Identification of reliable reference genes for gene expression studies in maternal reproductive tissues and fetal tissues of pregnant cows

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Abstract

Background: The relationship between the conceptus and the maternal uterine environment is crucial for the successful establishment and maintenance of pregnancy in cattle. Gene expression analysis of the conceptus and maternal reproductive tissues is a favorable method to assess the embryonic maternal interaction. The reliability of the commonly used method reverse transcription-quantitative polymerase chain reaction (RT-qPCR) depends on proper normalization to stable reference genes (RGs). The objective of this study was to determine the expression stability of ten potential RGs (*SUZ12*, *CNOT11*, *ACTB*, *RPL19*, *RPS9*, *GAPDH*, *TBP*, *HPRT1*, *SDHA* and *PPIA*) in maternal reproductive tissues and fetal tissues, and to analyze the effect of RG selection on the calculation of the relative expression of target genes.

Results: The expression stability of ten potential RGs was analyzed in eight different tissues (caruncular endometrium, intercaruncular endometrium, corpus luteum, ovary, oviduct, mammary gland, embryonic disc and trophoblast) from three pregnant dairy cows. Three programs—GeNorm, NormFinder and Bestkeeper—were used to identify the best RGs. According to all three programs, the most stable RG was *CNOT11*, whereas the least stable RGs were *GAPDH* and *HPRT1*. GeNorm analysis showed that a combination of five RGs (*SDHA*, *PPIA*, *CNOT11*, *RPS9* and *RPL19*) was necessary for appropriate data normalization. However, NormFinder analysis indicated that the combination of *CNOT11* and *PPIA* was the most suitable. When target genes were normalized to these RGs, the relative expression of the Radical Sadenosyl methionine domain containing 2 gene was not affected by the choice of RGs, whereas a large difference was observed in the expression profile of the Nuclear erythroid2-related factor 2 gene between the most stable RGs and least stable RGs.

Conclusions: The results indicate that careful selection of RGs is crucial under different conditions, especially for target genes with relatively small fold changes. Furthermore, the results provide useful information for the selection of RGs for evaluating genes affecting bovine reproduction.

Background

Reproductive performance substantially affects the profitability of dairy production. High rates of pregnancy loss, including both embryonic and fetal mortality during early gestation, are a key factor that decreases reproductive efficiency. The fertilization rate of dairy cattle is above 90%, whereas the average calving rate is approximately 55%; most pregnancy loss is thought to occur during the pregnancy recognition/pre-implantation period [1, 2]. During the critical window, day 19 post insemination is a key time point for the initiation of implantation and the end of the maternal recognition of pregnancy [3]. The trophoblast cells are apposed to microvilli of the luminal epithelium of the endometrium on days 19-20 of pregnancy, and the trophoblast loosely communicates with the luminal epithelium on day 18 [4, 5]. The ideal elongation of the embryo involves a complex interaction between the conceptus and the maternal system [6]. Notably, increased embryo loss during the pre-implantation period may be associated with alterations in conceptus-endometrium crosstalk. Investigating the potential interaction mechanisms between the conceptus and associated maternal tissues would not only improve understanding of

pregnancy establishment and maintenance, but also aid in the development of management to decrease pregnancy loss.

The study of gene expression within the conceptus and maternal reproductive tissues is a favorable method to detect the embryonic maternal interaction in cattle. However, most previous studies have focused on either the conceptus or the maternal endometrium, but few studies have investigated the crosstalk between mother and conceptus, except for two pioneering studies using RNA-Seq [7] and microarray hybridization technologies [8]. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is currently the most accurate and rapid method to validate high throughput transcriptomic results; it is also commonly used alone for gene expression measurement, even for genes with low transcription levels [9]. Because of the difficulty in determining the optimal normalization strategy to address the inherent variations in RT-qPCR studies, reference genes (RGs), also known as internal control genes or housekeeping genes, are commonly used in gene expression analysis [10]. An ideal RG should be consistently expressed in the samples regardless of the tissue, experimental condition or treatment. The choice of stably expressed genes for target gene normalization is crucial for accurate analysis of experimental results [11]. Unfortunately, no RG is generally appropriate in all contexts, and even the best RGs vary among different tissues [12]. Therefore, the selection of optimal RGs is an important prerequisite for RT-qPCR experiments.

Studies aimed at exploring the most suitable RGs in bovine species have been performed in specific tissues of the maternal reproductive system, such as the endometrium [13], endometrial explant [14], ovary [15], corpus luteum [16], myometrium [17] and mammary gland [18], as well as in embryos tissue under different experimental conditions [19, 20]. However, RGs for expression analysis in both maternal reproductive tissues and fetal tissues have not been examined. Thus, the aim of this study was to assess the expression stability of ten candidate RGs (polycomb repressive complex 2 subunit (*SUZ12*), CCR4-NOT transcription complex subunit 11 (*CNOT11*), actin beta (*ACTB*), Ribosomal protein L19 (*RPL19*), Ribosomal protein S9 (*RPS9*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA box binding protein (*TBP*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and peptidylprolyl isomerase A (*PPIA*)) by using 24 samples representing maternal reproductive and fetal tissues (caruncular endometrium, intercaruncular endometrium, corpus luteum, ovary, oviduct, mammary gland, embryonic disc and trophoblast) from three pregnant dairy cows and to identify reliable RGs through three commonly used programs: GeNorm, Normfinder and Bestkeeper. Furthermore, two target genes, Nuclear erythroid2-related factor 2 (*NRF2*) and Radical S-adenosyl methionine domain containing 2 (*RSAD2*) were normalized to these RGs on the basis of expression stability, and the effect of RG selection on expression calculations for target genes involved in establishment and maintenance of pregnancy in bovine was also examined.

Results

The amplification efficiencies and specificity of the candidate RGs

To evaluate the expression stability of the ten selected genes under pregnancy conditions, we collected both maternal and fetal samples. In total, eight different types of tissues from three pregnant dairy cows were used for gene expression analysis with RT-qPCR. The RNA integrity and purity for each tissue were measured (Additional file 1: Fig. S1–Additional file 2: Table S1), and qualified samples were used for RT-qPCR. The melting curve for each gene showed a single peak, thus indicating a single real time PCR product (date not shown). The amplification efficiencies of the primers varied from 85.0% to 100.4%. The coefficient of determination varied between 0.917 and 0.999 (Table 1). The quantification cycle (Cq) values for all tissues ranged from 16.79 and 29.98 (Fig. 1). The Cq values for caruncular endometrium tissue ranged from 21.01 to 28.16, whereas the Cq values for trophoblast tissue ranged from 16.79 to 27.92 (Fig. 1b, 1g).

Gene expression stability analysis

GeNorm software ranked the RGs according to their *M* values, from the largest to smallest, and identified *RPL19* as the most stably expressed gene, followed by *RPS9* and *CNOT11*, the commonly used reference gene *GAPDH*, and *HPRT1*, which was the least stably expressed gene (Fig. 2a). With the cut-off value of 0.15, five or six of the best GeNorm RGs were suggested to normalize the gene expression results (Fig. 2b). Thus, *SDHA*, *PPIA*, *CNOT11*, *RPS9* and *RPL19* were selected as sufficient RGs by using GeNorm.

According to NormFinder, *PPIA* was the least variable gene, with a stability value of 0.394, followed by *CNOT11* (0.422) and *RPL19* (0.453) (Table 2). Similarly to the GeNorm analysis results, *GAPDH* and *HPRT1* performed poorly as compared with all the other RGs, and had the highest stability values of 0.687 and 0.597, respectively. Additionally, *CNOT11* and *PPIA* were identified as the best RG combination, with a stability value of 0.264.

Bestkeeper calculated an SD > 1 for *ACTB* (1.47), *GAPDH* (1.84), *SDHA* (1.04), *HPRT1* (1.05) and *PPIA* (1.13). The other five RGs showed SD ≤ 1 and were used for further calculations: *RPS9* (1.00), *CNOT11* (0.81), *TBP* (0.90), *RPL19* (0.79) and *SUZ12* (0.71). Pair-wise correlation analysis was performed with Pearson correlation coefficients and *P*-values (Table 3). The results showed a strong correlation ($r > 0.8$) for *RPS9*, *CNOT11* and *TBP* as well as high significance levels ($P = 0.001$), and a medium correlation for *RPL19* and *SUZ12*, with high significance levels ($P = 0.001$ and 0.004, respectively). Therefore, *RPS9* and *CNOT11* were identified as the best RGs according to Bestkeeper.

To combine the results of GeNorm, NormFinder and Bestkeeper programs, we calculated the average rank. According to Bestkeeper, *ACTB*, *GAPDH*, *SDHA*, *HPRT1* and *PPIA*, with a high SD, were excluded from the calculation, and the average ranking of the remaining five candidates *SUZ12*, *CNOT11*, *RPL19*, *RPS9* and *TBP* in the three programs was 6.67, 2.33, 2.67, 2.33 and 4.67, respectively (Table 4). The results indicated that *CNOT11* and *RPS9* were the most stable RGs in the examined tissues, followed by *RPL19*.

Effect of RG on target gene relative quantification

To evaluate the effects of the above RG selections, we used RGs with various stability (Fig. 3) to calculate the relative expression of *NRF2* and *RSAD2* in both maternal reproductive tissues and fetal tissues. After normalization to the most stable gene (*CNOT11*) in all three programs, the optimum pair (*CNOT11* and *PPIA*) of RGs according to NormFinder analysis, and the combination of the five best genes (*SDHA*, *PPIA*, *CNOT11*, *RPS9* and *RPL19*) according to GeNorm analysis, the relative expression of *NRF2* was lower in corpus luteum tissue and higher in the other tissues (Figure. 3a). However, the expression of *NRF2* was lower in embryonic disc and trophoblast tissue as well as the corpus luteum when the least stable gene (*GAPDH*) was used (Figure. 3b). When both the most stable RGs and the least stable RGs were used to normalize the expression of *RSAD2*, the relative expression of *RSAD2* was low in embryonic disc and trophoblast tissues but higher in caruncular and intercaruncular endometrium tissues (Figure. 3c, d). The choice of control genes had no significant effect on the *RSAD2* expression pattern, whereas it did affect the relative expression of *NRF2* among all the tissues.

Discussion

The selection of suitable RGs for tissues of interest is critical for experimental design. Nevertheless, identifying universal RGs stably expressed in various types of tissues is difficult. Using RGs with varying expression may result in misinterpretation of gene expression data. Therefore, we evaluated the expression stability of ten candidate RGs in maternal reproductive tissues and fetal tissues of pregnant cows in the present study. Stability rankings obtained with the GeNorm, Normfinder and Bestkeeper algorithms revealed that *CNOT11* and *RPS9* were the most stable genes among the ten candidates. They were also used as suitable RGs in other tissues. Previous RT-qPCR studies have shown that *C2orf29*, which is currently listed as *CNOT11* in the NCBI database, has the highest stability in the bovine endometrium [13], corpus luteum [16] and myometrium [17], whereas *RPS9* is the best RG in ovary [15] and mammary tissues [21]. The ranks of *CNOT11* and *RPS9* differed slightly among the three algorithms. *CNOT11* was ranked second by both NormFinder and Bestkeeper, but third by GeNorm. *RPS9* was identified as the most stable gene by Bestkeeper, but second and fourth by GeNorm and NormFinder, respectively. Given the variability of the ranking, we recommend *CNOT11* as the best RG in maternal reproductive tissues and fetal tissues of pregnant cows.

As mentioned above, the most stable RGs identified in RT-qPCR studies may differ depending on the statistical program used [22]. In our study, it should be emphasized that Normfinder selected *PPIA* as the best RG alone and as well as *PPIA* and *CNOT11* as the best gene combination, whereas Bestkeeper determined a high SD for *PPIA*, indicating the least stability. Additionally, GeNorm showed moderate stability of *PPIA*. The different expression stability revealed by all of the three programs may have been because Normfinder considers the overall stability as well as any groups present in the tested samples, and it is not significantly influenced by co-regulation of candidate RGs [23]. Furthermore, it should be noted that the application of *PPIA* as RG was different in other tissues. *PPIA* has been found to be stable in the adipose tissue and mammary gland in goats [24], and it has been recommended as an RG for gene expression analysis of oocytes [25]. However, it is the most unstable gene in bovine adipose tissue, muscle, mammary gland and liver [18]. In agreement with our findings, *PPIA* expression was detected in

many types of tissues. Our results highlight that validation of RGs in RT-qPCR studies is necessary through comparing the stability of potential genes according to the experiment, even if they have been used in previous studies.

GAPDH and *HPRT1* were identified as the least stable genes as ranked by all the statistical programs. *GAPDH* is one of the most frequently used RGs, but we do not recommend it because of its potential regulation in various physiological states. Its expression is changed by the presence of estrogens [26], and it has low expression stability in bovine ovaries [15], corpus luteum [16] and myometrium [17]. *HPRT1*, a metabolism-related gene, has also been identified as the least stable gene in bovine ovaries [15] and myometrium [17]. Our results were in agreement with the findings of these studies: both *GAPDH* and *HPRT1* showed the lowest stability among the candidates. However, Lesage-Padilla et al. have suggested that the combination of *GAPDH*, *CNOT11* and *SLC30A6* is appropriate for accurate normalization in bovine endometrium [27]. Luchsinger et al. have reported that a combination of *GAPDH*, *HMBS* and *EEF1A2* is optimal to normalize gene expression in bovine blastocysts [20]. In addition, *HPRT1* together with other RGs has been used for normalization in cattle embryos [28]. The results suggest that the use of *GAPDH* or *HPRT1* as the sole RG in maternal reproductive tissues and fetal tissues may not be feasible. Whether the two genes can be used in conjunction with other RGs requires further research.

In addition, GeNorm can indicate the optimal number of RGs that should be used in studies of gene expression (Fig. 2b). Our results indicated that V5/6 pairwise variation was below 0.15, and five RGs were required for normalization of RT-qPCR data. It has been suggested that a minimum of three RGs be used between samples from the same tissue [29]. Cieslak et al. have proposed that a combination of four RGs is necessary for normalization in equine milk somatic cells [30]. Even more RGs are needed for analysis of oocyte gene expression from buffalos (seven) and cattle (nine) [25]. The samples detected in those studies involved one type of tissue or cell under different conditions. When the number of tissue types or species increases in GeNorm analysis, the number of RGs required for normalization may increase [18]. Saremi et al. have suggested that five RGs are required for data normalization in bovine adipose tissue and mammary glands [31], and the same number of RGs is also necessary for gene normalization in bovine adipose tissue, muscle, mammary glands and liver [18], in agreement with our results. In our study, the tested samples consisted of eight different tissues including maternal and fetal subgroups, and five RGs were needed to lower the *V* and *M* values and consequently decrease the technical standard error.

Finally, to confirm the effect of the selection of proper types and numbers of RGs in the gene expression studies, we assessed the expression of *NRF2* and *RSAD2* in maternal reproductive and fetal tissues of pregnant dairy cows by using four different groups of RGs with the most and least stability. We observed similar tendencies for *NRF2* and *RSAD2* after normalization to *CNOT11* (the best single gene according to the three programs); *CNOT11* and *PPIA* (the best combination according to NormFinder); or *SDHA*, *PPIA*, *CNOT11*, *RPS9* and *RPL19* (the best combination according to GeNorm). Interestingly, after normalization to the best single gene, *CNOT11*, and the worst single gene, *GAPDH*, there was a difference between *NRF2* and *RSAD2*. Although the relative expression of *RSAD2* was low in embryonic disc and trophoblast tissues regardless of the RGs used, the relative expression of *NRF2* was low in embryonic disc and

trophoblast tissues only when *GAPDH* alone was used as the RG. *RSAD2* is one of the 20 most abundant genes in endometrium tissues in early pregnancy in bovines, and its expression differs significantly between the endometrium and conceptus [7], thus possibly explaining why the relative expression of *RSAD2* was not affected by the choice of RGs. In agreement with our results, the expression of *PGR* and *OXTR* among group comparisons has not been found to differ when RGs with different stability are used [13, 17]. *NRF2* plays an important role in protecting bovine mammary epithelial cells and bovine endometrial cells against oxidative damage [32, 33]. However, the relative expression in maternal reproductive tissues and fetal tissues during early pregnancy is unknown. Our study is the first to investigate the relative expression of *NRF2*, and the results showed that *NRF2* was low in the corpus luteum and higher in the other tissues after normalization to the most stable RG or combinations of RGs. *NRF2* was low in the corpus luteum, embryonic disc and trophoblast after normalization to the least stable RG. The expression values of *NRF2* in the embryonic disc and trophoblast were 1.80 and 2.16, respectively, after normalization to *CNOT11*. The values were 0.39 and 0.30, respectively, after normalization to *GAPDH*. The difference in the expression pattern of *NRF2* confirmed that careful selection of the optimal RGs is important for the interpretation of the RT-qPCR results, especially for genes with low fold changes.

Conclusions

Under the conditions of the present study, the stability of the ten candidate RGs varied in the outcomes of the programs used. According to GeNorm, NormFinder and Bestkeeper analysis, *CNOT11* is recommended as the best single RG. In contrast, *GAPDH* or *HPRT1* should not be used as the sole RG for data normalization in maternal reproductive tissues and fetal tissues. The combination of five RGs (*SDHA*, *PPIA*, *CNOT11*, *RPS9* and *RPL19*) is necessary for appropriate data normalization according to GeNorm analysis, whereas the combination of *CNOT11* and *PPIA* is most suitable according to NormFinder analysis. When the target genes were normalized to these RGs, a large difference was observed in the expression pattern of the *NRF2* gene between the most stable and the least stable RGs, thus confirming that careful selection of the best RGs is critical for each experimental condition, especially when there are small differences in gene expression. The results obtained from this study provide valuable information for future gene expression analysis in bovines during pregnancy.

Methods

Animals and treatments

All animal procedures were performed according to protocols approved by The Hubei Province for Biological Studies Animal Care and Use Committee. Protocols were carried out in accordance with Hubei Provincial Regulation on Administration of Laboratory Animals (10/1/2005). License for all experimental animals was approved by Hubei Provincial Department of Science and Technology: SYXK(Hubei) 2014-0081.

Six multiparous dairy cows were selected from a standard dairy farm of Wuhan Jinxi Animal Husbandry Science and Technology Development Co. Ltd., China. The breeding system and fertility of those cows were normal, as confirmed by veterinary examination. Estrous *synchronization and a* superovulation program were implemented on the 9th day of the estrus cycle. Pituitary follitropin (FSH; Ningbo Sansheng Pharmaceutical Co., Ltd., Ningbo, China) was injected intramuscularly at a dosage of 70 IU (a.m.), 70 IU (p.m.), 60 IU, 60 IU, 60 IU, 60 IU and 50 IU, 50 IU every day for 4 days (total dose, 480 IU per cow). Cloprostenol sodium (Ningbo Sansheng Pharmaceutical Co., Ltd., 0.5 mg) was administered 60 h after the FSH treatment began, and Gonadorelin (Ningbo Sansheng Pharmaceutical Co., Ltd., 200 µg) was administered 108 h after the FSH treatment began. The animals were assessed for signs of estrus after the end of the injection, and five cows that came into standing estrus at similar times were used. All animals were kept in the same environment and inseminated with the semen from one bull.

Tissue collection

To ensure pregnancy success, we detected the steroid hormone progesterone (P4) in the peripheral blood once daily from day 0 to day 7 after estrus and from day 14 until slaughter. The pregnancy diagnosis on day 18 post insemination was confirmed by the detection of interferon stimulated genes (*ISGs*) expression in combination with P4 concentration (Additional file 3: Table S2). Three pregnant cows were head-only electrically stunned and subsequently slaughtered on day 19 at a local abattoir. The reproductive tract of each cow was collected within 30 min of slaughter and immediately placed on ice. The mammary gland tissues were sampled simultaneously. After the sample collections, an innocuity treatment for the dairy cows were performed following the standard operating rules of Hubei Provincial Regulation on Administration of Laboratory Animals. In the laboratory, the uterine horns ipsilateral to the ovary containing the corpus luteum were flushed with warm phosphate buffered solution (PBS). The collected embryos were observed under a stereomicroscope; for morphologically normal embryos (n=24; 8 recovered from each cow), the embryonic disc and the trophoblast were sampled and stored separately. The flushed uterine horns were opened longitudinally, and strips of endometrium (caruncular and intercaruncular) were carefully removed with curved scissors. The ovaries were cut longitudinally, the part of the zona parenchymatosa surrounding the corpus luteum was collected, and the corpus luteum tissues were also separated. The oviducts were separated from surrounding tissues, washed with sterile PBS, and transferred to Petri dishes. Subsequently, they were cut into three parts (isthmus, ampulla and infundibulum), and part of the ampulla was sampled. All tissues were cut to a maximum thickness of 0.5 cm in any one dimension, then immersed in five volumes of RNAlater (Sigma, St. Louis, MO) and stored for 24 h at 4°C. Subsequently, these samples were transferred from RNAlater to RNA-free tubes and stored at -80°C until RNA extraction.

RNA extraction and cDNA synthesis

The fetal tissues were washed with PBS three times before RNA extraction, and the maternal reproductive tissues were directly sectioned into smaller pieces and submerged in RNA isolation lysis solution. Total RNA was extracted with an RNAPrep Pure Tissue kit (Tiangen Biochemical Technology Co. Ltd., Beijing,

China). RNA concentration and purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI USA), which measures the optical density (OD) at the absorption ratio $OD_{260nm}:OD_{280nm}$ ($OD_{260nm}:OD_{280nm}$ ranged from 1.9 to 2.1); RNA integrity was detected through 1.2% agarose gel electrophoresis. After extraction, cDNA was synthesized from 500 ng of total RNA with a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania), the total reaction volume was 50 μ L.

Reverse transcription-quantitative polymerase chain reaction

Ten RGs were selected to analyze gene expression stability (Table 1). The candidate RGs were selected because they had been used as RGs in bovines in previous studies [14, 15, 17, 19, 28, 34, 35]. The primers of the *PPIA* gene were designed with the online software Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and the other genes were referenced from previous studies [15-18, 35, 36]. The primers were synthesized by Tsingke Biotechnology Co. Ltd. (Wuhan, China). RT-qPCR, including the pre-experiment and formal experiment. In the pre-experiment, a standard curve for each primer pair was calculated. Briefly, cDNA from pooled samples was subjected to preamplification at the optimal annealing temperature. Standard curves were generated by RT-qPCR using five ten-fold serial-dilutions of the preamplified products as templates. Amplification efficiencies (E) were determined for all candidate RGs through serial dilution with the formula $E = [10^{(1-\text{slope})} - 1] \times 100$. The coefficient of determination indicated the linearity of the standard curve. The formal RT-qPCR was conducted in 20 μ L reactions containing 10 μ L 2 $\times$ SYBR Green, 0.5 μ L (10 μ mol/L) forward and reverse primers, 0.5 μ L cDNA template, DNase and RNase-free water (PCR-grade water). The cycling conditions were: 95°C for 60 s; 95°C for 15 s, annealing temperatures (Table 1) for 15 s, and 72°C for 30 s for 40 cycles. For the melting curve and signal acquisition, the fluorescent quantitative PCR products were incubated for 5 s at each step with an increase in temperature of 0.5°C from 65°C to 95°C in each cycle. Each sample was repeated three times, and RT-qPCR was performed with a CFX Connect fluorescence quantitative PCR Detection System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Three algorithms—GeNorm [29], Normfinder [37] and Bestkeeper [10]—were used to evaluate the expression stability of the ten candidate RGs. GeNorm and Bestkeeper use raw C_q values, whereas in Normfinder analysis, the raw C_q values are transformed to relative quantities through the delta- C_t method. The GeNorm algorithm was implemented in QBASEPLUS software. The GeNorm algorithm determines the gene expression stability factor (M), which was calculated as the average pairwise variation of one RG compared with all other RGs. M -values allow for ranking and sorting of the candidate RGs according to their expression stability. Lower values indicate the most stable genes. The lowest number of RGs needed for the optimal determination of gene expression can also be calculated as the GeNorm V value [25]. The Normfinder software estimates not only the variation of the candidate normalization genes but also the variation between the sample subgroup of the sample set. Therefore, all tissues included two groups of maternal reproductive tissues and fetal tissues. This program can also

identify the pair of genes with the highest expression stability [26]. Bestkeeper, an Excel-based tool, calculates the standard deviation (SD) for each RG [10]. Pairwise correlation analysis was used to study the inter-gene relations of all RG combinations. The examined RGs with SD > 1 were considered the least stably expressed. A Bestkeeper index was created from the stably expressed RGs (SD < 1) as a geometric mean of the Cq values from a maximum of ten RGs, which was then compared for each individual gene through pairwise correlation analysis, with each combination assigned a Pearson correlation coefficient value. The average rank was also calculated on the basis of the combined results of the GeNorm, NormFinder and Bestkeeper programs.

Expression profile of target genes *NRF2* and *RSAD2*

NRF2 and *RSAD2* were used as the target genes to determine the effects of RG selection on gene expression. CFX Connect software was used to perform expression profile analysis of target genes in maternal and fetal tissues of pregnant dairy cows. Expression of *NRF2* and *RSAD2* was determined with the most stable RGs and the least stable RGs, which were identified by all of the three programs. The reaction system and cycling conditions were the same as those for the RGs.

Abbreviations

RT-qPCR: reverse transcription - quantitative polymerase chain reaction; RGs: reference genes; *SUZ12*: polycomb repressive complex 2 subunit; *CNOT11*: CCR4-NOT transcription complex subunit 11; *ACTB*: actin beta; *RPL19*: Ribosomal protein L19; *RPS9*: Ribosomal protein S9; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *TBP*: TATA box binding protein; *HPRT1*: hypoxanthine phosphoribosyltransferase 1; *SDHA*: succinate dehydrogenase complex flavoprotein subunit A; *PPIA*: peptidylprolyl isomerase A; *NRF2*: Nuclear erythroid2-related factor 2; *RSAD2*: Radical S-adenosyl methionine domain containing 2; Cq: quantification cycle; P4: progesterone; *ISGs*: interferon stimulated genes; PBS: phosphate buffered solution; SD: standard deviation.

Declarations

Ethics approval and consent to participate

All animal procedures were performed according to protocols approved by The Hubei Province for Biological Studies Animal Care and Use Committee. Protocols were carried out in accordance with Hubei Provincial Regulation on Administration of Laboratory Animals (10/1/2005). License for all experimental animals was approved by Hubei Provincial Department of Science and Technology: SYXK(Hubei) 2014-0081.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LC and SHZ designed the study; LC wrote the paper; LC, JY, MX, YX, BFT and DFW carried out the experiments; XZH and XYD participated in data collection and analysis; SHZ and HBC conducted the study and prepared the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Primers for selected candidate RGs and target genes.

Genes	GenBank accession NO.	Primer sequences 5'-3'	Annealing temperature/ °C	Product length/bp	Efficiencies (%)	Determination Coefficient
<i>SUZ12</i>	NM_001205587	Forward: GAACACCTATCACACACATTCTTGT Reverse: TAGAGGCGGTTGTGTCCACT	60	130	92.1	0.925
<i>CNOT11</i>	XM_002691150.5	Forward: TCAGTGGACCAAAGCCACCTA Reverse: CTCCACACCGGTGCTGTTCT	60	169	93.8	0.996
<i>ACTB</i>	NM_173979.3	Forward: CGGCATCGAGGACAGGAT Reverse: CATCGTACTCCTGCTTGCTGAT	60	169	85.3	0.917
<i>RPL19</i>	NM_001040516.2	Forward: TCGCTGTGGCAAGAAGAAAGTCTGG Reverse: AGCCCATCTTTGATCAGCTTCCG	60	102	100.4	0.999
<i>RPS9</i>	NM_001101152.2	Forward: CGAGTATGGGCTCCGGAACAAACG Reverse: GGGTCTTTCTCATCCAGCGTCAGC	60	105	97.3	0.953
<i>GAPDH</i>	NM_001034034.2	Forward: AAGGGCATTCTAGGCTACACTGAGG Reverse: ACAAAGTGGTCGTTGAGGGCAATGC	60	113	94.6	0.991
<i>TBP</i>	NM_001075742.1	Forward: CCTAAAGACCATTGCACTTCG Reverse: CTTCCTCTTGGCTCCTGTG	60	146	85.9	0.999
<i>HPRT1</i>	NM_001034035.2	Forward: GGATTACATCAAAGCACTGAACA Reverse: CATTGTCTTCCCAGTGCAATT	60	194	94.5	0.990
<i>SDHA</i>	NM_174178.2	Forward: GCAGAACCTGATGCTTTGTG Reverse: CGTAGGAGAGCGTGTGCTT	60	185	85.0	0.960
<i>PPIA</i>	NM_178320.2	Forward: GCAAGCACGTGGTACTTTGG Reverse: TTGTCCACAGTCAGCAATGG	60	120	98.3	0.989
<i>NRF2</i>	NM_001011678.2	Forward: CCAGTACCAGCAACGGCAT Reverse: TGTGTGCTTTCAGGGTGGT	60	134	90.0	0.975
<i>RSAD2</i>	NM_001045941.1	Forward: GCTGAAAGAAGCAGGTATGGA Reverse: CGTACTTCTGGAACCACTCT	58	176	95.0	0.969

Table 2 Ranking of the stability of the RGs determined by NormFinder.

Gene	stability	Rank
<i>PPIA</i>	0.394	1
<i>CNOT11</i>	0.422	2
<i>RPL19</i>	0.453	3
<i>RPS9</i>	0.453	4
<i>TBP</i>	0.476	5
<i>ACTB</i>	0.495	6
<i>SUZ12</i>	0.575	7
<i>SDHA</i>	0.577	8
<i>HPRT1</i>	0.597	9
<i>GAPDH</i>	0.687	10
<i>CNOT11 + PPIA</i>	0.264	Best combination

Table 3 Ranking of the stability of the RGs determined by Bestkeeper.

Gene	Standard deviation (SD)	coefficient of variance (CV)	Correlation coefficient (r)	P-value	Rank
<i>RPS9</i>	1.00	4.67	0.834	0.001	1
<i>CNOT11</i>	0.81	3.05	0.831	0.001	2
<i>TBP</i>	0.90	3.26	0.807	0.001	3
<i>RPL19</i>	0.79	3.82	0.773	0.001	4
<i>SUZ12</i>	0.71	2.70	0.567	0.004	5

Table 4 Ranking of the stability of the RGs determined by the three programs.

Gene	Programs			Average Rank
	GeNorm	NormFinder	Bestkeeper	
<i>SUZ12</i>	8	7	5	6.67
<i>CNOT11</i>	3	2	2	2.33
<i>RPL19</i>	1	3	4	2.67
<i>RPS9</i>	2	4	1	2.33
<i>TBP</i>	6	5	3	4.67

Figures

Figure 1

Box plot of Cq values. The variations in the quantification cycle (Cq) values are shown for all detected genes from maternal and fetal tissues of pregnant cows. Each box represents 50% of the most frequent Cq value. Whiskers depict the 25% minimum and maximum Cq values. The median is represented by the line in each box. (a), all tissues. (b), caruncular endometrium. (c), intercaruncular endometrium. (d), oviduct. (e), corpus luteum. (f), ovary. (g), trophoblast. (h), embryonic disc. (i), mammary gland.

Figure 2

GeNorm calculations. (a) Stability of reference gene expression (M) calculated in GeNorm software. Low M values indicate the best RGs, and high M values indicate the worst RGs. (b) Determination of the optimal number of RGs for normalization. The GeNorm V value shows the pairwise variation between the normalization factors of RGs (V_n/V_{n+1}), and the optimal number of RGs is indicated by values of GeNorm V below 0.15.

Figure 3

Relative gene expression results for NRF2 and RSAD2 after normalization to reference genes with different stability in maternal and fetal tissues of pregnant dairy cows (in contrast to ovary tissue). (a), (c) The relative gene expression of NRF2 or RSAD2 gene after normalization to the most stable gene (CNOT11) identified by the three programs, the optimum pair (CNOT11 and PPIA) of RGs identified by NormFinder, and the five best genes according to GeNorm (SDHA, PPIA, CNOT11, RPS9 and RPL19). (b), (d) The relative gene expression of NRF2 and RSAD2 after normalization to the most stable gene (CNOT11) and the least stable gene (GAPDH) identified by the three programs.

Supplementary Files

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