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Identification of Optimal Reference Genes for Examination of Gene Expression in Different Tissues of Fetal Yaks

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ABSTRACT

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Reverse transcription quantitative real-time PCR (RT-qPCR) is widely used to study the relative abundance of mRNA transcripts because of its sensitivity and reliable quantification. However, the reliability of the interpretation of expression data is influenced by several complex factors, including RNA quality, transcription activity, and PCR efficiency, among others. To avoid experimental errors arising from potential variation, the selection of appropriate reference genes to normalize gene expression is essential. In this study, 10 commonly used reference genes – *ACTB*, *B2M*, *HPRT1*, *GAPDH*, *18SrRNA*, *28SrRNA*, *PPIA*, *UBE2D2*, *SDHA*, and *TBP* – were selected as candidate reference genes for six fetal tissues (heart, liver, spleen, lung, kidney, and forehead skin) of yak (*Bos grunniens*). The transcription stability of the candidate reference genes was evaluated using geNorm, NormFinder, and BestKeeper. The results showed that the combination of *TBP* and *ACTB* provided high-quality data for further study. In contrast, the commonly used reference genes *28SrRNA*, *SDHA*, *GAPDH*, and *B2M* should not be used for endogenous controls because of their unstable expression in this study. The reference genes that could be used in future gene expression studies in yaks were indentified.

Keywords: transcription stability; RT-qPCR; *TBP* gene; *ACTB* gene

Reverse transcription quantitative real-time PCR (RT-qPCR) is a rapid and powerful method for the examination of the expression levels of transcripts. Relative quantification by RT-qPCR can determine if the transcript level changes in given samples relative to control samples (Chen et al. 2015). The accuracy of RT-qPCR largely depends on the stability of the reference gene for normalization, which allows for the elimination of potential variants in RNA quality (Huggett et al. 2005), transcription activity (Vandesompele et al. 2002), PCR efficiency (Rekawiecki et al. 2012), and run-to-run variation during multistage experimental processes (Zeng et al. 2016).

The most frequently used reference genes include *glyceraldehyde 3 phosphate dehydrogenase*

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(*GAPDH*), *β actin* (*ACTB*), *hypoxanthine guanine phosphoribosyl transferase 1* (*HPRT1*), *β2 microglobulin* (*B2M*), *peptidylprolyl isomerase A* (*PPIA*), *TATA box binding protein* (*TBP*), *18S ribosomal RNA* (*18SrRNA*), *28S ribosomal RNA* (*28SrRNA*), *ubiquitin conjugating enzyme E2 D2* (*UBE2D2*), and *succinate dehydrogenase complex subunit A* (*SDHA*). It is generally accepted that the expression level of reference genes is constant across all cells, tissues, and environmental conditions (Svingen et al. 2015). However, there has been an increase in the number of studies that have evaluated the suitability of classic reference genes because of differences in age (Touchberry et al. 2006), tissue types (Sakai et al. 2014), thermal stress (Purohit et al. 2015), hormones (Das et al. 2013), and other treatment protocols (Young et al. 2006; Mihi et al. 2011). Studies in different tissue types have revealed that the most commonly used reference genes vary with the tissues studied (Zhang et al. 2013; Zeng et al. 2016). The application of unproven reference genes could lead to erroneous conclusions. For many important target genes, which are typically expressed at a low level, minor fluctuations in the reference gene could result in erroneous findings (Das et al. 2013). Taken together, it is extremely important to validate one or more suitable reference genes for normalizing the RT-qPCR data under every experimental condition across a range of samples or tissues.

Yaks (*Bos grunniens*) are important livestock animals, which are mainly distributed in the Qinghai-Tibetan Plateau and the adjacent alpine regions. Yaks provide milk, meat, fuel, service labour, and other daily necessities for local pastoralists. A number of studies on growth (Hu et al. 2016), cloned embryo development (Pan et al. 2015), and hypoxia (Wu et al. 2015) in yak have used expression analysis with RT-qPCR. In these studies, and in fact in most studies using RT-qPCR methodologies, normalization of expression data was conducted using a single, traditional reference gene as an internal standard. To the best of our knowledge, the reliability of reference genes was only evaluated for yak mammary tissue (Jiang et al. 2016), and no research has been conducted on other yak tissues. Therefore it is essential to identify the optimal reference genes for normalizing the RT-qPCR data.

In this study, we examined the stability of 10 commonly used reference genes (*ACTB*, *B2M*, *HPRT1*, *GAPDH*, *18SrRNA*, *28SrRNA*, *PPIA*, *UBE2D2*, *SDHA*, and *TBP*) in six different tissues of fetal yaks. This work will certainly facilitate future research on gene expression in yaks.

MATERIAL AND METHODS

Sample collection and preservation. Six fetuses of Datong yaks were collected at a slaughterhouse in the Qinghai Tibetan Plateau of Datong County. The fetuses were selected at 80–90 days (as estimated based on the crown-rump length of fetus) (Liu et al. 2010). Heart, liver, spleen, lung, kidney, and forehead skin were collected and frozen in liquid nitrogen until analysis. The entire study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, China.

RNA extraction and reverse transcription. Total RNA was extracted from the collected tissues using the TRIzol reagent (Invitrogen, USA). RNA purity and quantity were detected by NanoDrop 2000 (Thermo Scientific, USA). RNA integrity was verified by 1% agarose gel electrophoresis. The complementary DNA (cDNA) was synthesized from 500 ng total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, China) following the manufacturer's protocol.

Quantitative real-time PCR. Ten reference genes (Table 1) commonly used as reference genes in RT-qPCR assays were selected for evaluation. The primers were designed using Primer Premier 5.0 software, and synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). The qPCR reaction was performed using a CFX-96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Each reaction (20 µl) consisted of 1 µl cDNA, 10 µl SYBR® Premix Ex Taq™ II (TaKaRa), 0.8 µl of each forward and reverse primers (10 µmol/l), and 7.4 µl ddH_2O. Standard amplification conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 55°C for 30 s. Next, melting curve analysis was conducted to determine the specificity of PCR products. Three PCR reactions were performed for each sample and then averaged. No template controls (NTC) were included for each primer. Plate controls were conducted on each plate to normalize the Ct value from multiple plates into a single study dataset. PCR efficiency of refer-

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ence genes was derived from a standard curve generated from serial dilution of pooled cDNA. The mean Ct value of each ten-fold dilution was plotted against the logarithm of the cDNA dilution factor. RT-qPCR efficiency was determined for each gene using slope analysis with a linear regression model. An estimate of PCR efficiency was derived from the formula $E = (10^{-1/\text{slope}} - 1) \times 100\%$ (Kubista et al. 2006).

SYNJ1 encodes synaptojanin 1, which is a key neural protein highly expressed in nerve terminals with an essential role in the regulation of synaptic vesicles. The *SYNJ1* gene was used as a target gene to evaluate the performance of candidate reference genes. The primers for the *SYNJ1* gene were designed using Primer Premier 5.0 software for RT-qPCR (Table 1), and the expression profile was assessed in the heart, liver, spleen, lung, kidney, and forehead skin tissue of six fetuses. Two different factors were used for selecting the reference: (1) a single reference gene with stable transcription levels, and (2) the geometric mean of the combination of stable reference genes. The reaction system and program was the same as that of the qPCR experiment. The PCR reaction was performed using a CFX-96 Touch™ Real-Time PCR Detection System (Bio-Rad).

Data analysis. The stability of the 10 reference genes was evaluated using the softwares geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004). The geNorm analysis calculated the gene stability measure (M value), which was arbitrarily required to be lower than 1.5 (with lower values indicating increased gene stability across samples), and pairwise variation (V value) for a single gene compared to all other reference gene candidates to determine the benefit of adding an extra reference gene to the normalization process. The arbitrary cut-off V value of 0.15 indicated acceptable stability of the reference gene combination. The NormFinder program was used to introduce raw data into a MS Excel spreadsheet following the manufacturer's instructions. The NormFinder algorithm used the least variation (intragroup and intergroup) to estimate expression stability between subgroups of the sample set, and lower values indicated increased stability in gene tran-

Table 1. The primer list of genes used in the study

Gene	Primers	Accession number	Amplicon length (bp)	PCR efficiency (%)
TBP – TATA box binding protein	F: GTCCAATGATGCCTTACGG R: TGCTGCTCCTCCAGAATAGA	NW_005395834.1	82	96.8
ACTB – β actin	F: ATTGCCGATGGTGATGAC R: ACGGAGCGTGGCTACAG	NW_005392900.1	177	97.6
UBE2D2 – ubiquitin conjugating enzyme E2 D2	F: TCATTTCCCAACAGATTACC R: AGTTAGTGCTGGAGACC	XM_005900181.1	133	95.5
PPIA – peptidylprolyl isomerase A	F: TTTTGAAGCATACAGGTCC R: CCACTCAGTCTTGGCAGT	XM_005891872.2	98	100.2
18SrRNA – 18S ribosomal RNA	F: GGACAGGATTGACAGATTGAT R: CCCAGAGTCTCGTTCGTTAT	NR_036642.1	117	95.3
HPRT1 – hypoxanthine guanine phosphoribosyl transferase 1	F: GTGATGAAGGAGATGGG R: ACAGGTCGGCAAAGAAC	NW_005397637.1	79	106.8
B2M – β2 microglobulin	F: CTGAGGAATGGGGAGAAG R: TGGGACAGCAGGTAGAAA	NW_005398298.1	80	108.9
GAPDH – glyceraldehyde 3 phosphate dehydrogenase	F: TCACCAGGGCTGCTTTTA R: CTGTGCCGTTGAACTTGC	EU195062.1	126	103.7
28SrRNA – 28S ribosomal RNA	F: TCTTCCTGGAGTTGGGTTGC R: GGTTTCACGCCCTCTTGC	NR_036644.1	145	101.2
SDHA – succinate dehydrogenase complex subunit A	F: GGGAACATGGAGGAGGACA R: CCAAAGGCACGCTGGTAGA	XM_005894659.2	188	97.1
SYNJ1 – synaptojanin 1	F: TGGTACGGTGTTGGTCTCAA R: ATTCAAGGCAGAGCTTCCCT	XM_005893341.2	171	103.3

PCR = polymerase chain reaction

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Table 2. Mean RT–qPCR threshold values (means ± standard deviation) of 10 reference genes in yak tissues

Gene	Heart	Liver	Spleen	Lung	Kidney	Forehead skin
TBP	25.27 ± 0.34	24.36 ± 0.33	24.63 ± 0.56	24.49 ± 0.78	24.12 ± 0.51	24.77 ± 0.36
ACTB	18.19 ± 0.32	17.15 ± 0.26	17.53 ± 0.85	17.46 ± 0.90	17.28 ± 0.60	17.74 ± 0.32
UBE2D2	22.14 ± 0.27	22.30 ± 0.32	22.24 ± 0.58	22.11 ± 1.01	21.48 ± 0.76	22.29 ± 0.40
PPIA	19.79 ± 0.44	18.82 ± 0.35	18.93 ± 0.28	19.44 ± 1.10	18.71 ± 0.58	20.22 ± 0.33
18SrRNA	5.23 ± 0.49	4.72 ± 0.42	5.28 ± 0.08	5.14 ± 0.41	5.26 ± 0.52	5.58 ± 0.48
HPRT1	22.60 ± 0.47	22.97 ± 0.39	23.47 ± 0.46	21.90 ± 0.68	23.47 ± 0.66	24.58 ± 0.45
B2M	21.80 ± 0.32	20.99 ± 0.29	19.80 ± 0.62	21.56 ± 0.83	21.54 ± 0.71	20.97 ± 0.77
GAPDH	18.97 ± 0.11	20.77 ± 0.30	20.27 ± 0.35	20.39 ± 0.68	20.28 ± 0.72	22.64 ± 0.12
28SrRNA	26.44 ± 0.81	26.01 ± 0.70	26.65 ± 0.43	26.62 ± 0.66	25.66 ± 0.78	28.85 ± 0.51
SDHA	21.37 ± 0.51	21.38 ± 0.33	23.874 ± 0.50	24.34 ± 1.14	21.62 ± 0.89	24.44 ± 0.39

scription. The BestKeeper software determined optimal reference genes by employing a pair-wise correlation analysis of all pairs of candidate genes and calculating the geometric mean of the best-suited genes. The raw Ct values were introduced directly into the MS Excel spreadsheet using a macro. The results were immediately calculated by the algorithm. The $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008) was used to analyze the relative expression of mRNA of each gene by quantitative fluorescence.

RESULTS

Transcript levels of candidate reference genes. Ten reference genes were amplified from different tissues of six yaks. Melting curves for each primer showed a single peak, confirming a single product from the RT-qPCR. Standard curves were generated using a ten-fold serial dilution of a cDNA pool, and these employed a linear correlation coefficient (R^2) of > 0.95. Based on the slopes of the standard curves, the estimated PCR amplification efficiencies ranged from 95.3 to 108.9% (Table 1). The mean Ct values are shown in Table 2. There was low inter-assay variation for the heart, liver, spleen, and forehead skin tissue, with most standard deviation (SD) values < 0.6 and the highest 0.85. For lung and kidney tissue, there was larger variation and all SD values were > 0.41, with those of *UBE2D2*, *PPIA*, and *SDHA* for lung tissue exceeding 1.0.

Transcript levels were used to establish three arbitrary categories: highly expressed *18SrRNA* (mean Ct values = 5.17), moderately transcribed (mean Ct values = 17.53–23.07), including the *ACTB*, *B2M*, *28SrRNA*, *HPRT1*, *SDHA*, *UBE2D2*,

Table 3. Results of stability among 10 candidate genes computed by 3 algorithms on all yak tissues

Gene	geNorm		NormFinder		BestKeeper	
	M value	rank	S value	rank	SD	CV
TBP	0.822	1	0.314	4	0.48	1.97
ACTB	0.841	2	0.336	5	0.55	3.12
UBE2D2	0.841	2	0.293	2	0.47	2.12
PPIA	0.847	3	0.287	1	0.64	3.33
18SrRNA	0.863	4	0.300	3	0.36	6.95
HPRT1	1.089	5	0.571	6	0.75	3.27
B2M	1.117	6	0.632	9	0.68	3.21
GAPDH	1.134	7	0.603	7	0.86	4.17
28SrRNA	1.148	8	0.617	8	0.88	3.30
SDHA	1.454	9	0.902	10	1.37	6.05

M value = gene stability measure, S value = stability value, SD = standard deviation, CV = coefficient of variation

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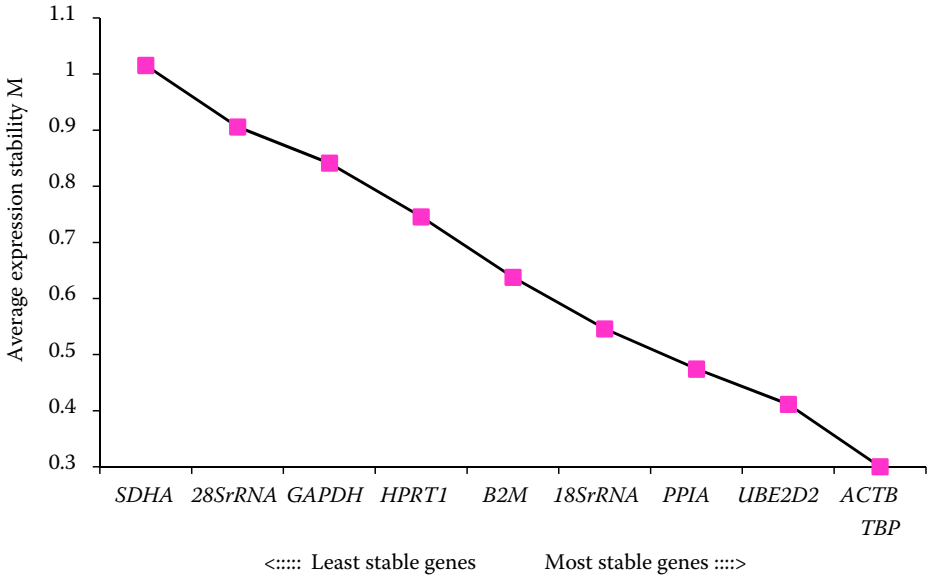

Figure 1. Stability of reference gene expression (M) calculated using the geNorm software. Low M values indicate the best reference genes and high M values the worst reference genes

and *PPIA* gene, and low transcript levels (mean Ct values > 24), including *GAPDH* and *TBP*.

Stability of reference gene expression. The stability ranking of the 10 reference genes was evaluated based on the entire dataset (Table 3). The *TBP* gene had the lowest M value. The geNorm algorithm also eliminated the least stable expressed gene and recalculated new M values for the remaining genes. Figure 1 illustrates the average expression stability values (M); *TBP* and *ACTB* were the most stable genes. Next, the geNorm algorithms calculated the normalization factor (NF) and used V values to determine the minimal number of genes mandatory for normalization. Additional

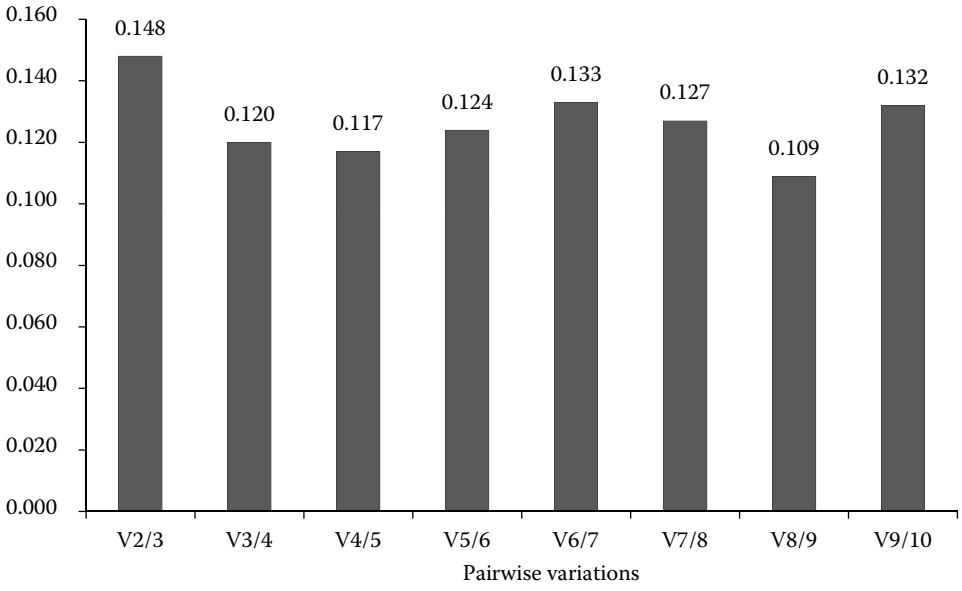

Figure 2. Determination of the optimal number of reference genes for gene expression normalization. To determine the number of stable reference genes needed for normalization, the pairwise variation Vn/n + 1 was calculated between the normalization factors NFn and NFn + 1 by the geNorm software. A threshold of pairwise variation V < 0.15 was suggested for the valid normalization

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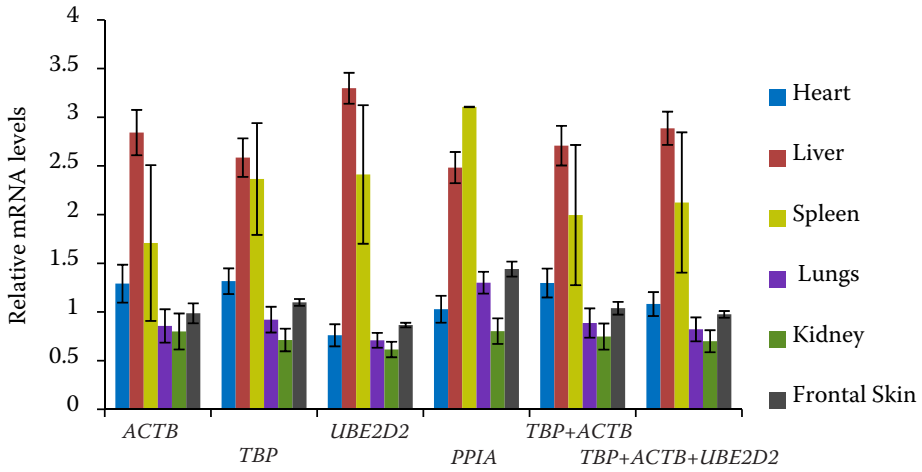

Figure 3. Validation of the recommended reference genes. Expression profiles of *SYNJ1* gene were investigated using different reference genes. The expression of *SYNJ1* was normalized using the different groups: *ACTB*, *TBP*, *UBE2D2*, *PPIA*, the combination of *ACTB* and *TBP*, the combination of *ACTB*, *TBP*, and *UBE2D2*. Bars represent the means and standard error of six biological replications

genes were included when V exceeded the cutoff value 0.15. Figure 2 shows the first V value < 0.15 (0.148) that emerged at V2/3, suggesting that the two reference genes (*ACTB* and *TBP*) were sufficient for reliable normalization.

NormFinder calculated the stability value (S value) based on a variance estimation approach. An upper S value of 0.5 indicated genes were relatively stable, and greater stability of gene expression was indicated by lower S values. The overall ranking of the genes from the most to the least stable was *PPIA*, *UBE2D2*, *18SrRNA*, *TBP*, *ACTB*, *HPRT1*, *GAPDH*, *28SrRNA*, *B2M*, and *SDHA* (Table 3).

BestKeeper measured the stability of reference genes according to the coefficient of variance (CV) and the standard deviation (SD) of the Ct values. The most stable reference gene exhibited the lowest CV and SD (CV ± SD) (Radonic et al. 2004). The results showed that the *TBP*, *UBE2D2*, *ACTB*, and *PPIA* genes had the most stable expression across all tissues, followed by *HPRT1*, *GAPDH*, and *28SrRNA*. The expression of most reference genes exhibited Ct variation less than 1 (Pfaffl et al. 2004) (0.36 < SD < 0.88), whereas *SDHA* exhibited high Ct variation (SD = 1.37 Ct) and was deemed unstable. The *18SrRNA* gene had the lowest SD (0.36) and the highest CV (6.95); therefore, we could not define its stability with this analysis. *B2M* displayed a moderate CV ± SD value (3.21 ± 0.68), but $P = 0.051$ indicating it should be excluded (Table 3).

Validation of the recommended reference genes. To examine the validity of the top ranked reference genes (*TBP*, *ACTB*, *UBE2D2*, and *PPIA*), the expression profile of the target gene *SYNJ1* was investigated in different tissues (Figure 3). Similar expression profiles of the target gene were observed using either two (*TBP* and *ACTB*) or three (*TBP*, *ACTB*, and *UBE2D2*) stable reference genes. The expression of the *SYNJ1* gene in spleen was numerically higher than that in liver with single *PPIA* gene normalization. This indicated that the *PPIA* gene was not suitable as a reference gene in this study.

DISCUSSION

Reference genes are defined as genes that are stably expressed across all tissues/cell types; however, no single gene is constitutively expressed in all tissues/cell types and under all experimental conditions (Andersen et al. 2004; Yang et al. 2015). Therefore, selection of suitable reference genes is a crucial precondition for a successful gene expression study based on RT-qPCR (Liu et al. 2014). In addition, numerous studies have focused on the expression stability of reference genes in cattle (Lisowski et al. 2008), goats (Zhang et al. 2013), pigs (Nygard et al. 2007), humans (Touchberry et al. 2006), and mice (Zeng et al. 2016). Little is known about the reference genes in yak tissues.

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Herein, we selected 10 traditional reference genes to identify the superior reference gene for six different tissues from yak fetuses. Although the geNorm, NormFinder, and BestKeeper softwares are widely used for assessing potential reference genes, it has been demonstrated that discrepancy typically exists among results obtained from these programs (Purohit et al. 2015). The top four ranked reference genes (*TBP*, *ACTB*, *UBE2D2*, and *PPIA*) obtained through geNorm were consistent with those of BestKeeper, although the ranking order of the second and third gene was different. Based on NormFinder, *PPIA* was identified as the most stable reference gene, followed by *UBE2D2* and *18SrRNA*, whereas in BestKeeper, *18SrRNA* was deemed unsuitable as a reference gene. The different software programs are based on different algorithms (Liu et al. 2014). Thus, the use of more than one algorithm for ranking is necessary.

Based on the aforementioned discrepancies, there was no scientific evidence to allow for determination of a single reference gene in this study. The V values calculated by geNorm software were used to determine the optional number of reference genes, and we must balance the trade-off between accuracy and practicality by using this value (Vandesompele et al. 2002). The V value of V2/3 was 0.148 (< 0.15), which indicated that two reference genes are needed for reliable normalization, and the addition of one more reference gene would not significantly improve reliability. Furthermore, the target gene validated this result in that the expression profile with the geometric mean of two reference genes was similar to that of three genes. This showed that the combination of *TBP* and *ACTB* would provide high quality data. In contrast, the results suggested that *28SrRNA*, *SDHA*, *GAPDH*, and *B2M* should be used for endogenous controls because of their unstable expression.

It has been demonstrated that utilization of different functional reference genes reduces co-regulation effects, which may affect the accuracy of pairwise comparison results (Andersen et al. 2004). The candidate reference genes *ACTB* and *TBP* were responsible for cell locomotion and trans-cription, respectively (Mihi et al. 2011). *ACTB* is an important actin isomer and cytoskeleton actin (Zeng et al. 2016), the basal level transcription of which is essential for all cellular physiological conditions. It has been widely used as an internal control for experimental testing (Mihi et al. 2011). *TBP* is an indispensable basal transcription factor, and the RNA polymerase II binds to the TBP-DNA complex to initiate transcription (Ponomarenko et al. 2016). A knockout (Martianov et al. 2002) or knockdown (Muller et al. 2001) of the *TBP* gene is lethal, and *TBP* expression might not be subjected to significant regulation. *TBP* was determined to be the optimal reference gene in low-abundance transcripts of goat (Zhang et al. 2013) and pig (Nygard et al. 2007) tissue expression studies. Appropriate reference genes were suggested to have the same transcript levels as the target gene to enhance the uniformity of the analysis (Spinsanti et al. 2006). In this study, *TBP* gene was classified in the low transcript level (mean Ct values > 24), and the transcript level of the *ACTB* gene (mean Ct values = 17.15–18.19) was higher than that of the *TBP* gene. Based upon these concepts, the *ACTB* would be the logical reference gene for studying high transcript-level target genes, and the combination of *ACTB* and *TBP* genes would be more appropriate for modest transcript-level studies. Thus, we recommend the geometric averaging of the *ACTB* and *TBP* genes to normalize the relative gene expression levels for further study.

CONCLUSION

This study firstly validated the suitable reference genes for data normalization for gene expression in yak splanchnic and skin tissues. The combination of the *ACTB* and *TBP* genes was optimal for determination of gene expression in this study. In conclusion, the result may provide methodological support for further candidate gene identification and gene-expression pattern studies. It also clearly advocates for a reference gene assessment prior to performing a target gene expression-level analysis.

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