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Internal Controls for Quantitative Polymerase Chain Reaction of Swine Mammary Glands During Pregnancy and Lactation

S. Tramontana,* M. Bionaz,†‡ A. Sharma,§ D. E. Graugnard,†‡ E. A. Cutler,†[1] P. Ajmone-Marsan,* W. L. Hurley,† and J. J. Loor†‡[2]
*Istituto di Zootecnica, Università Cattolica del Sacro Cuore, 29100 Piacenza, Italy
†Mammalian NutriPhysioGenomics, Department of Animal Sciences, University of Illinois, Urbana 61801
‡Division of Nutritional Sciences, University of Illinois, Urbana 61801
§Animal Genetics Division, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh-243122, India

ABSTRACT

High-throughput microarray analysis is an efficient means of obtaining a genome-wide view of transcript profiles across physiological states. However, quantitative PCR (qPCR) remains the chosen method for high-precision mRNA abundance analysis. Essential for reliability of qPCR data is normalization using appropriate internal control genes (ICG), which is now, more than ever before, a fundamental step for accurate gene expression profiling. We mined mammary tissue microarray data on >13,000 genes at –34, –14, 0, 7, 14, 21, and 28 d relative to parturition in 27 crossbred primiparous gilts to identify suitable ICG. Initial analysis revealed *TBK1*, *PCSK2*, *PTBP1*, *API5*, *VAPB*, *QTRT1*, *TRIM41*, *TMEM24*, *PPP2R5B*, and *AP1S1* as the most stable genes (sample/reference = 1 ± 0.2). We also included 9 genes previously identified as ICG in bovine mammary tissue. Gene network analysis of the 19 genes identified *AP1S1*, *API5*, *MTG1*, *VAPB*, *TRIM41*, *MRPL39*, and *RPS15A* as having no known co-regulation. In addition, *UXT* and *ACTB* were added to this list, and mRNA abundance of these 9 genes was measured by qPCR. Expression of all 9 of these genes was decreased markedly during lactation. In a previous study with bovine mammary tissue, mRNA of stably expressed genes decreased during lactation due to a dilution effect brought about by large increases in expression of highly abundant genes. To verify this effect, highly abundant mammary genes such as *CSN1S2*, *SCD*, *FABP3*, and *LTF* were evaluated by qPCR. The tested ICG had a negative correlation with these genes, demonstrating a dilution effect in the porcine mammary tissue. Gene stability analysis identified *API5*, *VABP*, and *MRPL39* as the most stable ICG in porcine mammary tissue and indicated that the use of those 3 genes was most appropriate for calculating a normalization factor. Overall, results underscore the importance of proper validation of internal controls for qPCR and highlight the limitations of using absence of time effects as the criteria for selection of appropriate ICG. Further, we showed that use of the same ICG from one organism might not be suitable for qPCR normalization in other species.
Key words: internal control gene, quantitative polymerase chain reaction

INTRODUCTION

Quantitative reverse transcription PCR is the method of choice for mRNA expression analysis of small number of genes and is commonly used for verification of microarray results. Although qPCR is an accurate technique, it is subject to errors during the steps from RNA extraction to mRNA quantitative analysis (Valasek and Repa, 2005). Normalization of qPCR data is required to take into account those errors, just as it is the case for traditional methods of mRNA quantification (e.g., Northern blot analysis, ribonuclease protection assay, competitive reverse transcription PCR). Genes that maintain a stable expression level (i.e., amount of mRNA/cell) regardless of the experimental condition, or cell and tissue type, are the most widely used internal control genes (**ICG**) for normalization, also referred as housekeeping genes (Vandesompele et al., 2002).

The classical approach for choosing ICG has been based solely on the absence of statistical effects of a treatment or physiological state on qPCR data. However, this approach is not ideal in studies in which a treatment or physiological adaptation is accompanied by large increases in constitutively abundant transcripts. In this scenario, the expression level of stably expressed genes will appear as downregulated (i.e., it is a dilution effect) and will render these genes unrelia-

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[1]Current address: 1383 Deerfield Dr., Allentown, PA 18104.
[2]Corresponding author: jloor@uiuc.edu

ble as ICG. Dilution effects often seen in metabolically active tissues such as mammary glands (Reinhardt and Horst, 1999; Bionaz and Loor, 2007) give rise to technical artifacts arising from the use of the same amount of starting RNA in each reaction. We recently demonstrated a dilution effect on 9 stably expressed genes in bovine mammary during lactation (Bionaz and Loor, 2007). We hypothesized that a similar situation occurs in porcine mammary gland. In support of this hypothesis, previous studies reported consistent increases of mammary RNA and RNA:DNA ratio between the end of pregnancy and the beginning of lactation in pigs (Hacker and Hill, 1972; Kensinger et al., 1982).

The use of pairwise ratio-stability comparison among several candidate genes (Vandesompele et al., 2002) was an appropriate method to select ICG in a longitudinal study of bovine mammary (Bionaz and Loor, 2007). The method effectively overcomes obvious limitations associated with a simple evaluation of time effects on gene expression and takes into account a potential dilution effect. The objective of this study was to select an appropriate set of ICG for normalization of qPCR data generated from longitudinal pig mammary gland gene expression studies.

MATERIALS AND METHODS

Animals and Tissue Collection

Mammary tissue from 4 crossbred primiparous gilts each on –34, –14, 7, 14, 21, and 28 d relative to parturition (DIM) and from 3 gilts at 0 DIM was used (Hurley et al., 1991; Kim et al., 1999). Tissue was harvested and immediately frozen in liquid N_2 and preserved at –80°C until RNA extraction.

RNA Extraction, RNA Quality Evaluation, qPCR, and Primer Design and Testing

Total RNA from 0.4 to 1.4 g of tissue was extracted with ice-cold Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (Loor et al., 2005). Quantity and purity of isolated RNA samples were analyzed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples (n = 27) with an RNA integrity number of 5 to 9 (median of 8), a range considered appropriate for downstream expression analysis (Fleige and Pfaffl, 2006), were used. Genomic DNA was removed with DNase using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the assessed RNA was diluted to 100 mg/L using DNase-RNase-free water before reverse transcription. Sufficient cDNA was prepared to run all selected genes. Each cDNA was synthesized by reverse transcription PCR and cDNA used for qPCR as described previously (Bionaz and Loor, 2007). Each sample was run in triplicate, and a 6-point relative standard curve plus the nontemplate control were used. The 4-fold-dilution standard curve was made using cDNA from a pool RNA of all the samples. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, Foster City, CA) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems).

Primer Express 3.0 software (Applied Biosystems), optimized for use with Applied Biosystems qPCR Systems, was used for primer design using default features, except for the amplicon length, which was fixed at a minimum of 80 bp. Primers were designed across exon junctions when possible to avoid amplification of genomic DNA. The exon junctions were uncovered blasting the pig sequence against human genome (Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences at the National Center for Biotechnology Information (2008) and University of California, Santa Cruz (Genome Browser Gateway, 2008). Before qPCR, primers were tested using the same protocol as for qPCR without the dissociation step in a 20-μL reaction. Part of the PCR product was run in a 2% agarose gel stained with ethidium bromide to assess presence of the product to an expected size and absence of primer-dimer, and the rest was purified using Qiaquick PCR purification kit (Qiagen) and sent to sequence at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. Only primers with high specificity evaluated by a single band on agarose gel, absence of primer-dimer, amplification of the right cDNA verified by sequencing (Table 1), and a unique peak in the dissociation curve after qPCR reaction were used.

Selection and Evaluation of ICG

Porcine mammary microarray data (Tramontana et al., 2007) were mined to identify a set of potential ICG. Ten genes were chosen among the most stably expressed in this data set following these criteria: 1) only genes with median intensity of 3 SD above background intensity were used; 2) filtered data were uploaded to GeneSpring GX software (Agilent Technologies) and normalized using Lowess; 3) only genes with expression ≥100 (i.e., medium-large mRNA abundance) relative

Table 1. Accession number, gene name, hybridization position, primer sequence, and amplicon size of primers used to analyze gene expression by qPCR

Accession no.	Gene	Primer[1]	Primer[2] (5′-3′)	Amplicon size (bp)	E-value[3]
DQ452569	*ACTB*	F.120	CTTCTGCCATTTTCCTAGGACTTTT	120	1.00E-23
		R.239	AACACCTAGTCAGAAAGGCAAACA		
CV865977	*AP1S1*	F.171	GGAGCTCATGCAGGTTGTTCT	100	2.00E-06
		R.270	GTAGAGGCTGGCGTATCTCTTATAGAC		
CV872150	*API5*	F.196	GCATTTTTAGTAGCATAGGCCCTTT	82	2.00E-10
		R.277	AACTTGAGGGAAGATTAACTGTGGAA		
NM_001004030	*CSN1S2*	F.876	ATGTTTGGAGAGGCCAAGACA	120	1.60e-27
		R.995	AAACCACAGTACAAACCCAGTATGTT		
AJ416019	*FABP3*	F.229	GATGACAGGAAGGTCAAGTCCATT	100	6.00E-12
		R.328	CTAGTTCCCGAACAAGCGTTGT		
AY306198	*LTF*	F.1486	TGCAAATTTGACGAATTCTTTAGTCA	100	1.00E-19
		R.1585	TGTTCACGCCCTGGTCATT		
AY610067	*MRPL39*	F.600	CAAAAGAGAACCTACATTCCTTCACA	100	4.00E-20
		R.699	TCTAATGCCACTTTTGCTTCAACT		
AK231290	*MTG1*	F.270	AGGAGCAGCAGAAAATTATGCAA	100	9.00E-16
		R.369	GGGATGATCTGCTTGATATTTTCA		
AK238842	*RPS15A*	F.444	TGCCGAAAAGAGAGGCAAAC	100	7.00E-24
		R.543	TTCGCCAATGTAACCATGCTT		
NM_213781	*SCD*	F.805	TGGTGATGTTCCAGAGGAGGTACTA	120	2.00E-26
		R.924	TGGCGACGAACAGGCTTT		
NM_033549	*TRIM41*	F.1432	TGTGGTGTGCCGAGAATCC	90	1.00E-11
		R.1521	CCCTGCAGTTTGGCCTTGTA		
NM_153477.1	*UXT*	F.277	GCGGGACTTGCGAAAGGT	100	1.00E-19
		R.376	AGCTTCCTGGAGTCGCTCAA		
NM_004738.3	*VAPB*	F.402	TGAAGACTACAGCACCACGTAGGT	100	4.00E-14
		R.501	TCGAAAGGCTGTAACATCACAGATA		

[1]Direction (F = forward; R = reverse) and hybridization position for each primer (5′–3′).

[2]Exon-exon junction in primer sequences are underlined.

[3]Results of PCR product sequencing were BLAST-searched against all known nucleotides in the NCBl database. The E-value is the score from global alignment of homology with the pig sequence deposited in NCBl; the lower the E-value, the higher the significance. All amplicons hit the appropriate porcine sequence with high homology (low E-value).

fluorescent units were chosen; and 4) only genes with expression ratio ~1.0 between sample/reference (RNA mixture from 5 different porcine tissues not including porcine mammary at the different time points) in at least 30 out of 35 microarrays were used (Figure 1).

Gene Stability Evaluation

geNorm software (Vandesompele et al., 2002; geNorm, 2008) was used for assessing gene expression ratio stability of potential ICG following the pairwise comparison method. geNorm also helps determine the optimal number of ICG and calculates a normalization factor (**NF**) to be used for normalization. The pairwise comparison requires evaluation of multiple genes to select appropriate internal controls. For this reason, we also included in our analysis 9 ICG (*ACTB*, *GAPDH*, *RPS23*, *RPS9*, *MTG1*, *ITGB4BP*, *MRPL39*, *RPS15A*, and *UXT*) previously tested in a longitudinal bovine mammary gland experiment (Bionaz and Loor, 2007). Critical for the use of the pairwise comparison method in selecting appropriate ICG is the absence of co-regulation [common upstream regulator factor(s) or direct regulation of transcript expression between gene products] among the genes tested (Vandesompele et al., 2002). Co-regulation was assessed using Ingenuity Pathways Analysis (Redwood City, CA). The qPCR data representing casein α_{S2} (***CSN1S2***), fatty acid-binding protein 3 (***FABP3***), lactotransferrin (***LTF***), and stearoyl-CoA desaturase (***SCD***) were normalized using the NF calculated with the most stable genes among the ones tested.

Statistical Analysis

A MIXED model with repeated measures (release 8.0; SAS Inst. Inc.,Cary, NC) was used to evaluate the effect of time on raw (i.e., nonnormalized) and normalized mRNA abundance. Compound symmetry was the most suitable covariate structure for repeated-measures analysis. The model included the fixed effect of time (–34, –14, 0, 7, 14, 21, and 28 DIM) and the random effect of gilt. Statistical correlations among nonnormal-

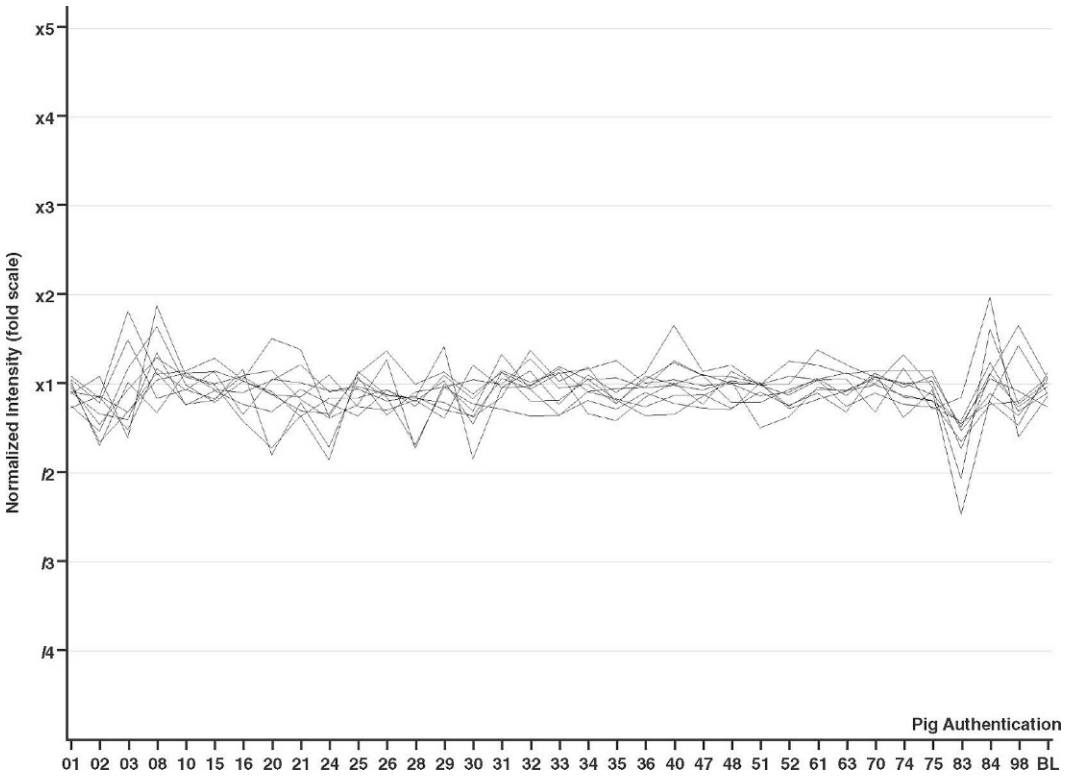

Figure 1. Expression ratio (sample/reference) for each single sample (i.e., gilt and time point) reported as *n*-fold for *AP1S1*, *API5*, *PCSK2*, *PPP2R5B*, *PTBP1*, *QTRT1*, *TBK1*, *TMEM24*, *TRIM41*, and *VAPB*. Figure generated by GeneSpring GX after application of the 4 filtering criteria described in detail in the Materials and Methods section.

ized qPCR data from all genes tested were performed using the PROC CORR procedure of SAS.

RESULTS AND DISCUSSION

ICG Selection and Evaluation

The approach to select ICG from microarray data uncovered *AP1S1*, *API5*, *PCSK2*, *PPP2R5B*, *PTBP1*, *QTRT1*, *TBK1*, *TMEM24*, *TRIM41*, and *VAPB* as the most stable genes among >10,000 annotated transcripts (Table 2). These represented 10 novel, potential ICG with stable expression (ratio sample/reference = 1.0 ± 0.2; Figure 1) throughout the entire lactation. None of the 9 ICG previously tested in bovine mammary tissue (Bionaz and Loor, 2007) were present in the list of potential porcine ICG uncovered by microarray analysis. There was larger variability among samples for these 9 ICG (ratio sample/reference = 1.27 ± 1.25) and a large difference in mRNA abundance between sample and reference for some of those ICG (e.g., *GAPDH* with 0.33 ± 0.16 and *RPS15A* with 2.58 ± 2.09), or both.

Co-regulation among the 10 selected genes from the microarray plus the additional 9 previously tested ICG in bovine mammary (Table 2) was assessed using Ingenuity Pathways Analysis. Ingenuity Pathways Analysis is a Web-based software that uses information from the published literature on humans and rodents (in vivo and in vitro) to generate relationships among genes or proteins. This analysis allowed identification of 10 genes without known co-regulation that served as candidates for stability analysis. Ingenuity Pathways Analysis identified co-regulation among *ACTB*, *GAPDH*, *ITGB4BP*, *PCSK2*, *PTBP1*, *RPS9*, *RPS23*, *TBK1*, and *UXT*, which are part of complex gene networks (Figure 2). These were excluded from further analysis, with the exception of *UXT* and *ACTB*. Apparently, *UXT* and *ACTB* are co-regulated by MYC. In fact, MYC directly affects expression of *ACTB* (Grandori et al., 2005) and also binds the promoter region of *UXT* (Mao et al., 2003). However, there is no direct evidence that MYC actively regulates *UXT* expression in vivo. Despite these apparent limitations, which can affect pairwise comparison analysis (i.e., geNorm), we kept *ACTB* and *UXT* in the subsequent analysis, because *ACTB* is one of the most widely used ICG for qPCR across cell types, tissues, and organisms, and UXT was one of the most suitable ICG in bovine mammary tissue (Bionaz and Loor, 2007).

Table 2. Gene description, cellular location, and main function or component, or both, of the 10 genes selected as the more stable genes with ~1 sample/reference ratio among a temporal porcine mammary array data set plus 9 previously tested internal control genes (Bionaz and Loor, 2007)

Gene	Description	Cellular location[1]	Main function/component[2]
ACTB	β-actin	Cytoplasm	Cytoskeleton component
AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	Cytoplasm	Part of coated vesicle involved in endocytosis and Golgi processing
API5	Apoptosis inhibitor 5	Cytoplasm	Regulation of apoptosis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Plasma membrane	Glycolytic enzyme
ITGB4BP[3]	Eukaryotic translation initiation factor 6	Cytoplasm	Protein synthesis
MRPL39	Mitochondrial ribosomal protein L39	Cytoplasm	Mitochondrial protein synthesis
MTG1	Mitochondrial GTPase 1 homolog (*Saccharomyces cerevisiae*)	Unknown	Unknown
PCSK2	Proprotein convertase subtilisin/kexin type 2	Extracellular space	Protein maturation
PPP2R5B	Protein phosphatase 2, regulatory sub B, β isoform	Cytoplasm	Protein phosphatase type 2A regulator activity
PTBP1	Polypyrimidine tract binding protein 1	Nucleus	mRNA metabolism and transport
QTRT1	Queuine tRNA-ribosyltransferase 1	Unknown	tRNA synthesis/function
RPS9	Ribosomal protein S9	Cytoplasm	Protein synthesis/40S subribosome
RPS15A	Ribosomal protein S15a	Cytoplasm	Protein synthesis/40S subribosome
RPS23	Ribosomal protein S23	Cytoplasm	Protein synthesis/40S subribosome
TBK1	TANK (TNF receptor-associated factor family member-associated NFκB activator)-binding kinase 1	Cytoplasm	Activation and nuclear translocation of the nuclear factor κB complex.
TMEM24	Transmembrane protein 24	Unknown	Unknown
TRIM41	Tripartite motif-containing 41	Unknown	Nuclear transport
UXT	Ubiquitously expressed transcript	Cytoplasm	Transcriptional activation
VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	Plasma membrane	Vesicle trafficking

[1]From Ingenuity Pathway Analysis (Redwood City, CA).

[2]From gene description in NCBI (National Center for Biotechnology Information, 2008).

[3]Also denominated *EIF6* in humans.

Pig-specific sequences for *QTRT1* and *TMEM24* were not available, and sequences of *Homo sapiens* genes were used instead to design specific primers (accession no. NM_031209 and NM_014807.3, respectively). However, those primers were unable to amplify any cDNA. Suitable primers were designed for the remaining 8 genes (*AP1S1*, *API5*, *MTG1*, *MRPL39*, *PPP2R5B*, *RPS15A*, *TRIM41*, and *VAPB*) plus *ACTB* and *UXT* (Table 1). Among these, *PPP2R5B* had very low expression in pig mammary tissue (median cycle threshold = 29.5) and was not considered for subsequent analysis.

ICG Expression Pattern

All ICG tested had the same pattern of expression across the time points studied (Figure 3). There was a progressive decrease in relative mRNA abundance from –34 to 28 DIM for all of the potential ICG measured. This apparent temporal decrease in mRNA abundance parallels previous observations of ICG in bovine mammary tissue (Bionaz and Loor, 2007). In that study, it was clearly demonstrated that this trend was an artifact of the qPCR protocol, which uses equal amounts of RNA for synthesis of cDNA, and the apparent reduction of stably expressed genes throughout lactation was a consequence of a dilution of their mRNA due to the large increase in RNA synthesis of milk-specific genes (e.g., *LALBA*). In support of our hypothesis, Kensinger et al. (1982) reported a sudden increase of RNA concentration in pig mammary at parturition, leading to large increases of tissue RNA/DNA ratio.

ICG and Dilution Effects

Based on the above premises and our data (Figure 3), we hypothesized that the reduction of stably expressed genes (i.e., potential ICG) in porcine mammary during lactation was apparent and could be explained by a dilution effect, as seen previously in bovine mammary (Bionaz and Loor, 2007). To verify the hypothesis, we selected 4 genes from the microarray data whose expression was not only abundant in mammary tissue but also increased markedly from farrowing through lactation: *CSN1S2*, *FABP3*, *LTF*, and *SCD*. All these genes were confirmed via qPCR to be highly expressed in lactating porcine mammary relative to nonlactating tissue (Table 3) and had an extremely large increase in expression at the onset of lactation (Figure 4). Correlation analysis revealed an inverse relationship between the expression of highly abundant genes and ICG (Table 4), particularly for *CSN1S2*, the most abundant transcript during lactation among the ones analyzed (Table 3). Overall, the data support the hypothesis of a dilution effect caused by a large surge in mRNA synthesis of highly abundant transcripts. Interestingly, *FABP3* had a minimal effect on the dilution of stably

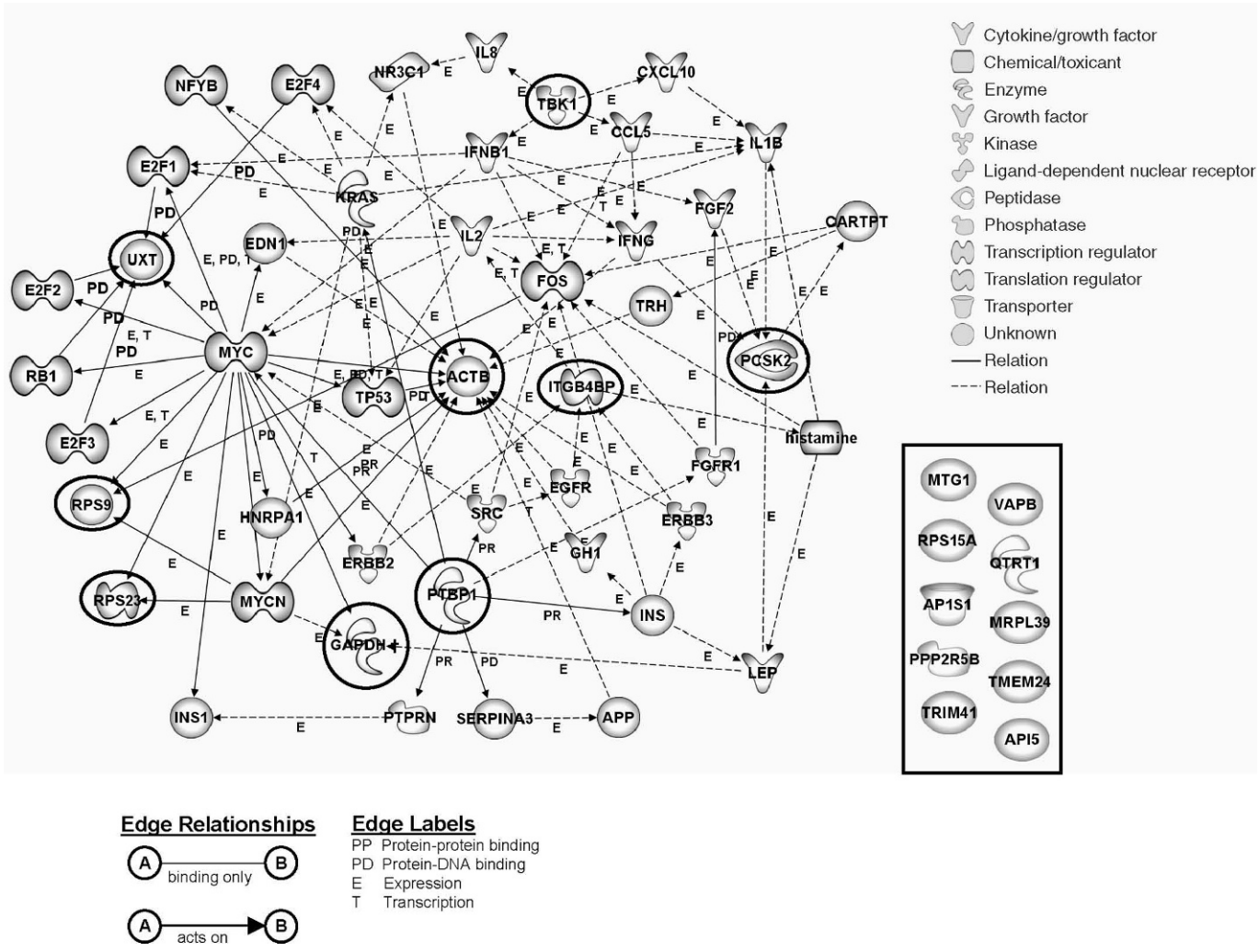

Figure 2. Known interactions that determine co-regulation (i.e., common upstream regulation) among internal control genes (ICG) under investigation. Potential ICG with known co-regulation are circled. The ICG without currently known interactions are framed in a box and were used for subsequent analysis.

expressed genes (Table 3) likely due to its relatively large mRNA abundance at –34 d relative to parturition (Table 3) and its behavior at 0 DIM, which corresponded with an increase in expression of some ICG (e.g., *AP1S1* and *UXT*; Figures 3 and 4).

A significant decrease in ICG mRNA abundance, as a consequence of the dilution effect, such as a pre- vs. postpartum comparison, clearly highlights the limitation in solely relying on a temporal effect as the primary criteria in evaluating the appropriateness of ICG. Thus, an alternative method is essential to overcome this limitation. The pairwise comparison, previously demonstrated to be suitable to identify ICG stability in bovine mammary (Bionaz and Loor, 2007), was applied in this study. The pairwise comparison method is based on the fact that all genes with stable expression are affected to a similar extent by errors during the steps from RNA extraction to qPCR analysis. Therefore, the expression ratios among them should remain constant across samples (Vandesompele et al., 2002). The most stable genes (i.e., lowest M value) were *VAPB* and *MRPL39*, whereas *AP1S1* and *MTG1* were the least stable, followed by *UXT* and *ACTB* (Figure 5). A low reliability of *ACTB* as ICG has been reported previously by others in cells (Glare et al., 2002) and bovine mammary tissue (Bionaz and Loor, 2007). In contrast, Bionaz and Loor (2007) reported *UXT* as an ideal ICG among those tested. Clearly, ICG in the same tissue from one organism might not be suitable for qPCR normalization in other species.

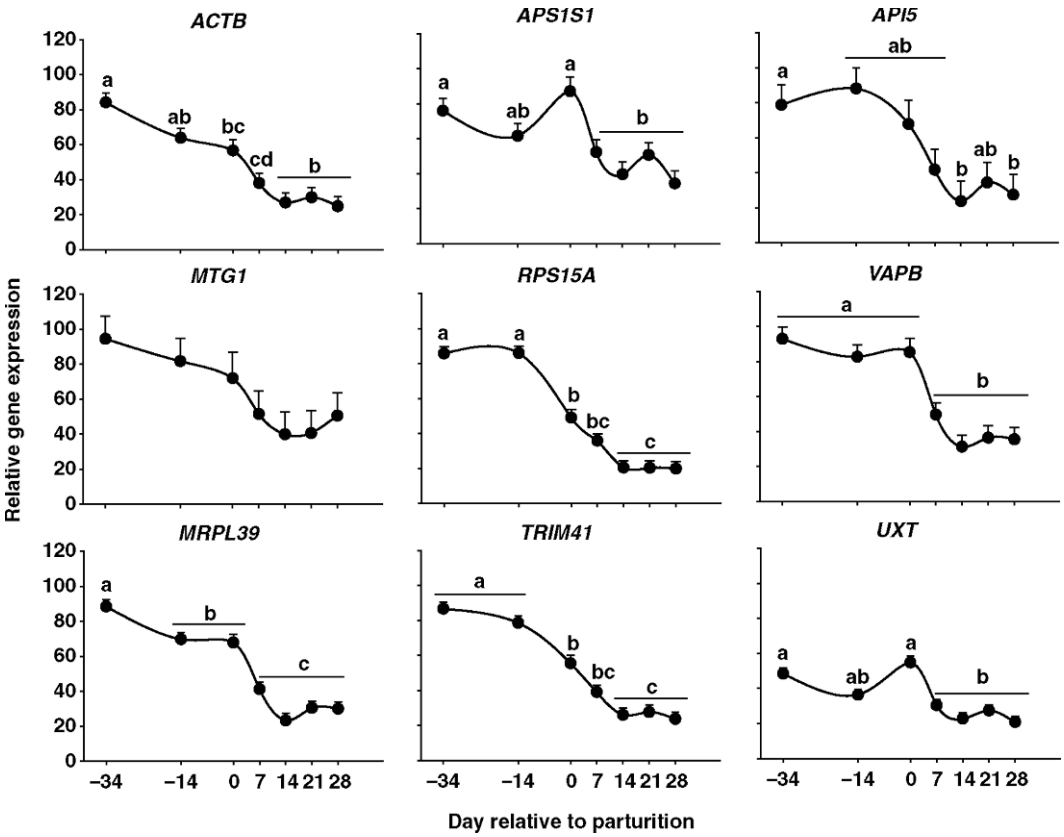

Figure 3. The mRNA expression patterns (mean ± overall SEM) of potential internal control genes. Data shown are from transformed cycle threshold values with a standard curve. Time effect was $P \leq 0.05$ for all genes. Different letters denote time effects among time points.

Table 3. Slope and coefficient of determination of the standard curve (R^2), efficiency (E), median cycle threshold or number of cycles required for fluorescent signal to cross the threshold (Ct), and mRNA abundance of the measured transcripts

Gene	Slope[1]	R^2	E^2	Median Ct	mRNA abundance (DIM)[3]	
					–34	28
ACTB	–3.34	0.993	1.99	22.4	1.0	1.0
API5	–3.05	0.998	2.13	22.5	0.8	1.2
APS1A1	–3.23	0.998	2.04	21.7	1.1	2.3
MRPL39	–3.05	0.994	2.13	20.6	3.1	3.7
MTG1	–3.12	0.998	2.09	22.8	0.6	0.9
RPS15A	–3.35	0.997	1.99	16.3	64	59
TRIM41	–2.83	0.999	2.26	22.0	1.2	1.6
UXT	–2.78	0.989	2.29	25.5	0.1	0.2
VAPB	–3.12	0.983	2.09	21.4	1.7	2.7
CSN1S2	–3.32	0.994	1.99	12.5	0.6	9,467
FABP3	–3.40	0.998	1.97	14.4	63	402
LTF	–4.07	0.998	1.76	17.6	4.5	26
SCD	–3.41	0.996	1.96	16.4	4.4	206

[1]Slope generated by 6-point standard curve.

[2]Efficiency = $10^{(-1/\text{slope})}$.

[3]The mRNA abundance is relative to *ACTB*. Abundance = $1/E^{(\text{median Ct})}$.

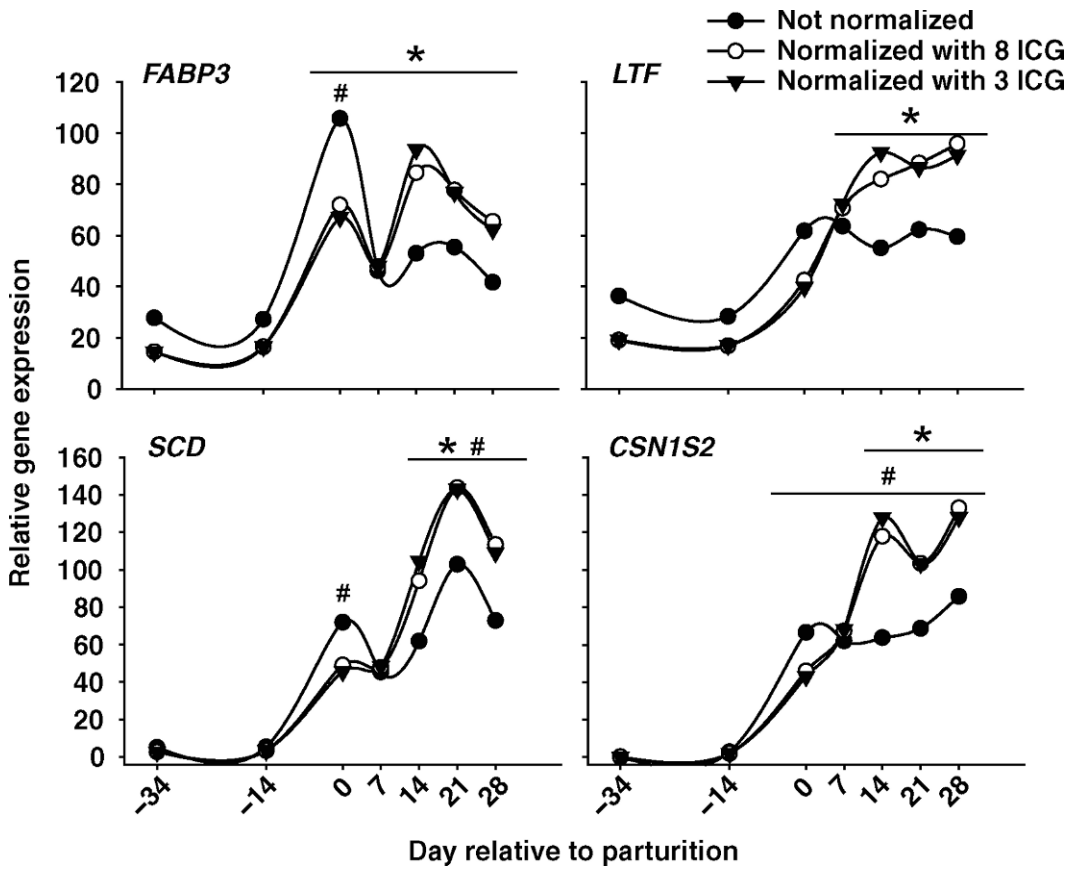

Figure 4. Patterns of *CSN1S2*, *FABP3*, *LTF*, and *SCD* before and after normalization using normalization factor calculated with the geometrical mean of 3 or 8 most stable genes from geNorm analysis. Time effect was $P < 0.01$ for all the genes. The SEM for genes normalized with 3 ICG were as follows: *CSN1S2* = 18.9; *FABP3* = 6.2; *LTF* = 9.3; *SCD* = 14.2. The symbols * and # denote significant ($P < 0.05$) effects relative to –34 DIM for normalized (3 ICG) and not normalized data, respectively.

Considerations for Selecting Optimal Number of ICG

A minimum of 3 genes should be used as ICG (Vandesompele et al., 2002). Results showed a large decrease in the variation of pairwise expression ratios when using 3 genes instead of 2, for instance, from 0.17—pairwise *VAPB* and *MRPL39* (top panel of Figure 5)—to 0.065 in V2/3 (lower panel in Figure 5). Thus, addition of the subsequent most stable gene increased the reliability of NF. The use of 4 genes instead of 3 resulted in a small increase in stability (0.065 vs. 0.060). The maximum reliability of NF would have been obtained using 8 of 9 genes tested (M = 0.044 at V7/8, Figure 5 lower panel). Based on both practicality and M < 0.10, the limit previously set as acceptable (Bionaz and Loor, 2007), use of the 3 most stable genes (i.e., *VAPB*, *API5*, and *MRPL39*) to calculate the NF was highly reliable.

To our knowledge, *VAPB*, *API5*, and *MRPL39* have never been used as ICG. The description, main function, and cellular location of all genes tested are re-

Table 4. Pearson correlation between nonnormalized qPCR data of *CSN1S2*, *LTF*, *FABP3*, and *SCD* with nonnormalized qPCR data of potential internal control genes (ICG) in pig mammary tissue across all time points analyzed[1]

Gene	*ACTB*	*APS1S1*	*API5*	*MRPL39*	*MTG1*	*RPS15A*	*TRIM41*	*UXT*	*VAPB*
CSN1S2	–0.69	–0.42	–0.60	–0.69	–0.39	–0.79	–0.81	–0.43	–0.62
FABP3	–0.15	0.43	–0.06	–0.03	0.03	–0.26	–0.22	0.40	0.11
LTF	–0.56	–0.16	–0.47	–0.53	–0.40	–0.66	–0.59	–0.22	–0.45
SCD	–0.54	–0.15	–0.46	–0.55	–0.28	–0.70	–0.68	–0.23	–0.49

[1]Comparisons for the best ICG are underlined (>0.38 corresponding to $P \leq 0.05$).

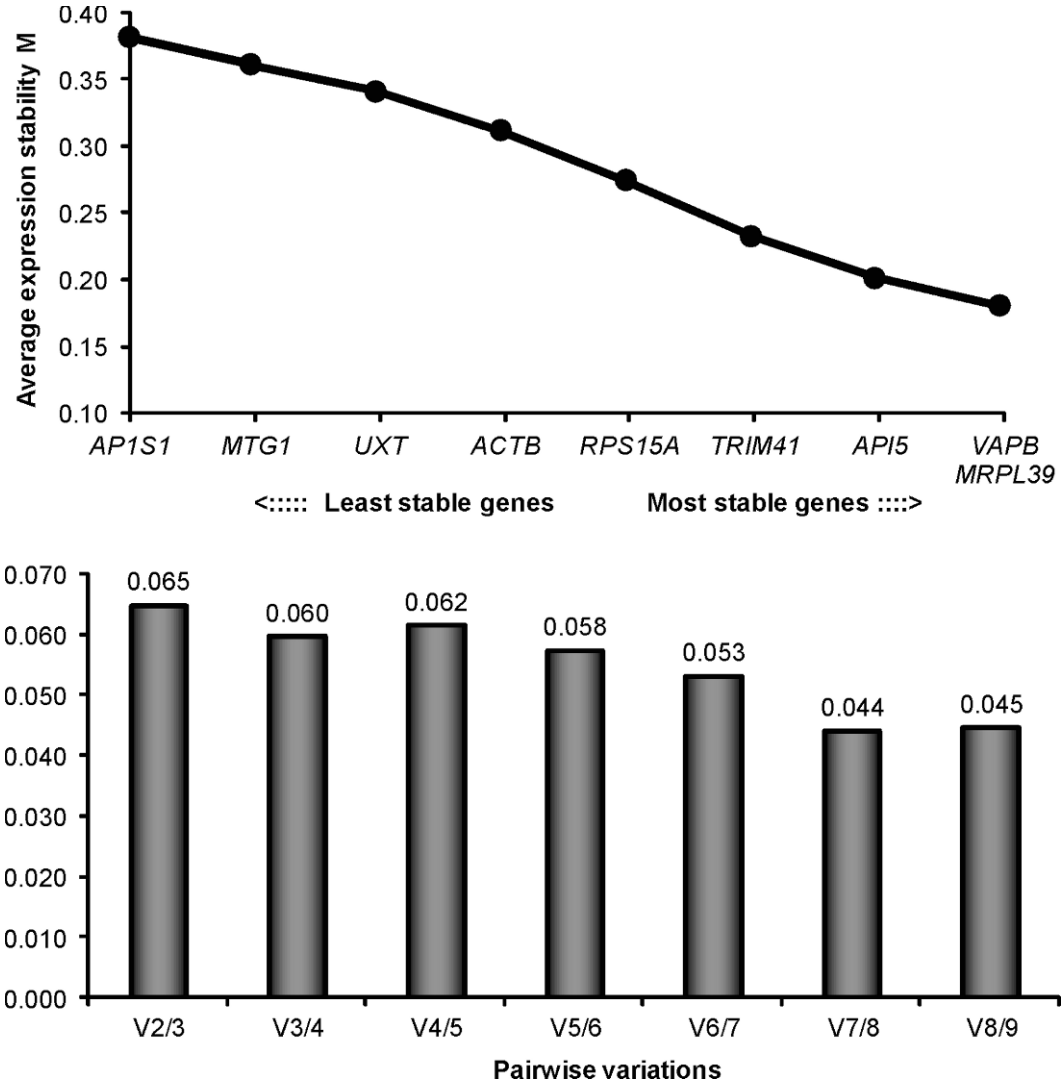

Figure 5. Top panel: geNorm results for the average expression ratio stability (M) of potential internal control genes (ICG) calculated via pairwise comparison. Bottom panel: determination of optimal ICG number for normalization. y-axis = pairwise variation V ($V_n/_{n+1}$) between the normalization factors NF_n and NF_{n+1}; x-axis = comparison between the use of n or $n + 1$ genes to calculate the normalization factor.

ported in Table 2. Vesicle-associated membrane protein (*VAPB*) encodes a membrane protein found in plasma membranes and intracellular vesicle membranes with a likely involvement in vesicle trafficking (Teuling et al., 2007). Apoptosis inhibitor-5 (*API5*) is a protein with potent antiapoptotic activity and with a crucial role in tumor cell survival (Morris et al., 2006). Mitochondrial ribosomal protein L39 (*MRPL39*) is a nuclear gene coding for a constituent of mitochondrial ribosomes, which are essential for the translation of mitochondrial genes mostly involved in oxidative phosphorylation (O'Brien, 2003).

The large discrepancy between nonnormalized and normalized qPCR data of *CSN1S2*, *FABP3*, *LTF*, and *SCD* highlights the importance of normalization (Figure 4). Comparing normalized expression using 8 vs. 3 most stable genes (Figure 5) confirmed that there was minimal difference in normalized qPCR results by having 5 additional ICG to calculate NF (i.e., stability increased minimally). Thus, using only the 3 most stable genes for normalization was most appropriate, and we suggest it is the minimal number of ICG that should be used to improve precision and relevance of qPCR data.

CONCLUSIONS

Results confirmed that a simple statistical analysis of mRNA data would have rejected our candidate ICG as inappropriate because of an apparent temporal ef-

fect on their expression. In addition, we showed that use of the same ICG from one organism might not be suitable for qPCR normalization in other species. This point is particularly important due to the extensive use of *ACTB* and *GAPDH* in the livestock scientific community. We demonstrated that the apparent temporal decrease in ICG between pregnancy through lactation was a consequence of the dilution effect caused by large increases in mRNA synthesis of tissue-abundant genes (e.g., *CSN1A2*, *LTF*, and *SCD*). Use of the pairwise comparison method was suitable for selecting reliable ICG. We identified *MRPL39*, *API5*, and *VAPB* as novel ICG for qPCR in temporal studies of pig mammary tissue.

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