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Selection of accurate reference genes in mouse trophoblast stem cells for reverse transcription-quantitative polymerase chain reaction

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Abstract. Mouse trophoblast stem cells (TSCs) form colonies of different sizes and morphologies, which might reflect their degrees of differentiation. Therefore, each colony type can have a characteristic gene expression profile; however, the expression levels of internal reference genes may also change, causing fluctuations in their estimated gene expression levels. In this study, we validated seven housekeeping genes by using a geometric averaging method and identified *Gapdh* as the most stable gene across different colony types. Indeed, when *Gapdh* was used as the reference, expression levels of *Elf5*, a TSC marker gene, stringently classified TSC colonies into two groups: a high expression groups consisting of type 1 and 2 colonies, and a lower expression group consisting of type 3 and 4 colonies. This clustering was consistent with our putative classification of undifferentiated/differentiated colonies based on their time-dependent colony transitions. By contrast, use of an unstable reference gene (*Rn18s*) allowed no such clear classification. *Cdx2*, another TSC marker, did not show any significant colony type-specific expression pattern irrespective of the reference gene. Selection of stable reference genes for quantitative gene expression analysis might be critical, especially when cell lines consisting of heterogeneous cell populations are used.

Key words: Gene expression, Mouse, Reverse transcription-quantitative polymerase chain reaction, Trophoblast stem cell

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Trophoblast stem cells (TSCs) are representative of the multipotent trophectoderm, showing extensive self-renewal ability in the presence of fibroblast growth factor (FGF) 4 and heparin [1]. Since the first establishment of TSCs in 1998, they have been widely used in the field of trophoblast study, providing invaluable information on the mechanisms of trophoblast proliferation and differentiation. However, unlike their embryonic counterparts—embryonic stem cells—TSCs are inherently prone to spontaneous differentiation *in vitro* [2, 3], and therefore, thought to be heterogeneous and contain both undifferentiated and differentiating trophoblast cells. Indeed, a few days after passage, TSCs occasionally contain trophoblastic giant cells, a terminally differentiated form of trophoblast cells. This unstable nature of TSCs results in a time-dependent transition of their colony morphology, which changes from the primary dome-like shape to a flattened, loose shape within a few days (types 1–4; see below). Therefore, it is reasonable to assume that the gene expression profiles of TSC colonies might reflect their undifferentiated/differentiation status. The colony-dependent changes in the expression levels of specific TSC marker genes can be traced by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) amplification. However, the accuracy of the gene expression levels provided by RT-qPCR highly depends on the selection of appropriate internal reference gene(s). As a matter of fact, commonly used reference genes are known to modulate their expression levels, in particular between distinct cell and tissue types [4, 5]. In the present study, we sought to identify stable reference genes that could be used for RT-qPCR analysis of different types of TSC colonies. For this purpose, we employed the geNorm algorithm [4, 6] to determine the most stable reference genes from a set of candidate reference genes in TSC colonies. Using this analysis, a gene-expression normalization factor was calculated for each sample, based on the geometric mean of a defined number of reference genes.

TSC colonies can be classified into four major types depending on their morphology (Fig. 1A): type 1, small, compact and dome-shaped; type 2, compact and flattened; type 3, similar to type 2 but with loose and multilayered cell clusters in their centers; and type 4, similar to type 3 but with an extensive multilayered area. There is also an additional type 5, with a sparse monolayered appearance that is observed rarely in the standard FGF4- and heparin-containing medium (see below). Therefore, this type was not analyzed here. Our time-lapse live-imaging observations revealed that type 1 colonies appeared predominantly after passaging, and that a single type 1 colony gave rise to all other types. During these colony transitions, type 2 colonies appeared at an earlier stage, followed by the formation of types 3 and 4. These colony transitions were mostly irreversible. Thus, we putatively designated types 1 and 2 as undifferentiated and types 3 and 4 as more differentiated. The proportions of each type appearing from type 1 colonies after passaging are shown in Fig.

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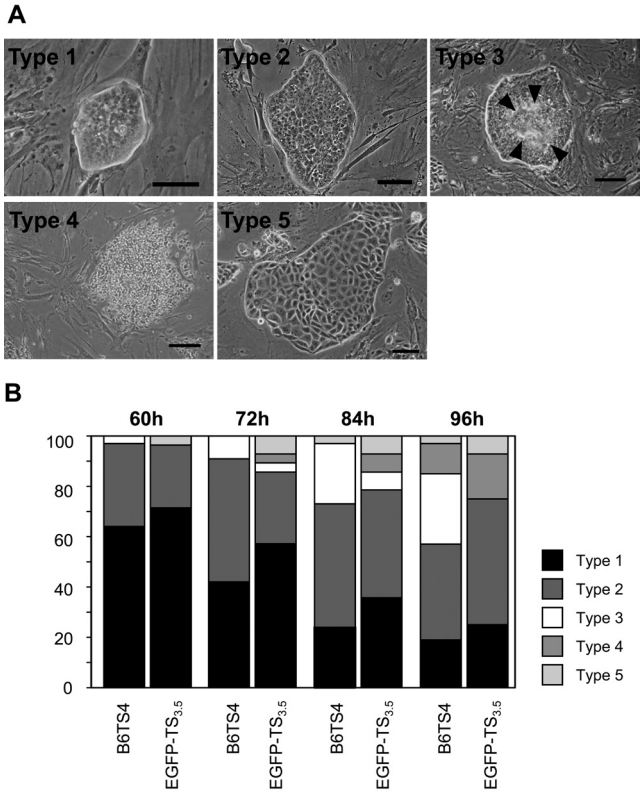

Fig. 1. A: Morphology of TSC colonies in the B6TS4 line. Type 1 colonies were small, compact and dome-shaped; type 2 colonies were compact and flattened; type 3 colonies were similar to type 2 but with loose and multilayered cell clusters (arrowheads) in their centers; type 4 colonies were similar to type 3 but with an extensive multilayered area; and type 5 colonies were similar to type 2 but with a sparse monolayered appearance. Scale bar = 100 μm. B: The percentages of colony types appearing from type 1 colonies after passaging. The percentages of type 1 colonies decreased while those of type 3 and 4 colonies increased with time. The same tendency was also found in the EGFP-$TS_{3.5}$ line, except for a relatively smaller population of type 3 colonies.

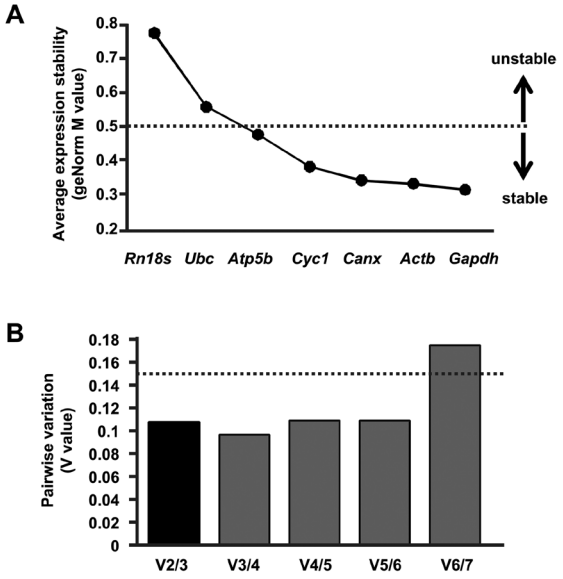

Fig. 2. A: Ranking of reference genes based on their stability (geNorm M value) provided by geNorm analysis. The higher geNorm M values (left) indicate lower stability and the lower M values (right) indicate higher stability. The dotted line indicates 0.5 as the cutoff value. B: This bar chart shows the pairwise variation (V value) that helps in determining the optimal number of reference genes for normalization. The V value is calculated between two sequential normalization factors from the most stable gene to the least stable one. A V value under the 0.15 threshold suggests a minimum required number of reference genes. The black bar indicates an optimal number of reference targets in this experiment. The dotted line at 0.15 indicates the threshold V value.

1B, illustrating a decrease in type 1 colonies and an increase in types 3 and 4 colonies along time after passaging. We found that another TSC line, EGFP-$TS_{3.5}$, commonly used in other TSC studies [7, 8], showed a colony transition pattern similar to that of B6TS4, except for a relatively smaller population of type 3 colonies (Fig. 1B).

To determine stable reference genes across different colony types in a mouse TSC line (B6TS4), we calculated the average expression stability (geNorm M value) with qbase^{Plus} software (Biogazelle, Gent, Belgium) (Fig. 2A). The reference genes used in the current study are listed in Table 1. The genes with lower M values are considered more stable and 0.5 is the threshold value between stable and unstable reference genes. Thus, *Atp5b*, *Cyc1*, *Canx*, *Actb*, and *Gapdh* were determined as stable genes. Among these, *Gapdh* was the most stable, followed by *Actb*. Next, we calculated pairwise variation (V value) by qbase^{Plus} software for determining the optimal number of reference genes. V values below 0.15 suggest a minimum required number of reference genes for normalization [4]. As a result, we found that a combination of the two most stable genes, *Gapdh* and *Actb*, was sufficient for normalization (Fig. 2B).

To evaluate the influence of reference genes on the expression profiles of target genes across different colony types, the mRNA levels of *Elf5* (E74-like factor 5) and *Cdx2* (caudal-related homeobox 2), undifferentiated TSC marker genes [9–12], were normalized against a single reference gene or combinations of selected reference genes. We chose to analyze these two genes because *Cdx2* is known as the key regulator for specification of the extraembryonic lineage [13] and *Elf5* is essential for the establishment of TSC lines by sustaining the self-renewal of mouse extraembryonic ectoderm cells [12]. To visualize the gene expression trends, the sample data have been arranged in order of expression level or plotted within the column corresponding to each colony type using the mean values (Figs. 3 and 4).

The relative expression levels of *Elf5* were divided into two groups when the combination of the two most reliable genes (*Gapdh* and *Actb*) was used for normalization: a higher expression group consisting of types 1 and 2 colonies and a lower expression group consisting of types 3 and 4 colonies. The same pattern was also obtained with the most stable gene, *Gapdh*, alone. However, the pattern was slightly changed with *Actb*, the second most stable gene, or with all seven reference genes, some samples switching their positions. With the

Table 1. The seven candidate reference genes

Official symbol	Full name	Function
Actb	Actin, beta	One of six different actin isoforms that play important roles in determining cell shape and controlling cell movement.
Atp5b	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner mitochondrial membrane during oxidative phosphorylation.
Canx	Calnexin	A calcium-binding, endoplasmic reticulum associated protein.
Cyc1	Cytochrome C1	A small heme protein that acts in the mitochondrial respiratory chain by transferring electrons from the Rieske iron–sulfur protein to cytochrome c.
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	An enzyme involved in energy metabolism and in the production of ATP and pyruvate through anaerobic glycolysis.
Ubc	Ubiquitin C	A polyubiquitin precursor conjugated to target proteins via an isopeptide bond as a monomer.
Rn18s	18S ribosomal RNA	A part of the ribosomal RNA. Ribosomal RNAs perform critical functions in the ribosome that allow protein synthesis to occur.

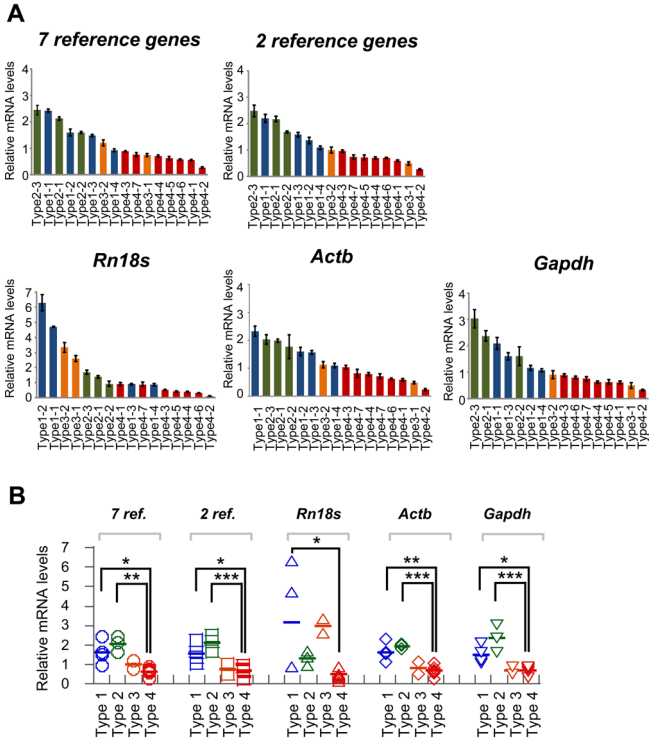

Fig. 3. Relative expressions levels of *Elf5* normalized against different reference genes in colony samples from all four colony types. A: The relative expression levels of *Elf5* in each colony sample are arranged from highest to lowest expression level. The samples were divided into two groups: an undifferentiated group (types 1 and 2) and a differentiated group (types 3 and 4) when the most stable reference gene (*Gapdh*) and the combination of the two most stable reference genes were used for normalization. However, the normalization with the least stable gene (*Rn18s*) resulted in no discrimination among colony types. B: Dot plots showing the *Elf5* expression levels of each sample and the mean values. There were significant differences between types 1 and 4 and between types 2 and 4, with an exception for the analysis using *Rn18s*. 7 reference genes, *Rn18s*, *Ubc*, *Atp5b*, *Cyc1*, *Canx*, *Actb*, and *Gapdh*. 2 reference genes, *Actb* and *Gapdh*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

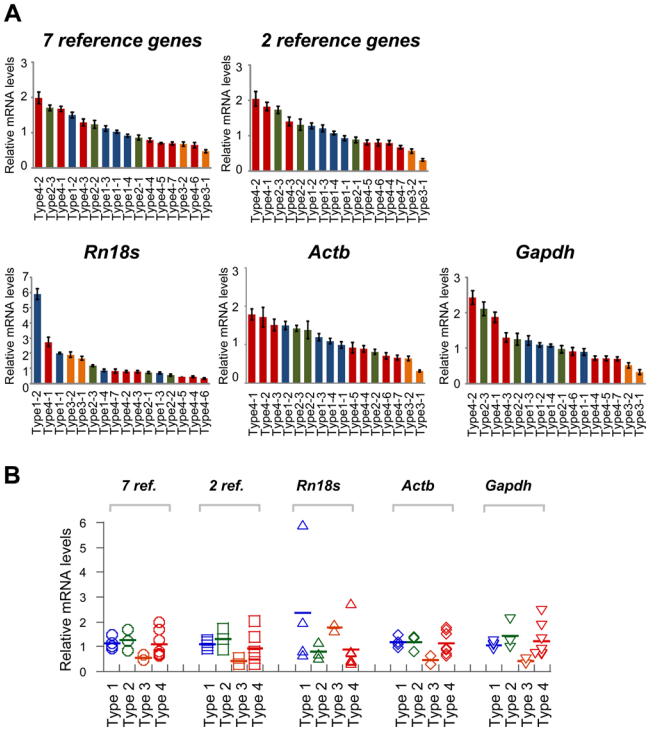

Fig. 4. Relative expression levels of *Cdx2* normalized against different reference genes in samples from all four colony types. A: The relative expression levels of *Cdx2* in each colony sample are arranged from highest to lowest expression level. There were no distinctive colony type-specific patterns, irrespective of the reference gene used. B: Dot plots showing the *Cdx2* expression levels of each sample and the mean values. There were significant differences between colony types. Type 1 colony included one exceptionally high sample when *Rn18s* was used. 7 reference genes, *Rn18s*, *Ubc*, *Atp5b*, *Cyc1*, *Canx*, *Actb*, and *Gapdh*. 2 reference genes, *Actb* and *Gapdh*.

most unstable gene, *Rn18s*, the order became more random (Fig. 3A). When the mean *Elf5* expression levels were compared between the colony types, there were significant differences between types 1 and 4 and between types 2 and 4, except when using *Rn18s* for the analysis (Fig. 3B). Thus, the use of reliable genes as references resulted in putative identification of undifferentiated colony types (1 and 2) and differentiated colony types (3 and 4) based on the expression levels of *Elf5*.

We also analyzed the expression levels of *Cdx2*, another TSC marker gene. When the combination of both the two most reliable genes was used for normalization, the relative expression levels were intermediate in types 1 and 2 colonies, and the lowest in type 3 colonies (Fig. 4A). Intriguingly, type 4 colonies were split into higher and lower expression groups. A similar pattern was obtained when either of these most reliable genes, *Gapdh* or *Actb*, were used. However, when a combination of all seven reference genes or the most unstable gene (*Rn18s*) was used, the samples spread more randomly (Fig. 4A). Furthermore, one extremely high expression sample appeared with the latter. Unlike *Elf5*, there were no significant differences in the mean expression levels of *Cdx2* between the colony types (Fig. 4B). Use of the unstable *Rn18s* resulted in a greater variation of type 1 colonies.

We did not expect the *Cdx2* expression levels of TSC colonies not to be correlated with their undifferentiated statuses because this is one of the most frequently used genes for marking undifferentiated trophoblasts [14]. Therefore, we examined whether further differentiation of TSCs would result in downregulation of *Cdx2*, using *Gapdh* as the internal reference gene. We cultured TSCs under the FGF4- and heparin-free condition, which is known to strongly induce the differentiation of TSCs (Fig. 5A). After the removal of FGF4 and heparin at 48 h after passaging, the colonies started to change morphology, resulting in the transformation of most colonies into type 5 by 96 h (48 h after the removal of FGF4 and heparin; Fig. 5B). This change was associated with downregulation of *Cdx2* as well as *Elf5* (Fig. 5C). Taken together, it is likely that both *Cdx2* and *Elf5* can be used as TSC markers, but *Elf5* might be a better indicator of the undifferentiated status of TSCs maintained under standard culture conditions including FGF4 and heparin.

These results were consistent with the initial analysis carried out for the identification of stable reference genes (M value): the most stable gene was *Gapdh* and the most unstable one was *Rn18s*. In general, geometric averaging methods are used to determine the minimum number of reference genes required to calculate a reliable normalization factor. However, in our case, the combination of two genes (*Gapdh* and *Actb*) was sufficient to obtain a reliable normalization. Furthermore, the use of the single gene (*Gapdh*) gave similar results to those based on a combination of both genes. This probably reflected the high stability of these two genes across the different colony types. When *Rn18s* was used, the results were highly variable for both *Elf5* and *Cdx2*, with a tendency for a relatively higher expression among type 3 colonies (Figs. 3A and 4A), probably indicating the biased normalization pattern specific to this gene.

Our findings suggest that the use of stable reference genes is critical in order to gain an accurate understanding of the gene expression profiles of cultured cell lines, especially when they are composed of heterogeneous cell populations. In addition, this study identified

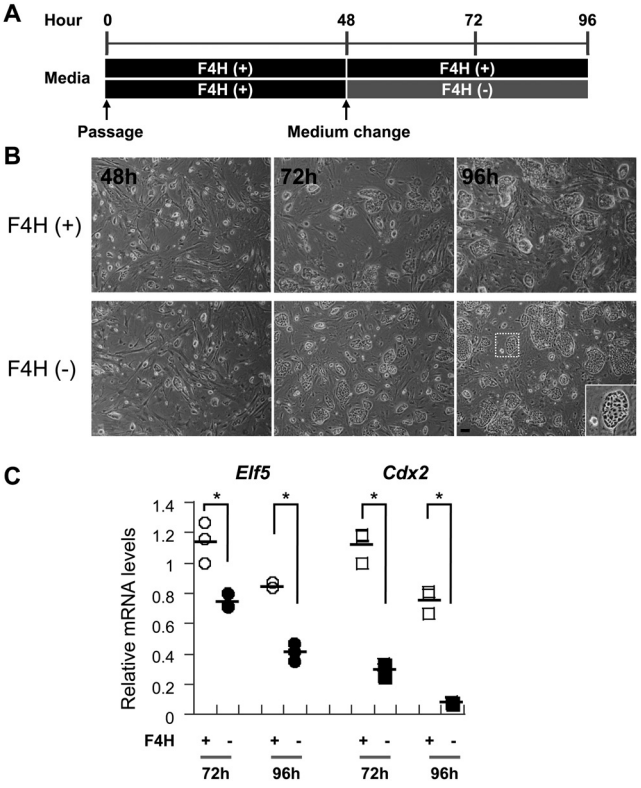

Fig. 5. Effect of the removal of FGF4 and heparin (F4H) on colony morphology and gene expressions in TSCs. A: Experimental design for the observation of the effect of the removal FGF4- and heparin-free culture conditions. B: Morphological changes in TSC colonies cultured with or without FGF4 and heparin. Type 5 colonies with a sparse monolayered shape became predominant in the absence of FGF4 and heparin by 96 h. (Inset) Higher magnification showing a typical type 5 colony enclosed by a dotted rectangle. Scale bar = 100 µm. C: Relative expression levels of *Cdx2* and *Elf5* in TSCs cultured with or without FGF4 and heparin. *Gapdh* was used as an internal control for normalization. * $P < 0.05$.

Elf5 as a more reliable indicator for the undifferentiated status of TSCs than *Cdx2*. It could be important to verify the applicability of this finding to other TSC lines as the ones currently available show highly variable characteristics in terms of their proliferation *in vitro* and differentiation *in vivo*.

Materials and Methods

Cell lines

The TSC lines used in this study were B6TS4 and EGFP-$TS_{3.5}$, which were derived from a blastocyst of the C57BL/6 and ICR mouse strain, respectively. TSCs were cultured as described previously [1]. In brief, cells were cultured on mitomycin-C (Sigma-Aldrich, St. Louis, MO, USA)-treated primary mouse embryonic fibroblasts in RPMI1640 medium (Thermo Fisher Scientific, San Jose, CA, USA) with 20% fetal bovine serum (Thermo Fisher Scientific), 25 ng/ml human recombinant FGF4 (Wako Pure Chemicals, Osaka,

Japan), 1 μg/ml heparin (Sigma-Aldrich), 100 μM 2-mercaptoethanol (Sigma-Aldrich), 1% GlutaMAX (Thermo Fisher Scientific), and 1 mM sodium pyruvate (Thermo Fisher Scientific).

Colony classification and sample collection

TSC colonies could be classified into four major types by their morphology. Representative colony morphologies are shown in Fig. 1, together with descriptions of their morphological characters. There was an additional type (type 5), but this was not analyzed here because it rarely emerged under normal TSC culture conditions. At 96 h after passaging, colonies were picked up using a glass capillary pipette under a dissecting microscope and used for RT-qPCR analysis. Each sample consisted of 60–80 colonies of the same colony type.

RT-qPCR

Total RNA for RT-qPCR was extracted with RNeasy Micro kits (Qiagen, Venlo, Netherlands) from TSC colonies. Following extraction, the first strand cDNA was synthesized with a SuperScript III reverse transcriptase reagent set (Thermo Fisher Scientific). Gene expression was assessed by qPCR on a StepOnePlus™ instrument (Thermo Fisher Scientific) using Quantitect SYBR Green PCR kits (Qiagen) according to the manufacturer's instructions. Samples were denatured at 95°C for 10 min, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 sec, and an annealing and extension step at 60°C for 1 min. Raw Cq (Ct) values (PCR cycles at which the fluorescence signal crosses threshold) were calculated using StepOne software (v. 2.1; Thermo Fisher Scientific) setting baseline and appropriate threshold values. All runs were performed in triplicate and an identical sample was used in each different runs as an inter-run calibration sample to correct for the technical variance between the runs and thus compare results from different plates [4, 6]. The primer sets used for TSC marker genes were the following: *Cdx2*, 5′–GCAGTCCCTAGGAAGCCAAG–3′ and 5′–GCAGCCAGCTCACTTTTCCT–3′; *Elf5*, 5′–GTGGCATCCTGGAATGGGAA–3′ and 5′–CACTAACCTC CGGTCAACCC–3′. For quantification of reference genes (*Atp5b*, *Canx*, *Cyc1*, *Gapdh*, *Ubc*, *Rn18s*), the primer sets from the Mouse geNorm Kit (Primerdesign Ltd., Southampton, UK) were used. *Actb* was analyzed as an additional reference gene using the primer set 5′–CTGTCGAGTCGCGTCCA–3′ and 5′–ACCCATTCCCACCATCACAC–3′.

Data analysis

The baseline cycles and cycle thresholds were established manually for each gene. To assess the stability of the reference genes, the raw Cq values of each reference gene were analyzed with the geNorm algorithmic tool using qbase^{Plus} software (Biogazelle). These values were converted into normalized relative quantities (NRQs) with multiple reference genes based on the modified classic Δ-Δ-Ct method [6] or a single reference gene. NRQs were scaled in relation to the mean across all samples for each target or to reference genes. We ranked the stability of the reference genes based on the mean expression stability (geNorm M value) provided by the geNorm program. To determine the optimal number of reference genes across different colony types, qbase^{Plus} was also used to calculate pairwise variation (V value). Normalization of the expression levels of TSC marker genes (*Cdx2* and *Elf5*) was performed by qbase^{Plus} software using reference gene(s) ranked as described above.

Statistical Analysis

To compare the gene expression levels between colony types within the TSC lines, normalized relative mRNA levels were analyzed with Kruskal-Wallis tests followed by Dunnett's multiple comparison tests; $P < 0.05$ was considered statistically significant.

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