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

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/differentiated 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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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-TS3.5, 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.

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⁺⁺ 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 the most 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⁺⁺ 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 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 differ-
entiation 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
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-TS3.5,
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 passage, 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 (Ci) 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′-GCAGTCCTCTAGAAGCCCAAG-3′ and 5′-GCACGCGCTCAGCTTTCTTCT-3′; Elf5, 5′-GTGGCAATCTGGAATGGGA-3′ and 5′-ACCCATTCCCACCATCACAC-3′. Cq values of each reference gene were analyzed with the geNorm for each gene. To assess the stability of the reference genes, the raw NRQs were scaled in relation to the mean expression stability (geNorm M value) provided by the geNorm program. To determine the optimal number of reference genes across different colony types, qbasePlus was also used to calculate pairwise variation (V value). Normalization of the expression levels of TSC marker genes (Cdx2 and Elf5) was performed by qbasePlus 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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