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APPROPRIATE REFERENCE GENE SELECTION FOR REAL-TIME PCR DATA NORMALIZATION DURING RAT MESENCHYMAL STEM CELL DIFFERENTIATION

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Abstract

Reverse transcription quantitative PCR (RT-qPCR) is one of the best methods for the study of mesenchymal stem cell (MSC) differentiation by gene expression analysis. This technique needs appropriate reference or housekeeping genes (HKGs) to normalize the expression of the genes of interest. In the present study the expression stability of six widely used HKGs including *Actb, Btub, Hprt, B2m, Gusb* and *Tfrc* was investigated during rat MSC differentiation into osteocytes, adipocytes and chondrocytes lineages using geNorm and NormFinder software. RT-qPCR data analyzed by geNorm revealed the different sets of suitable reference genes for each cell type. NormFinder also showed similar results. Analysis of the combined data of MSCs with each differentiated cell type revealed the considerable shift in expression of some reference genes during differentiation; for example *Gusb* and *B2m* were among the least stable genes in MSCs but the most stable in chondrocytes. Normalization of specific genes for each lineage by different reference genes showed considerable difference in their expression fold change. In conclusion, for the appropriate analysis of gene expression during rat MSC differentiation and also for monitoring differentiation procedures, it is better to consider precisely the reference gene stability and select suitable reference genes for each purpose.

Key words: Mesenchymal stem cell, Housekeeping gene, Quantitative PCR, normalization.

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INTRODUCTION

MSCs are adult stem cells with a high replication capacity and the ability to differentiate into multiple cell lineages including osteocytes, adipocytes and chondrocytes (30). There is great hope for the application of these cells and their derivatives in future regenerative medicine and they are promising candidates for use in the regeneration of skeletal tissue injuries (7, 38). Furthermore, osteogenic and chondrogenic culture of MSCs would be an appropriate model for study of the development of bone and cartilage in-vitro.

During development, as a result of different extracellular signals, MSCs undergo complex differentiation procedures and phenotypic changes. To realize the potential of these cells, it is necessary to understand the processes that govern their differentiation.

One approach to investigating cellular processes during development of this lineage, is transcriptomic methods which are widely used as an initial step for analyzing the gene expression at mRNA level (27). Several techniques can be used to quantify the target gene expression, including high throughput techniques such as hybridization or sequence based microarray (18, 26) and low throughput techniques like RNase protection, Northern blot and semi-quantitative RT-PCR; among these the RT-qPCR has a distinguished position (3). While conventional methodologies provide quantification of mRNA abundance in a narrow dynamic range, low sensitivity and time consuming manner, RT-qPCR provides higher sensitivity, specificity, and a broad quantification range. The sensitivity of RT-PCR makes it a powerful tool for molecular diagnostics and gene expression measurement, especially when sample quantities are limited or a transcript is expressed at a low level, and for validating the results of high-throughput microarray data of a smaller set of genes (5, 41).

To avoid any misinterpretations in gene expression analysis by all above-mentioned methods, several variables need to be controlled. There are many sources of variation including the amount of starting material (tissue mass or cell number), RNA extraction efficiency, mRNA integrity, DNase treatments, enzymatic efficiency during cDNA synthesis and differences between tissues or cells in overall transcriptional activity. Especially with the techniques that have higher sensitivity like RT-qPCR, great care must be taken because small variations can have a significant impact on the results. Although various strategies have been applied to normalize these variations (19), in each case there are some drawbacks that make their usage unsuitable. For example, the normalization of the amount of cells is a problem when tissue samples are used and any variation in cell counting can result in a large variation in data. Normalization to total RNA is exposed to the variation in determining the concentration of RNA especially when only minimal RNA quantities are available. The use of rRNAs, which constitute 80–90% of total RNA, can be problematic because of imbalance between mRNA and rRNA fractions (37).

Normalization can be achieved using internal standards or exogenous controls (16, 28). Endogenous reference genes,

also called housekeeping genes or maintenance genes are the most commonly used internal standards in real-time qPCR. Reference genes are required for basal cellular function and maintenance (9, 42) and their expressions in the samples studied are assumed to be constant and stable, non-regulated and not influenced by experimental conditions or treatments (4, 32) . In addition, if the reference gene and the target gene have similar ranges of expression, it will be better for precise quantifying (21).There are some reports that have demonstrated that the expression of these genes in some experimental conditions is altered significantly due to differences in tissue types or other experimental treatments (12, 18, 34, 39) . It is believed that, in some cases, housekeeping proteins are not only involved in the basal cell metabolism but also have other functions in the cells. For example, beta-actin as a structural protein and GAPDH as a protein involved in the metabolism cycle have been used broadly for normalization. However, during cell division and differentiation, genes that are involved in the modulation of the cytoskeleton or metabolic pathways are often changed which makes these genes uncertain for normalization purposes (20, 35, 36). According to the recently published data, it is necessary to identify appropriate reference genes for each individual experiment (12, 17, 31, 33, 43).

In this study, we carried out an evaluation of six commonly used housekeeping genes in rat MSCs and their differentiated adipocytes, chondrocytes and osteocytes. Despite the importance of such differentiation, to our knowledge, there is no report on the determination of optimal reference genes to study gene expression profiling using these culture conditions. The software applications geNorm (40) and NormFinder (2) were used to calculate the most stably expressed reference genes and to determine the optimal number of them required for reliable normalization of gene expression data. To show the importance of reference gene selection in our experiment, we tested whether the choice of any of these affected the results of our lineage-specific target gene expression.

MATERIALS AND METHODS

MSC culture

This study was performed according to protocols approved by the Animal Care and Use Committee of Royan Institute (Tehran, Iran). Bone marrow was collected from the femurs of ten Wistar rats, eight to ten weeks old. Separate cultures were established for each animal. About 10^6 bone marrow cells/ml were plated in 75 cm^2 culture flasks in DMEM (Dulbecco's Modified Eagle Medium; Gibco, UK) supplemented with 15% FBS (Fetal Bovine Serum, Gibco, UK), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, UK). After 48 hours, non-adherent cells were removed by medium replacement. The cultures were fed twice weekly for two weeks. By the time of confluency, the cells were lifted by tripsin/EDTA (Gibco) and split into two fresh 75 cm^2 flasks as passage-1 cells. With further successive subcultures, MSC population was increased to a number sufficient to conduct the next stages of the experiment.

Osteogenic, Chondrogenic and Adipogenic differentiation

To induce osteogenic differentiation, confluence passage-3 cells were cultured in the DMEM medium supplemented with 50 µg/ml ascorbic2-phosphate (Sigma, USA), 10 nM dexamtasone (Sigma, USA) and 10 µM β-glycerol phosphate (Sigma, USA) for three weeks. At the end of this period, alizarin red staining was used to observe the matrix mineralization.

For adipogenesis, DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma,USA) was used to induce the differentiation in the confluence culture of the cells. At the end of the differentiation period, the culture was stained with Oil Red for lipid droplets within the adipogenic cell cytoplasm.

Cartilage differentiation was induced by a micro mass culture system. For this purpose, 2.5×10^5 passaged-cells were pelleted under 1200g for five minutes and cultured in a DMEM medium supplemented by 10 ng/ml transforming growth factor-β3, 10 ng/ml bone morphogenetic protein-6, 50 ng/ml insulin transferin selenium$^+$ premix and 1.25 mg bovine serum albumin and 1% fetal bovine serum. At the end of the differentiation period the pellets were histologically prepared and five micrometer thick sections were obtained and stained with toluidine blue for metachromatic matrix.

To determine the percentage of differentiation in osteogenic, chondrogenic and adipogenic culture quantitative analyses were done on photomicrographs acquired through light microscope (BX71, Olympus) by using Adobe Photoshop CS5.1 and image analysis system (Image Pro Plus 6.0 by Media Cybernetics Inc., USA). We used the measurements of range statistics of Image Pro Plus to determine the red area at osteogenic and adipogenic cultures on several microscopic field and purple area at chondrogenic cultures on sequential sections prepared from chondrogenic pellet.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from independent biological replicates of MSC (n=3), chondrocytes (n=3), Adipocytes (n=6) and osteocytes (n=6) using the RNX™-Plus (RN7713C; CinnaGen Inc., Tehran, Iran), following the manufacturer's instructions. To eliminate any contaminating DNA, the isolated RNA was treated with 1U of RNase-free DNaseI (EN0521; Fermentas, Germany) per 1 µg of RNA in the presence of 20 U of ribonuclease inhibitor (E00311; Fermentas, Germany) and 1X reaction buffer with MgCl2 for 30 minutes at 37°C. To inactivate the DNaseI, 1 µl of 25mM EDTA was added and incubated at 65°C for ten minutes. RNA concentration and purity were determined in duplicate samples spectrophotometrically. Purity of samples was assessed by measuring the absorbance A260nm/A280nm ratio with expected values between 1.8 and 2.0 using a Biowave II spectrophotometer (WPA, Biochrom, Cambridge, UK). RNA quality was verified by electrophoresis on ethidium bromide-stained 1% agarose gels with two intact bands corresponding to 28s and 18s rRNA. On the basis of 1 µg of RNA, the first strand cDNA was synthesized by using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1632, Fermentas) and 0.2 µg random hexamer primer per reaction, according to the manufacturer's instructions.

For every reaction set, one RNA sample was prepared without RevertAid™M-MuLV Reverse Transcriptase (RT reaction) to provide a negative control in the subsequent PCR. To minimize variation in the RT reaction, all RNA

Table 1. Reference genes evaluated in this study and the characteristics of primers used to detect them.

Gene Symbol; Name	Transcript ID	Function	Genomic Localization	Primers sequence	Product size (bp)	Anchoring Exons
Actb; Beta actin	ENSRNOT00000042459 NM_031144.2	Cytoskeletal structural protein	12p11	F: CTATGTTGCCCTAGACTTCG R: AGGTCTTTACGGATGTCAAC	228	(E4-E5)
B2m; Beta-2-microglobulin	ENSRNOT00000023017 NM_012512.1	Beta-chain of major histocompatibility complex class I molecules	3q35	F: TCTGGTGCTTGTCTCTCTGG R: ATTTGAGGTGGGTGGAACTG	138	(E1-E2)
Tfrc; Transferrin receptor protein1	ENSRNOT00000002407 NM_022712.1	Cellular iron uptake	11q22	F: TGGCTTTCCTTCTTTCAACC R: AGCGGTCTGGTTCCTCATAG	272	(E8-E9)
Gusb; β-Glucuronidase	ENSRNOT00000001215 NM_017015.2	Lysosome exoglycosidase	12q13	F: CCAGCCACTATCCCTACTCG R: TAGGTGGTGCCGAAGAGAC	134	(E8-E9)
Hprt1; Hypoxanthine phosphoribosyl-transferase1	ENSRNOT00000045153 NM_012583.2	Purine synthesis in salvage pathway	Xq36	F: CAGACTTTGCTTTCCTTGG R:TCCACTTTCGCTGATGACAC	207	(E7-E10)
Btub; Tubulin beta-5 chain	ENSRNOT00000001095 NM_173102.2	structural proteins	20p12	F:GGAAGAGGATTTCGGAGAGG R:GGACAGAGGCAGCAGAAAGA	125	(E4)

Table 2. Ranking of the candidate reference genes based on their stability values calculated from NormFinder.

Gene Symbol	Stability Value						
	MSC	Chondrocytes	Adipocytes	Osteocytes	MSC& Chondrocytes	MSC & Adipocytes	MSC & Osteocytes
Gusb	0.242(4)	0.090(1)	0.070(1)	0.303(3)	0.464(1)	0.290(4)	0.464(4)
Tfrc	0.526(6)	0.525(5)	0.290(5)	0.551(5)	0.504(2)	0.506(6)	0.385(3)
Btub	0.111(3)	0.233(3)	0.212(4)	0.167(1)	0.559(4)	0.217(2)	0.484(5)
B2m	0.388(5)	0.113(2)	0.193(3)	0.700(6)	0.522(3)	0.501(5)	0.525(6)
Actb	0.082(1)	0.364(4)	0.551(6)	0.259(2)	0.711(6)	0.269(3)	0.119(1)
Hprt	0.103(2)	0.742(6)	0.088(2)	0.408(4)	0.670(5)	0.071(1)	0.378(2)
Best combination of two genes					*Gusb* and *Btub*	*Gusb* and *Btub*	*Gusb* and *Hprt*
Stability value for best combination of two genes					0.160	0.118	0.119

Lowest value equals highest stability. Bracketed numbers show the ranking of the stability.

samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 µl cDNA, 1X PCR buffer (AMS^{TM}, CinnaGen Co., Iran), 200 µM dNTPs, 0.5 µM of each antisense and sense primer and 1U Taq DNA polymerase. The primers used for conventional RT-PCR are listed in Table 3.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Quantitative PCR was performed on a Rotor-Gene 6000 (Corbett Life Science) using the following program: stage 1: 95°C for 10 minutes, stage 2: 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 20 seconds, for 40 cycles. The primers were designed by PerlPrimer software (23). All primers except *Btub*, listed in Table 1, were designed to span introns in order to detect any genomic DNA contamination.

The PCR mix in each well included 10 µl of SYBR®Premix Ex Taq™ II (RR081Q, Takara Bio. Inc.), 6 µl dH_2O, 1 µl each of the forward and reverse primers (5 pmol/µl), 2 µl of single strand cDNA (16 ng/µl) in a final reaction volume of 20 µl. For relative quantification, a standard curve was generated in every individual run by serially diluting (50, 10, 2, 0.4 and 0.08 ng) the pool of cDNA samples with a high expression value. The standard curve was calculated by plotting the log value of the starting concentration versus the threshold cycle (Ct). Amplification efficiencies were determined based on the slope of the standard curve using the following equation: $E\% = [10^{(-1/slope)} - 1]\%$, and the correlation coefficient (R2) was calculated by Rotor-Gene 6000 analysis software (Corbett Life Science, version 1.7). The standard samples were included in every PCR run to control intra-assay variability. The efficiency was ≈ 95-105%. At the end of the run, the melting profile

Table 3. Primers used in this study for evaluating lineage specific target genes.

Genes	Primer sequences (5´-3´)	Annealing Temperature (°C)	Size (bp)	Gene bank code
Osteocalcin (bone gamma-carboxyglutamate protein 2)	F: GGAGGGCAGTAAGGTGGTG R: GCTGTGCCGTCCATACTTTC	58	293	**NM_013414.1**
	F: GAGGGCAGTAAGGTGGTGAA (qPCR) R: GTCCGCTAGCTCGTCACAAT (qPCR)	60	135	
Osteopontin (secreted phosphoprotein 1 (Spp1))	F: AGCAGGAATACTAACTGC R: GATTATAGTGACACAGACTATT	58	287	**NM_012881**
Col1a2 (collagen, type I, alpha 2)	F: ATGCTCAGCTTTGTGGATAC R: CAGCAAAGTTCCCAGTAAGAC	60	253	**NM_053356**
Runx2 **(**runt-related transcription factor 2**)**	F: GGACGAGGCAAGAGTTTCAC (qPCR) R: GAGGCGGTCAGAGAACAAAC (qPCR)	60	165	**NM_053470.2**
Ppara (peroxisome proliferator activated receptor alpha)	F: CCCTGCCTTCCCTGTGAAC R: GGGACTCATCTGTACTGGTGG	61	387	**NM_013196.1**
	F: CTGCTATAATTTGCTGTGGAGA (qPCR) R: TTGGGAAGAGAAAGGTATCATC (qPCR)	60	135	
Pparg (Rattus norvegicus peroxisome proliferator-activated receptor Gamma)	F: ATGCTGTTATGGGTGAAACT (qPCR) R: GATGTCAAAGGAATGGGAGTG (qPCR)	60	194	**NM_013124.3**
Lpl (lipoprotein lipase)	F: GCATTTGAGAAAGGGCTCTG R: CTGACCAGCGGAAGTAGGAG	60	370	**NM_012598.2**
Col10a1 (collagen, type X, alpha 1)	F: AACAGGCAGCAGCACTATG R: TGAAGCCTGATCCAAGTAGC	58	183	**AJ131848.1**
Col2a1 (collagen, type II, alpha 1)	F: CCCAGAACATCACCTACCAC R: GTCAACAATGGGAAGGCG	58	235	**NM_012929.1**
	F: CCAGAACATCACCTACCACT (qPCR) R: CCCTCATCTCCACATCATTG (qPCR)	60	109	
Acan (Aggrecan)	F: GGCAACCTCCTGGGTGTAAG R: TCGCACCACCAGGTCCTC	58	444	**NM_022190.1**
	F: ATGGTGACAAGGACGAGTTC (qPCR) R: CTCACCCTCCATCTCTTCAG (qPCR)	60	101	

was determined to demonstrate the synthesis of a single PCR product, as instructed by the manufacturer. The PCR products were also examined by gel electrophoresis to verify correct sizes. All samples were run in duplicate and the mean value of each duplicate was used for all further calculations. RT minus samples and no template controls were run together with test samples.

Candidate reference gene selection

Six commonly used candidate reference genes, *Actb, Btub, Hprt, B2m, Gusb* and *Tfrc* (Table 1), were selected for analysis based on literature review. All genes are assumed to be constitutively expressed across the experimental samples and have independent functions in cellular maintenance. Only *Actb* and *Btub* share similar biochemical process in the cytoskeleton of the cell. For stability comparisons of candidate reference genes, the output data generated by Rotor-Gene 6000 analysis software (Corbett Life Science, version 1.7), were transferred to Microsoft Excel for analysis and transformed to linear values by transforming Ct values into quantities using standard curves or the comparative Ct method ($Q = 2^{(\min Ct - \text{sample } Ct)}$) (34), setting the highest relative quantity for each gene to 1, and scaling the expression value for all other samples to a proportion relative to this highest value. The data obtained were converted into correct input files, according to the requirements of the particular software, and analyzed using geNorm (version 3.5) (40) and NormFinder (2). The experimental groups and the number of independent biological replicates included in input files for each program were: MSC (n=3), chondrocytes (n=3), Adipocytes (n=6), osteocytes (n=6), MSCs and chondrocytes combination (n=6), MSCs and adipocytes combination (n=9) and MSCs and Osteocytes combination (n=9).

The geNorm program uses a gene-stability measure M. Its estimation of the most stable genes is based upon pairwise comparisons of sample variability. This algorithm assumed that the expression ratio of two ideal reference genes is the same for all samples, regardless of the experimental conditions or cell types. The two most stable genes are identified and a normalization factor calculated. NormFinder analyzes the stability of the candidate genes based on an estimate of the variation in each subgroup (intra-group) and for combination of all subgroups (inter-group) then combines both results in a stability value for each investigated gene. The program then ranks genes based on a stability value, with the lowest value indicating the most stably expressed gene. The option to define sub-groups in NormFinder was applied in the three groups of study which contained combination of MSCs and their differentiated lineages.

Effect of using the different reference genes to normalize data for lineage-specific target genes

For the analysis of the differentiation of the MSCs and to show how using the different endogenous control genes for normalization can affect the quantification of genes of interest, we representatively analyzed_the expression of the lineage-specific genes, aggrecan (*Acan*) and type 2 collagen (*Col 2*) for chondrocytes, osteocalcin and runt-related transcription factor (*Runx*) for osteocytes and two isoforms of peroxisome proliferator activated receptor (*Ppara* and *Pparg*) for adipocytes (Table 3).

Relative expression levels were determined using a $2^{-\Delta\Delta Ct}$ method (22) where $\Delta\Delta C_t = (C_t$ target gene, test sample $- C_t$ endogenous control, test sample) - (C_t target gene, calibrator sample - C_t endogenous control, calibrator sample). In this experiment MSC was considered as a calibrator.

RESULTS

To identify suitable reference genes for gene expression analysis during differentiation of rat MSCs towards chondrocytes, adipocytes and osteocytes, a set of six commonly used reference genes was evaluated by geNorm and NormFinder softwares which employ different statistical models to define the most reliable reference genes for normalization.

To see if there was any shift in the expression stability of reference genes in the transition from MSCs to differentiated osteocytes, adipocytes and chondrocytes, we performed analysis first for these four populations of cells separately and then combined MSCs with each of the differentiated cells.

To determine the optimal number of reference genes for normalization purposes in quantifying gene expression, we compared pairwise variation (Vn/Vn+1) calculated by geNorm between each combination of sequential normalization factors (NFn and NFn+1) for all samples in the group. We applied a default threshold (geNorm recommended threshold = 0.15) for cut-off (40) below which inclusion of additional reference genes is not necessary.

MSC culture and expression stability of candidate reference genes

In the early days, the primary cultures consisted mainly of fibroblastic cells and a few small clear cells. Two weeks after culture initiation, all available surfaces of the culture dish were covered with a monolayer in which the bundles of fibroblastic cells extended in different directions (Fig. 1A). During subcultures, the cells maintained their fibroblastic morphology and approached confluency within ten days. According to geNorm analysis of reference genes, *Actb* and *Btub* were the most stably expressed genes and *Tfrc* the least (Fig. 2G). NormFinder also ranked *Actb* and *Tfrc* as the most and least stable reference genes, respectively (Table 2). GeNorm determined two most stable genes as an optimal number of reference genes for calculating the normalization factor (Fig. 2H).

Osteogenic differentiation and expression stability of candidate reference genes

In the osteogenic culture, nodule-like aggregations appeared. Upon alizarin red staining, these nodules stained red indicating that they were mineralized during the induction period (Fig. 1B). According to the measurements more than 90% of osteogenic culture tended to stain red by alizarin red. RT-PCR analysis indicated that the mRNA of bone-specific proteins including osteocalcin, osteopontin and collagen I were produced in the culture (Fig. 1E).

To identify suitable reference genes for gene expression studies of osteogenic cells, differentiated cells analyzed by geNorm showed the least variation in expression levels of *Actb* and *Gusb* and the most variation in expression of the *B2m* gene (Fig. 2A). However, NormFinder introduced *Btub* as the most stable gene and *Actb* and *Gusb* as second and third. NormFinder was in agreement with geNorm about the least stable gene (Table 2). For osteogenic cells

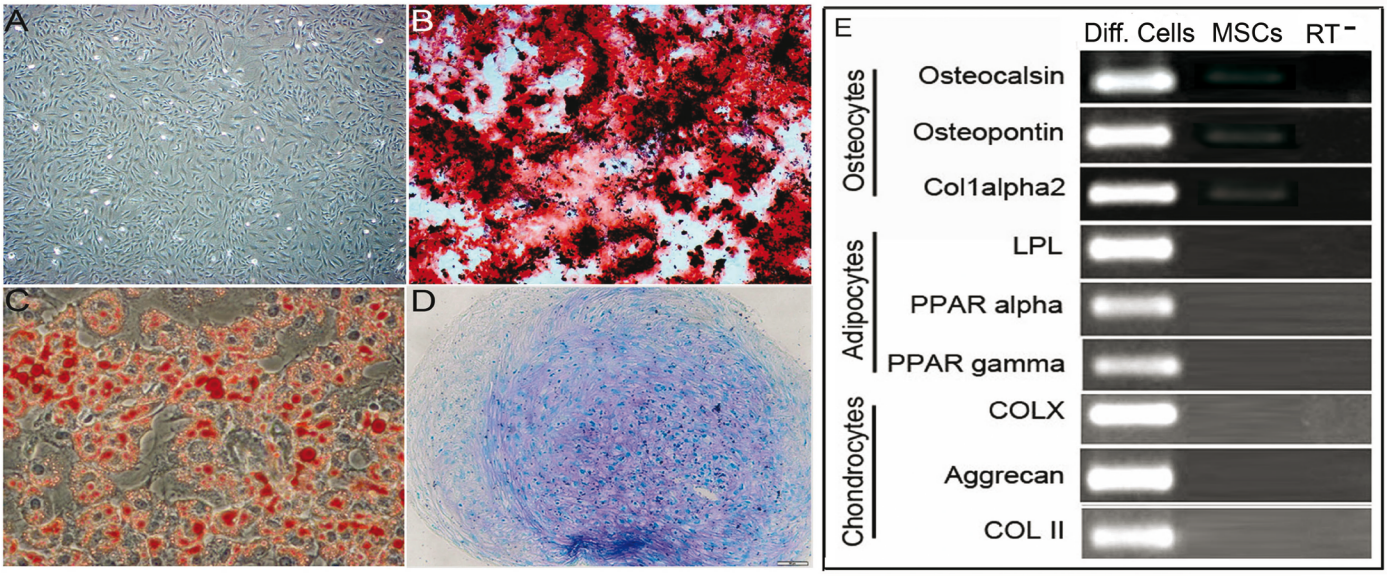

Figure 1. MSCs and histochemical staining of MSC-derived chondrogenic, osteogenic and adipogenic cells and RT-PCR result for confirmation of differentiation. **A)** MSCs. **B)** Osteogenic cells stained with alizarin red. **C)** Staining with Oil Red for lipid droplets within adipogenic cell cytoplasm. **D)** Chondrogenic cells stained with toluidine blue for metachromatic matrix. **E)** RT-PCR result which confirmed the differentiation by analyzing expression of specific gene for each lineage.

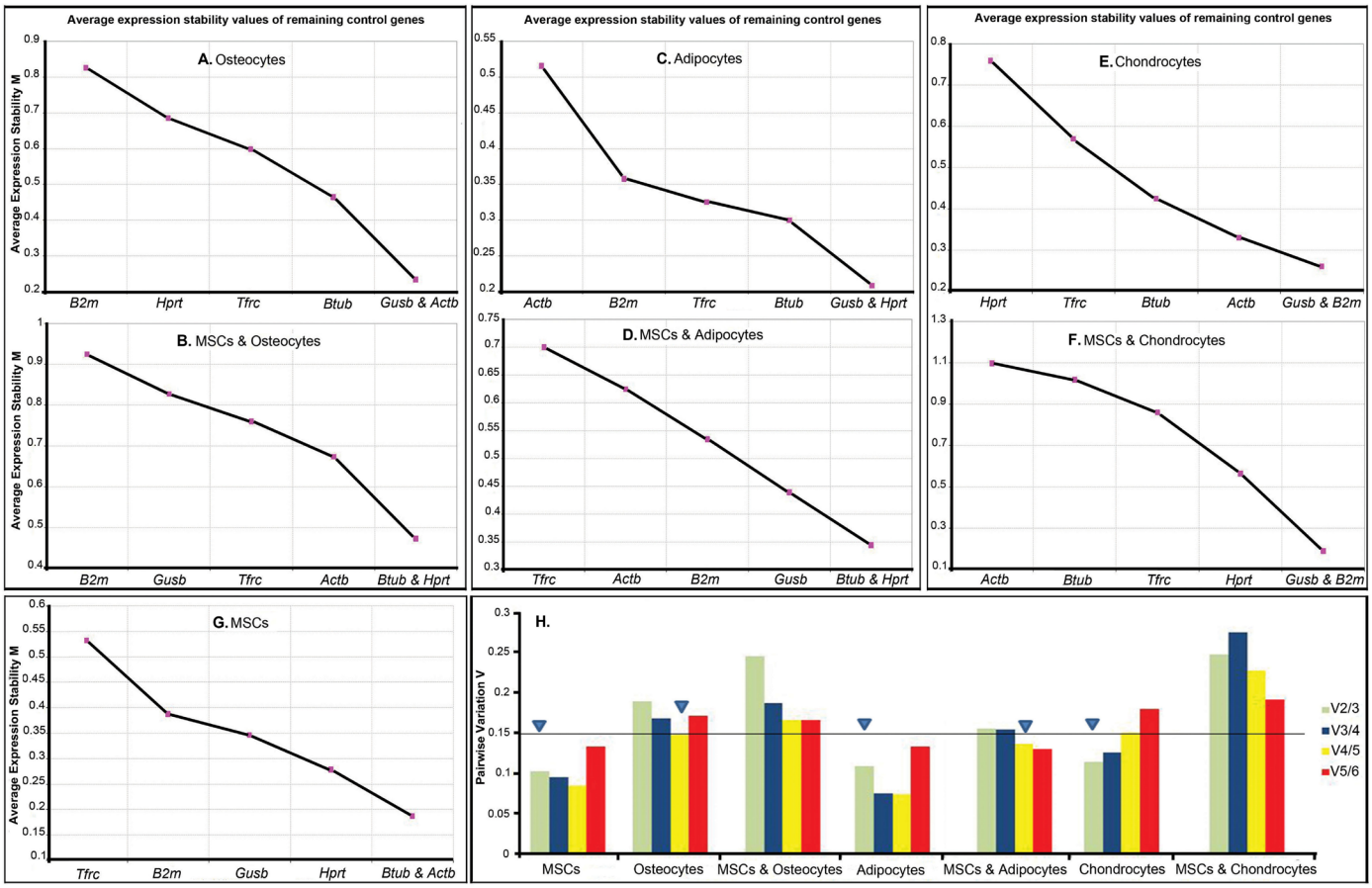

Figure 2. Average gene expression stability (M) of six candidate reference genes and determination of the optimal number of them for normalization calculated by geNorm. Expression stability was plotted in Osteocytes (n=6) **(A)**, MSCs and Osteocytes combination (n=9) **(B)**, adipocytes (n=6) **(C)**, MSCs and adipocytes combination (n=9) **(D)**, chondrocytes (n=3) **(E)**, MSCs and chondrocytes combination (n=6) **(F)**, and MSCs(n=3) **(G)**. The least stable reference gene (higher M value) is on the left and the most stable combination (lower M value) is on the right of the plot. Pairwise variation (*Vn*/*n*+1) analysis between the normalization factors NF_n and NF_{n+1} to determine the number of reference genes required for accurate normalization (arrowhead shows the optimal number of reference genes for normalization) **(H)**.

the value of V4/5 was 0.149. This shows the need for at least four most stable genes for precise normalization (Fig. 2H).

To see if there was any change in the expression stability of reference genes during differentiation from MSCs to osteocytes, we continued our study in the second group, a combination of MSCs and osteocytes. Interestingly, geNorm proposed *Btub* and *Hprt* as stable genes (Fig. 2B) and NormFinder selected *Gusb* and *Hprt* as the best combination of two genes (Table 2). As we can see from the results *Hprt* was second least stable gene in the case of osteocytes whereas it was one of the most stable genes in MSCs and one of the two most stable genes when we combined osteocytes with MSCs. For the combination of these two cell populations, all pairwise variations (Vn/Vn+1) calculated by geNorm were above the threshold (recommended threshold = 0.15) (Fig. 2H).

Adipogenic differentiation and expression stability of candidate reference genes

About ten days after induction initiation, the first lipid droplets appeared inside the cells and increased in number as time progressed. These droplets were positively stained red with Oil Red staining for adipocyte detection (Fig. 1C). Quantitative image analyses which were done on photomicrographs showed that about 70% of adipogenic culture tended to stain red by Oil Red. RT-PCR analysis further confirmed the expression of adipocyte marker genes including *Ppar-alpha*, *Ppar-gamma2* and *Lpl* (Fig. 1E).

For adipogenic cells, using geNorm analysis, we ranked the six candidate reference genes by the average expression stability (M) as follows: *Atcb* > *B2m* > *Tfrc* > *Btub* > *Gusb* and *Hprt,* in which the latter ones were the most stable (Fig. 2C). NormFinder assessed the most and least stable genes like geNorm (Table 2). Interestingly *Actb*, as one of the two most stable genes in MSCs, had become a least stable gene in adipocytes. Again to study the differentiation procedure effect on reference gene stability ranking we studied the combination of MSCs and their differentiated adipogenic cells. GeNorm determind *Btub* and *Hprt* as a stable pair (Fig. 2D) and NormFinder showed *Gusb* and *Btub* (Table 2). Among the six reference genes *Btub* had the least expression variation from MSCs to adipocytes.
According to our data, for adipocytes and combined data, pairwise variation (V) showed V2/3 of 0.110 and V4/5 of 0.137, respectively, which were below the cut-off value of 0.15 (Figure 3). It showed that the top two most stable genes would be chosen for accurate normalization of RT-qPCR data of adipocytes and top four most stable genes for the combination of MSCs and adipocytes (Fig. 2H).

Chondrogenesis differentiation and expression stability of candidate reference genes

The sections from micromass cultures for cartilage differentiation were stained metachromatically purple with toluidine blue staining (Fig. 1D). The percentage of purple area at sections prepared from chondrogenic pellet appeared to be 84%. RT-PCR analysis revealed that the mRNA of collagen II, collagen X and aggrecan were produced in the differentiated cells (Fig. 1E).

To determine the stability of candidate reference genes in chondrocytes, geNorm analysis showed that *Gusb* and *B2m* were the two most stable genes and *Hprt* the least stable (Fig. 2E). A similar result was obtained by NormFinder. *Gusb* and *B2m* were among the least stable genes in MSCs but the most stable in chondrocytes. During differentiation of MSCs into chondrocyte derivatives, the reference genes that were at the top of the stability ranking were *Gusb* and *B2m* by geNorm (Fig. 2F) and *Gusb* and *Btub* by NormFinder (Table 2).

The pairwise variation values Vn/Vn+1 between two sequential normalization factors NFn and NFn+1 (Fig. 2H) showed that for chondrocytes, the V2/3 was only 0.115. It was below the threshold and by including further reference genes the V value increased so there was no need to include more than two genes into the normalization factor. For analyzing the combination of MSCs and their differentiated chondrocytes, pairwise variations of all sequential normalization factors were above the threshold.

Lineage-specific gene expression varies according to the reference genes employed for normalization

To evaluate the necessity of analyzing and selecting the best reference gene(s) for the study of a target gene of interest, we performed RT-qPCR on RNAs extracted from differentiated osteocytes, adipocytes and chondrocytes from rat MSCs. For each lineage, two specific genes whose expression increased during differentiation from MSCs were chosen: *Osteocalcin* and *RunX* for osteocytes, *Ppara* and *Pparg* for adipocytes, and *Aggrecan* and *Collagen II* for chondrocytes (Table 3).

In osteogenic differentiation, although expression of osteocyte markers in differentiated cells had been shown by RT-PCR (Fig. 1E), expression of *Osteocalcin* and *RunX* in RT-qPCR were also shown to be upregulated in comparison with MSCs but with considerable variation in the fold change regarding which reference genes had been used for normalization (Fig. 3A,B). The fold increases in *Osteocalcin* compared to control MSCs using these reference genes, *Btub* and *Actb,* were 126.03 and 100.89-fold, respectively, which were significantly higher values compared with those obtained using the *Tfrc, Hprt, Gusb* and *B2m* as reference genes (Fig. 3A). In the case of *RunX,* although its expression was not upregulated as much as *Osteocalcin,* normalization with *Btub* and *Actb* showed 2.26 and 1.8-fold increases compared to MSCs where it appears to be downregulated when *B2m,* which was one of the least stable genes, is used for calculations (Fig. 3B)

The effects of using different normalizer genes on the expression levels of the tested adipogenic markers (*Ppara* and *Pparg*) are shown in Fig. 3C, D. Although expression of these two genes in differentiated adipocytes was greater than in MSCs regardless of which endogenous control gene was used, marked differences in the expression levels were observed for the different normalization strategies that were applied. For *Pparg*, it was overestimated by *Tfrc* and underestimated by *B2m* normalization, as two of the least stable genes, up to 18622 and 2045-fold, respectively. Similar patterns were seen in *Ppara* expression but with different fold change values (Fig 3C, D).

To evaluate the effects of different normalization strategies on chondrogenic markers, *Aggrecan* and *Collagen* expression were studied. After normalization with each of our candidate reference genes, for both markers we had the same pattern of expression. Normalization with *Tfrc*, *Actb* and *Btub* showed a higher magnitude of fold change in comparison with MSCs, but normalization with *B2m, Gusb* and *Hprt* showed a lower value of upregulation (Fig.

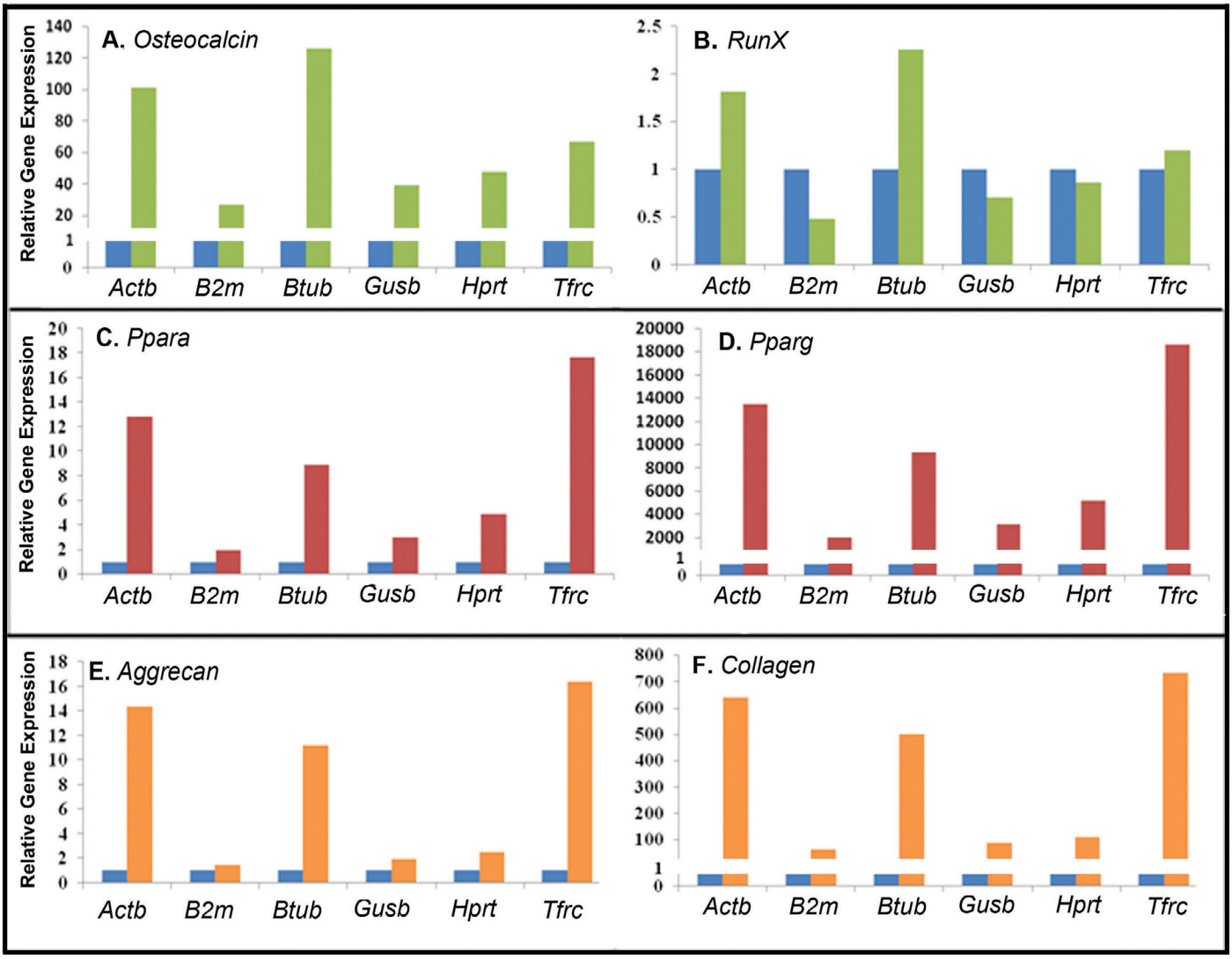

Figure 3. Relative quantification of lineage-specific genes expression in differentiated cells to MSCs depends on different reference genes: Osteocalcin **(A)** and runX **(B)** as Osteogenic markers, Ppara **(C)** and Pparg **(D)** as adipogenic markers, Aggrecan **(E)** and Collagen **(F)** as chondrogenic markers. Blue, green, red and orange colored-column represents MSCs, osteocytes, adipocytes and chondrocytes respectively. Representative results from one experiment.

3E, F).

DISCUSSION

MSCs hold great promise in the future of regenerative medicine for bone and cartilage defects. Understanding these cells and their differentiation potential has an important role in conducting them to the desired cell types. Until now different culture conditions have been examined to promote differentiation of MSCs towards osteocytes, chondrocytes and adipocytes. To evaluate the rate of these differentiation procedures, measuring the mRNA level of specific genes for each lineage is necessary.

In this case real-time RT-PCR, due to its high sensitivity and accuracy, would be the method of choice. However, the validity of the real-time PCR results is largely dependent on the reference genes used for normalization of data. Several studies have indicated that the traditionally used internal control genes like *Gapdh* and *B-actin* do not have stable expression; therefore determination of the appropriate reference gene for normalization purposes becomes necessary.

To our knowledge, there are no reports about the validation of reference genes for gene expression assays of rat MSCs during osteogenic, adipogenic and chondrogenic differentiation. There are some similar studies which have used reference genes like *Gapdh*, *Actb* and 18s rRNA (1, 6, 8, 14, 15, 24). However, very few of those studies provided any validation for the use of these genes as reference genes prior to their application. In one study, Fink et al. evaluated the gene expression levels of twelve widely used reference genes during passaging of primary human adipose-derived stem cells and hypoxic culture, osteogenic, adipogenic and chondrogenic differentiation. Finally they recommended normalizing transcription levels to the geometric mean of the three most stable genes, *Ymhaz*, *Tbp* and *Gusb*, in experiments like theirs which use adipose-derived stem cells (12). Although their suggestion would be useful for the same type of experiment, they have combined samples of all experimental groups and evaluated reference gene stability. This kind of evaluation suffers from the point that stability ranking for all samples is not the same as for part of them. For example if in one experiment only ASCs and osteogenic cells are available, that ranking may be different from the calculated ones.

In some other studies, investigators preferred to make

a distinction between experimental groups for calculating the most stable reference genes. Foldger et al. found the same candidate reference genes in their experimental groups, but Rho et al. introduced a different combination of reference genes for each purpose (13, 33).

In the present study, we studied the stability of six commonly used reference genes over differentiation of rat MSCs towards osteocytes, adipocytes and chondrocytes. For precise selection and to show alterations in the expression pattern of reference genes during specific lineage differentiation, we set our analysis in different groups. In the first set of analysis, four populations of cells, MSCs, osteocytes, adipocytes and chondrocytes were studied separately to identify most stable genes in each one.

In every experiment which comprises differentiation procedures, investigators study the pattern of expression of specific genes in experimental samples relative to a control sample which is named calibrator. In the case of our experiment the MSC group was defined as a calibrator sample. So in the second set of analysis we combined the data of each differentiated lineage with the MSC group.

Among the several statistical programs developed to identify optimal reference genes, we chose geNorm and NormFinder, which are the most common softwares.

In the first set of analysis, in all cases the results of geNorm and NormFinder were in close agreement. For MSCs, *Btub* and *Actb*, for osteocytes, *Gusb* and *Actb*, for adipocytes, *gusb* and *Hprt*, and for chondrocytes *Gusb* and *B2m* were identified as most stable reference genes. GeNorm also determined the two most stable genes as an optimal number of reference genes for normalization. Although *Gusb* was one of the stable genes in three differentiated cell types, ranking of reference gene stability was different for each cell type. These results indicate the necessity of examining reference gene stability across all experimental groups as it can be different for every cell type.

In the second set of our analysis, by combining MSCs with each differentiated cell type, new combinations of stable reference genes were revealed. The results showed a considerable shift in the expression stability of several reference genes in the transition from MSCs to differentiated progenies.

Furthermore, the combination of the two most stable genes introduced by geNorm and NormFinder was not the same. But in each case one of the reference genes was identical, *Hprt* for the MSCs and osteocytes group, *Btub* for the MSCs and adipocytes group and *Gusb* for the MSCs and chondrocytes group. The difference in methodologies which rank the stability of genes could explain the different results obtained by these two algorithms. Determination of groups prior to analysis is one of the advantages of NormFinder because it takes into account if there were any grouping effects on gene expression. However, Mehta et al. argue that the sample size has an effect on NormFinder but not on geNorm results (25). Based on pairwise variation between two sequential normalization factors NFn and NFn+1, for combination of MSCs with osteocytes and also with chondrocytes, all pairwise variation (Vn/Vn+1) was above the threshold (recommended threshold = 0.15)(40). But for the combination of MSCs with adipocytes, the value in V4/5 was below the threshold and this indicates that the top four most stable reference genes should be enough for calculating the normalization factor. Although the defined threshold by geNorm is not mandatory, altogether the pairwise variation results may show the need for the inclusion of other new reference genes in our sets.

To assess the importance of selecting suitable reference genes for use in normalization of real-time PCR data, in our final analyses we measured the expression of two genes of interest for each differentiated lineage. In all cases the dramatic difference between normalized expressions of genes of interest regarding which reference genes are used for normalization show how reference gene expression can affect the final results. These data are in agreement with several other published data in different areas of biology which emphasize the importance of this crucial selection step in gene expression assay by real-time PCR (10, 11, 43).

Overall, our study indicated that, for appropriate analysis of gene expression during rat MSC differentiation into bone, cartilage and adipose lineages, looking for appropriate reference genes is necessary and it is better to consider precisely the reference gene stability during differentiation. We would also like to emphasize that sometimes a new set of reference genes would be appropriate rather than the set we have chosen based on literature review, and the stable genes which we introduced here for each cell type or differentiation procedure should be used only in experiments similar to ours.

Finally to find the best reference genes we would like to recommend the following two steps. First, according to the results of high throughput gene expression profiles such as microarrays which are related to our experiments, stably expressed genes should be identified. Second, a selected number of potential references genes can then be validated experimentally by using statistical algorithms, for example, geNorm (40), Best keeper (29) and NormFinder (2). However, this is not always feasible especially in experiments with limited amounts of cells or tissue. In these situations it is proposed to have a precise literature review to find reported stable reference genes in similar conditions to our experiments, and then to carry out normalization of data with at least two of them to see if there is any difference in their results. If they do not show significant difference we can proceed with further analysis based on one of them or on the normalization factor calculated for both of them.

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