doi: 10.17221/8782-CJAS

Combination of *let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p* is suitable normalizer for studying microRNA expression in skin tissue of Liaoning cashmere goat during hair follicle cycle

W.L. Bai[1], Y.L. Dang[1], R.H. Yin[1], R.L. Yin[2], W.Q. Jiang[1], Z.Y. Wang[1], Y.B. Zhu[1], J.J. Wang[1], Z.H. Zhao[3], G.B. Luo[1]

[1]College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang, P.R. China
[2]Research Academy of Animal Husbandry and Veterinary Medicine Sciences of Jilin Province, Changchun, P.R. China
[3]College of Animal Science and Veterinary Medicine, Jilin University, Changchun, P.R. China

ABSTRACT: The microRNAs are non-coding RNA molecules of approximately 20–22 nucleotides that are found to be implicated in a wide range of physiological processes. In this study, the suitability of 10 candidate reference RNAs was evaluated for microRNA expression data in the skin tissue of Liaoning cashmere goat including 1 small nuclear RNA (snRNA; *RNU6B*), 1 small nucleolar RNA (snoRNA; *Z30*), 1 rRNA (*5S*), 1 transfer RNA (tRNA; *Met-tRNA*), and 6 microRNAs (miR; *let-7d-5p*, *miR-15a-5p*, *miR-26a-5p*, *miR-125a-5p*, *miR-214-3p,* and *miR-221-3p*). Based on geNorm and NormFinder algorithms, we identified *let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p* as the most stable reference RNAs. Also, three reference RNAs (*let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p*) were sufficient for the normalization of microRNA expression data in the skin of this breed. We further assessed the suitability of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* in a combination as reference RNAs through detecting the relative expression of *miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p* as putative genes of interest. Significant differences were revealed in the relative expression of *miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p* at telogen stage of hair follicle cycle when a combination of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* vs a single *let-7d-5p* were used as reference RNA. Based on the results from this study, we suggested that the combination of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* as normalizers for microRNA expression data would be more reliable than that of single *let-7d-5p*, and the geometric mean of these three microRNAs (*let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p*) can be used for the normalization of microRNAs expression data in the skin of Liaoning cashmere goat.

Keywords: cashmere goat skin; microRNA expression reference; expression stability

INTRODUCTION

Liaoning cashmere goat, the most productive cashmere breed in China, is mainly raised in the Buyun Mountains of Liaotung Peninsula (McGregor et al. 1991). The goats of this breed are white with horns in both sexes. It lives in a humid environment with mean temperature of 7–8°C, rainfall of 700–900 mm, and frost free period of 150–170 days. There are around 1.5 million individuals of this

Supported by the Foundation of Natural Science Project of Liaoning Province, China (No. 2015020758), the Foundation of Education Department of Liaoning Province, China (No. L2014263), the Foundation for University Talents of Liaoning Province of China (LJQ2013070), and the Projects of Tianzhushan Person of Outstanding Ability of Shenyang Agricultural University.

doi: 10.17221/8782-CJAS

breed. It is adapted well to the local environment. Although it has a relatively good meat-producing ability with average adult weight being 91.6 kg (male) and 43.8 kg (female), the main use of this breed is cashmere with the average adult cashmere yield of 540 g (male) and 470 g (female) (Bai et al. 2012). It is of great economic importance to local residents.

The microRNAs (miRNAs) are endogenous single-stranded non-coding RNA molecules of approximately 20–22 nucleotides in length. They are able to downregulate gene expression at post transcriptional levels by binding to the 3'-untranslated region of target coding mRNA, an event that usually involves mRNA cleavage or inhibition of mRNA translation (Baek et al. 2008; Selbach et al. 2008). Over the past few years, miRNAs are found to be implicated in a wide range of physiological processes, such as embryonic development, cell proliferation, differentiation, and apoptosis (Bartel 2004; Kloosterman and Plasterk 2006; Filipowicz et al. 2008).

In recent years, a large number of miRNAs have been identified in skin tissue of cashmere goat (Wenguang et al. 2007; Liu et al. 2012; Yuan et al. 2013). For further insight into their potential functional roles in cashmere fibre growth, it would be important to elucidate the expression characteristics in the skin tissue of cashmere goat during hair follicle cycling. Although various different approaches were used for measuring miRNA expression level, such as northern blotting (Sempere et al. 2004), bead-based flow cytometry (Lu et al. 2005), and microarray technology (Miska et al. 2004), the relative quantification based on real-time PCR (qPCR) technique has received particular attention for measuring microRNA expression (Latham 2010; Wessels et al. 2011; Wang et al. 2012) due to its high sensitivity, reproducibility, wide dynamic range, and low template requirements (Davoren et al. 2008). In qPCR technique, however, the accurate normalization of qPCR data is crucial to reduce the possible errors (Wessels et al. 2011; Gu et al. 2012). Moreover, the applicability of multiple references instead of a single one is recommended to obtain accurate expression levels in the miRNAs of interest (Vandesompele et al. 2002; Davoren et al. 2008). To date, however, there is not a well-defined set of references for miRNA expression analysis in the skin of cashmere goat. Thus, the aim of the present work was to identify suitable normalizers for studying miRNA expression in the skin tissue of Liaoning cashmere goat during hair follicle cycling.

MATERIAL AND METHODS

Samples, RNA extraction, and cDNA synthesis. Tissue biopsies of skin of approximately 1 cm^2 were collected from six female Liaoning cashmere goats in June (early anagen), October (anagen), January (catagen), and March (telogen). The animals sampled for skin tissue were in good general health, and avoided common traceable genetic relationships. The skin tissue samples collected were immediately put into Sample Protector (TaKaRa Biotechnology Co. Ltd., Dalian, China). They were transported to laboratory in low temperature condition and frozen at –80°C before RNA extraction. All experimental procedures were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University.

Using the RNAiso reagent kit (TaKaRa) we isolated total RNA from different samples according to the manufacturer's instructions. The integrity of total RNA extracted was verified by 1.5% agarose gel electrophoresis (Figure S1). Based on the ultraviolet spectrometer, we assessed the purity and quantity of the total RNA with the ratio of OD_{260}/OD_{280} being 1.8–2.0 for each sample. The total RNA was further treated with DNase I (TaKaRa) to exclude the contamination from residual genomic DNA. Lack of genomic DNA contamination was confirmed by a null result from PCR amplification reaction using the digested RNA as a template. Using the One Step PrimeScript miRNA cDNA synthesis kit (code D350; TaKaRa), the first strand of cDNA was synthesized for each sample following the manufacturer's instructions. The reactions of reverse transcription were prepared in a total volume of 20 µl containing: 2× miRNA Reaction Buffer Mix (10 µl), 0.1% BSA (2 µl), miRNA PrimeScript® RT Enzyme Mix (2 µl), Total RNA (1 µl, 1 µg/µl), and RNase Free dH_2O (5 µl) (all TaKaRa). The reaction conditions were as follows: 37°C for 60 min followed by 85°C for 5 s. Absence of inhibitors was tested by different dilutions.

Candidate reference selection, primer design, and qPCR. A total of 10 candidate reference RNAs were selected including 1 small nuclear RNA (snRNA; *RNU6B*), 1 small nucleolar RNA (snoRNA; *Z30*), 1 rRNA (*5S*), 1 transfer RNA (tRNA; *Met-tRNA*), and 6 microRNAs (miR; *let-7d-5p*, *miR-15a-5p*, *miR-26a-5p*, *miR-125a-5p*, *miR-214-3p,* and *miR-221-3p*). Also, four miRNAs (*miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p*) were used as interest miRNA to validate the identified

doi: 10.17221/8782-CJAS

Table 1. Information on the primers and characterization of real-time PCR reactions

Gene symbol[1]	Full gene name	Reference in GenBank or miRBase[2]	NcRNA species	Primer sequence[3] (5'-3')	T_a (°C)	Slope	R^2	E[4] (%)
RNU6B	*U6 small nuclear 2 RNA*	NR_002752	snRNA	F: AAAATTGGAACGATACAGAGA R: AAATATGGAACGCTTCACGAA	54	–3.351	0.998	98.8
Z30	*Z30 small nucleolar RNA*	AJ007733	snoRNA	F: GCGATGATGAGTGAAGTAGAG R: TCAGAGAGAAGATTAAGAGAT	53	–3.326	0.999	99.8
5S	*5S ribosomal RNA*	X57170	rRNA	F: CGGCCATACCACCCTGAAC R: AGGCGGTCTCCCATCCAAGT	59	–3.392	0.998	97.2
Met-tRNA	*Transfer RNA-Met*	K00328	tRNA	F: AGCAGAGTGGCGCAGCGG R: CGGCAGAGGATGGTTTCGAT	60	–3.222	0.995	104.3
let-7d-5p	*MicroRNA-let-7d-5p*	MIMAT0000383	miRNA	F: CGAGAGGTAGTAGGTTGCATAGTT	62	–3.371	0.997	98.0
miR-15a-5p	*MicroRNA-15a-5p*	MIMAT0000526	miRNA	F: CGTAGCAGCACATAATGGTTTGTG	62	–3.388	0.996	97.3
miR-26a-5p	*MicroRNA-26a-5p*	MIMAT0000533	miRNA	F: CGTTCAAGTAATCCAGGATAGGCT	61	–3.218	0.992	104.5
miR-125a-5p	*MicroRNA-125a-5p*	MIMAT0000135	miRNA	F: TCCCTGAGACCCTTTAACCTGTG	62	–3.409	0.998	96.5
miR-214-3p	*MicroRNA-214-3p*	MIMAT0000661	miRNA	F: ACAGCAGGCACAGACAGGCAGT	63	–3.347	0.997	99.0
miR-221-3p	*MicroRNA-221-3p*	MIMAT0000669	miRNA	F: GAGCTACATTGTCTGCTGGGTTTC	62	–3.384	0.998	97.5
miR-24-3p	*MicroRNA-24-3p*	MIMAT0000219	miRNA	F: TGGCTCAGTTCAGCAGGAACA	61	–3.377	0.999	97.8
miR-29a-3p	*MicroRNA-29a-3p*	MIMAT0000535	miRNA	F: CGTAGCACCATCTGAAATCGGTTA	63	–3.401	0.997	96.8
miR-145a-5p	*MicroRNA-145a-5p*	MIMAT0000157	miRNA	F: GTCCAGTTTTCCCAGGAATCCC	63	–3.366	0.999	98.2
miR-205-5p	*MicroRNA-205-5p*	MIMAT0000238	miRNA	F: TCCTTCATTCCACCGGAGTCTG	63	–3.355	0.998	98.6

NcRNA = noncoding RNA, snRNA = small nuclear RNA, snoRNA = small nucleolar RNA, rRNA = ribosomal RNA, tRNA = transfer RNA, miRNA = microRNA, T_a = annealing temperature, E = efficiency of the real-time PCR

[1]primers for *RNU6B, Z30, 5S,* and *Met-tRNA* were designed in our previous study (Bai et al. 2013), and their amplicon lengths were 79-, 91-, 91-, and 72-bp, respectively. Amplicon length of the 10 microRNAs analyzed is not available because of the use of universal reverse primer obtained from the kit (code D350; TaKaRa Biotechnology Co. Ltd., Dalian, China)

[2]GenBank: http://www.ncbi.nlm.nih.gov/; miRBase: http://www.mirbase.org/

[3]F = forward, R = reverse; the anti-sense primer of all the miRNAs analyzed was obtained from the kit (code D350; TaKaRa), being a universal miRNA reverse primer

[4]calculated as $E = 10^{(-1/\text{slope})} - 1$

doi: 10.17221/8782-CJAS

optimal reference RNAs for microRNA expression analysis in the skin tissue of Liaoning cashmere goat. The information of primers used was shown in Table 1.

In a LightCycler 480 Real Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany), we carried out the qPCR amplification reaction with SYBR® Green I assay (TaKaRa). Six-point standard curve was produced for each reference RNA by the 10-fold serial dilutions of cDNA. Reactions were prepared in a total volume of 20 µl containing: 2.0 µl cDNA, 0.8 µl of each 10µM primer, 10 µl SYBR® Premix Ex Taq™ (TaKaRa), and 6.4 µl RNase/DNase-free sterile water. The cycle conditions were set as follows: 95°C for 5 min, 40 cycles of 95°C for 10 s, annealing temperature 53–63°C (Table 1) for 10 s, and 72°C for 15 s. For specificity testing, the last cycle was followed by a melting curve analysis ranging from 56 to 95°C with temperature increasing steps of 0.5°C every 10 s. Each reaction was run in triplicate. A no-template control was included in each assay. Using the equation: $E = 10^{(-1/\text{slope})} - 1$, the qPCR efficiency ($E$) was calculated by the given slope from the instrument software.

Stability evaluation of candidate reference RNAs. Two popular algorithms, geNorm, Version 3.5 (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), were used to evaluate the stability of 10 candidate reference RNAs following the authors' instructions. The geNorm calculates the expression stability measure M as the average pairwise variation for the reference RNA with all other tested reference RNAs. Stepwise exclusion of the reference RNA with the highest M allows ranking of the tested reference RNAs based on their M value. NormFinder estimates expression stability based on intra- and inter-group variations for candidate reference RNAs. We also evaluated the correlation coefficient (r) between the geNorm M value and the NormFinder stability value using SPSS software (Version 16.0.2, 2008). The geNorm algorithm was used to determine the optimal number of reference RNAs for miRNA data normalization (Vandesompele et al. 2002).

Evaluation and validation of identified suitable reference RNAs for normalization. The validation of the identified suitable normalizers for studying miRNA expression was evaluated through detecting the relative expression of 4 miRNAs of interest including *miR-24-3p*, *miR-29a-3p*, *miR-145a-5p*, and *miR-205-5p*. The reaction solution of qPCR was prepared in a final volume of 20 µl as described above, with an annealing temperature of 61 or 63°C (Table 1). The *let-7d-5p* alone and the geometric mean of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* were used to normalize the expression level of 4 miRNAs of interest (*miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p*), respectively.

Normalized qPCR data are presented as $\log_2$ n-fold change relative to early anagen. In order to estimate standard errors at early anagen and prevent possible biases in statistical analysis, we transformed normalized data to obtain a perfect average of 1.0 at early anagen, leaving the proportional difference between the biological replicates. The same proportional change was calculated at anagen, catagen, and telogen to obtain a fold change relative to early anagen. Finally, the dataset was analyzed using a mixed model in SPSS software to evaluate the effect of different stages of hair follicle cycling. The mixed model included the fixed effect of different stages (early anagen, anagen, catagen, and telogen) and the random effect of goat. Means were compared using the Tukey's test, and differences between means of different stages of hair follicle cycling were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Based on the geNorm analysis, the expression stabilities of the 10 candidate reference RNAs were found to be different with the M values ranging from 0.259 to 0.648 below the default limit of 1.5 accepted by geNorm (Vandesompele et al. 2002). The average M value of the pair *miR-26a-5p* and *let-7d-5p* was the lowest, and this pair was identified as the most suitable reference RNA with average M values of < 0.3. The *miR-15a-5p* was ranked as the third most stable reference RNA. The M value of *5S* was the highest among the candidate reference RNAs at 0.648, and was found to be the worst performing normalizer for miRNA expression analysis in the skin tissue of Liaoning cashmere goat during hair follicle cycling (Figure 1A). The NormFinder algorithm was also used to evaluate the expression stabilities of the 10 candidate reference RNAs, and the results obtained were presented in Figure 1B. Throughout the hair follicle cycling, *let-7d-5p* was found to be the most stably expressed reference RNA, followed by *miR-26a-5p* and *miR-15a-5p*,

doi: 10.17221/8782-CJAS

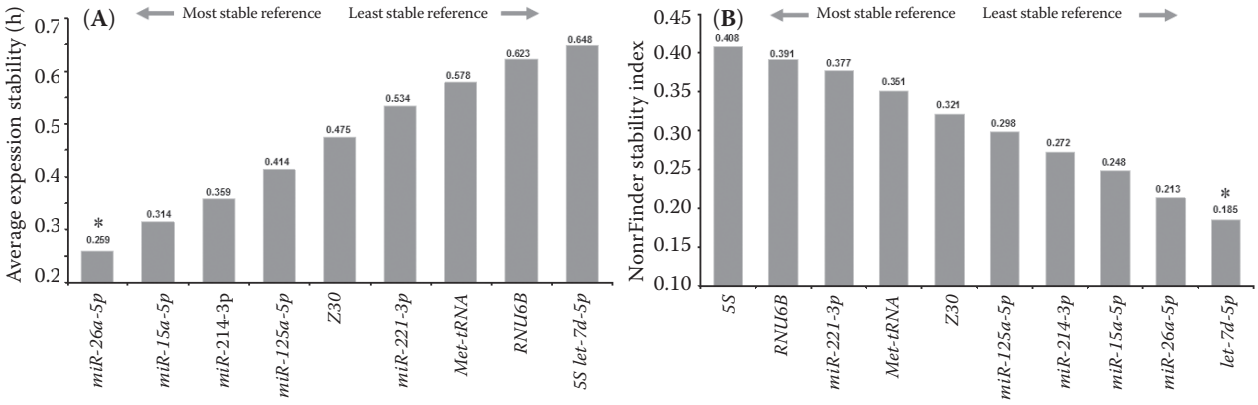

Figure 1. Expression stability of 10 candidate reference RNAs in the skin tissue of Liaoning cashmere goat

(**A**) output charts for average expression stability (M) of 10 candidate reference RNAs based on the geNorm algorithm where M value was calculated by stepwise exclusion of the least-stable candidate RNA. A lower M value means higher expression stability. The most stable RNA pair (*let-7d-5p* and *miR-26a-5p*) was indicated by asterisk, (**B**) output charts for stability index values of 10 candidate reference RNAs based on the NormFinder algorithm. A lower index value of stability indicates higher expression stability. The miRNA *let-7d-5p* was identified as the most stable reference RNA that was indicated by asterisk

whereas the *5S* and *RNU6B* exhibited the least expression stability. On the whole, the results from NormFinder algorithm were consistent with that of geNorm analysis with an exception of two candidate reference RNAs *miR-221-3p* and *Met-tRNA*, the ranking of which were inverted. On the other hand, we noted a robust correlation (r = 0.986, $P < 0.01$) between the geNorm M value and the NormFinder stability value by analyzing the correlation coefficient (Figure 2A), which further confirmed the reliability of the results obtained from the present study.

Previously, *5S* was used as normalizer for miRNA expression analysis in the skin of cashmere goat (Fu et al. 2012). In the present work, however, it was remarkable that *5S* was ranked as the least-stable reference RNA in the skin of Liaoning cashmere goat, based on both geNorm and NormFinder

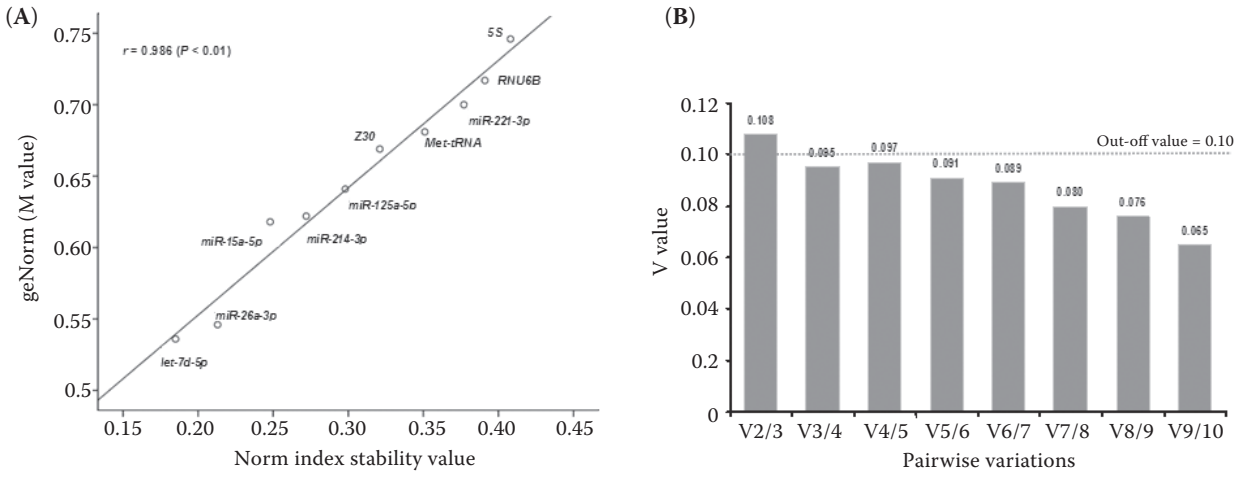

Figure 2. Correlation analysis between M values (geNorm) and stability values (NormFinder) and determination of the optimal number of reference RNAs

(**A**) correlation of M value and stability value that was evaluated by the coefficient of determination with SPSS software (Version 16, 2008), (**B**) determination of the optimal number of reference RNAs for the normalization of miRNAs expression data that was performed by pairwise variation analysis based on geNorm algorithm. *Y*-axis indicated pairwise variation V ($V_{n/n+1}$) between the normalization factors NF_n and NF_{n+1}. When cut-off value of 0.10 was used, the three most stable microRNAs (*let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p*) were sufficient to provide a reliable normalization factor in the skin of Liaoning cashmere goat

doi: 10.17221/8782-CJAS

computational programs (Figure 1). Therefore, it appeared that *5S* was unsuitable for the normalization of miRNA expression data in the skin tissue of Liaoning cashmere goat during hair follicle cycling. *RNU6B* was previously used as normalizer for miRNA expression analysis in other organs of ruminants, such as mammary gland of cattle (Li et al. 2012; Wang et al. 2012) and goat (Ji et al. 2012). Also, it was used in the normalization of miRNA expression data in the skin of alpaca (He et al. 2010). In this study, however, *RNU6B* exhibited the second-lowest stability in both geNorm and NormFinder analysis (Figure 1). Additionally, *Met-tRNA* (along with *RNU6B*) was also used as reference RNA for microRNA expression data in different tissues of human and mouse (Liu et al. 2004). In the present work, however, *Met-tRNA* exhibited the third- and fourth-lowest stability in geNorm and NormFinder algorithms, respectively (Figure 1). Similarly, it was demonstrated that *Met-tRNA* was inappropriate as normalizer for miRNA expression data in the mammary gland of yak (Bai et al. 2013) and pig (Gu et al. 2012).

In the qPCR-based expression analysis of miRNA, the reference RNAs used should share similar properties with miRNA molecules, such as size. Numerous noncoding small RNA species (below 200 bp) are found to be closer in size to microRNA including tRNA, rRNA, snRNA, and snoRNA, and have previously been used as reference RNAs in the normalization of miRNA qPCR data (Davoren et al. 2008). In this study, the 10 candidate RNAs were selected mainly based on the following considerations. Firstly, *RNU6B*, *5S,* and *Z30* were previously used as normalizers for miRNA expression analysis in the skin of alpaca (He et al. 2010), goat (Fu et al. 2012), and human (Xia et al. 2013), respectively. Secondly, *Met-tRNA* was not typically used in skin tissue related experiments of goats, however, it was used as normalizer for miRNA expression data in various tissues of human and mouse (Liu et al. 2004), and was explored as candidate reference RNA in other organs of ruminants, such as the mammary gland of yak (Bai et al. 2013). Thirdly, the expressions of the 6 candidate reference miRNAs (*let-7d-5p*, *miR-15a-5p*, *miR-26a-5p*, *miR-125a-5p*, *miR-214-3p,* and *miR-221-3p*) were found to be relatively stable in the skin of mouse during hair follicle cycling (Mardaryev et al. 2010).

Over the past few decades, it was demonstrated that the use of a single reference appears to be insufficient in the normalization of qPCR data (Tricarico et al. 2002; Vandesompele et al. 2002; Pfaffl et al. 2004), and the use of multiple references was recommended for qPCR data normalization (Brinkhof et al. 2006; Peters et al. 2007; Varshney et al. 2012). Using geNorm algorithm, therefore, we further determined the optimal number of reference RNAs required for data normalization of miRNA quantitation through calculating the pairwise variation ($V_{n/n+1}$) between each combination of sequential normalization factors (NF_n and NF_{n+1}). An additional inclusion of a less stable reference RNA resulting in a large variation suggests that this reference should not be included in the calculation for a reliable analysis. In the present work, a lower variation was observed in the expression stability of the tested miRNAs (Figure 1), therefore the cut-off was reduced from 0.15 to 0.10 as suggested by Vandesompele et al. (2002). As shown in Figure 2B, it appeared that three RNAs ($V_{2/3}$) were suitable for miRNA data normalization in the skin of Liaoning cashmere goat during hair follicle cycling. Factually, addition of a fourth gene ($V_{3/4}$) would increase reliability in normalization but it was not justified given the lower cut-off value of 0.10 used (Vandesompele et al. 2002; Bionaz and Loor 2007). As shown in Figure 1, *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* were ranked as top three most stable references by both geNorm and NormFinder algorithms. Therefore, the use of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* in a combination would be sufficient for an accurate normalization of microRNA expression data in the skin of Liaoning cashmere goat during hair follicle cycling.

In the present work, we further evaluated the suitability of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* as combined normalizers for miRNA expression data in the skin of Liaoning cashmere goat. Using a combination of three miRNAs (*let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p*) against a single one (*let-7d-5p*) as references, the relative expression of four putative interest miRNAs (*miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p*) was investigated in the skin of Liaoning cashmere goat during hair follicle cycling. The relative expressions of *miR-24-3p*, *miR-29a-3p*, *miR-145a-5p,* and *miR-205-5p* are presented in Figure 3. On the whole, the expression patterns of the 4 microRNAs analyzed were similar to those recorded in the skin of mouse during hair follicle cycling (Mardaryev et al. 2010). On the other hand, although a highly similar change

doi: 10.17221/8782-CJAS

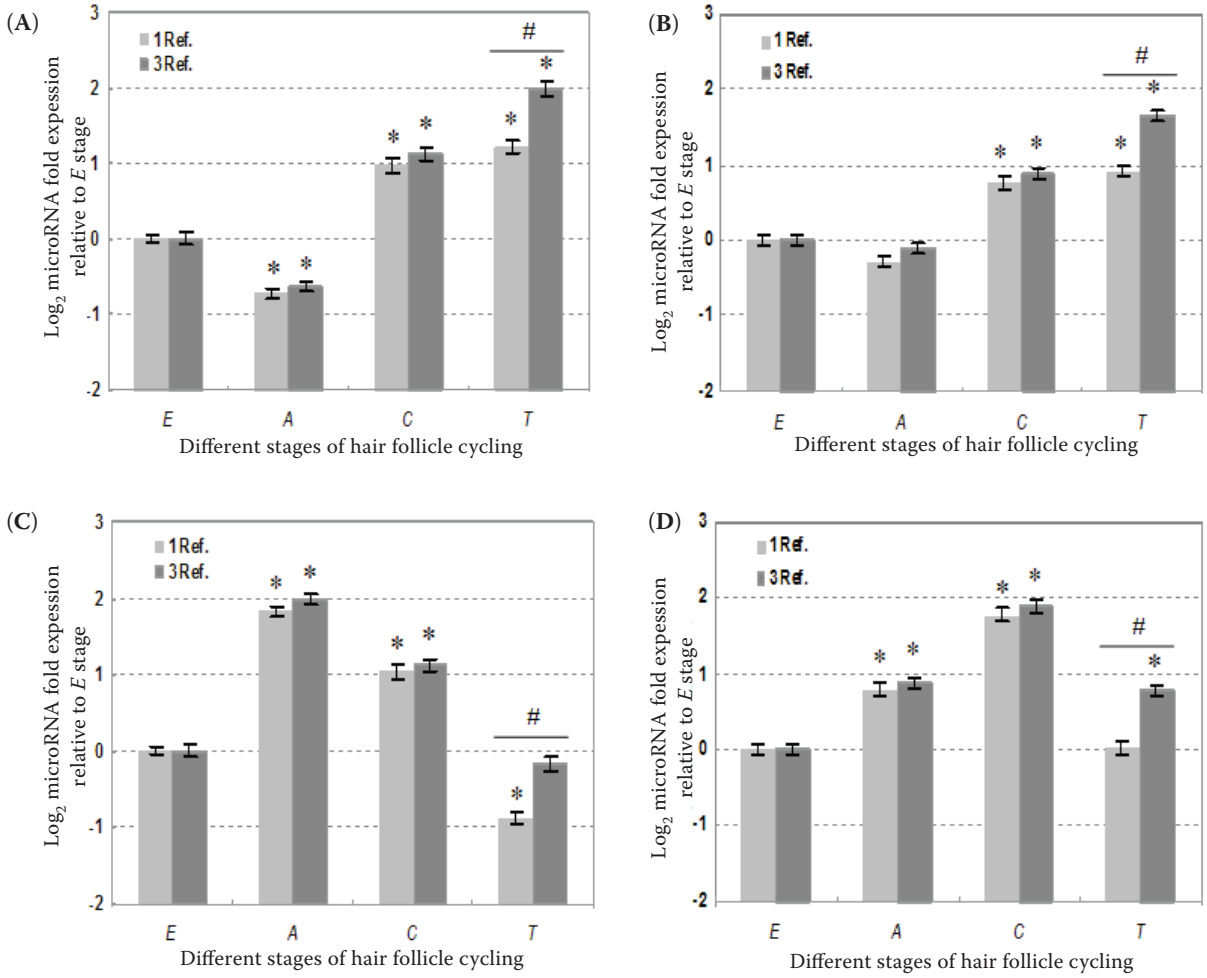

Figure 3. Relative expression of putative interest miRNAs *miR-24-3p* (**A**), *miR-29a-3p* (**B**), *miR-145a-5p* (**C**), and *miR-205-5p* (**D**) in the skin tissue of Liaoning cashmere goat during hair follicle cycling compared with *E* stage, taking one reference RNA (*let-7d-5p*) and the geometric mean of three reference RNAs (*let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p*) as normalizers

E = early anagen, *A* = anagen, *C* = catagen, *T* = telogen

*significant difference ($P < 0.05$) in comparison with *E* stage

#significant difference ($P < 0.05$) between one (*let-7d-5p*) and three (*let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p*) reference RNAs used as normalizers at the same stage of hair follicle cycling

error bar indicates SEM within the group

trend was observed for each microRNA analyzed between using 3 combined references (*let-7d-5p*, *miR-26a-5p*, and *miR-15a-5p*) and one single reference (*let-7d-5p*), significant differences were revealed for each microRNA in the relative expression at telogen stage ($P < 0.05$) (Figure 3). Moreover, at telogen stage, a significant difference was revealed in the relative expression of *miR-145a-5p* compared with early anagen stage ($P < 0.05$) when using 1 single reference, but not when using 3 combined references (Figure 3C). Also, in comparison to early anagen stage, a significant difference was recorded in the relative expression of *miR-205-5p* at telogen stage when using 3 combined references, but not when using 1 single reference (Figure 3D). In fact, compared with mRNA, more rigorous strategies should be introduced for the normalization of miRNA expression data, in that miRNA might regulate multiple targets. As a result, their effects could be amplified in a pathway (Croce and Calin 2005). In that case, even a small change in the miRNAs expression level might mean significant biological effects (Davoren et al. 2008; Peltier and Latham 2008).

doi: 10.17221/8782-CJAS

Therefore, it would be a better choice to use three combined references (*let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p*) instead of a single *let-7d-5p* to obtain an accurate normalization of miRNAs expression data in the skin of Liaoning cashmere goat.

CONCLUSION

In the present work, we evaluated the suitability of 10 candidate reference RNAs for normalization of miRNA expression data in the skin tissue of Liaoning cashmere goat. Three miRNAs (*let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p*) were identified as the most suitable reference RNAs. Based on the results from the present work, we recommended that the combination of *let-7d-5p*, *miR-26a-5p,* and *miR-15a-5p* could be used for the normalization of miRNA expression data in the skin of Liaoning cashmere goat.

REFERENCES

Andersen C.L., Jensen J.L., Orntoft T.F. (2004): Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research, 64, 5245–5250.

Baek D., Villen J., Shin C., Camargo F.D., Gygi S.P., Bartel D.P. (2008): The impact of microRNAs on protein output. Nature, 455, 64–71.

Bai W.L., Yin R.H., Jiang W.Q., Luo G.B., Yin R.L., Li C., Zhao Z.H. (2012): Molecular characterization of prolactin cDNA and its expression pattern in skin tissue of Liaoning Cashmere goat. Biochemical Genetics, 50, 694–701.

Bai W.L., Yin R.H., Yang R.J., Khan W.A., Ma Z.J., Zhao S.J., Jiang W.Q., Wang Z.Y., Zhu Y.B., Luo G.B., Zhao Z.H. (2013): Identification of suitable normalizers for microRNA expression analysis in milk somatic cells of the yak (Bos grunniens). Journal of Dairy Science, 96, 4529–4534.

Bartel D.P. (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116, 281–297.

Bionaz M., Loor J.J. (2007): Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. Physiological Genomics, 29, 312–319.

Brinkhof B., Spee B., Rothuizen J., Penning L.C. (2006): Development and evaluation of canine reference genes for accurate quantification of gene expression. Analytical Biochemistry, 356, 36–43.

Croce C.M., Calin G.A. (2005): miRNAs, cancer, and stem cell division. Cell, 122, 6–7.

Davoren P.A., McNeill R.E., Lowery A.J., Kerin M.J., Miller N. (2008): Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. BMC Molecular Biology, 9, 76.

Filipowicz W., Bhattacharyya S.N., Sonenberg N. (2008): Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews, 9, 102–114.

Fu S.Y., Zhao D.C., Zhao H.L., Li J.Q., Zhang W.G. (2012): A method for screening microRNA target genes in goat skin by microRNA seed sequence mediated PCR. Yi Chuan, 34, 919–926.

Gu Y.R., Liang Y., Gong J.J., Zeng K., Li Z.Q., Lei Y.F., He Z.P., Lv X.B. (2012): Suitable internal control microRNA genes for measuring miRNA abundance in pig milk during different lactation periods. Genetics and Molecular Research, 11, 2506–2512.

He X.Y., Hao H.Q., Liu D.D., Fan R.W., Cao J., Zhu Z.W., Dond Y.J., Xing H.Q. (2010): Difference of microRNA expression in the ear and back skin of young alpaca (Lama pacos). Chinese Journal of Biochemistry and Molecular Biology, 26, 1016–1022.

Ji Z., Wang G., Xie Z., Wang J., Zhang C., Dong F., Chen C. (2012): Identification of novel and differentially expressed microRNAs of dairy goat mammary gland tissues using Solexa sequencing and bioinformatics. PLoS One, 7, e49463.

Kloosterman W.P., Plasterk R.H. (2006): The diverse functions of microRNAs in animal development and disease. Developmental Cell, 11, 441–450.

Latham G.J. (2010): Normalization of microRNA quantitative RT-PCR data in reduced scale experimental designs. Methods in Molecular Biology, 667, 19–31.

Li Z., Liu H., Jin X., Lo L., Liu J. (2012): Expression profiles of microRNAs from lactating and non-lactating bovine mammary glands and identification of miRNA related to lactation. BMC Genomics, 13, 731.

Liu C.G., Calin G.A., Meloon B., Gamliel N., Sevignani C., Ferracin M., Dumitru C.D., Shimizu M., Zupo S., Dono M., Alder H., Bullrich F., Negrini M., Croce C.M. (2004): Proceedings of the National Academy of Sciences of the United States of America, 101, 9740–9744.

Liu Z., Xiao H., Li H., Zhao Y., Lai S., Yu X., Cai T., Du C., Zhang W., Li J. (2012): Identification of conserved and novel microRNAs in cashmere goat skin by deep sequencing. PLoS One, 7, e50001.

Lu J., Getz G., Miska E.A., Alvarez-Saavedra E., Lamb J., Peck D., Sweet-Cordero A., Ebert B.L., Mak R.H., Ferrando A.A., Downing J.R., Jacks T., Horvitz H.R., Golub T.R. (2005): MicroRNA expression profiles classify human cancers. Nature, 435, 834–838.

doi: 10.17221/8782-CJAS

Mardaryev A.N., Ahmed M.I., Vlahov N.V., Fessing M.Y., Gill J.H., Sharov A.A., Botchkareva N.V. (2010): MicroRNA-31 controls hair cycle-associated changes in gene expression programs of the skin and hair follicle. FASEB Journal, 24, 3869–3881.

McGregor B.A., An M., Jiang Y. (1991): Fleece metrology of Liaoning cashmere goats. Small Ruminant Research, 4, 61–71.

Miska E.A., Alvarez-Saavedra E., Townsend M., Yoshii A., Sestan N., Rakic P., Constantine-Paton M., Horvitz H.R. (2004): Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biology, 5, R68.

Peltier H.J., Latham G.J. (2008): Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA, 14, 844–852.

Peters I.R., Peeters D., Helps C.R., Day M.J. (2007): Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. Veterinary Immunology and Immunopathology, 117, 55–66.

Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P. (2004): Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnology Letters, 26, 509–515.

Selbach M., Schwanhausser B., Thierfelder N., Fang Z., Khanin R., Rajewsky N. (2008): Widespread changes in protein synthesis induced by microRNAs. Nature, 455, 58–63.

Sempere L.F., Freemantle S., Pitha-Rowe I., Moss E., Dmitrovsky E., Ambros V. (2004): Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biology, 5, R13.

Tricarico C., Pinzani P., Bianchi S., Paglierani M., Distante V., Pazzagli M., Bustin S.A., Orlando C. (2002): Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Analytical Biochemistry, 309, 293–300.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3, research0034.1–0034.11.

Varshney N., Mohanty A.K., Kumar S., Kaushik J.K., Dang A.K., Mukesh M., Mishra B.P., Kataria R., Kimothi S.P., Mukhopadhyay T.K., Malakar D., Prakash B.S., Grover S., Batish K. (2012): Selection of suitable reference genes for quantitative gene expression studies in milk somatic cells of lactating cows (Bos indicus). Journal of Dairy Science, 95, 2935–2945.

Wang M., Moisa S., Khan M.J., Wang J., Bu D., Loor J.J. (2012): MicroRNA expression patterns in the bovine mammary gland are affected by stage of lactation. Journal of Dairy Science, 95, 6529–6535.

Wenguang Z., Jianghong W., Jinquan L., Yashizawa M. (2007): A subset of skin-expressed microRNAs with possible roles in goat and sheep hair growth based on expression profiling of mammalian microRNAs. OMICS, 11, 385–396.

Wessels J.M., Edwards A.K., Zettler C., Tayade C. (2011): Selection and validation of reference genes for miRNA expression studies during porcine pregnancy. PLoS ONE, 6, e28940.

Xia J., Joyce C.E., Bowcock A.M., Zhang W. (2013): Noncanonical microRNAs and endogenous siRNAs in normal and psoriatic human skin. Human Molecular Genetics, 22, 737–748.

Yuan C., Wang X., Geng R., He X., Qu L., Chen Y. (2013): Discovery of cashmere goat (Capra hircus) microRNAs in skin and hair follicles by Solexa sequencing. BMC Genomics, 14, 511.

Received: 2014–12–08
Accepted after corrections: 2015–09–21

Corresponding Author

Dr. Rong H. Yin, Shenyang Agricultural University, College of Animal Science and Veterinary Medicine, Dongling Road 120, Shenyang 110866, P.R. China
Phone: +86 24 884 871 56, e-mail: yinronghuan@163.com