An RNA spiking method demonstrates that 18S rRNA is regulated by progesterone in the mouse uterus

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Abstract: Identifying suitable housekeeping genes for quantitative RT–PCR in the uterus is problematic, as this tissue undergoes significant structural and functional alterations during the oestrous cycle and pregnancy in response to circulating hormones. The suitability of 18S rRNA as a housekeeping gene in mouse uterus was investigated by introducing an ‘RNA spike’ standard into the reverse transcription reaction. 18S rRNA levels increased by Day 4 of pregnancy and after progesterone administration in ovariectomized mice. We conclude that 18S rRNA is not a suitable housekeeping gene for quantitative RT–PCR analysis in progesterone-responsive tissues, and the RNA spiking method provides a suitable alternative.

Key words: 18S rRNA / housekeeping gene / progesterone / quantitative real-time RT–PCR / uterus

Introduction

Mouse uterine tissues undergo significant hormone-mediated changes during the oestrous cycle and pregnancy. These tissues are used extensively as models for the study of angiogenesis (Walter et al., 2005), decidualization (Kaitu’u et al., 2005), implantation and pregnancy (Jones et al., 2006). In these studies, gene expression is often compared using quantitative (real-time) RT–PCR (QRT–PCR). A housekeeping gene is routinely used to normalize expression during QRT–PCR analysis, accounting for potential differences in the amount of sample and the efficiency of the reverse transcription reaction. This method assumes that the housekeeping gene is expressed at the same level in each sample, and is unaffected by experimental treatment or changes in cell type and activity. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin, two commonly used housekeeping genes, are unsuitable for QRT–PCR studies in the uterus as they are regulated by estrogen (Durrer et al., 2005). Since 18S rRNA expression is considered to be stable in many studies (Banda et al., 2008; Murthi et al., 2008) it was investigated as a suitable housekeeping gene for the mouse uterus during QRT–PCR analysis.

Materials and Methods

Experimental models

18S rRNA levels were measured in mouse uteri collected from two experimental models: early pregnancy and progesterone treatment following ovariectomy (described in Fig. 1). Mice used in these experiments were 6–8-week-old females from the first generation of a C57BL/6J × CBA cross. Samples from early pregnancy were taken on Days 1–5 of pregnancy, where the appearance of a vaginal plug after mating was designated Day 1 of pregnancy. In the progesterone treatment model, mice were bilaterally ovariectomized and left for 7 days to allow the endometrium to regress. On the eighth day following surgery, animals were primed with subcutaneous injection of 17β-estradiol (100 ng in 100 μl peanut oil) (Sigma-Aldrich, St Louis, MO, USA). After a further 48 h, three consecutive daily injections of progesterone (1 mg in 100 μl peanut oil) (Sigma-Aldrich) were given, on Days 10, 11 and 12 after ovariectomy. Three control groups were included, which were treated with either 17β-estradiol only, progesterone only or vehicle only (Walter et al., 2005). Uteri were collected for RNA extraction, from all mice on Day 13 after ovariectomy. Animal experiments were approved by the Monash Medical Centre Animal Ethics Committee.
RNA isolation and cDNA synthesis

Total RNA was extracted from whole uteri using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s method. Contaminating genomic DNA was removed by treatment with 4 U TurboDNase Free (AppliedBiosystems, Foster City, TX, USA). RNA solutions were quantified using nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA), and 1 μg of total RNA was converted to cDNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Equal amounts of a standard RNA spike (described below) were added to each reverse transcription reaction by inclusion in the master mix.

Design and construction of RNA spike

The RNA spike was generated by in vitro transcription of a PCR product amplified from a 300 bp region of the pBluescript phagemid (Stratagene, La Jolla, CA, USA) as previously described (Winnall et al., 2008). This PCR product was amplified using a forward primer of 5′-GTAATACGACT-CACATATG TGC-3′ and reverse primer of 5′-GCAGCTGGCACCACATCCAGGAAA-3′, which were designed based on the pBluescript phagemid sequence. The reverse primer inserted a short poly A region into the final spike product which makes amplification of cDNA using an oligo dT primer possible (although not used in the experiments described herein). The spike PCR product was purified using a PCR purification kit (Qiagen, Hilden, Germany) and in vitro transcribed using T7 RNA polymerase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Following in vitro transcription, PCR product cDNA was removed from the RNA spike solution by treatment with DNasefree (Ambion Inc., Austin, TX, USA) according to the manufacturer’s instructions. The RNA spike was stored in undiluted 1 μl aliquots at −80 °C. 1 μl of a 1000-fold dilution of spike solution per sample was added to the reverse transcription master mix for cDNA synthesis. As equal amounts of RNA spike were added to each cDNA synthesis reaction, only a difference in reverse transcription efficiency would lead to different levels of RNA spike after QRT–PCR analysis. Identical patterns of 18S rRNA and RNA spike detection across experimental groups would indicate that any differences in 18S levels between samples were due to changes in reverse transcription reaction efficiency, and would validate use of 18S as a housekeeping gene in the mouse uterus. Alternatively, differing 18S and RNA spike patterns would indicate a change in 18S levels between experimental groups.

QRT–PCR and statistical analyses

18S rRNA was detected during QRT–PCR using sense 5′-GGCTTACCACATCGAAGGAAA-3′ and antisense 5′-GCTGGAATTACCAGGCGGCT-3′ primers (Ponnampalam et al., 2004), and the RNA spike was detected using 5′-ACTCTATAGGCGGATTGGA-3′ and 5′-GAGCGGATAACATTTCCACA-3′ which amplify an internal region of the RNA spike sequence (Winnall et al., 2008). QRT–PCR was performed using Power SYBR (Applied Biosystems, Foster City, CA, USA) and analysed by an ABI7900 Real-time PCR System. Data were analysed using absolute quantification, presented as fold change compared with a calibrator sample (Ratio(calibrator)/test) = 2−ΔCt, where ΔCt = Ct(calibrator) − Ct(test)). The calibrator was the mean of the Ct values from Day 1 of pregnancy or vehicle-treated mice during the progesterone treatment model. Additionally, relative quantification was used to normalize 18S levels to the RNA spike, using the 2−ΔΔCt method (Livak and Schmittgen, 2001). Data were analysed using a one-way ANOVA with a Tukey’s post-hoc test or two-tailed t-test (GraphPad Software Inc., San Diego, CA, USA). Where Bartlett’s test for equal variances found significant differences in variance, data were log10 transformed prior to analysis by ANOVA.

Results

18S rRNA was significantly increased on Days 4 and 5 of pregnancy compared with Day 1 (P < 0.001, Fig. 2A), consistent with an increase in progesterone levels (Walter et al., 2005). In the progesterone treatment model, 18S rRNA levels were elevated following progesterone treatment, with or without estrogen-priming, compared with vehicle or estrogen alone (P < 0.001, Fig. 2B). These data indicated that either 18S rRNA levels are increased by hormone treatment in the mouse uterus, or that relatively more cDNA was made during the reverse transcription reaction in these samples. In order to examine these possibilities, 18S rRNA levels were compared with those of an RNA spike, which controls for the efficiency of cDNA synthesis in the reverse transcription reaction.

Uterine samples from Days 1 and 5 of early pregnancy and the progesterone model were used to compare 18S rRNA levels to those of the RNA spike. There was no change in the amount of RNA spike detected between Days 1 and 5 of pregnancy (Fig. 2C), nor after estrogen only, or estrogen-primed progesterone treatment relative to vehicle in the estrogen-primed progesterone model (Fig. 2D), as measured by absolute quantification. There was a 7-fold increase in RNA spike levels detected in the progesterone-only group compared with the vehicle (Fig. 2D), indicating that the reverse-transcription reaction occurred more efficiently with these samples, therefore more cDNA was present. However, the increase in 18S levels in these samples was considerably greater (88-fold, Fig. 2B). The RNA spike data were then used for normalization during the calculation of 18S rRNA levels in early pregnancy (Fig. 2E) and progesterone treatment model (Fig. 2F) by relative quantification. In the progesterone model, a 15-fold increase in 18S rRNA levels in the progesterone-treated samples (P < 0.001), and a 10-fold increase in 18S in the estrogen-primed progesterone-treated samples (P < 0.05), were observed (Fig. 2F). These results are consistent with the absolute quantification data in Fig. 2C and take into account the predicted change in reverse transcription efficiency that could occur with the progesterone only treatment. Relative quantification also showed a significant 2.5-fold increase in 18S levels on Day 5 of pregnancy compared with Day 1 (P < 0.05, Fig. 2E), consistent with the absolute quantification data in Fig. 2B. These data demonstrate, firstly, that 18S rRNA levels increase with progesterone exposure, and second, that an RNA spike is useful tool for determining the stability of
expression of potential housekeeping genes during QRT–PCR analysis. Importantly, the RNA spiking method can be used to normalize expression data in samples of tissues where no appropriate housekeeping gene is available.

**Discussion**

Our results predict that progesterone regulates the expression of 18S rRNA in the mouse uterus. 18S rRNA is therefore an inappropriate...
housekeeping gene for QRT–PCR experiments in the hormone-treated mouse uterus, as has previously been demonstrated for GAPDH and β-actin (Durrer et al., 2005). During pregnancy, and with progesterone treatment, the mouse uterus undergoes significant changes which include proliferation of endothelial cells (Walter et al., 2005) and proliferation and decidualization of stromal cells (Rider, 2002). These changes could lead to the regulation of rRNA synthesis in the pregnant or progesterone-treated mouse uterus seen in the present study.

Transcription of 18S rRNA is regulated in a number of cell and tissue types when substantial changes in protein biosynthesis are required (Hannan et al., 1998). The transcription of rRNA is increased with cell growth (Jacob, 1995) and hormonal treatments (Hannan et al., 1995), and decreased during terminal differentiation of muscle cells and a monocyte cell line (Cavanaugh et al., 1995), and during withdrawal of rat hepatocytes from nutrients (Antonetti et al., 1993). It is therefore not surprising that 18S levels change in the uterus with the considerable structural and functional changes occurring during pregnancy and with progesterone treatment. However, it is surprising that the potential for 18S levels to change is often overlooked in real-time PCR studies; many publications use 18S levels for normalization without determining if expression levels are stable.

Endothelial cell proliferation within the progesterone-treated uterus (Walter et al., 2005) is a possible contributor to the increases in 18S levels seen in our results. Although we have extracted RNA from whole uteri, an equal amount of RNA was used in the reverse transcription reaction. Therefore the changes seen should not be attributed to an increased number of cells being used to derive our RNA, and are more likely to have occurred due to changes in the functional state of the cells present.

Our findings have important implications for previous studies, where gene expression data generated from uterine tissue exposed to progesterone has been normalized to 18S rRNA levels, as they indicate that such normalization would be inaccurate. The RNA spiking method has proved valuable in showing that 18S rRNA does not make a good housekeeping gene in this tissue, as well as determining the fold change in uterine 18S rRNA levels by relative quantification. Although the use of an RNA spike is common in microarray experiments where it is used as a quality control for sample amplification and labelling (Yang, 2006), it’s rarely used during relative quantification QRT–PCR analyses (Livak and Schmittgen, 2001; Bettegowda et al., 2006; Bower et al., 2007). The results from the present study suggest an RNA-spiking method should be more widely adopted by researchers during QRT–PCR analysis of gene expression.

In summary, these data should discourage the use of 18S rRNA as a housekeeping gene for QRT–PCR in the progesterone-treated or pregnant mouse uterus. The data further suggest that care should also be taken in the use 18S as a housekeeping gene of any progesterone-responsive tissue. We have also demonstrated that the RNA spiking approach is an easy and useful method to determine the validity of a housekeeping gene for QRT–PCR analyses and can be used during relative quantification of gene expression.

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