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ORIGINAL PAPER

Estrogen modulates expression of putative housekeeping genes in the mouse uterus

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Abstract Estrogens regulate gene expression and cell proliferation in target tissues. In studies of estrogen-regulated gene expression, identification of appropriate housekeeping genes (HKGs), reference genes whose expression is not altered by treatment, is difficult. The goal of this study was to define HKGs unaltered by estrogen in the mouse uterus. Ovariectomized C57BL6 mice were dosed with 20 micrograms/kg ethinylestradiol and the uterus was collected at 6, 24, and 72 h later to bracket the biphasic time course of estrogen action in the rodent uterus. RNA was isolated, cDNA synthesized and equal amounts of cDNA were added to real-time PCR reactions. The expression of seven out of nine putative HKGs was altered by estrogen in the mouse uterus. Estrogen induced four gene expression profiles, expression of: (1) *Actb* and *Hsp90ab1* were up-regulated early, (2) *B2m* and *Gusb* were up-regulated late, (3) *Gapdh*, *Hprt1*, and *Ppia* were up regulated at all time points, and (4) *Rpl13a* and *18srRNA* were unaltered. This highlights the need to empirically determine the appropriate HKG for each experimental condition. Based on these results, we suggest using *Rpl13a* or *18srRNA* as HKGs for xenoestrogen studies in the mouse uterus and as good candidates to test under different experimental conditions.

Keywords Estrogen · Xenoestrogen · Uterus · Housekeeping genes · Gene expression · RT-PCR

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Introduction

Classically, estrogens regulate gene expression and cell proliferation in many cell types. Both estrogen receptors (ER alpha and beta) are ligand dependent, DNA binding transcription factors [1]. Ligand-bound ER associates with estrogen response elements (ERE) located within the promoter region of target genes to regulate transcription [2]. The number and sequence of EREs vary from gene to gene [2, 3]. For example, the consensus ERE characterized from the vitellogenin A1 promoter is a 13 bp palindrome, whereas the human c-fos gene contains two imperfect EREs [4, 5]. In addition, ERE flanking sequences alter ER activity [6]. ER also regulates gene expression through other pathways besides direct binding to DNA. For example, ER regulates transcription by tethering to other transcription factors, such as AP-1 and SP-1, which associate with their respective response elements [7]. As ER can regulate transcription through dozens of DNA response elements, finding all estrogen-regulated genes based only on the presence of EREs is not possible.

Estrogen has different effects on gene transcription depending on the tissue and promoter context and also the time course of exposure [7–10]. ER associates with co-regulators to initiate or repress transcription. One mechanism of tissue-specific ligand-induced activity is thought to depend on the population of co-activators or co-repressors expressed [11]. Along with tissue-specific gene regulation, estrogen-induced gene expression can be time dependent. Hewitt et al. characterized the time course of estrogen-stimulated gene expression in the mouse uterus as a biphasic response with some genes uniquely regulated during the early (2–6 h) and/or late response (12–24 h) [12].

Real-time, reverse transcription polymerase chain reaction (RT-PCR) is a very sensitive method to quantify the

expression of specific target genes. As RT-PCR is so sensitive, it is subject to artifacts related to quality and quantity of input RNA and efficiency of the cDNA reaction. For example, the amount of RNA can vary from 10 to 100% of the total nucleic acid measured at Abs260 if total RNA is not treated with DNAse to remove contaminating DNA [13]. To minimize these artifacts, target gene expression is often normalized to a housekeeping gene (HKG), a reference gene whose expression is not altered by treatment. Stable expression of HKGs across treatment groups allows the investigator to normalize the expression of target genes thereby factoring out differences in starting RNA concentrations and other technical variation.

Many commonly used HKGs (Table 1) were originally selected because of their ubiquitous expression in an array of different cell types, necessity for cell survival or putative constitutive expression. However, many studies have shown altered expression of putative HKG genes with vital cell functions, in different cell types and by experimental treatment [14–16]. For example, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* has been used extensively as a HKG. Barber et al. demonstrated in a comprehensive analysis of 72 tissues that the expression of *Gapdh* varied by 15-fold across tissues [17]. Experimental treatment also alters the expression of *Gapdh*. Filby et al. showed that estrogen down-regulated expression of *Gapdh* by 5-fold in the fathead minnow liver [18]. These studies clearly demonstrate the need to validate HKG expression for each tissue and experimental condition.

Although the importance of validating HKGs is now recognized, this step is often omitted in gene expression studies. As HKGs must be empirically determined, it can be time consuming to find a gene that is not altered by experimental treatment, and many times researchers rely on HKGs used by other investigators.

This study is the first report of a systematic examination of potential estrogen-regulation of HKGs in the murine uterus across the biphasic time course. We undertook this study to identify HKGs that are not regulated by estrogen in the adult mouse uterus and to illustrate the consequences of normalizing to putative HKGs without validation. This information will help investigators choose the correct HKG when conducting estrogen exposure experiments at different time points in the mouse uterus.

Fig. 1 Estrogen stimulation of positive control endpoints in the mouse uterus. Four animals per group were dosed with either vehicle or 20 μg/kg ethinylestradiol (EE_2), the uterus was collected and weighed 6, 24, or 72 h (3 daily injections) later. Estrogen stimulated uterine wet weight gain (**a**), uterine epithelial cell proliferation, average of Ki67 positive cells per field in four fields (**b**), and complement 3 (*C3)* gene expression (**c**). Fold change in gene expression relative to the vehicle control at each time point was calculated using the Pfaffl method [27]. Representative graphs are shown. Error bars represent standard deviation. Comparisons between groups were made with ANOVA and Specified Contrasts post hoc (*$p < 0.01$)

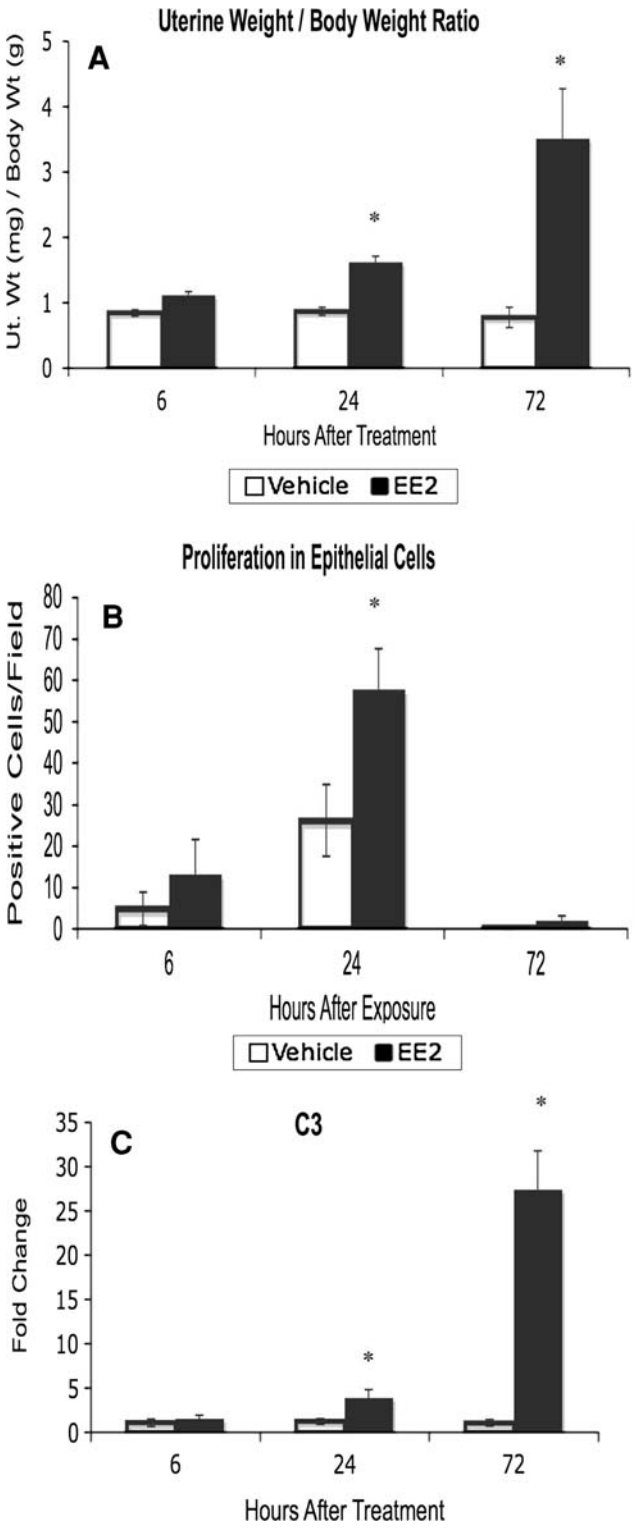
Results

Estrogen stimulated uterine wet weight gain and proliferation (Fig. 1)

Estrogens are known to stimulate uterine weight gain through water imbibition (early response-6 h) and cell

Table 1 Putative HKG descriptions

Abbreviation	Gene name	Description
Actb	Actin, beta, cytoplasmic	One of the two non-muscle cytoskeletal actins. Highly conserved protein; involved in cell motility, structure and integrity.
B2m	Beta-2 Microglobulin	A component of MHC class I molecules present on almost all cells of the body.
C3	Complement component 3	Plays a key role in the activation of the classical and alternative pathways in lysis response to foreign pathogens.
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	An enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules.
Gusb	Glucuronidase, beta	Located in lysosomes and plays an important role in recycling cellular components by cleaving glucuronide moieties from proteins.
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	The enzyme primarily functions to salvage purines from degraded DNA to renewed purine synthesis.
Hsp90ab1	Heat shock protein 90 kDa alpha (cytosolic), class B member	Displays chaperone activity; binds to peptides and facilitates their appropriate transport and/or secondary structure.
Ppia	Peptidylprolyl isomerase A	PPIases catalyze the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins.
Rpl13a	Ribosomal protein L13a	A component of the 60S subunit. Belongs to the L13P family of ribosomal proteins located in the cytoplasm.
18srRNA	18srRNA gene, clones 5a,6,7	Central component of the ribosome. Provides a mechanism for decoding mRNA into amino acids.



proliferation (late response 24 h and/or 72 h) and these endpoints were used as positive controls of our estrogen treatment. Four animals per treatment group were dosed with either vehicle or 20 μg/kg ethinyl estradiol (EE_2), the uteri were collected 6 and 24 h and after three daily injections (72 h). Treatment with EE_2 increased uterine wet weight (uterine weight/body weight) by 1.3, 1.9, and 4.4-fold at 6, 24, and 72 h, respectively (Fig. 1a).

Estrogen-induced cell proliferation was measured by immunohistochemical staining of the proliferation marker Ki67 (Fig. 1b). EE_2 significantly increased cell proliferation in the uterine epithelium by 2.5-fold at 24 h. Expression of Ki67 protein was not significantly elevated in the epithelium at 6 or 72 h or in the stroma at any time point (stromal cell data not shown).

Complement 3 (*C3*) is a known estrogen-regulated gene, and we used *C3* as a positive control estrogen-regulated gene in this study. EE_2 upregulated *C3* expression by 1.3, 2.5, and 23.5-fold, at 6, 24, and 72 h, respectively (Fig. 1c).

Relative expression levels of putative HKGs in the uterus

Of the nine putative HKGs we investigated, all were expressed at medium to very high levels in mouse uterus from cycling animals. *18srRNA* was very highly expressed with an average cycle threshold value (Ct) of six in the mouse uterus. Peptidylprolyl isomerase A (*Ppia*), *Rpl13a*, *Actb*, *Gapdh*, Beta-2 microglobulin (*B2m*), and Heat shock protein 90 kDa alpha, class B member (*Hsp90ab1*) all showed strong expression with Cts ranging from 16.9 to 19.1. *Gusb* showed the lowest level of expression with a Ct of 25.5. All primer sets showed very low expression in the negative control reactions (noRT = mock cDNA synthesis reaction without reverse transcriptase) with noRT Cts at least 17 cycles later than the corresponding RT reactions.

Estrogen regulation of common HKGs

RT-PCR was performed to determine the fold change in gene expression between the control and EE_2-treated groups. Estrogen regulation of gene expression exhibited four distinct profiles based on the time course of regulation: (1) unaltered at all time points, (2) altered at early time points (6 and/or 24 h only), (3) altered at later time points (24 and/or 72 h) or (4) altered at all time points.

Identification of genes not altered by estrogen in the mouse uterus (Fig. 2)

We found only two genes whose expression was unaltered by EE_2 at all time points. Only the expression of *18srRNA* and *Rpl13a* genes was unaltered at all three exposure times (Fig. 2).

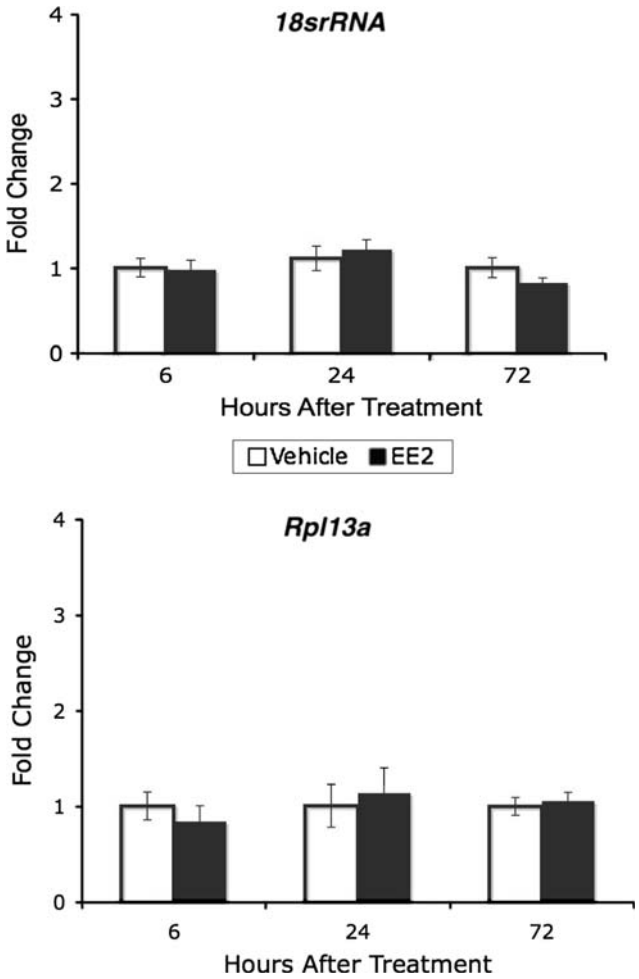


Fig. 2 Expression of putative HKGs unaltered by estrogen. The fold change in *18srRNA* (**a**) and *Rpl13a* (**b**) gene expression in mouse uterus after treatment with vehicle or 20 μg/kg EE_2 ($N = 4$/group). Fold change in gene expression was calculated relative to vehicle control (for 6 and 24 h relative to 6 h control, for 72 h relative to 72 h control). Representative graphs are shown. Error bars represent standard deviation. Comparisons between groups were made with ANOVA and Specified Contrasts post hoc ($^*p < 0.01$)

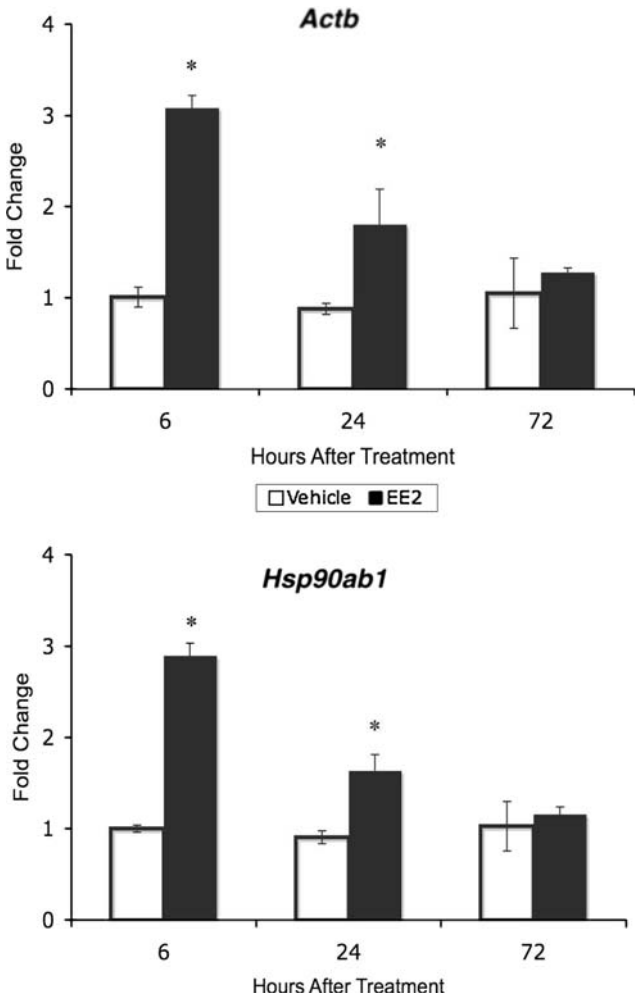


Fig. 3 Expression of putative HKGs altered at early time points by estrogen. The fold change in *Actb* (**a**) and *Hsp90ab1* (**b**) gene expression in mouse uterus after treatment with vehicle or 20 μg/kg EE_2 ($N = 4$/group). Fold change in gene expression was calculated relative to vehicle control (for 6 and 24 h relative to 6 h control, for 72 h relative to 72 h control). Representative graphs are shown. Error bars represent standard deviation. Comparisons between groups were made with ANOVA and Specified Contrasts post hoc ($^*p < 0.01$)

Gene expression altered early (Fig. 3)

The expression of *Actb* and *Hsp90ab1* genes was up-regulated by approximately 2.5-fold at 6 h and 2-fold at 24 h after EE_2 exposure. After 3 daily injections of EE_2 the expression of both genes was unaltered between the control and EE_2-treated groups.

Gene expression altered late (Fig. 4)

B2m gene expression was up-regulated after 3 daily injections of EE_2 by 2-fold with no differences in expression in the control and EE_2-treated uteri at 24 h. Interestingly, *B2m* was slightly downregulated at 6 h. *Gusb* gene expression was up-regulated at 24 and 72 h by 3.3 and 3.1-fold respectively with unaltered expression at 6 h of exposure.

Gene expression altered early and late (Fig. 5)

The expression of *Gapdh*, which has been one of the most widely used HKGs, was altered by EE_2 at all three time points tested by 2.4, 3.9, and 2.4-fold at 6 and 24 h and after 3 daily injections, respectively. Similarly, Hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) was up-regulated by 2.6, 1.9, and 1.6-fold; and *Ppia* was up-regulated by 1.8, 2.4, and 1.5-fold at 6 and 24 h and after 3 daily injections, respectively.

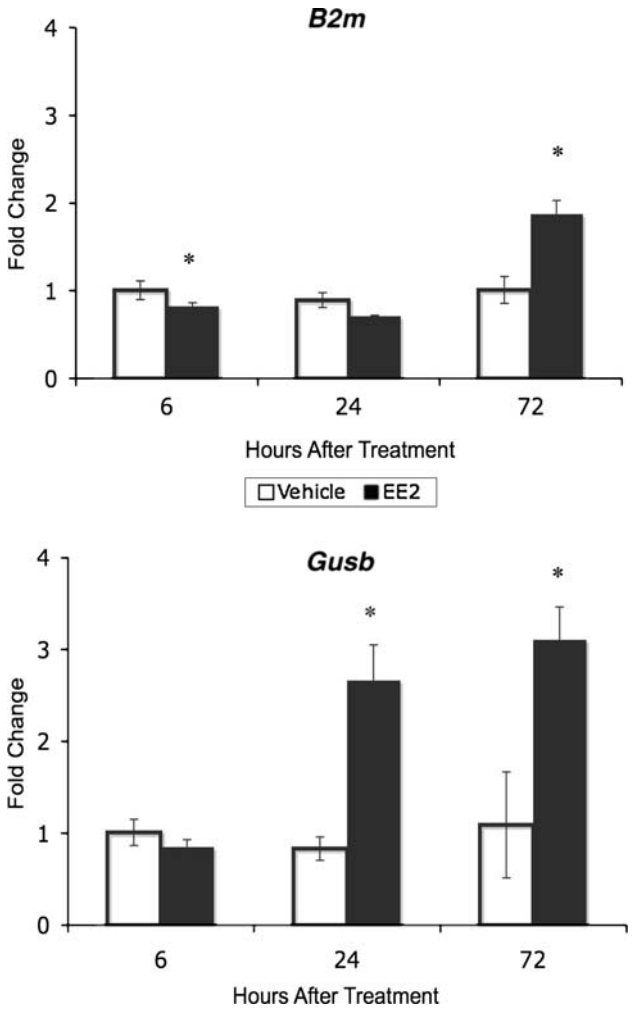


Fig. 4 Expression of putative HKGs altered at later time points by estrogen. The fold change in *B2m* (**a**) and *Gusb* (**b**) gene expression in mouse uterus after treatment with vehicle or 20 μg/kg EE_2 ($N = 4$/group). Fold change in gene expression was calculated relative to vehicle control (for 6 and 24 h relative to 6 h control, for 72 h relative to 72 h control). Representative graphs are shown. Error bars represent standard deviation. Comparisons between groups were made with ANOVA and Specified Contrasts post hoc (*$p < 0.01$)

The effect of normalizing to putative HKGs on the calculated *C3* gene expression (Fig. 6)

For this study the concentration of cDNA was measured and equal amounts were added to each RT-PCR reaction. Under these conditions, *C3* gene expression was induced 2.5-fold at 24 h by EE_2. In order to examine the effect of normalizing *C3* expression to the putative HKGs in this study, *C3* expression was normalized to each of the HKGs. The observed induction of *C3* at 24 h was lost when expression data were normalized to *Actb*, *Gapdh*, *Gusb*, *Hprt1*, *Hsp90ab1*, and *Ppia* (Fig. 6).

Use of equal amounts of cDNA as an alternative strategy to use of HKG

cDNA concentration was measured and equal amounts were used in each RT-PCR reaction. To validate this approach, first, *B2m* expression was calculated from data where an equal volume of cDNA reaction was added to each RT-PCR reaction. The coefficient of variation (CV) for fold change within groups was 14.1% without normalization to a HKG. When the cDNA concentration was measured and equal amounts of cDNA were added to each RT-PCR reaction, the CV was reduced to 8.25%.

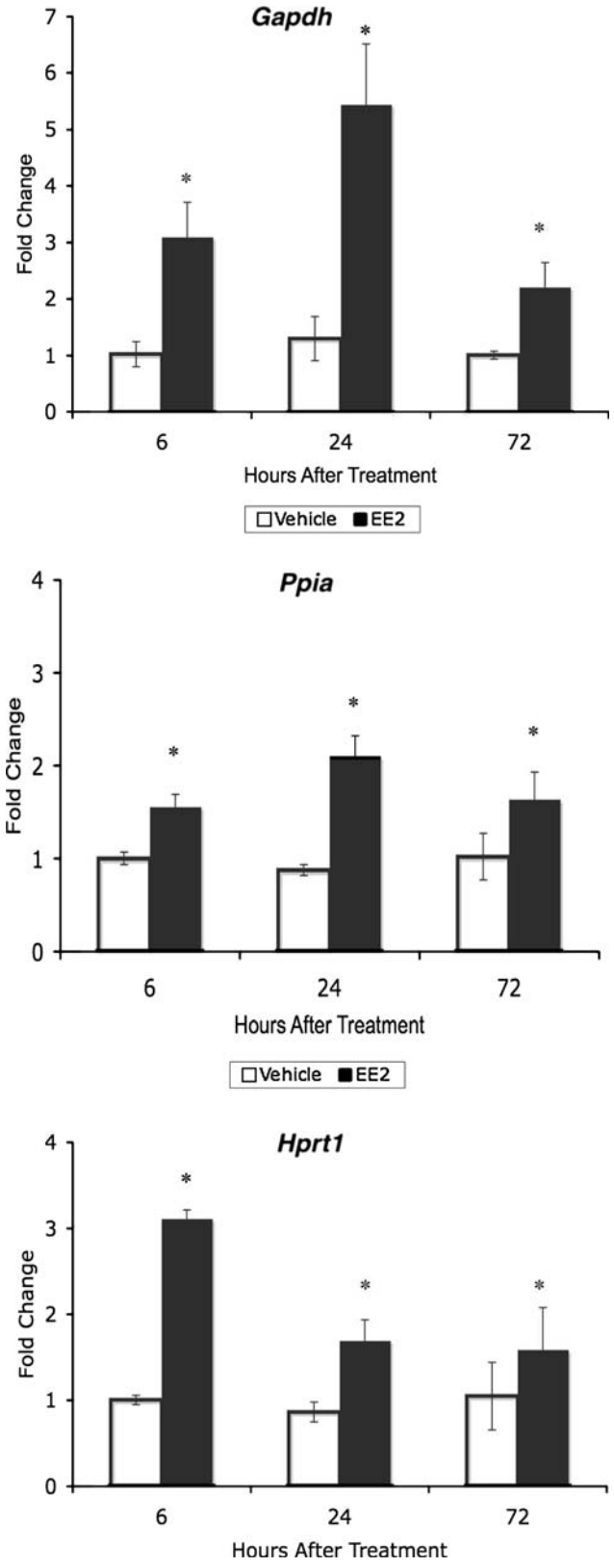
Discussion

In this study nine genes commonly used as HKGs in RT-PCR were examined for potential estrogen-regulation across the biphasic response in the mouse uterus. Of these genes only two were found to be unaltered at all three exposure times tested: *18srRNA* and *Rpl13a* both ribosomal RNAs. Four of the genes were altered at one or two exposure times and three were altered at all three exposure times. Taken together, these results highlight the critical need to validate each candidate HKG with each specific experimental condition.

Estrogen-regulated gene expression

Estrogen receptor (ER) ligands can have unique tissue and species-specific gene regulation. Filby et al. showed that estrogen down-regulated *Actb* and *Gapdh* in the fat head minnow liver, while we found that estrogen up-regulated both genes in the mouse uterus [18, 19]. These studies show that while estrogen exposure alters HKG expression, it is not always possible to predict altered expression from other species or tissues. In addition selective ER ligands may induce different gene expression profiles, and a potential limitation of the current study is the use of only a full ER agonist to define appropriate control genes. However, use of HKGs to normalize gene expression data altered by a positive control estrogen, like EE_2, would be inappropriate in studies of estrogen or xenoestrogen action in the mouse uterus.

The altered gene expression of HKGs in this study may be the result of direct estrogen-ER gene regulation or indirect stimulation as a result of increased cell proliferation. EREs can confer estrogen regulation from target gene promoters or from within the target gene itself [20]. The genes under investigation in this study that were altered by estrogen do not contain classical EREs; however, they contain ERE half sites. For example, within the *Gapdh* gene there are seven classical (GGTAC) and nine other



◀ **Fig. 5** Expression of putative HKGs altered at all time points by estrogen. The fold change in *Gapdh* (**a**), *Ppia* (**b**), and *Hprt1* (**c**) gene expression in mouse uterus after treatment with vehicle or 20 μg/kg EE_2 ($N = 4$/group). Fold change in gene expression was calculated relative to vehicle control (for 6 and 24 h relative to 6 h control, for 72 h relative to 72 h control). Representative graphs are shown. Error bars represent standard deviation. Comparisons between groups were made with ANOVA and Specified Contrasts post hoc (*$p < 0.01$)

ERE half sites (GGTGG) within the gene and one putative ERE. In addition to EREs, estrogens can regulate gene transcription by tethering to other transcription factors, such as AP-1. Seven out of nine genes in the current study contain AP-1 response elements. This is another potential pathway for direct estrogen regulation through the ER, but independent of EREs. In addition to direct gene regulation, indirect estrogen regulation by downstream targets is also likely as a result of estrogen-induced cell proliferation. For example, both *Gapdh* and *Actb* have been shown to be up-regulated in proliferating collateral arteries [21].

Identification of appropriate HKGs for RT-PCR studies

A good HKG allows the researcher to control not only for the initial amount of RNA but also the reverse transcription reaction efficiency. There is a growing literature on what constitutes an appropriate HKG. HKGs should be expressed by the specific tissue of interest and the threshold value should appear in about the same range as the target gene [22]. For this reason, we suggest the use of *Rpl13a* as a good candidate HKG. *18srRNA* has an extremely high level of expression and may require the use of a different threshold from the target gene of interest to calculate cycle threshold values, which could potentially introduce error. The high level of expression may also potentially reduce the ability to measure differences in cDNA concentration between samples. Several reports have suggested the use of *Rpl13a* as a good candidate HKG in other tissues. *Rpl13a* expression was found to be unaltered in mouse prostate tissue explants, human prostate cancer (LNCaP) cells and human breast cancer (T-47D and ZR-75-1) cells following treatment with the histone deacetylase inhibitor Trichostatin-A and in a rat model of cerebral artery occlusion [23, 24].

Treatment-induced changes in HKG expression can greatly alter the interpretation of gene expression data. *C3* is up regulated in the presence of estrogen in the mouse and rat uterus within hours of exposure [25, 26], and *C3* expression was used as a positive control gene in the current study. We used *C3* gene expression to illustrate the impact of normalizing to putative HKGs altered by estrogen treatment. When equal amounts of cDNA were added to the RT-PCR reaction, *C3* expression was upregulated by 2.5-fold after 24 h of EE_2 exposure. Strikingly, normalization of *C3* expression to six of the nine HKGs tested resulted in no apparent up-regulation of gene expression at 24 h. In fact, when normalized to *Gapdh*, *C3* expression

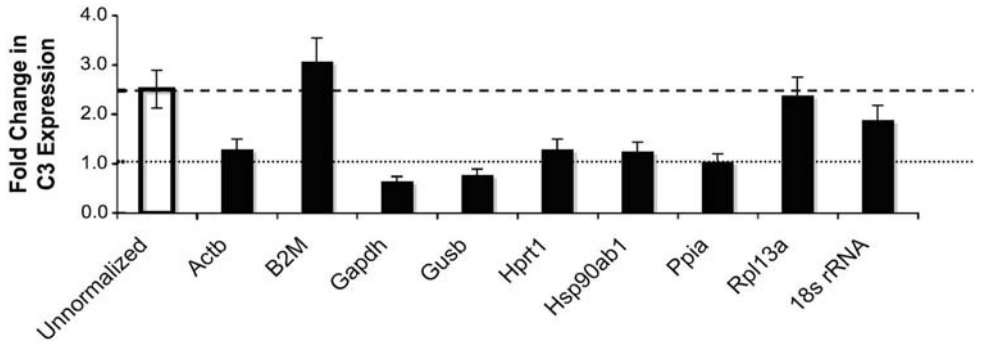


Fig. 6 The effect of normalizing estrogen-regulated gene expression to putative HKGs. About 24 h after EE_2 exposure the induction of complement 3 (*C3*) gene expression was 2-fold over vehicle using the same amount of cDNA added to each RT-PCR reaction. When the *C3* RT-PCR data were normalized to the expression of the other putative HKGs, only three yielded a 2-fold induction: *Rpl13a*, *18srRNA* and *B2m*, and the remaining resulted in a false negative result. The loss of significance in this figure indicates that normalization to each HKG resulted in *C3* gene expression that was no longer greater than vehicle. Error bars represent standard deviation. $*p < 0.02$ by one-way ANOVA, control versus EE_2 for each HKG normalization

appeared to be down-regulated (Fig. 6). This further highlights the need to validate HKG expression for each experimental condition and, when known, to include a positive control in each study.

While it is desirable to identify a HKG not altered by experimental treatment, an alternative strategy is to quantify cDNA concentration and to use equal amounts of cDNA in each reaction. One of the key features of this study that allowed for the accurate analysis of altered HKG expression was the measurement of cDNA concentration and the addition of equal amounts of cDNA to each RT-PCR reaction. This takes into account the amount of starting RNA quantity and quality and the efficiency of the cDNA reaction, which are the key reasons to use a HKG. To illustrate the utility of this approach, in the analysis of *B2m* gene expression use of equal amounts of cDNA versus use of an equal volume of cDNA reaction mix resulted in a >50% reduction in variation within treatment groups.

EE_2 altered the expression of seven out of nine commonly used HKGs in the mouse uterus. Importantly, *Gapdh*, which has been one of the most widely used HKGs, was altered by EE_2 at all three time points tested by up to 4-fold over vehicle control. These results highlight the need to empirically determine the appropriate HKG for each experimental condition for use in RT-PCR data normalization. Based on these results, we suggest using *Rpl13a* as a HKG for xenoestrogen studies in the mouse uterus and as a good candidate to test under different experimental conditions.

Materials and methods

Animals

Investigations were conducted in accordance with the Guide for Care and Use of Laboratory Animals (copyright 1996, National Academy of Science). C57BL6 were originally purchased from Jackson Labs, but mice for this study were bred in house. Female mice were group housed in polysulfone cages. All mice were fed Purina 5008 chow, given acidified water ad libidum and were on a 12-h light schedule.

Dosing and tissue collection

Experiment #1: Three-month old C57BL6 females were ovariectomized and dosed subcutaneously with either vehicle (tocopherol stripped corn oil, MP Biomedicals) or 20 μg/kg EE_2 (Sigma) 8 to 9 days later. EE_2 is found in most oral contraceptive pills prescribed to women in the U.S. and used in many studies looking at xenoestrogen regulation of gene expression in the mouse uterus. Four animals per dose group were euthanized by CO_2 asphyxiation and the uterus was collected and weighed 6 or 24 h after dosing.

Experiment #2: Female C57BL6 mice (2–6 months old) were ovariectomized and dosed subcutaneously with either vehicle (1:1 DMSO:PBS) or 20 μg/kg EE_2 for three consecutive days. Four animals per dose group were euthanized by CO_2 asphyxiation and the uterus was collected and weighed 24 h after the last dose and 72 h after the first dose.

RNA isolation

The whole uterus was weighed and 10–15 mg of tissue was directly added to 500 μl lysis binding solution (Ambion), immediately homogenized and stored at −80°C until RNA isolation. Total RNA was isolated using RNAqueous (Ambion). Total RNA was treated with DNAse using TURBO-DNA free (Ambion), according to the manufacturer's protocol, and ethanol precipitated.

Quantity and purity of total RNA was measured by UV absorbance using a Nanodrop 3.0.1 (Coleman Technologies) and quality was determined on an ethidium bromide stained 1% agarose gel. ImageJ 1.38X (National Institute of Health) was used to determine the intensity of the 28S and 18S bands from gel electrophoresis. The ratio of 28S to 18S was used as a measure of RNA quality. Only samples with a ratio greater than or equal to 1.0 were used for further analysis. We determined that this cut-off correlates strongly with the determination of high quality RNA using an Agilent Bioanalyzer (DNA Core, University of Missouri-Columbia).

cDNA synthesis

Total RNA (200 nanograms) was reverse transcribed with SuperScript III First-Strand Synthesis Kit (Invitrogen) as per the manufacturer's recommendations. The cDNA synthesis reaction contained both oligo(dT)20 and random hexamer primers. *Escherichia coli* RNase H (Invitrogen) and RNAse cocktail (Ambion) incubation was performed at 37°C for 30 min and at 70°C for 10 min. cDNA was column purified using RNAqueous-Micro columns (Ambion), denaturation solution (Ambion) and 100% ethanol. Columns were washed twice with 80% ethanol and RNA was eluted in 60°C ultra-pure water. For each sample, a mock reaction (noRT) was prepared identically but without reverse transcriptase or ribonuclease inhibitor.

Quantification of cDNA

We measured the concentration of purified cDNA after synthesis using Oligreen (Molecular Probes), a sensitive fluorescent dye that binds to both single and double stranded DNA, as per manufacture's instructions. Briefly, 6.5% of each sample cDNA was diluted with 1X TE and Oligreen reagent (1:200) was added directly before reading the samples at ex500/em525. A standard curve of an 18 bp M13 primer starting at 0.8 μg/ml was used to determine the concentration of cDNA in each sample. Three separate batches of cDNA were made and following quantification, each sample was brought to a final concentration of 1 ng/μL and 10 ng cDNA was added to each RT-PCR reaction. After identifying *Rpl13a* as a good HKG, the third batch of cDNA was normalized to *Rpl13a* using the method described by Pfaffl et al. [27].

Real-time reverse transcription PCR

RT-PCR was performed using SYBRgreen technology with primers designed in Vector NTI (Invitrogen) and synthesized by IDT DNA. cDNA dissociation curve analysis and visualization of amplification products with gel electrophoresis was performed to ensure that primers amplified only target cDNA.

Primer sequences, concentrations, and efficiencies (E) used are summarized in Table 2. Primer set efficiencies

Table 2 Primer characteristics

Gene	Accession #		Sequence	Junctiona	[Primer]	Efficiency
Actb	NM_007393	Sense	GATGACCCAGATCATGTTTGAGACC	Exon 3/4	140 nM	89%
		Antisense	AGATGGGCACAGTGTGGGTGA		140 nM	
B2m	NM_009735	Sense	GCTCGGTGACCCTGGTCTTT	Exon 1/2	140 nM	90%
		Antisense	TGTTCGGCTTCCCATTCTCC		140 nM	
C3	M35659	Sense	GCGTCTCCATCAAGATTCCAGCCA	Exon 1/2	140 nM	91%
		Antisense	CACCACCGTTTCCCCGAAGTTTG		140 nM	
Gapdh	NM_008084	Sense	TGTGATGGGTGTGAACCACGAGAA	–	140 nM	93%
		Antisense	GAGCCCTTCCACAATGCCAAAGTT		140 nM	
Gusb	NM_010368	Sense	TGGGCATTGTGCTACCTCAGAG	Exon 7/8	140 nM	97%
		Antisense	CACATCACAACCGCAGGGTG		140 nM	
Hprt1	NM_013556	Sense	CATGGACTGATTATGGACAGGACTG	Exon 2/3	70 nM	88%
		Antisense	ATCCAGCAGGTCAGCAAAGAACT		70 nM	
Hsp90ab1	NM_008302	Sense	GCTTCAGATGCCCTGGACAA	Exon 2/3	70 nM	94%
		Antisense	GGTCATGCCAATGCCTGTGT		70 nM	
Ppia	NM_008907	Sense	TGCTGGACCAAACACAAACGGTTC	–	140 nM	91%
		Antisense	CAAAGACCACATGCTTGCCATCCA		140 nM	
Rpl13a	NM_009438	Sense	TACCAGAAAGTTTGCTTACCTGGG	Exon 6/7	140 nM	88%
		Antisense	TGCCTGTTTCCGTAACCTCAAG		140 nM	
18srRNA	K01364	Sense	TTCCTTACCTGGTTGATCCTGCCA	–	140 nM	86%
		Antisense	AGCCATTCGCAGTTTCACTGTACC		140 nM	

were determined for all primer pairs prior to use by triplicate runs of four serial 10-fold dilutions of cDNA using the equation $E = 10^{[-1/slope]}$ [27]. Only primer sets with greater than 85% efficiency over at least three dilution points were used.

RT-PCR reactions were performed in an ABI 7500 sequence detector using SYBR-Green PCR core reagents (Invitrogen) in the presence of the designed primers, according to the manufacturer's instructions, except that the reaction volume was reduced to 25 µl. PCR amplification was performed under the following conditions: 2 min at 50°C, 2 min at 95°C, followed by a total of 40 cycles of 15 s at 95°C and 31 s at 60°C. Each assay included duplicate sample reactions (RT) and one noRT for each sample. Each assay was repeated three times for each gene. Across 20 plates of 40 sets of duplicates, the duplicate coefficient of variation was 7.2% (standard deviation of duplicate CTs/average CT).

Immunohistochemistry

A small piece of the uterus from each animal was formalin-fixed and paraffin embedded (RADIL, University of Missouri). Five micron sections were mounted on Superfrost Plus slides (Fisher), de-waxed and dehydrated using washes of xylene and decreasing concentrations of ethanol. For immunostaining, sections went through antigen retrieval at 95°C in citrate buffer (10 mM), and were then washed with PBS overnight and blocked for 5–7 h with 4% goat serum (Vector) and 4% BSA (Pierce) in saline. The primary antibody was rabbit anti-rat Ki67 (Neomarkers, RB-1510-P0) diluted 1:300 and incubated overnight at 4°C. Ki67 is a nuclear protein and a marker of cell proliferation. The secondary antibody was goat anti-rabbit labeled with Alexa 488 (Invitrogen, A11034) diluted 1:2000 and incubated for 2 h at room temperature. DNA was stained with 7 µM Dapi added with the secondary antibody incubation. Slides were mounted with Mowiol (Calbiochem) and pictures were taken on a Zeiss, Axiophot microscope with epifluorescence at 400X in three different fields of the uterus. Total number of proliferating cells was quantified in each of the three fields per tissue using the program Metamorph (Molecular Devices).

Statistics

Three independent RT-PCR reactions per gene were performed and averaged. One-way ANOVA followed by Specified Contrasts were performed to detect differences between EE_2 and vehicle at each time point.

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