

Effect of Culture Conditions on Reference Genes Expression in Placenta-derived Stem Cells

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Background and Objectives: Normalization with valid reference genes is crucial for gene expression analysis with quantitative real-time reverse transcription PCR (qRT-PCR). This is especially relevant when stem cells are investigated with respect to gene expression in the differentiation process. Due to the plasticity of the stem cells, the variation of reference gene expression may cause misinterpretation of the target gene expression. **Methods and Results:** In this study, we investigated the gene expression stability of commonly used 32 reference genes in placenta-derived stem cells, which were cultured with or without exogenous epidermal growth factor. The influence of unstable reference gene expression on the data interpretation was also demonstrated with stem cell marker gene expressions on the placenta-derived stem cells. Statistical validation analysis of reference genes revealed the stability of each gene. Commonly used β -actin, 18S and GAPDH expression were relatively instable. The cell cycle relating house keeping genes, PPIA, POLR2A, and POP4 were most stable in the compared culture conditions. Reference genes were divided into the following three groups and statistically analyzed; 1) unstable genes, 2) stable genes, and 3) commonly used genes. The results indicate that the interpretation of the experiments was significantly different depending on the stability of the reference genes. **Conclusions:** In the stem cell experiments, even minor differences in the culture conditions influenced the expression of reference genes. Thus, the identification of valid reference genes must be determined at each experimental setting. We recommend performing a stepwise screening process to determine valid reference genes.

Keywords: Quantitative real-time PCR, Stem cells, Reference genes, Placenta, Human

Introduction

In recent biology research, quantification of mRNA using real-time reverse transcription PCR (qRT-PCR) is one of the most frequently used technologies to quantify relative gene expression (1). Based on the principle of polymerase chain reaction and fluorescent reporter system, the technology enables to quantify low abundance messenger RNA (mRNA). The method is reliable, affordable, and relatively easy to perform to investigate mRNA expressions

at a particular time point, or in interested relevant cells or tissue types.

There are several methods to quantify and compare the gene expression (2). Most of these methods use house-keeping gene expressions as reference to obtain relative values of interested gene expressions. The strategy stands on the stability of reference gene expression. Selection of the appropriate reference gene is the key for accuracy of the relative value data. Commonly, housekeeping genes such as β -actin, 18S, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are used as reference genes (3). In general cell biology, these genes are stable and may not be of concern as long as the same cell types are investigated. However, the selection of reference genes must be carefully addressed in stem cell biology. Most of the time, qRT-PCR technology is used to evaluate either stem cell specific gene expression or interested cell type or cell line-

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age specific gene expression. Unlike other primary cells or cell line cells, stem cells are unique for the plasticity in any aspect, including gene expressions. Along with the morphological changes, the cell structural gene (e.g. β -actin) expressions may be altered. The metabolic activity-related gene (e.g. GAPDH) expressions may also change during the differentiation. However, often times, the importance of selecting appropriate reference genes is undervalued.

In this present study, we investigated the effect of culture conditions on reference gene expressions in placenta-derived stem cells (4). Previously we have shown that human placenta contains stem cells, which express stem cell surface marker and stem cell specific marker genes, such as OCT4, NANOG (5). Under certain culture conditions these cells showed differentiation into all three germ layer cells (5-7). Based on the plasticity, naïve human amniotic epithelial cells, which contain the placenta-derived stem cells, are considered a suitable material to evaluate effects of minimal difference of culture conditions on the reference gene expression and the data interpretation.

Materials and Methods

Cell isolation and culture conditions

Human placentae were obtained with the approval of the University of Pittsburgh institutional review board (IRB), after uncomplicated elective caesarean deliveries from healthy mothers. Amniotic epithelial (AE) cells were enzymatically dissociated with 0.05% trypsin containing 0.53 mM EDTA-4Na as described previously (8). Viability of the AE cells was determined by exclusion of trypan blue dye and counted with a hemocytometer. Five million AE cells were plated on 100 mm diameter cell culture dishes in culture media with or without 10 ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ) for 7 days. The cell culture media was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acid, 55 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% antibiotic-antimycotic (All from Invitrogen/Gibco, Grand Island, NY).

RNA isolation and cDNA synthesis

Total RNA was isolated from the cell samples at day 0, and day 7 with or without EGF with an mRNA isolation kit (QIAGEN, Valencia, CA). The RNA concentration of each sample was fluorometrically measured (Qubit, Invitrogen). The integrity of RNA samples was

further confirmed with the 28S/18S ratio (>1.0) on 1% agarose gel using Gel Logic 100 Imaging System (Kodak Molecular Imaging Systems, New Haven, CT). Two micrograms of total RNA were used to synthesize cDNA (Promega, Madison, WI). The reaction mixture was incubated at 42°C for 60 minutes, followed by heat inactivation of the enzyme at 95°C for 5 minutes. After cooling on ice for 5 minutes, the cDNA was stored at -20°C until analysis.

Quantitative real-time PCR

The TaqMan[®] Express Human Endogenous Control Plate (Applied Biosystems, Foster City, CA) that contains 32 genes in triplicate were used to evaluate commonly used reference genes (Table 1). The cDNA samples (5 ng) were subjected to quantitative real-time PCR (qPCR) using an ABI PRISM 7,000 (Applied Biosystems) according to the instructions provided by the manufacturer. As experimental target genes, three additional stem cell specific genes were chosen for analysis. The pre-designed TaqMan probe and primer sets for OCT4 (Hs03005111_g1), NANOG (Hs02387400_g1), and KLF4 (Hs00358836_m1), were selected from the library of TaqMan Gene Expression Assays (Applied Biosystems).

Statistical analysis

The relative variations of multiple reference genes were calculated by statistical algorithms using the geNorm software package (9). In the first analysis the software returns the internal control gene stability measure (M) for all candidate genes. Furthermore, the software plots two types of graphs. In one of these graphs it plots the average expression stability values of remaining reference genes during stepwise exclusion of the least stable reference gene. This process is repeated until two genes remain. In a second graph the minimal number of reference genes for normalization was determined. This determination is based on the stepwise inclusion of reference genes in the calculation of pairwise variation (V) of a normalization factor (NF). A large variation indicates the added gene has a significant effect and should be included.

The relative expression software tool (REST) was used to quantify mRNA expression of each target gene (10). The REST analysis provides statistical information suitable for comparing groups of treated versus untreated samples by using simple statistical randomization tests with the consideration of multiple reference genes. In this study, gene expression of the day 0 (starting point) samples were used as control group and the samples of EGF(+) and EGF(-) of day 7 samples were statistically

Table 1. List of the tested reference genes

Symbol	Gene name	TaqMan assay ID
18s	Eukaryotic 18S rRNA	Hs99999901_s1
GAPDH	Glyceradehyde-3-phosphate dehydrogenase	Hs99999905_m1
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1	Hs99999909_m1
GUSB	Glucuronidase, beta	Hs99999908_m1
ACTB	Actin, beta	Hs99999903_m1
B2M	Beta-2-microglobulin	Hs99999907_m1
HMBS	Hydromethylbilane synthase	Hs00609297_m1
IPO8	Importin 8	Hs00183533_m1
PGK1	Phosphoglycerate kinase 1	Hs99999906_m1
RPLP0	Ribosomal protein, large	Hs99999902_m1
TBP	TATA box binding protein	Hs99999910_m1
TFRC	Transferrin receptor	Hs99999911_m1
UBC	Ubiquitin C	Hs00824723_m1
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Hs00237047_m1
PPIA	Peptidylprolyl isomerase A	Hs99999904_m1
POLR2A	Polymerase II polypeptide A	Hs00172187_m1
CASC3	Cancer susceptibility candidate 3	Hs00201226_m1
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Hs00355782_m1
CDKN1B	Cyclin-dependent kinase inhibitor 1B	Hs00153277_m1
GADD45A	Growth arrest and DNA-damage-inducible 45 alpha	Hs00169255_m1
PUM1	Pumilio homolog 1	Hs00206469_m1
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	Hs00197826_m1
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha	Hs00426752_m1
PES1	Pescadillo homolog 1, containing BRCT domain	Hs00362795_g1
ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	Hs00245445_m1
ELF1	E74-like factor 1	Hs00152844_m1
MT-ATP6	Mitochondrially encoded ATP synthase 6	Hs02596862_g1
MRPL19	Mitochondrial ribosomal protein L19	Hs00608519_m1
POP4	Processing of precursor 4, ribonuclease P/MRP subunit	Hs00198357_m1
RPL37A	60S ribosomal protein L37a	Hs01102345_m1
RPL30	Ribosomal protein l30	Hs00265497_m1
RPS17	Ribosomal protein S17	Hs00734303_g1

compared. Detailed information and free downloading for these statistical software, geNorm and REST, are available at the following websites:

<http://medgen.ugent.be/~jvdesomp/genorm/>

http://www.corbettlifescience.com/control.cfm?page=REST_2008

<http://rest.gene-quantification.info/>

Results

Statistical validation on 32 reference genes by geNorm

To identify the most stably expressed reference genes in samples from the starting point and two different culture conditions, the expression of a total of 32 genes (Table 1) was measured by quantitative RT-PCR. Data (Ct values) were further analyzed using the geNorm software. The first output showed that M values of all 32 genes were less than 1.5, which is considered as a cut-off value by the

geNorm software to use a gene as reference. The average expression stability output showed that there were some reference genes that led to a great variation (Fig. 1A). The exclusion of genes like GUSB or EIF2B1 led to a dramatic decrease in the average. From this analysis resulted that the most unstable reference gene was GUSB (11) and the most stable genes were PPIA (12) and POLR2A (13). The optimal number of reference genes was assessed calculating the pairwise variation (V) between the normalization factor (NF) with a given number of reference genes and the NF value obtained after the inclusion of the following less stable genes (Fig. 1B). The V values were plotted to determine where the inclusion of the additional gene has little effect on the normalization. As seen in Figure 1B, the inclusion of the first three genes as opposed to two was 0.007. According to Vandesompele, et. al. (9), the suggested cut-off value was 0.15 and at least 3 genes must be used as reference. Therefore, in this ex-

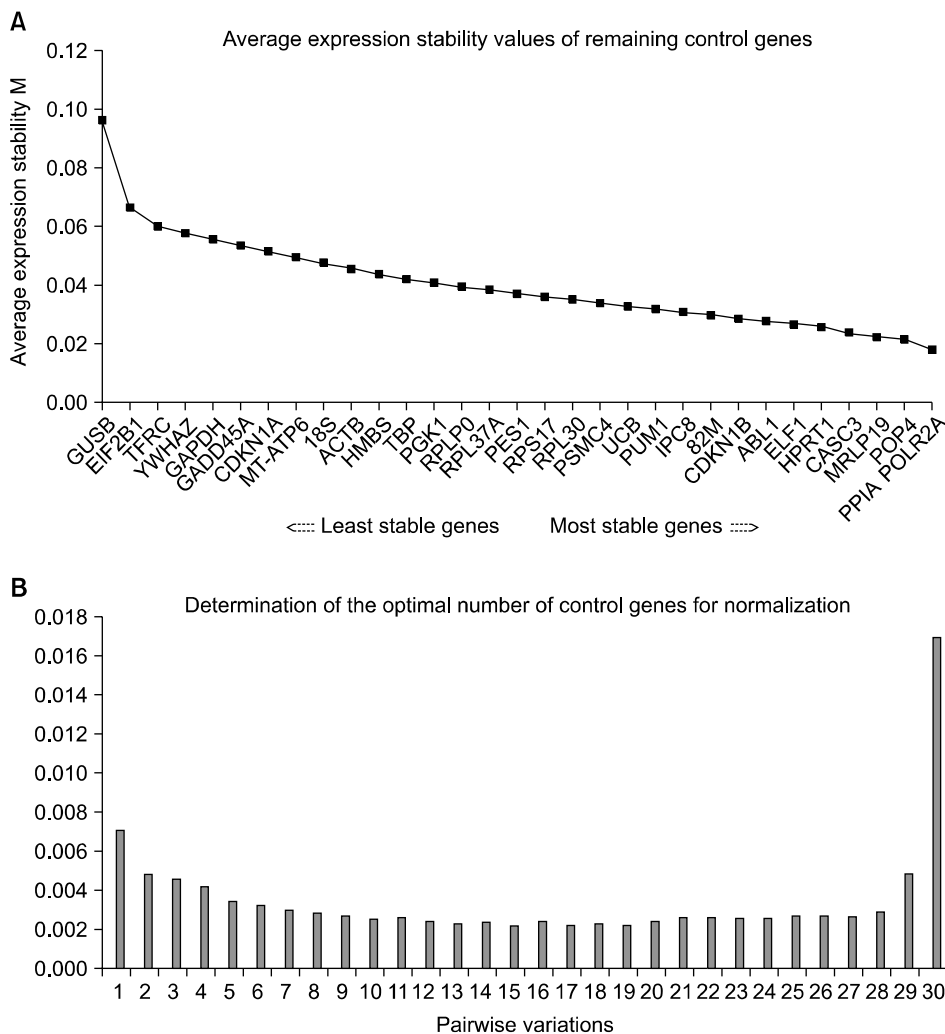


Fig. 1. Determination of the best reference gene. (A) Average expression stability values (M) of remaining control genes during step-wise exclusion of the least stable control gene. All 32 genes are ranked in order of their expression stability. Lower M value of average expression stability indicates more stable expression. (B) Determination of optimal number of control genes for normalization. The graph reports the pairwise variation ($V_{n/n+1}$) of the normalization factors (NFs). The first value corresponds to $V_{2/3}$.

perimental condition more than three genes were not necessary to perform the normalization. In this present study, we investigated the influence of three reference genes on the interpretation of expression data. It is important to note that the geNorm software determination ($M < 1.5$) has to be considered as the initial screening. Further evaluation is necessary to confirm the valid reference genes.

The influence of reference gene selection on the interpretation of stem cell specific gene expression

Expression profiles of the three stem cell specific genes, OCT4, NANOG, and KLF4, were statistically analyzed to investigate the influence of reference gene selection. A total of 9 reference genes were selected and divided into the following three groups based on their expression stability; Group 1: most unstable reference genes, GUSB, EIF2B1, TFRC (Fig. 2A), Group 2: most stable reference genes, PPIA, POLR2A, POP4 (Fig. 2B), and Group 3: commonly

used reference genes, GAPDH, 18S, β -actin (Fig. 2C). Each stem cell specific gene expression of EGF(+) and EGF(-) culture conditions was plotted as relative expression to the gene expression of the starting material (day 0). Although the profile of the graph looks similar, the statistical differences were completely different depending on the selection of the reference genes. With the most unstable reference gene, GUSB, the result indicates OCT4 expression was significantly increased in both culture conditions (Fig. 2A). However, when the considerably stable reference genes were used to analyze the data, the OCT4 expression was not significantly increased whereas the KLF4 expression was significantly decreased (Fig. 2B). The results based on the stable 3 reference genes were identical and the interpretation was confident. On the other hand, using some commonly used reference genes and less stable gene group, the statistical data analysis became variable and different from the data obtained with stable

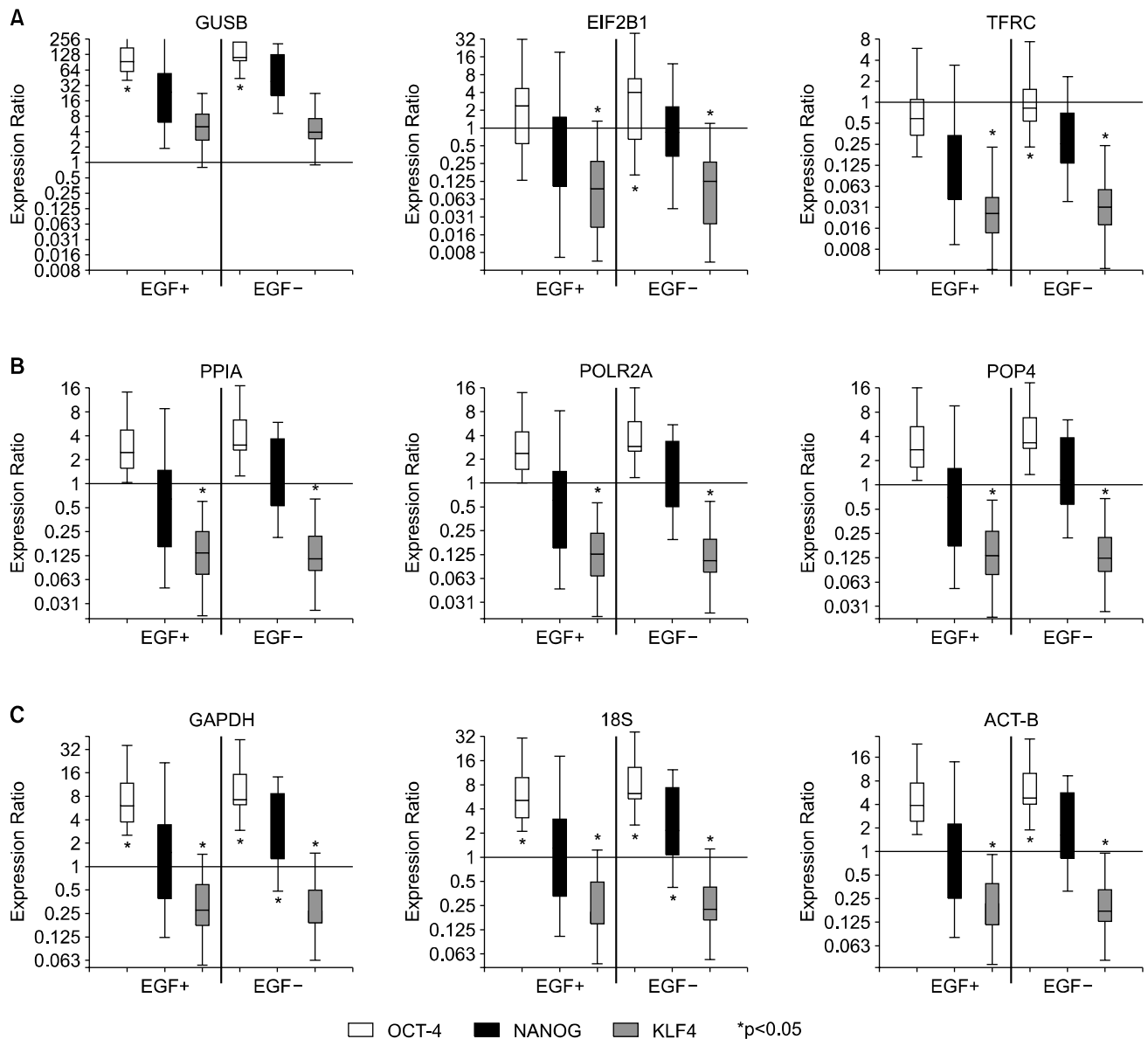


Fig. 2. Influence of the reference gene on the data statistical interpretation. Graphs show the expression of OCT4, NANOG and KLF4 in the placenta-derived stem cells cultured with and without EGF relative to the starting material. Statistically significant up/down regulation is marked by an asterisk ($p < 0.05$). Three groups of reference genes are shown: less stable genes group, more stable genes group based on the geNorm analysis (A and B, respectively) and the most commonly used genes group (C).

genes (Fig. 2A, 2C). When GAPDH and 18S were used as reference genes, the data indicates that OCT4 and KLF4 gene expressions were significantly changed. The significance of OCT4 gene expression in EGF treated groups was not detected when the most popular reference gene, β -actin, was used. The statistical results indicate that the selection of reference genes critically influences the gene expression analysis interpretation.

Discussion

In the present study, 32 commonly used reference gene expressions were investigated in human placenta-derived stem cells. Freshly isolated human placenta-derived stem cells were cultured in two different conditions for 7 days. The gene expression was statistically analyzed using the gene expression value of day 0 as the control group. Although the difference of the culture condition was minimum and the culture period was relatively short, the refer-

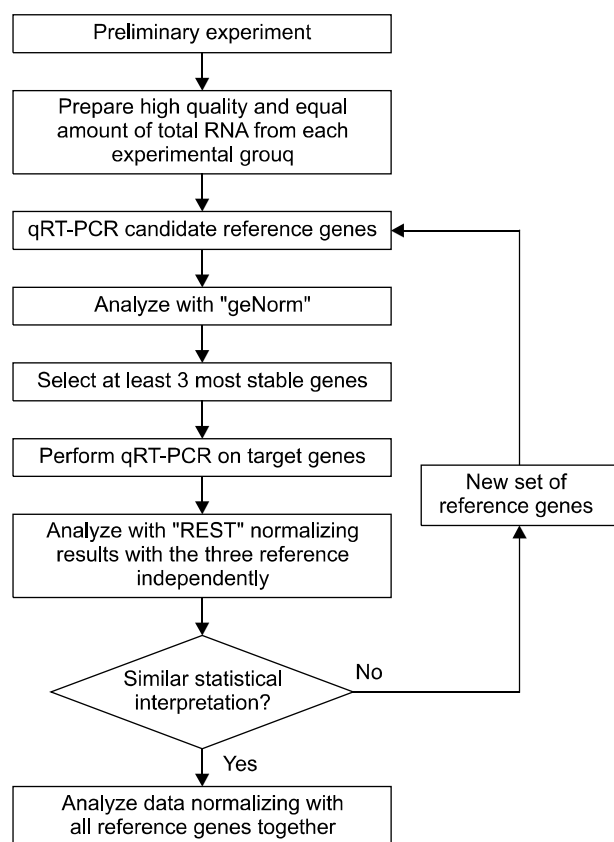


Fig. 3. Methodological flow chart. Stepwise procedure for performing selection of reference genes and gene expression analysis.

ence gene expressions were varied. And the selection of the reference gene significantly influenced the interpretation of the target gene expression. Influence of the selection of reference genes on the normalization has been reported in several cell types (14) including other types of stem cells such as embryonic stem cells (15, 16) and adipose-derived stem cells (17) but not placenta-derived stem cells. Most of the reports concluded that multiple reference genes might overcome the problems. However, in the present study we demonstrated that random selection of commonly used reference genes not only misleads the data interpretation, but may also cause confusion.

In consideration of the unique characteristics of stem cells, the selection of reference genes for expression analysis in stem cell research must be carefully performed. One of the strategical approaches is to identify valid reference genes by stepwise screening processes (Fig. 3). We recommend initial screening followed by multiple reference gene setup for each experiment with final confirmation. The selection of 12 reference genes will be sufficient for the initial screening of each experimental culture condition prior to the experimental analysis. For the initial screening, the

samples must be prepared with all different experimental conditions in use. Using the geNorm software, the nature and optimal number of reference genes will be determined. In each experiment, the selected reference genes will be used to normalize the expression of the target genes using the REST software. We suggest performing a crosschecking independently calculating the relative expression of the target genes using one reference gene each time. The analysis should return the same statistical interpretation for each reference gene used. At this point it will be possible to re-calculate the expression data using all reference genes together, which will yield results as accurate as possible. Failing in crosschecking will require repeating the reference gene selection and expanding the starting gene set.

In conclusion, the identification of valid reference genes is critically important to investigate the gene expression in stem cell research. We propose a methodological approach that considers not only stable reference gene selection but also the influence of the selection on the statistical interpretation of the results.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

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