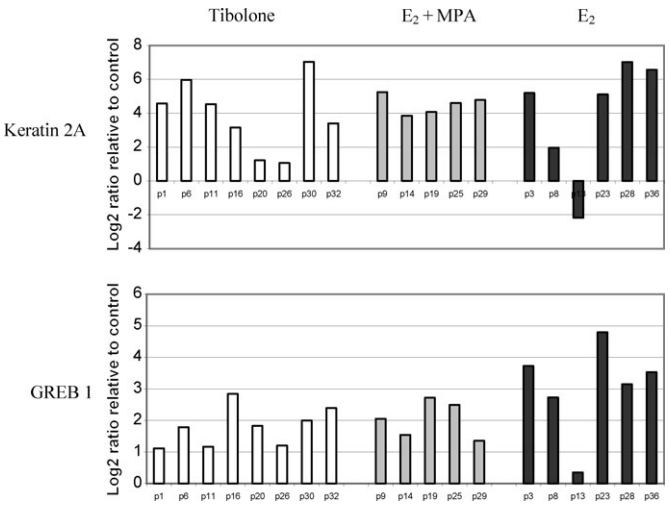

Figure 3: Real-time qPCR verification of upregulation of KRT2A and GREB1 in vaginal tissues. Real-time qPCR was performed on all vaginal RNA samples and was corrected for β-actin expression in the same samples
In the direction of the Y-axes, the Log2 ratio of the level of expression relative to control is indicated, on the X-axes the findings in the individual patient-tissues in the different treatment groups

qPCR was performed on two genes observed to be regulated by E_2, E_2 + MPA and tibolone treatment in the vagina: for KRT2A as well as GREB1, up-regulation by the three treatments could readily be confirmed (Fig. 3).

Biological classification of genes in endometrium and myometrium

Here, we will further refine the signalling analysis at the level of regulation of predefined biological processes using various databases. Furthermore, because treatment-induced signalling in the upper vagina was found to be very limited, we will concentrate our analysis on the endometrium and myometrium.

All significantly differentially regulated genes were classified in predefined biological function-categories. After reviewing this distribution of regulated genes, three significantly regulated biological functions were selected for a more detailed analysis, cell death, cell cycle and signal transduction. In Table II, data are presented on 'cell death', 'cell cycle' and 'signal transduction'. From Table II, it becomes clear that E_2 only treatment by far has the highest impact on gene expression in the endometrium as well as in the myometrium. Furthermore, reviewing the overlap in genes between endometrium and myometrium, the overlap turns out to be very small. In other words, the endometrium and myometrium use completely different sets of genes to regulate similar biological processes. Reviewing the actual numbers of genes, it is clear that E_2 regulation of 'cell cycle' genes is much higher in the endometrium than in the myometrium, in contrast to 'cell death' and 'signal transduction'.

The question now becomes whether these findings actually have a biological meaning. Therefore, proliferation was assessed using Ki67 staining as a marker, and cell death (apoptosis) was assessed using the TUNEL assay. The results of the endometrium have been published previously (Klaassens *et al.*, 2006; Hanifi-Moghaddam *et al.*, 2007) and are here compared with those of the myometrium. Figure 4 shows that proliferation in the endometrium is far more pronounced than in the myometrium. Furthermore, in both tissues E_2 only treatment has the highest impact on proliferation.

Comparing the effects of tibolone with E_2 + MPA, it was observed that in the myometrium these two hormone therapy drugs have a similar stimulatory effect on proliferation, whereas in the endometrium there are differences: both preparations stimulate proliferation of stromal cells while only E_2 + MPA inhibits proliferation of glandular cells, compared

Table II. Biological classification of significantly differentially expressed genes in endometrium and myometrium by functional category.

	Endometrium			Myometrium			Overlapping genes Endo–Myo
	E_2	E_2 + MPA	Tib	E_2	E_2 + MPA	Tib	
Cell death	32	3	5	26	8	9	2
Cell cycle	108	10	20	37	9	5	1
Signal transduction	159	36	40	133	31	36	4

Genes showing significant differential expression relative to control identified by SAM were classified into the biological processes 'cell death', 'cell cycle' and 'signal transduction' using the Panther database (panther.appliedbiosystems.com). To the right of the table the number of genes shared between endometrium and myometrium in the regulation of a particular process are indicated.

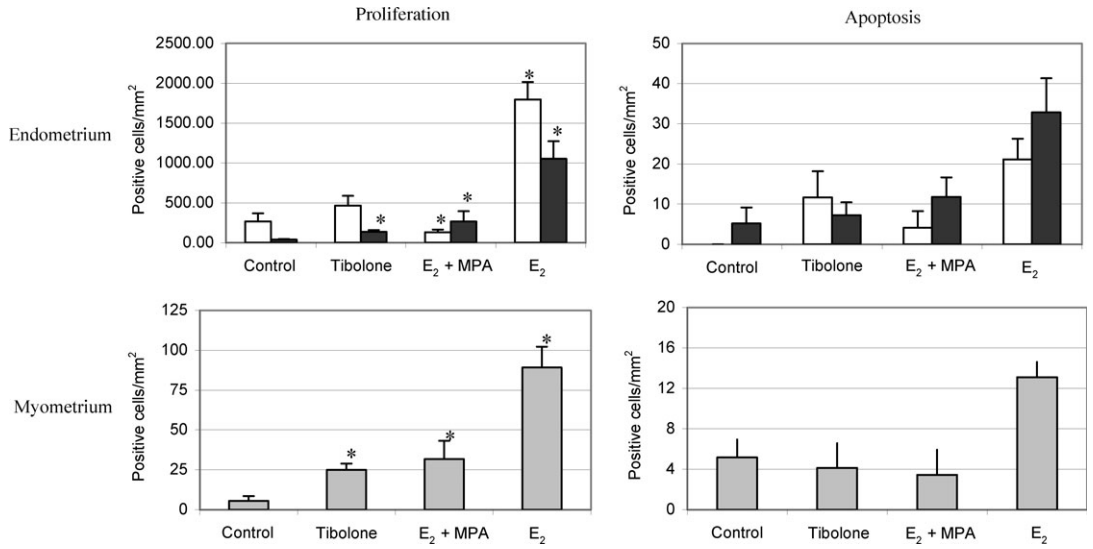

Figure 4: Proliferation and apoptosis in endometrium and myometrium
Proliferation was measured using Ki67 staining and apoptosis was measured using the TUNEL assay. For the endometrium two different compartments were assessed: glandular epithelium (open bars) and stroma (black bars). The following number of patients was included in each group: control, $n = 6$; tibolone, $n = 5$; E_2, $n = 6$; E_2 + MPA, $n = 5$. Averages were calculated $\pm$SEM, and significance was measured using student *t*-tests: $^*P < 0.05$ in comparison to control. The data on Ki67 staining in the endometrium were taken from our earlier work (Klaassens *et al*., 2006)

with controls. It is clear from the TUNEL-assay data that whenever proliferation is increased, apoptosis is also stimulated. Furthermore, since the number of positive staining cells is low, SEMs are high and small differences between the treatments can not be identified.

Discussion

The human endometrium and myometrium are both clearly estrogen-responsive tissues. For the endometrium this is not a new finding (Klaassens *et al*., 2006; Hanifi-Moghaddam *et al*., 2007), but for the myometrium, the large number of estrogen-regulated genes is an interesting new observation. In the endometrium, as expected, the estrogenic response is effectively counterbalanced by the addition of a progestagenic compound (MPA) and, interestingly, in the myometrium progesterone also readily counterbalances the actions of estrogen. Tibolone, from which both estrogenic as well as progestagenic metabolites can be formed, also shows a progestagenic counterbalance of its estrogenic properties in endometrium as well as myometrium although there are differences between the two tissues.

Upon a more careful examination of the effects of the different treatments, it becomes clear that tibolone treatment of the endometrium results in a gene expression profile which shows more resemblance to the E_2 induced endometrial expression profile than to the E_2 + MPA induced endometrial expression profile. For the myometrium, the situation is different: here the gene expression profile induced after tibolone treatment is more similar to the profile after E_2 + MPA treatment and quite distinct from the E_2-induced myometrial expression profile. This observation is strengthened when myometrial tibolone- and E_2 + MPA-treatments are directly compared: there are only four genes differently regulated by the two treatments. The observation that tibolone behaves in a more estrogen-like manner in the endometrium than in the myometrium under the current circumstances, illustrates its tissue-selective modulatory properties (Reed and Kloosterboer, 2004).

More puzzling are the data obtained with the upper vagina. In the current experimental setup, the vagina is not responding very profoundly to stimulation with estrogens, estrogens combined with progestagens or tibolone. Literature is very clear about this: the human vagina expresses ERs (Schwartz, 2000) and is highly responsive to estrogen-fluctuations (Utian *et al*.,

2001; Gorodeski, 2005; Gorodeski *et al*., 2005; Johnson *et al*., 2005; Utian *et al*., 2005; Farage and Maibach, 2006). Our data only show regulation of a limited number of genes but among these genes we could identify some that have been reported in literature as regulated by estrogens: iGF-1 (Gielen *et al*., 2005) GABRP (Symmans *et al*., 2005), LEPR (Bryzgalova *et al*., 2006), SFRP2 (Hayashi and Spencer, 2006), CYP3A5 (Williams *et al*., 2004), S100P (Schor *et al*., 2006), MUC4 (Gollub *et al*., 1995), C3 (Puy *et al*., 1993), GREB1 (Rae *et al*., 2005) and ANXA1 (Castro-Caldas *et al*., 2001). Furthermore, real-time qPCR could indeed confirm regulation of two chosen genes (KRT2A and GREB1). In conclusion, there is a response of the vagina to the different treatments, the magnitude of the response, however, is limited.

There are a number of possible reasons for the reduced response observed in the vagina under the current treatment conditions. First, the current treatment is only for 21 days, while in literature almost all data are collected at three or more months after start of treatment. Secondly, here we used hormone therapy drugs administered orally while most reports in literature are on estrogens, which are administered locally (Suckling *et al*., 2006). Local estrogens are reported to work much faster than systemic estrogens (Palacios *et al*., 2005). It should be mentioned here that we do measure increased estrogen levels in the upper vagina of these women with E_2 or E_2 + MPA treatment (Verheul *et al*., 2007). Third, women treated in the experiments described here had been post-menopausal for, on average, 10 years and Bachmann (2005) and Gorodeski (2005) suggest that the potency of post-menopausal vaginal cells to respond to estrogen-treatment is significantly reduced in post-menopausal women with permanent estrogen depletion.

In summary, 21-days of treatment of post-menopausal women with E_2, E_2 + MPA or tibolone imposes clear differential gene expression profiles on the endometrium and myometrium. Treatment with only E_2 results in the most pronounced effect on gene expression, proliferation and apoptosis, both in endometrium and myometrium. Tibolone, potentially metabolized to both estrogenic and progestagenic metabolites, shares some resemblance to E_2 signalling in the endometirum and, in contrast, significant resemblance to E_2 + MPA signalling in the myometrium.

In the vagina the situation is entirely different; all three hormonal treatments result in regulation of some genes, but the numbers are small in comparison to signalling in endometrium and myometrium. Possibly, the short duration of treatment, the fact that the drugs were administrated orally or the fact that most women had been post-menopausal for many years is at the basis of this finding.

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References

Bachmann G. Quantifying estrogen treatment effect on vagina tissue: cellular age matters. *Menopause* 2005;**12**:656–657.

Bryzgalova G, Gao H, Ahren B, Zierath JR, Galuska D, Steiler TL, Dahlman-Wright K, Nilsson S, Gustafsson JA, Efendic S *et al.* Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia* 2006;**49**:588–597.

Castro-Caldas M, Duarte CB, Carvalho AR, Lopes MC. 17beta-estradiol promotes the synthesis and the secretion of annexin I in the CCRF-CEM human cell line. *Mediators Inflamm* 2001;**10**:245–251.

Claver JA, Colillas OJ, Travi BL. Hostological and microbiological aspects of the vagina in captive howler monkeys. *Primates* 1984;**25**:110–116.

Deng L, Shipley GL, Loose-Mitchell DS, Stancel GM, Broaddus R, Pickar JH, Davies PJ. Coordinate regulation of the production and signaling of retinoic acid by estrogen in the human endometrium. *J Clin Endocrinol Metab* 2003;**88**:2157–2163.

Esplin MS, Fausett MB, Peltier MR, Hamblin S, Silver RM, Branch DW, Adashi EY, Whiting D. The use of cDNA microarray to identify differentially expressed labor-associated genes within the human myometrium during labor. *Am J Obstet Gynecol* 2005;**193**:404–413.

Farage M, Maibach H. Lifetime changes in the vulva and vagina. *Arch Gynecol Obstet* 2006;**273**:195–202.

Gielen SC, Hanekamp EE, Blok LJ, Huikeshoven FJ, Burger CW. Steroid-modulated proliferation of human endometrial carcinoma cell lines: any role for insulin-like growth factor signaling? *J Soc Gynecol Investig* 2005;**12**:58–64.

Gollub EG, Waksman H, Goswami S, Marom Z. Mucin genes are regulated by estrogen and dexamethasone. *Biochem Biophys Res Commun* 1995;**217**:1006–1014.

Gorodeski GI. Effects of estrogen on proton secretion via the apical membrane in vaginal-ectocervical epithelial cells of postmenopausal women. *Menopause* 2005;**12**:679–684.

Gorodeski GI, Hopfer U, Liu CC, Margles E. Estrogen acidifies vaginal pH by up-regulation of proton secretion via the apical membrane of vaginal-ectocervical epithelial cells. *Endocrinology* 2005;**146**:816–824.

Hanifi-Moghaddam P, Boers-Sijmons B, Klaassens AH, van Wijk FH, den Bakker MA, Ott MC, Shipley GL, Verheul HA, Kloosterboer HJ, Burger CW *et al.* Molecular analysis of human endometrium: short-term tibolone signaling differs significantly from estrogen and estrogen + progestagen signaling. *J Mol Med* 2007;**85**:471–480.

Hayashi K, Spencer TE. WNT pathways in the neonatal ovine uterus: potential specification of endometrial gland morphogenesis by SFRP2. *Biol Reprod* 2006;**74**:721–733.

Horwitz RI, Feinstein AR. Estrogens and endometrial cancer. Responses to arguments and current status of an epidemiologic controversy. *Am J Med* 1986;**81**:503–507.

Johnson SR, Ettinger B, Macer JL, Ensrud KE, Quan J, Grady D. Uterine and vaginal effects of unopposed ultralow-dose transdermal estradiol. *Obstet Gynecol* 2005;**105**:779–787.

Klaassens AH, van Wijk FH, Hanifi-Moghaddam P, Sijmons B, Ewing PC, Ten Kate-Booij MJ, Kooi GS, Kloosterboer HJ, Blok LJ, Burger CW. Histological and immunohistochemical evaluation of postmenopausal endometrium after 3 weeks of treatment with tibolone, estrogen only, or estrogen plus progestagen. *Fertil Steril* 2006;**86**:352–361.

Levens E, Luo X, Ding L, Williams RS, Chegini N. Fibromodulin is expressed in leiomyoma and myometrium and regulated by gonadotropin-releasing hormone analogue therapy and TGF-beta through Smad and MAPK-mediated signalling. *Mol Hum Reprod* 2005;**11**:489–494.

Mangioni S, Vigano P, Lattuada D, Abbiati A, Vignali M, Di Blasio AM. Overexpression of the Wnt5b gene in leiomyoma cells: implications for a role of the Wnt signaling pathway in the uterine benign tumor. *J Clin Endocrinol Metab* 2005;**90**:5349–5355.

Masui F, Kurosaki K, Mori T, Matsuda M. Persistent trefoil factor 1 expression imprinted on mouse vaginal epithelium by neonatal estrogenization. *Cell Tissue Res* 2006;**323**:167–175.

Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S. In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod* 2005;**20**:2104–2117.

Miyagawa S, Suzuki A, Katsu Y, Kobayashi M, Goto M, Handa H, Watanabe H, Iguchi T. Persistent gene expression in mouse vagina exposed neonatally to diethylstilbestrol. *J Mol Endocrinol* 2004;**32**:663–677.

Morris EP, Wilson PO, Robinson J, Rymer JM. Long term effects of tibolone on the genital tract in postmenopausal women. *Br J Obstet Gynaecol* 1999;**106**:954–959.

Otun HA, MacDougall MW, Bailey J, Europe-Finner GN, Robson SC. Spatial and temporal expression of the myometrial mitogen-activated protein kinases p38 and ERK1/2 in the human uterus during pregnancy and labor. *J Soc Gynecol Investig* 2005;**12**:185–190.

Palacios S, Castelo-Branco C, Cancelo MJ, Vazquez F. Low-dose, vaginally administered estrogens may enhance local benefits of systemic therapy in the treatment of urogenital atrophy in postmenopausal women on hormone therapy. *Maturitas* 2005;**50**:98–104.

Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* 2004;**10**:879–893.

Punyadeera C, Dassen H, Klomp J, Dunselman G, Kamps R, Dijcks F, Ederveen A, de Goeij A, Groothuis P. Oestrogen-modulated gene expression in the human endometrium. *Cell Mol Life Sci* 2005;**62**:239–250.

Puy LA, Kuivanen PC, DeSombre ER. Immunohistochemical localization of the oestrogen-responsive 110 kDa and 74 kDa polypeptides and complement component C3 in the rat genital tract after oestrogen treatment and during the oestrous cycle. *J Reprod Fertil* 1993;**99**:385–394.

Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME. GREB 1 is a critical regulator of hormone dependent breast cancer growth. *Breast Cancer Res Treat* 2005;**92**:141–149.

Reed MJ, Kloosterboer HJ. Tibolone: a selective tissue estrogenic activity regulator (STEAR). *Maturitas* 2004;**48**(Suppl. 1):S4–S6.

Sagsoz N, Yucel A, Noyan V, Bozdogan O. The effects of hormone therapy, estrogen therapy and tibolone on apoptosis and cyclin D1 expression in postmenopausal vaginal epithelium. *Eur J Obstet Gynecol Reprod Biol* 2005;**121**:61–66.

Schor AP, Carvalho FM, Kemp C, Silva ID, Russo J. S100P calcium-binding protein expression is associated with high-risk proliferative lesions of the breast. *Oncol Rep* 2006;**15**:3–6.

Schwartz PE. The oestrogen receptor (ER) in vulva, vagina and ovary. *Eur J Cancer* 2000;**36**(Suppl. 4):S31–S32.

Seidlova-Wuttke D, Christoffel J, Rimoldi G, Jarry H, Wuttke W. Comparison of effects of estradiol with those of octylmethoxycinnamate and 4-methylbenzylidene camphor on fat tissue, lipids and pituitary hormones. *Toxicol Appl Pharmacol* 2006;**214**:1–7.

Suckling J, Lethaby A, Kennedy R. Local oestrogen for vaginal atrophy in postmenopausal women. *Cochrane Database Syst Rev* 2006:CD001500.

Symmans WF, Fiterman DJ, Anderson SK, Ayers M, Rouzier R, Dunmire V, Stec J, Valero V, Sneige N, Albarracin C *et al.* A single-gene biomarker identifies breast cancers associated with immature cell type and short duration of prior breastfeeding. *Endocr Relat Cancer* 2005;**12**:1059–1069.

Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA *et al.* Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;**147**:1097–1121.

Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001;**98**:5116–5121.

Utian WH, Shoupe D, Bachmann G, Pinkerton JV, Pickar JH. Relief of vasomotor symptoms and vaginal atrophy with lower doses of conjugated equine estrogens and medroxyprogesterone acetate. *Fertil Steril* 2001;**75**:1065–1079.

Utian WH, Speroff L, Ellman H, Dart C. Comparative controlled trial of a novel oral estrogen therapy, estradiol acetate, for relief of menopause symptoms. *Menopause* 2005;**12**:708–715.

Verheul HA, Blok LJ, Burger CW, Hanifi-Moghaddam P, Kloosterboer HJ. Levels of tibolone and estradiol and their nonsulfated and sulfated metabolites in serum, myometrium, and vagina of postmenopausal women following treatment for 21 days with tibolone, estradiol, or estradiol plus medroxyprogestrone acetate. *Reprod Sci* 2007;**14**:160–168.

de Vries FA, de Boer E, van den Bosch M, Baarends WM, Ooms M, Yuan L, Liu JG, van Zeeland AA, Heyting C, Pastink A. Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. *Genes Dev* 2005;**19**:1376–1389.

Williams ET, Leyk M, Wrighton SA, Davies PJ, Loose DS, Shipley GL, Strobel HW. Estrogen regulation of the cytochrome P450 3A subfamily in humans. *J Pharmacol Exp Ther* 2004;**311**:728–735.

Yin Y, Ma L. Development of the mammalian female reproductive tract. *J Biochem (Tokyo)* 2005;**137**:677–683.

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