

Variation in stability of housekeeping genes in endometrium of healthy and polycystic ovarian syndrome women

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BACKGROUND: The use of housekeeping genes (HKG) as internal controls for real-time qPCR studies of gene expression is based on the assumption of their inherent stability. However, it is unclear whether this stability is maintained in disease states. In order to test this, the present study investigated the expression of specific HKG in the endometrium of healthy and polycystic ovarian syndrome (PCOS) women.

METHODS: Endometrial tissue samples were taken from women with PCOS ($n = 9$) and controls ($n = 10$). The stability of nine candidate reference genes in the endometrial tissues were evaluated; four encode mitochondrial proteins [*ATP5B*, succinate dehydrogenase complex subunit A (*SDHA*), cytochrome *c*-1, glyceraldehyde-3-phosphatedehydrogenase], two encode ribosomal protein genes (*18s ribosomal RNA*, ribosomal protein *L13A*), one for cell structure (*SDHA*), one for cell signalling (beta actin, *ACTB*) and one involved in DNA repair (topoisomerase I, *TOP1*). The expression stability of these HKGs was calculated using geNORM qbasePLUS software, with stability defined by *M*-values, where higher *M*-value indicating less stability. In addition, changes in their cycle threshold values were analysed to determine direction of change between groups, and a Mann–Whitney *U*-test was used to determine statistical differences in expression.

RESULTS: The most stable HKGs observed across both PCOS endometrium were found to be *YWHAZ*, *CYC1* and *ACTB*. Further *TOP1* demonstrated higher gene expression in the endometrium from PCOS women compared with those from healthy women.

CONCLUSIONS: Of the nine HKGs examined, only *YWHAZ*, *CYC1* and *ACTB* were stable in both control and PCOS endometrium: these should therefore be used as internal controls for quantitative reverse transcription-polymerase chain reaction analysis. Published discrepancies between endometrial gene expression studies may therefore be due in part to in the inappropriate HKG selection, and future gene expression studies should be based on HKG of known stability in both the disease and healthy states to avoid erroneous interpretation of results.

Key words: housekeeping genes / PCOS / endometrium / *TOP1*

Introduction

Comparative gene expression studies in the normal and pathological human endometrial tissue provide insights into molecular mechanisms of gynaecological disorders such as polycystic ovarian syndrome (PCOS), recurrent miscarriage, menorrhagia, endometriosis, hyperplasia and cancer. Endometrial gene expression profiles are used to better understand the molecular mechanisms behind gynaecological conditions. Endometrial growth and function evolve throughout the menstrual cycle in a succession of stages, under the influence of hormones and growth promoting factors, as well as programmed cell

death and survival factors (Knobil 1980; Kokawa *et al.*, 1996; Torry *et al.*, 1996). Within endometrial cells, these processes are mediated by changes in gene expression (Carson *et al.*, 2002). Thus, measuring gene expression levels in endometrial tissues must take into account physiological and pathological variations. Quantitative analysis of gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) involves the use of constitutively expressed internal controls, known as 'housekeeping' genes (HKG), which are used for the normalization of the target gene expression (Vandesompele *et al.*, 2002). This is a crucial step in accurately determining gene

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expression levels. The essential prerequisites for all HKG suitable for use as internal controls include being adequately expressed in the target tissue, and demonstrating minimal variability and high stability irrespective of physiological or pathological conditions. Previous studies investigating gene expression in gynaecological tissues have conventionally used the HKG glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*), beta actin (*ACTB*) or *18s ribosomal RNA (18s rRNA)* as reference genes (Quezada et al., 2006; Burney et al., 2007; Narayan et al., 2007; Tone et al., 2008; Kim et al., 2009; Lee et al., 2009; Xie et al., 2009; Margarit et al., 2010). As with all physiological processes, the menstrual cycle is subjected to environmental and pathological stressors. Work examining the effect of a hypoxic environment has shown altered gene expression within endometrial tissue (Sharkey et al., 2000; Milne et al., 2001). In addition, a number of gynaecological pathologies such as cancer and endometriosis have resulted in changes to gene expression in endometrium (Burney et al., 2007; Tanabe et al., 2008). It is therefore likely that such conditions could also affect expression levels of HKG. Investigations in other human tissues have shown that *GAPDH* and *ACTB*, which were previously thought of as stably expressed, are variably expressed in the placenta at various stages of pregnancy (Patel et al., 2002; Meller et al., 2005). To our knowledge, there has been no published data examining HKG stability in endometrial tissue. In this study, we investigated the expression of HKG in endometrium of reproductively healthy women and those with PCOS. This will provide useful information for other researchers carrying out gene expression studies on endometrial tissues in choosing the appropriate HKG.

Materials and Methods

All patients provided informed consent to participate in this study under a protocol approved by the Research and Development Committee at the University of Southampton and the Southampton University Hospitals

NHS Trusts and the local Ethics committee. Endometrial tissues were collected from 19 patients by endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France). The control group ($n = 10$) was composed of women with regular menstrual cycles (25–35 days) and who have not received any hormonal preparation in the 3 months preceding biopsy collection. PCOS patients ($n = 9$) were defined according to the revised 2003 Rotterdam Consensus Criteria (2004), where two out of the three identified features constituted PCOS. According to menstrual dates given by patients, specimens were classified as proliferative (Day 8–14) or secretory (Day 15–28). The selection criterion was aimed at reducing the possible confounding factors that may potentially alter gene expression. The age selection criterion of 18–45 years of age was used to ensure that subjects had reached menarche and were not past menopause. The exclusion criterion included subjects who were pregnant, had given birth in the past 12 months, had known or suspected malignancy, were taking hormone medication and those with a history of gynaecological pathology for PCOS. All control women entering the study had a normal pelvis examined by ultrasound.

Total RNA was extracted from the endometrial tissue block using TRIzol Reagent (Invitrogen, Paisley, UK). The A260/280 ratio for the samples was measured by spectrophotometry and the total RNA concentration for each sample was calculated. Agarose gel electrophoresis was carried out to check the integrity of the RNA. Complimentary DNA was synthesized from total RNA using M-MLV reverse transcriptase in the presence of random oligo primers, PCR nucleotides (Deoxynucleoside triphosphates) and RNase inhibitors. Primers and probes specific for nine human HK genes, namely succinate dehydrogenase complex subunit A (*SDHA*), *18s rRNA*, topoisomerase I (*TOP1*), *ACTB*, *GAPDH*, zeta polypeptide (*YWHAZ*), ATP synthase subunit (*ATP5B*), cytochrome c-I (*CYC1*) and ribosomal protein *L13A (RPL13A)*, were designed using the PerfectProbe software and made by Primer Design Ltd (Southampton, UK). These genes are also used as reference HK genes in the geNorm™ Reference Gene Selection Kit. PCR was performed in duplicate containing 25 ng of cDNA in each reaction. Reactions were performed on an Applied Biosystems 7500 real-time PCR using optimized cycling conditions of 50°C, 2 min; 95°C, 10 min; followed by 50 cycles of 95°C, 15 s and 50°C, 30 s and 72°C, 15 s.

Table 1 Expression levels and geNorm ranking and stability of HKGs tested in PCOS and control endometrium.

Gene	Calibrated normalized relative quantities: 10 ³									PCOS		Control	
	1	2	3	4	5	6	7	8	9	M-value stability result ^b	Rank ^a	M-value stability result ^b	Rank ^a
<i>CYC1</i>	1022	954	1128	1117	584	1110	963	1144	1138	0.22	3	0.34	4
<i>TOP1</i>	821	1140	761	1197	1322	833	1141	750	1239	0.39	6	0.21	1
<i>18s rRNA</i>	1175	1006	732	913	1788	1151	1041	675	873	0.46	8	0.70	8
<i>ACTB</i>	682	1260	1066	1010	1211	672	1138	1177	990	0.35	5	0.22	3
<i>RPL13A</i>	1044	699	963	1151	691	1303	914	1322	1135	0.22	1	0.53	7
<i>SDHA</i>	1417	1220	1104	607	722	1441	1195	1113	622	0.50	9	0.50	6
<i>YWHAZ</i>	981	947	1256	1020	889	833	859	1222	1079	0.25	4	0.21	2
<i>ATP5B</i>	994	782	1141	1162	816	1056	779	1190	1215	0.22	2	0.46	6
<i>GAPDH</i>	1029	1147	979	977	1648	844	1049	687	685	0.43	7	0.80	9

^aStability ranking of nine HKG within human endometrium of women diagnosed with PCOS.

^bEach target gene underwent logarithmical transformation according to geNorm software to calculate a control gene-stability measure, M. This is defined as the pairwise variation of a particular gene with all other control genes.

A number of methods are used to determine the stability of HKG. One of the more popular is the use of a computer algorithm called geNorm (Vandesompele *et al.*, 2002). This software ranks the HKG in order of stability in a specified tissue. The qRT-PCR data were transformed and processed using the geNorm program for gene stability analysis. We used qbasePLUS software version 1.5 based on geNorm and qbase technology. From this, a stability measure (M) is generated by geometric averaging of multiple target genes and mean pairwise variation of a gene from all other target genes in a given sample (Vandesompele *et al.*, 2002). It is based on the principle that the expression ratio between two ideal control genes is observed in all samples independent of tissue type or experimental conditions. Control genes with the lowest M values are deemed the most stable. In order to identify the most stable and therefore most suitable HKG across different tissue type, the geNorm program introduces a pairwise variation (V) which determines the number of HKG required for accurate normalization per experiment. Pairwise variation, $V (n/n + 1)$ determines the benefit gained from additional HK genes. In most cases, geNorm recommends the use of three reference genes as a valid method of an accurate normalization strategy, compared with a single non-validated reference gene.

Relative gene expression was calculated from normalized cycle threshold (C_T) values using the geNorm software. The value of stability (M) was given a cut off of 1.5 as determined by geNorm. A C_T value provides an indication to the level of gene expression detected during RT-PCR. The value is inversely proportional to the level of gene expression as it indicates the number of cycles required for the machine to detect a fluorescent signal. The difference between two C_T values is described as the delta C_T (ΔC_T). This will also give an indication of the direction of change between the PCOS and control groups. Non-parametric data were analysed using Mann-Whitney U -test. Data were represented as box plot with mean bars and SEM indicated, interquartile ranges are also provided.

Results

The menstrual cycle phase for the PCOS cohort were $n = 4$ patients in the proliferative phase and $n = 5$ in the secretory phase. In the control group, $n = 4$ women were in the proliferative phase and $n = 6$ were in the secretory phase of the menstrual cycle. Further analysis of the androgenaemic profile revealed the mean testosterone profile for PCOS and controls were 3.0 ± 1.2 and 1.6 ± 0.5 nmol/l, respectively.

All nine HKG demonstrated an M value below 1.5 in both tissue types (Table I). The three most stable HKG in the endometrial tissues taken from the PCOS group were $RPL13A > ATP5B > CYC1$ (M values; 0.21, 0.22, 0.22, respectively, see Fig. 1a and Table I). In endometrial tissue for control women, the three most stable HKG were $TOP1 > YWHAZ > ACTB$ (M values; 0.22, 0.22, 0.22, see Fig. 1b and Table I). However, when taking into consideration the endometrial tissue from both control and PCOS groups, the three most suitable HKG, as determined by their expression levels and minimal fluctuation, were $YWHAZ$, $ACTB$ and $CYC1$ (control tissue M 0.34).

These results indicate that the stability of certain HKG in the endometrium differed between the control group and those with PCOS. As to the type of pathology, $TOP1$ was found to be less stable in endometrial tissues from the PCOS women compared with those from control group, while $RPL13A$ appears to become increasingly stable in this tissue type. Few of the HKG displayed stability in the endometrium across the two groups.

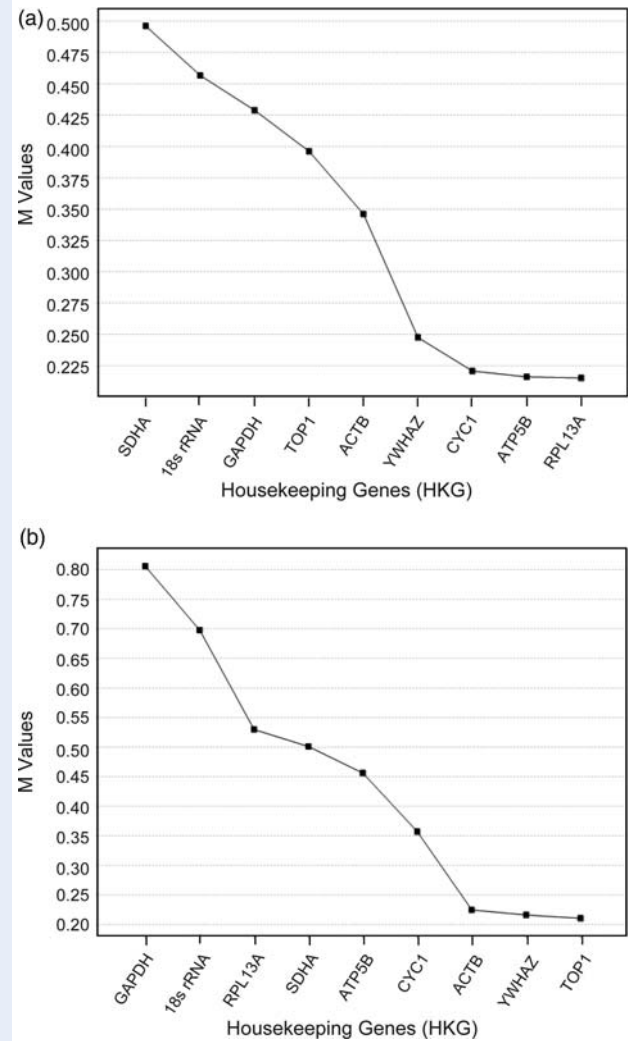


Figure 1 Stability ranking of HKG tested from PCOS (a) and control (b) endometrium. The expression stability value (M -value) was determined using the geNorm program. Genes are ranked left to right in order of increasing expression stability, indicated by lower M values.

Table II Pairwise variation within endometrial tissue.

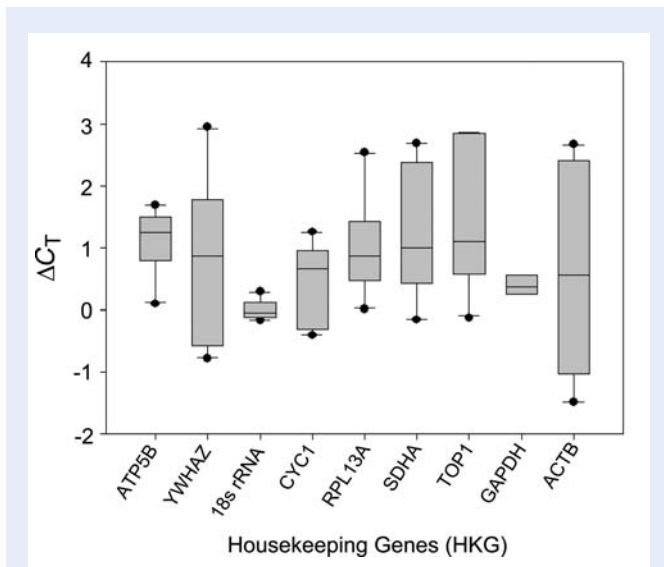
Optimal control gene number		
geNorm V	Control	PCOS
V2/3	0.07	0.07
V3/4	0.12	0.06
V4/5	0.11	0.1
V5/6	0.08	0.07
V6/8	0.07	0.06
V7/8	0.14	0.06
V8/9	0.12	0.06

Table III Mean change in C_T values (mean ΔC_T) of individual HKG expression within the PCOS and control endometrium.

HKG	<i>APT5B</i>	<i>YWHAZ</i>	<i>18S rRNA</i>	<i>CYC1</i>	<i>RPL13</i>	<i>SDHA</i>	<i>TOP1</i>	<i>GAPDH</i>	<i>ACTB</i>
Mean ΔC_T ^a	1.09 ± 0.53	0.87 ± 1.45	-0.01 ± 0.15	0.41 ± 0.66	1 ± 0.85	1.29 ± 1.29	1.51 ± 1.18	0.37 ± 0.16	0.64 ± 1.68
Percentile ^b									
25	0.8	-0.6	-0.1	-0.3	0.5	0.4	0.6	0.2	-1.0
50	1.3	0.9	-0.1	0.7	0.9	1.0	1.1	0.4	0.5
70	1.5	2.5	0.1	1.0	1.4	2.4	2.8	0.5	2.4

^aMean ΔC_T of HKG expression between PCOS and control endometrium with SD calculation.

^bQuartile range given in three divisions.

**Figure 2** Mean changes in C_T values (ΔC_T values) of HKG between the PCOS and control endometrium. Values are means ± SEM.

The optimal number of HKG (reference genes) can be determined when V drops below 0.15 (see Table II). The pairwise variation analysis data show that for endometrial tissue from the PCOS group, the optimum number of reference HKG targets is two (geNorm $V < 0.15$ when comparing a normalization factor based on the two or three most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets *APT5B* and *RPL13A*. A similar picture was seen for healthy endometrium where the optimal number of reference targets was again two (geNorm $V < 0.15$ when comparing a normalization factor based on the three most stable targets). The optimal normalization factor can be calculated as the mean of reference targets *YWHAZ* and *TOP1*.

Further examination of direction of change in C_T values (ΔC_T) of each HKG was calculated against its expression in both groups (Table III and Fig. 2). This value provides information on the direction of change of HKG between PCOS and control endometrium. *TOP1* and *APT5B* demonstrated the largest changes from control to PCOS. ΔC_T values demonstrated higher gene expression levels for *TOP1* in the endometrium of PCOS compared with healthy control

endometrium (C_T of 24.1 ± 0.38 versus 26.6 ± 0.1 in PCOS versus controls, respectively; $P = 0.003$). Table III provides detailed analysis with inter-quartile range for the non-parametric data set.

Discussion

This study sets out to determine the most stable HKG to use for accurate normalization of gene expression data on endometrium from healthy control women and those with PCOS. Our results indicate that HKG stability varies between endometrium from controls and that from PCOS. Within the PCOS group, *RPL13A* > *APT5B* > *CYC1* were found to be most stable compared with *TOP1* > *YWHAZ* > *ACTB* in healthy control tissue. These novel findings are consistent with recent studies indicating that both the experimental conditions and the presence of pathology can impact on the expression of these commonly used internal controls (Savli et al., 2003; Jiang et al., 2009; Quiroz et al., 2010). The assumption that HKG are equally stable under all conditions must therefore be questioned.

Previous studies of gene expression in the endometrium have employed a variety of HKG. Our study demonstrates that the previously described HKG, *18S rRNA* and *GAPDH*, are less suitable for normalization of endometrial tissue (Kim et al., 2009). It also shows that *YWHAZ*, *CYC1* and *ACTB* are the most stable HKG common to both PCOS and healthy endometrium. Thus, results from pairwise variation recommend the use of two of these HKG when normalizing gene expression in healthy and PCOS endometrium.

It is beyond the scope of this paper to examine the mechanisms behind the variation in mRNA expression of the internal controls. Nevertheless, a general statement about the possible reason for a drop in *TOP1* stability can be made. The variable expression of *TOP1* in PCOS tissue is of particular interest. Much work has examined the association between PCOS and endometrial carcinoma (Hardiman et al., 2003). In addition to increasing the risk of developing endometrial carcinoma, PCOS could also influence the prognosis of women with this tumour. *TOP1* inhibitors have long been used in chemotherapeutic agents for a range of tumours including ovarian carcinoma (Kellner et al., 2002). In recent times, focus has shifted towards the benefit of *TOP1* inhibitors in the treatment of advanced recurrent endometrial carcinoma (Wadler et al., 2003). These studies have found a clinical role for *TOP1* inhibition as a chemotherapeutic medication in endometrial carcinoma. Our findings that *TOP1* becomes variably expressed in PCOS tissue compared with normal

endometrium may add to this knowledge. Furthermore, the increased expression of *TOPI* that we have observed in PCOS endometrium, coupled with studies showing the successful use of *TOPI* inhibitors in the treatment of ovarian and endometrial cancer, and increased risk of developing endometrial carcinoma in PCOS women, suggest that *TOPI* could be a candidate early biomarker for endometrial cancer. Further work is needed to examine the association between *TOPI* in PCOS and subsequent development of endometrial carcinoma.

Gene expression studies in the endometrium have highlighted a variation in expression of genes responsible for physiological and pathological processes (Horcajadas *et al.*, 2007). Among many factors, the variation in HKG used in different studies could account for these changes (Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003). It is likely that the stability of HKG may be altered by various supra-physiological conditions such as control ovarian stimulation during assisted conception and/or the presence of reproductive endocrinological pathology. Future work into endometrial gene expression should be aware of the pitfalls associated with HKG expression instability when interpreting normalized data.

In conclusion, our results indicate HKG stability varies between healthy patients and those with PCOS. If one was conducting gene expression studies on endometrial tissue from PCOS patient and comparing them from healthy subjects, our results indicate that at least two of the three HKG, namely *YWHAZ*, *CYCI* and *ACTB*, should be used as the internal reference for data normalization.

Authors' roles

K.H.S. conceptualized idea, collected samples and performed laboratory work. Analysed data and wrote the paper. F.R.C. conceptualized the idea and assisted in writing the paper. K.D.B. performed laboratory work and analysed data. N.S. collected samples. N.M. analysed data and assisted in writing the paper. Y.C. analysed data and assisted in writing the paper.

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